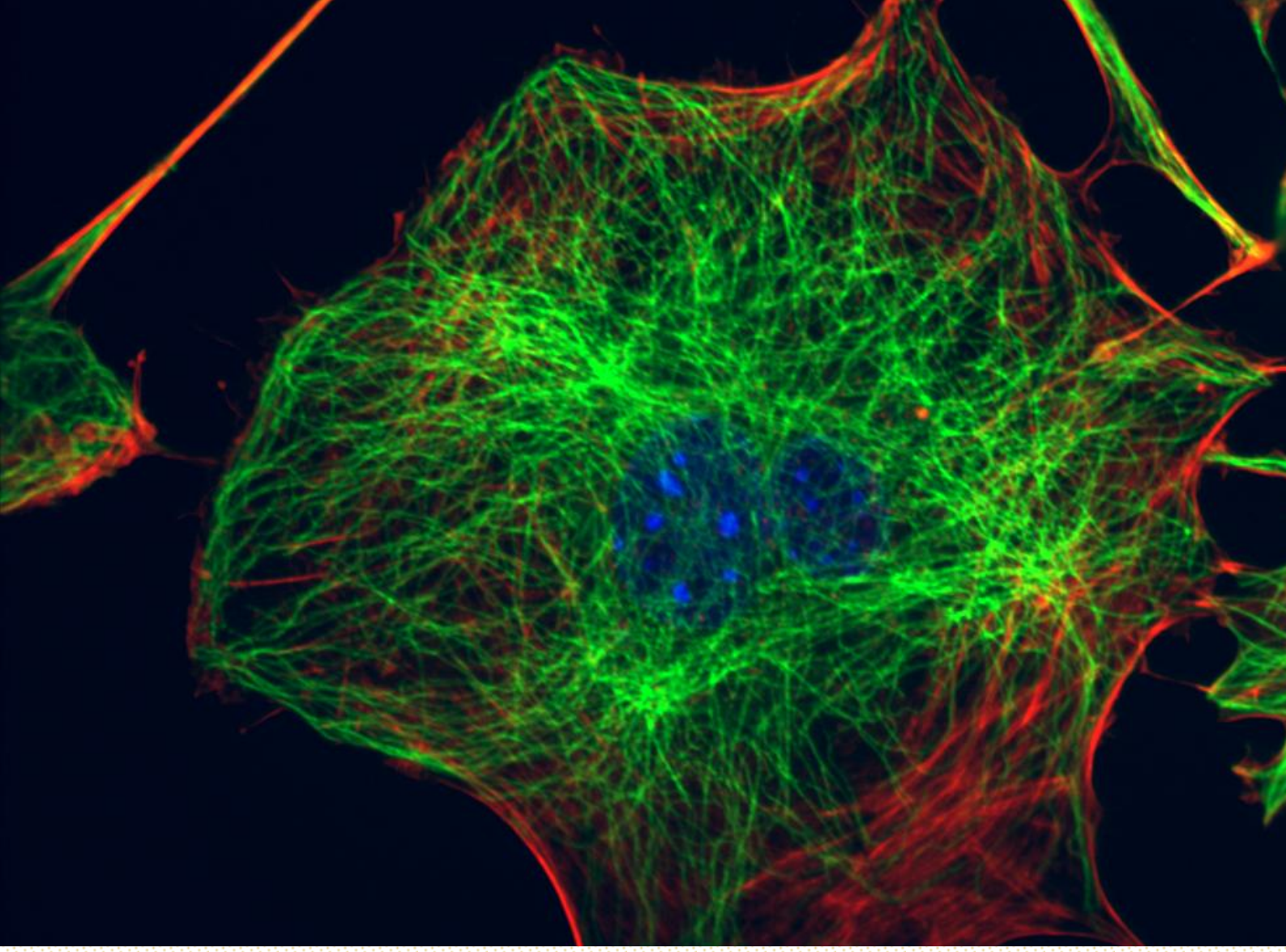

Paula Margarida dos Santos Laranjeira

Immunomodulation by mesenchymal stromal cells: relevance to mesenchymal stromal cells-based therapies

Tese de Doutoramento em Biociências na especialização de Biologia Celular e Molecular, orientada pelo Professor Doutor Carlos Bandeira Duarte e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

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Mesenchymal stem cells from umbilical cord matrix, adipose tissue and bone marrow exhibit different capability to suppress peripheral blood B, NK and T cells

Andreia Ribeiro*, Paula Laranjeira*, Sandrine Mendes, Isabel Velada, Cristiana Leite, Pedro Andrade, Francisco Santos, Ana Henriques, Mário Grãos, Carla Cardoso, António Martinho, M.Luísa Pais, Cláudia Lobato da Silva, Joaquim Cabral, Hélder Trindade, Artur Paiva

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* These authors contributed equally to this work and should be considered as co-first authors.

Human bone marrow-derived mesenchymal stromal cells differentially inhibit cytokine production by peripheral blood monocytes subpopulations and myeloid dendritic cells

Paula Laranjeira, Joana Gomes, Susana Pedreiro, Mónia Pedrosa, António Martinho, Brígida Antunes, Tânia Ribeiro, Francisco Santos, Rosário Domingues, Manuel Abecasis, Hélder Trindade, Artur Paiva

Stem Cells International 2015; 2015:819084

Effect of human bone marrow mesenchymal stromal cells on cytokine production by peripheral blood naive, memory and effector T cells

Paula Laranjeira, Mónia Pedrosa, Susana Pedreiro, Joana Gomes, António Martinho, Brígida Antunes, Tânia Ribeiro, Francisco Santos, Hélder Trindade, Artur Paiva

Stem Cell Research & Therapy 2015; 6:3

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Abbreviations

ABBREVIATIONS

A	
AC	Amcyan
ADA	Adenosine deaminase
ADORA2A	A_{2A} adenosine receptor
APC	Allophycocyanin
APC-H7	Allophycocyanin hillite 7
AT	Adipose tissue

B	
BAFF	B-cell activating factor
BCR	B cell receptor
BDCA	Blood dendritic cell antigen
BDNF	Brain-derived neurotrophic factor
BLIMP-1	B-lymphocyte-induced maturation protein 1
BM	Bone marrow

C	
CFU-F	Fibroblast colony forming units
CIA	Collagen-induced arthritis
CIITA	Class II transactivator
CLA	Cutaneous lymphocyte antigen
CLR	
CLIR	C-lectin-type-inhibitory receptors
CM	Central memory
COX	Cyclooxygenase
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4

D	
DAMP	Damage-associated molecular patterns
DC	Dendritic cells
DNAM-1	DNAX accessory molecule-1

E	
EAE	Experimental autoimmune encephalitis
EGF	Epidermal growth factor
EM	Effector memory
EP	E prostanoid receptors
Eph	Erythropoietin-producing hepatocellular receptor
ERK	Extracellular signal-regulated kinase

F	
FACS	Fluorescence-activated cell sorting
FasL	Fas ligand
FITC	Fluorescein isothiocyanate
FGF	Fibroblast growth factor 2
FoxP3	Forkhead box P3
FSC	Forward scatter

G	
G-CSF	Granulocyte-colony stimulating factor
GATA3	GATA binding protein 3
GD2	Neural ganglioside 2
GM-CSF	Granulocyte macrophage-colony stimulating factor
GSK3β	Glycogen synthase kinase-3 beta
GvHD	Graft-*versus*-host disease

H	
3-HAA	3-hydroxyanthranilic acid
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
HO-1	Heme oxygenase-1
HPC	Hematopoietic precursor cells
HSC	Hematopoietic stem cells

I	
ICAM	Intercellular adhesion molecule
ICOS	Inducible costimulatory
ICOSL	Inducible costimulatory ligand
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor-binding protein
IL	Interleukin
ILT	Immunoglobulin-like transcript
IL-1Ra	Interleukin-1 receptor antagonist
iNKT	Invariant natural killer T
iNOS	Inducible nitric-oxide synthase
IP-10	Interferon inducible protein 10
IRF-4	Interferon regulatory factor 4
ITAC	Interferon-inducible T-cell a-chemoattractant

J	
JAK	Janus kinase
JNK	Jun N-terminal kinase

K

KIR	Killer immunoglobulin-like inhibitory receptors

L

LAIR1	Leukocyte associated Ig-like receptor 1
LFA	Lymphocyte function-associated antigen
LIF	Leukemia inhibitory factor
LMP	Low molecular mass polypeptides
LPS	Lipopolysaccharide

M

M-CSF	Macrophage-colony stimulating factor
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemotactic protein
MDC	Macrophage-derived chemokine
mDC	Myeloid dendritic cell
MECL	Multicatalytic endopeptidase complex subunit
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MICA/B	MHC class I-related chain A /B
MIG	Monokine induced by gamma
MIP	Macrophage inflammatory protein
MLR	Mixed lymphocyte reaction
MMP	Matrix metalloproteinase
MNC	Mononuclear cells
MSC	Mesenchymal stromal/stem cells
MSCA-1	Mesenchymal stem cell antigen-1
mTOR	Mammalian target of rapamycin

N

NCR	Natural cytotoxicity receptors
NF-AT	Nuclear factor of activated T-cells
NF-kB	Nuclear factor-kappa B
NGF	Nerve growth factor
NGFR	Nerve growth factor receptor
NK	Natural killer cells
NKG2D	Natural killer group 2, member D receptor
NKT	Natural killer T cells
NO	Nitric oxide
NOS	Nitric-oxide synthase

O

OSM	Oncostatin M

P

PacB	Pacific blue
PacO	Pacific orange
PAMP	Pathogen-associated molecular pattern
PAX-5	Paired box gene 5
PBMC	Peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PC7	Phycoerythrin cyanin 7
PD-1	Programmed cell death 1
PD-L1	Programmed cell death ligand 1
pDC	Plasmacytoid dendritic cell
PDGF	Platelet-derived growth factor
15-PGDH	15-hydroxyprostaglandin dehydrogenase
PE	Phycoerythrin
PE-Cy5	Phycoerythrin cyanin 5
PE-Cy7	Phycoerythrin cyanin 7
PerCP-Cy5.5	Peridinin chlorophyll protein- cyanine 5.5
PG	Prostaglandin
PHA	Phytohemagglutinin
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
Poly(I:C)	Polyinosinic–polycytidylic acid
PRR	Pattern recognition receptors
PSGL-1	P-selectin glycoprotein ligand 1
PVR	Poliovirus receptor

R

RANTES	Regulated upon activation, normal T cell expressed and secreted
RNS	Reactive nitrogen species
RORc	Retinoic acid receptor-related orphan receptor C
RORγt	Retinoic acid receptor-related orphan receptor gamma-T
ROS	Reactive oxigen species
RT-PCR	Real time polymerase chain reaction

S

SCF	Stem cell factor
SDF-1	Stromal cell-derived factor 1
SSC	Side scatter
SSEA-4	Stage specific embryonic antigen-4
STAT	Signal transducer and activator of transcription
STRO-1	Stromal-derived factor-1

T

TAP1	Transporter associated with antigen processing-1
TARC	Thymus and activation-regulated chemokine
T-bet	T-box transcription factor
TCR	T cell receptor
Tfh	Follicular helper T cells
TGF	Transforming growth factor
Th	Helper T cells
TLR	Toll like receptor
TNF	Tumor necrosis factor
TPA	12-otetradodecanoyl-phorbol-13-acetate
TRAIL	Tumor necrosis factor-related apoptosis inducing ligand
Treg	Regulatory T cells
TSG-6	TNF-α stimulated gene/protein 6

U

UC	Umbilical cord
UCB	Umbilical cord blood
UCM	Umbilical cord matrix
ULBP	UL16 binding protein

V

VCAM	Vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VLA-4	Very late antigen 4

W

WHO	World Health Organization

X

XBP1	X-box binding protein 1

Z

ZAP-70	Zeta-chain-associated protein kinase of 70 kDa

ABSTRACT/RESUMO

ABSTRACT

Introduction: The clinical benefits of mesenchymal stromal/stem cells (MSC)-based therapies for immune disorders and degenerative diseases are based on their ability to modulate the immune system and secrete growth factors. However, some essential issues concerning the immunosuppressive properties of MSC, such as the differences among MSC derived from different tissues and the influence of MSC on the function of specific immune cell subpopulations (namely the recently described monocyte subsets, and T cell functional compartments) are not well elucidated.

Aim: Here, we compared the suppressive effect of human MSC derived from bone marrow (BM), adipose tissue (AT), and umbilical cord matrix (UCM), on lymphocyte activation, proliferation, and mRNA expression of genes with an important role in T cell and NK cell function; investigated the influence of human BM-MSC on monocyte and myeloid dendritic cell (mDC) activation/maturation, and on cytokine expression by peripheral blood mDC, monocyte subpopulations, and the naturally occurring functional compartments of $CD4^+$ and $CD8^+$ T cells, as well as on $CD4^+$ and/or $CD8^+$ T cells producing IL-17, IL-9, and IL-6.

Material and Methods: To investigate the ability of MSC to suppress immune cells, peripheral blood mononuclear cells (PBMC) were cultured in the presence or absence of MSC and treated with stimulating agents; cell cultures of non-stimulated PBMC, in the presence or absence of MSC, were also carried out. To compare the effect of MSC derived from BM, AT, and UCM, MSC were co-cultured with PHA-stimulated PBMC; T, B and NK cell activation and proliferation were evaluated by flow cytometry; while RT-PCR was used to quantify mRNA expression on purified T and NK cells' activation compartments. To evaluate the effect of BM-MSC on mDC, classical, intermediate, and non-classical monocytes, BM-MSC were co-cultured with PBMC stimulated with LPS+IFNγ. Cell activation and cytokine production were assessed by flow cytometry; and cytokine mRNA expression quantified by RT-PCR in purified monocyte subsets and mDC. Co-culture of BM-MSC with PMA+ionomycin-stimulated PBMC was carried out to evaluate MSC effect on $CD4^+$ and $CD8^+$ T cell functional compartments: naive, central memory (CM), effector memory (EM), and effector. Cytokine expression within each functional compartment and the percentage of $CD4^+$ and $CD8^+$ T cells producing IL-17, IL-9, and IL-6 were assessed by flow cytometry; cytokine mRNA expression was quantified in purified $CD4^+$ and $CD8^+$ T cells by RT-PCR.

Results: MSC derived from BM, AT, or UCM were able to inhibit $CD4^+$ and $CD8^+$ T cell proliferation and activation; BM- and AT-MSC also prevented B cell proliferation and activation, and $CD56^{dim}$ and $CD56^{bright}$ NK cell activation. AT-MSC always exerted the strongest suppressive action. In turn, UCM-MSC hampered activation of $CD56^{dim}$ NK cells, but had no effect on $CD56^{bright}$ NK cell activation, B cell activation or proliferation. Moreover, MSC co-culture reduced TNF-α and perforin mRNA levels in activated NK cells. In the purified T cell activation compartments, we observed increased mRNA levels of FoxP3 and T-bet by PHA-stimulated T cells in the presence of MSC.

BM-MSC effectively inhibited TNF-α and CCL4 protein expression in monocytes and mDC, without affecting CCR7 and CD83 expression. Of note, BM-MSC-driven inhibition was more pronounced for mDC, and the reduction of TNF-α expression was less marked for non-classical monocytes. MSC also decreased mRNA levels of IL-1β and IL-6 in classical monocytes, CCL3, CCL5, CXCL9, and CXCL10 in classical and non-classical monocytes, and IL-1β and CXCL10 in mDC.

In the same line, BM-MSC reduced the percentage $CD4^+$ and $CD8^+$ T cells producing TNF-α, IFNγ, and IL-2, as well as TNF-α and IFNγ mean fluorescent intensity (MFI), among all the four functional compartments, except for naive $CD4^+IFN\gamma^+$ T cells, where MSC had no inhibitory effect. While BM-MSC-driven inhibition of TNF-α and IL-2 production was higher for $CD4^+$ T cells, inhibition of IFNγ secretion was more pronounced for $CD8^+$ T cells. Accordingly, a decreased percentage of $CD4^+$ and $CD8^+$ T cells expressing IL-17, IL-17 and TNF-α, and IL-9, and of $CD4^+IL\text{-}6^+$ T cells, was induced by MSC. Interestingly, BM-MSC also increased IL-4 and TGF-β1, while reduced IL-10 mRNA levels, for $CD4^+$ T cells; and enhanced IL-4, while diminished IL-10 and TGF-β1 mRNA, for $CD8^+$ T cells. Analyzing the functional compartments, we found that, for $CD4^+$ and $CD8^+$ T cells producing TNF-α, EM and effector compartments were the most resistant to MSC suppressive effect; the degree of inhibition of $CD4^+$ T cells producing IL-2 and IFNγ was similar among all the compartments; and EM and effector CD8+ T cells displayed the lowest degree of inhibition for IL-2, and the highest for IFNγ.

Conclusions: MSC derived from either BM, AT, or UCM were able to inhibit T cell and $CD56^{dim}$ NK cell activation, and T and B cell proliferation; however, at different extents. In turn, UCM-MSC were unable to inhibit B cell and $CD56^{bright}$ NK cell activation, conversely to BM and AT-MSC. These important differences detected should be taken into account when choosing the MSC source for research or therapeutic purposes. We also found that BM-MSC didn't impair the expression of maturation markers in monocytes and mDC under our experimental

conditions, nevertheless, they hampered the pro-inflammatory function of monocytes and mDC, which may impede the development of inflammatory immune responses. Finally, we reported that the functional compartments of $CD4^+$ and $CD8^+$ T cells were differentially regulate by BM-MSC, which may impact the therapeutic effect of MSC in immune disorders with a distinct distribution of T cells among activation/differentiation compartments. Also, the influence of MSC on IL-9 can extend the research field of MSC in allergic inflammation.

RESUMO

Introdução: Os benefícios clínicos da utilização terapêutica das células estaminais mesenquimais (MSC) em doenças do foro imunológico devem-se à sua capacidade de modular o sistema imune. Contudo, algumas questões essenciais relativas às suas propriedades imunossupressoras, como as diferenças entre MSC isoladas de diferentes tecidos ou a sua influência sobre a função de subpopulações específicas de células do sistema imune (nomeadamente as subpopulações de monócitos e os compartimentos funcionais das células T), não se encontram totalmente esclarecidas.

Objectivo: Neste estudo, comparámos o efeito supressor de MSC humanas isoladas da medula óssea (BM), tecido adiposo (AT) e matriz do cordão umbilical (UCM), na activação e proliferação de linfócitos e na expressão de mRNA de genes com um papel relevante na função das células T e NK; investigámos a influência das BM-MSC na activação/maturação de monócitos e células dendríticas mielóides (mDC), e na expressão de citocinas em mDC, subpopulações de monócitos, e compartimentos funcionais das células T $CD4^+$ e T $CD8^+$.

Material e Métodos: Cultivaram-se células mononucleares do sangue periférico (PBMC), na presença ou ausência de MSC, bem como de agentes estimuladores. Para comparar MSC isoladas da BM, AT e UCM, as MSC foram cultivadas com PBMC estimuladas com PHA; a activação e proliferação das células T, B e NK foram avaliadas por citometria de fluxo; a quantificação de mRNA em células purificadas dos diferentes compartimentos de activação das células T e NK foi feita por RT-PCR. Para avaliar o efeito das BM-MSC em mDC, monócitos clássicos, intermédios e não-clássicos, as BM-MSC foram cultivadas com PBMC estimuladas com LPS+IFNγ. A activação celular e a produção de citocinas foram avaliadas por citometria de fluxo; a expressão de mRNA de citocinas foi quantificada por RT-PCR em subpopulações de monócitos e mDC purificadas. As BM-MSC foram também cultivadas com PBMC estimuladas com PMA+ionomicina para se avaliar o seu efeito nos compartimentos funcionais de células T $CD4^+$ e T $CD8^+$: naive, memória central (CM), memória efectora (EM) e efector. A expressão de citocinas dentro de cada compartimento funcional e a percentagem de células T $CD4^+$ e T $CD8^+$ a expressar IL-17, IL-9 e IL-6 foram avaliadas por citometria de fluxo; a expressão de mRNA de citocinas foi quantificada por RT-PCT em células T $CD4^+$ e T $CD8^+$ purificadas.

Resultados: As MSC com origem na BM, AT e UCM inibiram a proliferação das células T $CD4^+$, T $CD8^+$, bem como a activação das células T $CD4^+$ e T $CD8^+$, e das células NK $CD56^{dim}$; as BM-MSC e AT-MSC inibiram igualmente a proliferação das células B e activação das células B e NK

$CD56^{bright}$, enquanto que as UCM-MSC não tiveram qualquer efeito nestas populações celulares. A acção supressora das AT-MSC foi a mais elevada. Além disso, as MSC diminuíram a expressão de mRNA de TNF-α e perforina em células NK activadas. Nos diferentes compartimentos de activação das células T, observou-se um aumento dos níveis de mRNA de FoxP3 e T-bet após estimulação com PHA e na presença de MSC.

As BM-MSC regulam a expressão de TNF-α e CCL4 em monócitos e mDC, sem inibirem a expressão de CCR7 e CD83. Esta inibição é mais acentuada nas mDC, e os monócitos não-clássicos são os mais resistentes à regulação da produção de TNF-α. As MSC também diminuem a expressão de mRNA de IL-1β e IL-6 em monócitos clássicos, CCL3, CCL5, CXCL9, e CXCL10 em monócitos clássicos e não-clássicos, e de IL-1β e CXCL10 em mDC.

De modo semelhante, as BM-MSC diminuem a percentagem de células T $CD4^+$ e T $CD8^+$ produtoras de TNF-α, IFNγ e IL-2, bem como a expressão de TNF-α e IFNγ, em todos os compartimentos funcionais, excepto nas células T naive $CD4^+IFN\gamma^+$, nas quais as MSC não têm qualquer efeito. A inibição da produção de TNF-α e IL-2 mediada pelas MSC, foi maior para as células T $CD4^+$, enquanto que a inibição de IFNγ foi mais acentuada nas células T $CD8^+$. De forma idêntica, observou-se que as MSC conduziam a uma redução da percentagem de células T $CD4^+$ e T $CD8^+$ a expressar IL-17, IL-17 e TNF-α, e IL-9, e de células T $CD4^+IL\text{-}6^+$. Relativamente à expressão de mRNA, verificou-se um aumento de IL-4 e TGF-β1, juntamente com uma diminuição de IL-10, em células T $CD4^+$; e o aumento de IL-4, com a diminuição de IL-10 e TGF-β1, em células T $CD8^+$, na presença de MSC. Na análise dos compartimentos funcionais, observou-se que, para as células T $CD4^+$ e T $CD8^+$ produtoras de TNF-α, os compartimentos EM e efector eram os mais resistentes à acção supressiva das MSC; o grau de inibição das células T $CD4^+$ produtoras de IL-2 e IFNγ era similar para todos os compartimentos; enquanto que os compartimentos EM e efector das células T $CD8^+$ apresentavam o menor grau de inibição para IL-2 e o mais elevado para IFNγ.

Conclusões: As MSC isoladas da BM, AT e UCM são capazes de inibir a activação das células T e NK $CD56^{dim}$, assim como a proliferação de células T; mas em diferentes graus. Por outro lado, as UCM-MSC não inibem a activação das células B e NK $CD56^{bright}$, nem a proliferação de células B, ao contrário das BM e AT-MSC. Estas diferenças devem ser tidas em conta aquando da escolha do tipo de MSC para trabalhos de investigação ou utilização clínica. Também se verificou que as BM-MSC não impediam a expressão de marcadores de maturação em monócitos e mDC sob as nossas condições experimentais; no entanto, elas inibiram a função pró-inflamatória dos monócitos e mDC, o que pode impedir o desenvolvimento de uma

resposta imune. Por fim, verificou-se que as BM-MSC regulam de forma distinta os compartimentos funcionais das células T $CD4^+$ e T $CD8^+$, o que poderá ter impacto no efeito terapêutico das MSC em patologias do foro imunológico em que as células T apresentem alterações na sua distribuição pelos compartimentos de activação/diferenciação. É ainda de salientar que, com base na influência das MSC sobre a IL-9 aqui descrita, se poderá ampliar o campo de investigação das MSC na inflamação alérgica.

KEYWORDS/ PALAVRAS-CHAVE

Keywords:

Mesenchymal stromal cells; mesenchymal stem cells; immunosuppression; T cells; B cells; NK cells; monocytes; myeloid dendritic cells; activation compartments; functional compartments; cytokine; chemokine.

Palavras-chave:

Células mesenquimais do estroma; células estaminais mesenquimais; imunossupressão; células T; células B; células NK; monócitos; células dendríticas mielóides; compartimentos de activação; compartimentos funcionais; citocina; quimiocina.

CHAPTER 1 | *INTRODUCTION*

After the identification of a plastic-adherent bone marrow (BM) stromal cell population in 1976 by Friedenstein and colleagues, and the first evidence of its multilineage potential [1], with subsequent confirmation of their stem cell nature, an increasing interest on these BM-derived mesenchymal stem/stromal cells (MSC) has emerged, mainly because of their promising therapeutic applications. By definition, a stem cell is an undifferentiated cell with the potential ability of self-renewal and the capability of differentiation along different cell lineages (multipotency). However, data published so far demonstrate that only a subset of BM-derived mesenchymal stromal cells are, in fact, multipotent [2,3]. At the present moment, there is no marker available to distinguish the multipotent cells from the more committed cell progenitors comprised in the BM-MSC population [2,3].

Human MSC can be isolated from adult tissues – such as BM, adipose tissue (AT), dental pulp, synovial membrane, lung, skin, menstrual blood, and peripheral blood, among others – and fetal tissues – including extra-embryonic structures of fetal origin, such as umbilical cord blood (UCB), umbilical cord matrix (UCM) or Wharton's Jelly, amniotic fluid, amnion, and placenta (this latter comprises MSC from both fetal and maternal origin) [2,3]. The use of extra-embryonic fetal MSC not only circumvent ethical issues concerning the use of fetal MSC, because these structures are discarded after partum, but also avoid invasive procedures necessary to obtain these cells from adult tissues. Despite the common features shared by MSC from different tissues, it is now widely accepted they display different characteristics which are ultimately reflected in their function, namely in their immune suppressive abilities. And, beyond the variability ascribed to MSC origin, it is also recognized that the culture conditions during cell expansion can influence MSC characteristics and functional behavior, which is not surprising, given their high sensibility to the microenvironmental conditions which, in fact, can be one feature that definitely contributes to their success, either in their natural role in maintaining homeostasis or when used as therapy. Notwithstanding, it is also observed a significant inter-donor variability concerning the immune suppressive ability of MSC. All these factors cause difficulties in the comparison of the results yielded by different studies, and raise important issues concerning the optimal protocol to isolate and expand MSC for clinical use.

MSC display several features that make them attractive for cell-based therapies: they can be found in numerous adult tissues, they are relatively easy to isolate, and have the capability to expand manyfold in culture without lose their stem cell properties. Moreover, when systemically transplanted, MSC have the ability to migrate to sites of injury and promote tissue repair, by producing growth factors or other soluble factors important to tissue

regeneration, as well as by undergoing cellular differentiation to replace the damaged cells [2-4]; such features explain the success of MSC transfusion therapy in genetic disorders affecting mesenchymal tissues [2,3]. Furthermore, MSC can suppress or modulate the immune response of a wide variety of immune cells, including T cells, B cells and plasma cells, naturat killer (NK) cells, NKT cells, dendritic cells (DC), monocytes and macrophages, neutrophils, eosinophils, and mast cells [2-8] (focused in chapter 1, section 3); and their benefic effect in patients' clinical outcome has already been proven in severe acute graft-*versus*-host disease (GvHD) [2].

The success obtained in refractory acute GvHD treatment, together with the promising results in animal models of distinct diseases, propelled clinical trials in a broad spectrum of disorders with distinct etiology and pathophysiology, such as osteogenesis imperfecta, spinal cord injury, multiple sclerosis, amyotrophic lateral sclerosis, myocardial infarction, cirrhosis, systemic lupus erythematosus, nonhealing wounds, among others [2-4] (detailed in chapter 1, section 2). But, when the therapeutic use of MSC is concerned, is necessary to have into accout all the variables that may influence MSC efficacy. Besides the variables inherent to the technical preparation of MSC (election of the tissue origin, isolation, expantion, and storage conditions), which are known to influence MSC gene expression, phenotype, and function (detailed in chapter 1, section 1.4), the clinical use of MSC introduce new variables which influence the final clinical outcome: cell dose and number of infusions, route of infusion, and optimal timing of administration.

1. Characterization of Mesenchymal Stromal/Stem Cells and Factors that Influence their Phenotype and Function

1.1. Characterization of mesenchymal stromal/stem cells

1.1.1. Phenotypic characteristics of mesenchymal stromal/stem cells

The lack of a specific cell marker for the identification and purification of MSC give rise to a multitude of different protocols and different strategies for MSC isolation, complicating the integration of results from different studies and the understanding of MSC biology. In an attempt of standardization, in 2006, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) established the minimal criteria to define human MSC [2], as follows: 1) MSC must be plastic-adherent when maintained in standard culture conditions; 2) MSC must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19, and human leukocyte antigen (HLA)-DR surface molecules; 3) MSC must have the ability to differentiate into osteoblasts, adipocytes, and chondroblasts *in vitro*.

MSC are a rare cell population in the human BM, being reported to represent approximately 0.01% of all nucleated BM cells [3,4], although it is assumed that their number declines with aging [3]. Our data point to a percentage ranging between 0.01% and 0.03% of all nucleated BM cells [4].

In a previous work, our group evaluated a panel of nineteen markers on human BM-derived MSC (BM-MSC) from freshly collected BM samples, aiming a better characterization of this cell population and to explore whether there were phenotypically distinct subpopulations within BM-MSC [4]. As a minor BM cell population easy to expand *in vitro*, it is attractive to characterize MSC immunophenotype after cell expansion. Nevertheless, characterizing BM-MSC directly in the freshly collected BM samples (without previous culture) enables an analysis closest to their physiological conditions, excluding the phenotypic alterations induced by the factors present in the culture medium and the number of passages. In addition, this direct approach allows an accurate quantification of MSC in BM. Also, this same strategy can be applied to MSC from other tissues.

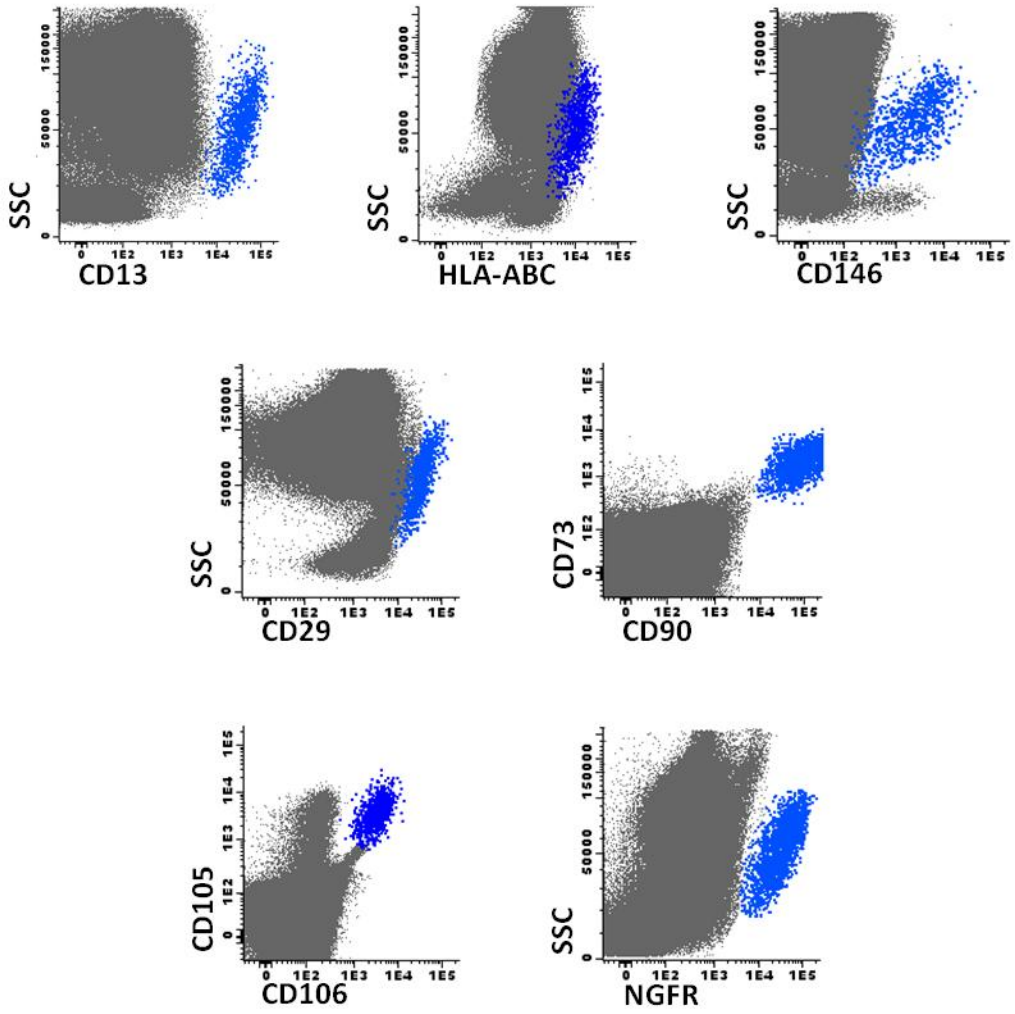

Figure 1. Immunophenotypic characteristics of MSC from freshly collected BM samples. BM-MSC are represented as blue events, the remaining BM nucleated cells correspond to the grey events.

Our study revealed that BM-MSC from freshly collected BM were uniformly positive to CD13, CD29, CD49e, CD90, CD106, CD146, CD73, CD271, CD105, and HLA-ABC (Figure 1); and negative to CD24, CD31, CD11b, CD14, CD15, CD34, CD45, CD133, and HLA-DR. Based on the expression profile of these markers, BM-MSC behave as one sole cell population, as all the studied markers were homogeneously expressed [4]. A former study from our group, carried out on freshly isolated human UCB samples, demonstrated that UCB-MSC express CD44, CD71, CD105, CD184, and CD271 [5].

In the literature it is described that, after cell culture expansion, human MSC derived from the BM, AT, UCM, UCB, and amniotic membrane stromal cells are positive for CD13, CD29, CD44, CD73, CD90, CD105, CD166, and HLA-ABC, and negative for CD14, CD19, CD24, CD34, CD36, CD38, CD45, CD49d, CD117, CD133, and HLA-DR [5-15]. Importantly, there are a

significant number of proteins that are not uniformly expressed by MSC: some are not express by all the cells comprised in the MSC population, while others display an heterogeneous expression, which translates a different amount of protein *per* cell within the MSC population. *In vitro* expanded human BM-MSC were shown to have heterogeneous expression of CD10, CD31, CD56, CD71, CD80, CD106, CD119, CD130, CD140a, CD146, CD173, CD271, CD273, CD274, Galectin 1, neural ganglioside 2 (GD2), mesenchymal stem cell antigen-1 (MSCA-1), and stage specific embryonic antigen-4 (SSEA-4); however, there are contradictory data published concerning the phenotypic characteristics of human MSC [5]. But, despite the inconsistencies regarding the phenotypic characterization of human MSC, there is an agreement that they do not constitute a homogeneous cell population but, instead, comprise phenotypically distinct subpopulations. Remarkably, a recent study analyzing single cell-derived clones from human AT-MSC proved they present different immunomodulatory properties over T cells and NK cells [6]. The expression of molecules with a role in MSC function, namely CD44, CD105, CD73, interleukin (IL)-1β, IL-4, and IL-5, was also shown to vary among the different clones analyzed [6].

Noteworthy, some phenotypical differences among MSC from the same tissue origin have been associated to a differential immunosuppressive ability. Accordingly, MSC expressing stromal-derived factor-1 (STRO-1), CD90, CD271, or are $CD39^{+}CD73^{+}$, display a higher immunosuppressive potential [6,7]. In the same line, other phenotypic differences are correlated to other MSC features: human BM-MSC preparations with higher expression, or higher percentage of cells expressing CD10, CD29, CD44, CD71, CD106, CD119, CD146, CD166, CD271, and HLA-ABC, possess a higher clonogenic potential [5]. Remarkably, it was reported that the subpopulation of human BM-MSC with trilineage potency present higher expression of CD146, have higher clonogenic potential and proliferate faster, compared their bipotent and unipotent counterparts [6]; nevertheless, it was also described that the proliferation capacity of human BM-MSC correlates negatively with the percentage of $CD146^{+}$ cells among the MSC population [5].

A special attention has been given to MSC expression of chemokines, chemokine receptors and adhesion molecules. These molecules are involved in MSC migratory and homing behavior, and enable the proximity between MSC and the target cells. Thus, the ability of MSC to actively migrate and integrate affected tissues or organs, and the capability to maintain a close interaction with target cells (either immune cells or others) are determinant for the successful use of MSC in cell-based therapies.

1.1.2. Chemokine receptors and chemokines expression by mesenchymal stromal/stem cells

The important role of chemokines and adhesion molecules expressed by MSC in their immunosuppressive action was clearly demonstrated in the studies carried out by Ren and colleagues [7,8] in mouse BM-MSC. These studies showed that the adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) [7], and the chemokines CXCL9 (or monokine induced by gamma, MIG) and CXCL10 (or interferon inducible protein 10, IP-10) [7], were upregulated in MSC after exposure to activated splenocytes or pro-inflammatory cytokines (interferon (IFN)γ plus tumor necrosis factor (TNF)-α, IL-1α, or IL-1β). The chemokines mediate MSC-driven chemoattraction of T cells, and the blockage of their receptor (CXCR3) abolished the immunosuppressive effect of MSC over T cell proliferation [7]. Similarly, blocking ICAM-1 or VCAM-1 also reversed the suppression of T cell proliferation [7].

Concerning the migratory ability of human BM-MSC, it is reported they express several chemokine receptors: CCR1, CCR2, CCR3, CCR4, CCR6, CCR7, CCR9, CCR10, CXCR4, CXCR5, CXCR6, and CX3CR1 (though contradictory data have been published). The chemotactic response of MSC towards chemokines and growth factors was demonstrated both *in vitro* and *in vivo* [7-10]. Additionally, human BM-MSC produce chemokines which can attract immune cells towards MSC, namely CCL2, CCL4, CCL5, CCL20, CXCL8, CXCL12, CXCL9, CXCL10, CXCL11, and CX3CL1 [7,8]. Of note, CXCL9, CXCL10, and CXCL11, are chemoattractant factors for T cells, being demonstrated that the neutralization of CXCR3 (a receptor for CXCL9, CXCL10, and CXCL11) reverts T cell immunosuppression mediated by MSC [7]. Human AT-MSC were shown to express CXCL12, CCL2, CCL5, and CCL20 [8]. Though the soluble factors released by these cells have been shown to be chemoattractant for B cells, CXCL12 and CCL2 do not seem to be essential for B cell migration towards MSC [8].

1.1.3. Adhesion molecules expression by mesenchymal stromal/stem cells

Besides possess the mechanisms necessary to migrate towards (and to induce the migration of) immune cells, MSC also have the means to interact in close proximity with the target cells by the surface expression of adhesion molecules. Adhesion molecules also play a determinant role in the exit of cells from the peripheral circulation and tissue homing, by allowing the interaction with endothelial cells and extracellular matrix.

Human MSC express CD54 (ICAM-1), CD102 (ICAM-2), CD106 (VCAM-1), CD58 (lymphocyte function-associated antigen 3, LFA-3), CD72, CD90 (Thy-1), CD146 (Muc18), and a multitude of integrins – α1-6 integrins (CD49a-f), αv integrin (CD51), β1 (CD29), β3, β4, and β7 integrins – which allow their stable binding to immune cells [4,7-9]. CD54 (ICAM-1) is dimly expressed and present only in a small percentage (≈ 10%) of human BM-MSC, but strongly enhanced or induced after TNF-α and IFNγ treatment [9]; it is the ligand of CD11a/CD18 (LFA-1), being demonstrated that CD54/activated LFA-1 interaction allows a stable binding between human BM-MSC and Th17 cells [9]. The blockage of ICAM-1 or VCAM-1 hampers MSC-mediated suppression of T cells, though the importance of these molecules seem to be greater for mouse than human MSC [7,10]. In the same line, the blockage of VCAM-1 and N-cadherin reverses the induction of tolerogenic DC by human BM-MSC, rendering the DC able to induce antigen-specific T cell proliferation [11]. CD29 (integrin β1-subunit) and CD106 (VCAM-1) are important for the efficient adhesion of MSC to endothelial cells [12-15]; and CD29, which when dimerized with CD49e (integrin α5-subunit) forms a receptor that binds to fibronectin and invasin, is likely to promote MSC-extracellular matrix interaction [16]. CD146 (Muc18) plays an important role in cell-cell and cell-extracellular matrix adhesion and an increased expression of these marker on tumor cells is associated with an increased cell motility and invasiveness/metastasis capability [17,18]. The glycoprotein CD90 (Thy-1) regulates as well cell-cell and cell-extracellular matrix interactions, being reported its involvement in adhesion to endothelial cells, migration, metastasis and tissue regeneration [19,20].

1.1.4. Other molecules expressed by mesenchymal stromal/stem cells

CD271 (nerve growth factor receptor, NGFR) is a growth factor receptor expressed in a wide variety of tissues and, depending on the cell type, signaling through this receptor regulates nuclear factor kappa B (NF-kB) activation, apoptosis, tissue regeneration, immune cell activation, proliferation, and cell differentiation [21,22]. CD105 (endoglin) is one of the receptors for transforming growth factor (TGF)-β, a growth factor involved in the regulation of development, maintenance, and proliferation of MSC [14], and also known to play an important role in tissue repair and immunosuppression. A broad set of immunosuppressive molecules is also constitutively and inducibly expressed by MSC; those are thoroughly described in chapter 1, section 4.

1.2. Differences among mesenchymal stromal/stem cells arising from different tissues

Despite sharing common phenotypic features and important functional characteristics, MSC arising from different tissues have differences. This is unequivocally demonstrated by studies that compared MSC isolated from different tissues of the same donor, specifically UCM-MSC *versus* UCB-MSC [23], and dental pulp *versus* periodontal ligament-derived MSC [24]. Some important differences between human fetal and adult tissue-derived MSC, and among human MSC isolated from different adult tissues, are described below.

1.2.1. Proliferation potential

Several studies demonstrated that, in human, UCM-MSC, followed by AT-MSC, had faster proliferation and greater expansion capabilities than BM-MSC; UCM-MSC also display longer survival than AT-MSC and BM-MSC [7,25-28]. Besides, as the passage number increases, UCM-MSC are able to maintain the rate of proliferation, while the proliferation rate of BM-MSC diminished [25]. Likewise, UCB-MSC possess higher proliferation capacity than AT-MSC and BM-MSC [29]. Similarly, fetal liver-derived MSC display faster growth, greater expansion ability, and longer telomeres than BM-MSC [30]. In turn, BM-MSC show signals of senescence in a lower passage number, compared to AT-MSC [28].

1.2.2. Phenotype

It was reported that, despite sharing a similar phenotype – expression of CD90, CD73, CD44, CD166, and HLA-ABC, and negative expression for CD34, CD31, CD45, and HLA-DR – human BM-MSC, AT-MSC, and UCM-MSC possess distinct phenotypic characteristics [31,32]. For instance, AT-MSC express lower levels of major histocompatibility complex (MHC)-I than BM-MSC [33], and higher levels of CD90, followed by BM-MSC and UCB-MSC, which display the lowest expression [29]; though contradictory results have been published for CD90 [27]. CD40 and CD34 is consistently negative in BM-MSC and UCM-MSC, whereas AT-MSC display a constitutive moderate expression of CD40, and the percentage of AT-MSC positive for CD34 increases with the number of passages [32]. Though CD105 is expressed by MSC arising from the BM, AT, and UCM, its expression is higher among BM-MSC [32], and the percentage of $CD105^+$ cells is lower for UCB-MSC than for AT-MSC and BM-MSC [27,29]. In the same line, the analysis of MSC from freshly collected BM and UCB samples reveal similar expression of CD71 and CD184 in both MSC types, whereas BM-MSC displayed a higher expression of CD105 and lower expression of CD44, compared to UCB-MSC [34]. The adhesion molecule CD54 is

constitutively expressed by UCM-MSC and AT-MSC, but not by BM-MSC; whereas CD146 is predominantly express by BM-MSC and at lower levels by UCM-MSC and AT-MSC [27,32,35]. AT-MSC express CD49d, in contrast to BM-MSC [28]; while the highest levels of CD106 expression are found in UCB-MSC, followed by BM-MSC and, finally, AT-MSC [28,29].

Human MSC can also produce several cytokines. A detailed study on the expression of cytokines, chemokines, and growth factors by human MSC from different origins showed that AT-MSC produce higher amounts of Th1 and Th2 cytokines than BM-MSC [36].

Considering molecules directly involved in MSC immunosuppressive functions, it was reported that UCM-MSC display the highest constitutive levels of CD274, hepatocyte growth factor (HGF), and prostaglandin (PG)E2, compared to AT-MSC and BM-MSC [26,32,37]; and UCM-MSC and AT-MSC were shown to produced higher levels of leukemia inhibitory factor (LIF), compared to BM-MSC [37,38]. Nevertheless, there is controversy concerning the type of MSC which express higher levels of PGE2, being reported as well that AT-MSC produce the highest levels of PGE2, followed by BM-MSC and, finally, UCM-MSC (which secrete the lowest levels) [26,39]. Of note, in comparison to UCB-MSC , BM-MSC secreted lower levels of PGE2 [40]. In turn, indoleamine 2,3-dioxygenase (IDO) activity is higher in human BM-MSC than UCM-MSC [39], and the protein expression of CD273 and TGF-β2 is higher among UCM-MSC, compared to BM-MSC [37]. Moreover, some authors reported that human UCM-MSC express higher levels of CD200 than BM-MSC [37,41], whereas other study showed that CD200 is not expressed by UCM-MSC, only by BM-MSC [42], making evident the influence of different experimental approaches in MSC phenotype and function.

1.2.3. Immunosuppressive ability

The results from studies concerning the differential immunosuppressive function of human MSC derived from different sources not always overlap. This is not surprising because, as MSC are highly sensitive to microenvironment, technical differences (MSC expansion conditions, ratio MSC:immune cells, types of cell present in MSC and immune cell co-culture, type of stimulus used, among others) are very likely to change the final outcome.

By comparing fetal and adult human MSC, it was demonstrated that BM-MSC lost their immunosuppressive action over T cell proliferation after 6-8 passages, whereas fetal liver-derived MSC maintained their immunoregulatory functions for at least 25 passages. This was associated to the decreased expression of HLA-G verified in BM-MSC after passage 8, which did not occur in fetal liver-derived MSC, at least before passage 30 [30]. Also, human embryonic

stem cells-derived mesenchymal progenitors were shown to be more resistant to the cytotoxic effects of activated NK cells, to have an increased inhibitory ability over NK cells, and to express a higher amount of HLA-G, compared to BM-MSC [43]. In turn, placenta-derived MSC were described as less effective in inhibiting T cell proliferation and expanding regulatory T (Treg) cell population than UCB-MSC and BM-MSC [44], but there are contradictory data concerning this subject [45].

Attending to the studies which aimed to compare human MSC arising from UCM, AT, and BM, there are contradictory results as well. Recently, our group described that, when co-cultured with peripheral blood mononuclear cells (PBMC) stimulated with phytohemagglutinin (PHA), UCM-MSC inhibited the activation and proliferation of $CD4^+$ and $CD8^+$ T cells, B cells, and $CD56^{dim}$ and $CD56^{bright}$ NK cells (only activativation was assessed for NK cells) to a lesser extent than AT-MSC and BM-MSC, and were unable to suppress B cell and $CD56^{bright}$ NK cell activation, and B cell proliferation, conversely to AT-MSC and BM-MSC [46]. In contrast, it was reported by others that UCM-MSC display a stronger inhibitory effect than BM-MSC over T cell proliferation induced by PBMC stimulation with PHA [39], mixed lymphocyte reaction (MLR) [39], and CD3/CD28 plus IL-2 [37]; being also described there were no significant differences among the suppressive effect of UCM-MSC and BM-MSC over T cell proliferation induced by MLR or PHA plus IL-2 stimulation of purified T cells, but UCM-MSC inhibit T cell activation to a greater extent than their BM counterparts [26]. Conversely, in PHA-stimulated PBMC, UCM-MSC have lowest ability to inhibit IFNγ secretion and CD28 expression, while induce higher levels of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) on T cells, compared to BM-MSC [39].

Notwithstanding, the studies comparing AT-MSC and BM-MSC are more consistent, indicating that AT-MSC display a higher immunosuppressive capacity. Indeed, AT-MSC exhibit a stronger inhibitory effect over $CD4^+$ and $CD8^+$ T cell proliferation and activation, in response to different stimuli, compared to BM-MSC [26,46]. Similarly, AT-MSC were more effective in suppressing B cell activation and proliferation, as well as $CD56^{dim}$ and $CD56^{bright}$ NK cell activation, than BM-MSC [46]. Remarkably, AT-MSC are also stronger inhibitors than BM-MSC of monocyte differentiation into DC and induce a higher secretion of IL-10 by these cells [47]. Besides, AT-MSC were more resistant to both NK cell lytic activity [33] and MHC-I-specific lysis by CD8+ T cells [48], compared to BM-MSC. The lower susceptibility to NK cell cytotoxicity may be associated to the lower expression of CD112 and CD155 (ligands for DNAX accessory molecule-1, DNAM-1) observed in AT-MSC [33].

The differences among the immunsuppressive activity of human MSC derived from BM, AT, and UCM over lymphocytes' proliferation and activation are discussed in more detail in chapter 5, section 1.

1.2.4. Other functional differences

Other functional differences are found among human MSC derived from different tissues. For instance, UCM-MSC are more skilled for neuroprotection and angiogenesis than BM-MSC [49], and UCM-MSC and AT-MSC have a inferior osteogenic differentiation potential than BM-MSC [35,50], whereas UCB-MSC exhibit a low adipogenic differentiation potential, conversely to AT-MSC and BM-MSC [29,51]. Notably, UCM-MSC display lower immunogenicity, compared to BM-MSC [37].

The differences among MSC from different sources impact their function. But, as described below, the expression pattern of factors that influence MSC immunosuppressive potential (chemokine, adhesion molecules, and immunosuppressive molecules) is not static. The *in vitro* experiments that subject MSC to pro-inflammatory cytokines, TLR ligands, etc, can give important clues about how MSC function will be conditioned after *in vivo* infusion, under non-homeostatic conditions, such as that experienced when tissue damage or infection occur.

1.3. DONOR-DEPENDENT VARIABILITY OF THE IMMUNOSUPPRESSIVE POTENTIAL OF MESENCHYMAL STROMAL/STEM CELLS

In recent years, an increasing body of evidence point to important differences among MSC isolated from different donors. Part of this donor-to-donor phenotypic variability among human BM-MSC was demonstrated by Siegel and colleagues [5] to be associated to donor age and gender. Importantly, the inter-individual heterogeneity underlines the utility of MSC potency tests prior to their clinical application.

When the donor's age is concerned, it was found that younger donors possess a higher proportion of $CD71^+$, $CD146^+$, and $CD274^+$ MSC, and a higher expression of CD71, CD90, CD106, CD140b, CD146, CD166, and CD274, than the older ones; in addition, MSC from younger donors express lower levels of IL-6 after exposure to activated T cells [5]. Differences concerning CD44, CD73, and CD105 expression were also observed among donors [52]. It is worth mentioning that some of these differentially expressed proteins are adhesion molecules (CD44, CD90, CD106, CD166) or immunosuppressive molecules (CD73 and CD274), thus, their

differential expression may imply important differences in the immunosuppressive potential of MSC arising from different donors.

Remarkably, concerning the immunosuppressive potential, the inter-individual heterogeneity is striking. A significant variability in the amounts of PGE2 secreted from human BM-MSC arising from different donors is described, being demonstrated a positive correlation between the levels of PGE2 produced and the degree of inhibition of DC differentiation by MSC [53]. In line with these findings, it was shown that human BM-MSC isolated from different donors (in co-culture with PBMC stimulated with anti-CD3/CD28) display distinct immunosuppressive potential over T cell proliferation and IFNγ production; interestingly, the immunosuppressive potential was found to be positively correlated with IDO mRNA [5,52] and protein [52] expression levels. Interestingly, the immunosuppressive ability over PHA-stimulated T cell proliferation is described to be stronger for BM-MSC isolated from female, compared to male donors [5]; gender differences in MSC immunosuppressive potential had formerly been describe in mice [54]. Furthermore, there was also observed donor-to-donor variability regarding the degree of inhibition of immunoglobulin (Ig)M, IgG and IgA production by B cells in MLR [55].

The inter-individual variability is also visible in other aspects of human MSC biology, such as the ability of BM-MSC to support neural growth *in vivo*, which is associated to the different amount of cytokines and growth factors that BM-MSC isolated from different donors produced [56]; and the potential for the formation of fibroblast colony forming units (CFU-F), and absolute progenitor numbers generated, which varied among menstrual fluid stem cells' donors [57]. Of note, in human BM-MSC, a higher number of CFU-F is found among women than men, and younger donors form more CFU-F than older donors [5]. The percentage of $CD119^+$ and $CD130^+$ MSC is higher among female donors, which also have MSC with a smaller diameter a lower population doubling time, compared to male donors [5]. Interestingly, the proliferation capacity of BM-MSC (and the cell size) were not correlated to donor age [5].

1.4. Microenvironment modulates mesenchymal stromal/stem cell action

MSC are highly sensitive to the microenvironment, which allow them to rapidly alter their protein expression profile in order to adapt their functions to the physiologic needs of the organism, giving their important contribute to the maintenance of the homeostasis. Thus,

expectedly, the culture conditions during MSC expansion or *in vitro* assays can change the cells' phenotype and function. Factors recognized to interfere with MSC phenotype and function include the ratio MSC:immune cells [26,58-60], the immune cells present in the cell culture [61], the activation status of immune cells [58,62], and the cytokines levels in the milieu. It is also generally recognized that hypoxia induce a higher proliferation rate, and upregulation of growth factors and anti-inflammatory molecules in MSC; pro-inflammatory conditions promote the production of immune modulating and anti-inflammatory factors; and three dimensional growth increases the production of anti-cancer factors and anti-inflammatory molecules by MSC, compared to monolayer culture [63-65].

Notwithstanding, other factors inherent to the MSC expansion procedures may exert an important influence over MSC phenotype and function [66]: the presence of platelet lysate from platelet-rich plasma in the culture medium significantly increased the intracellular levels of HLA-G in human BM-MSC [67], the number of passages changes MSC phenotype [68], wherein a high number of passages results in the loss of the surface expression of chemokine receptors and adhesion molecules [69], and the level of cell confluence affect MSC gene expression [70].

1.4.1. Cytokines

TNF-α treatment increases the proliferation rate of human BM-MSC [39]. Remarkably, a significant number of chemokines and chemokine receptors are upregulated after human BM-MSC exposure to TNF-α, namely CXCL8 (IL-8), CXCL9 (MIG), CXCL10 (IP-10), CCL3 (or macrophage inflammatory protein (MIP)-1α), and CXCR4, CCR2, CCR3, and CCR4, among many others [71,72]. As expected, TNF-α increases human BM-MSC migration toward CCL5 [71]. In turn, it is also observed an increased secretion of PGE2 in BM-MSC and UCM-MSC [39], and upregulation of the growth factors and immunosuppressive molecules vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF2), HGF, insulin-like growth factor (IGF)-1, and galectin-9 (mRNA levels for this latter) in BM-MSC, under the influence of TNF-α [73,74].

Likewise, IFNγ also promotes the expression of immune suppressive molecules by human MSC, namely IDO [7,39,75-77], programmed death ligand 1 (PD-L1 or B7-H1) [7,78], PD-L2 (B7DC) [7,78], TGF-β [76], CD73 [79], and galectin-9 (at mRNA level) [7,74],while reduce PGE2 expression [76]. Similar results are described for murine MSC [80]. Accordingly, IFNγ-stimulated human MSC were shown to be more resistant to NK cell lysis than non-stimulated MSC [76]. The chemokine receptor CXCR4 is also upregulated in human BM-MSC upon exposure to IFNγ [79]. Notwithstanding, low levels of IFNγ induce antigen-presenting cell

properties in MSC, upregulating MHC-I (including HLA-E) and MHC-II expression, and making MSC acquire allogeneic potential. Nevertheless, higher IFNγ levels drives to MHC-II downregulation and loss of T cell allostimulation potential [7,39,81-83].

CD54 (or ICAM-1) expression is enhanced or induced after human BM-MSC and UCM-MSC treatment with TNF-α and IFNγ, either alone or in combination, showing a synergistic effect [9,39]. In the same line, the increase of adhesion molecules had been reported in mouse MSC under the influence of pro-inflammatory cytokines, which contribute to their efficient interaction with immune cells [10]. TNF-α and IFNγ, acting individually, also increase HLA-ABC expression in both human BM-MSC and UCM-MSC [39]. Accordingly, exposure to either TNF-α or IFNγ was shown to increase human BM-MSC and UCM-MSC immunogenicity [39]. Of note, galectin-9 and TGF-β are also induced in human MSC by IL-1α and IL-1β [7,74,84].

In vivo, cells are exposed to various pro-inflammatory cytokines and not to each cytokine individually. Interestingly, the combination of different pro-inflammatory cytokines frequently results in a synergistic effect over the target cells, including MSC. Therefore, many authors opt to use a cocktail of pro-inflammatory cytokines, instead of individual cytokines and, though the insight of the role of each cytokine is lost, the assays become more close to the physiologic conditions. Using this approach, it was reported that a cocktail of pro-inflammatory cytokines (TNF-α, IFNγ, and IL-6) upregulates the mRNA expression of immune suppressive factors (HLA-G, HGF, and IDO); MHC-I and MHC-II; CD40; and several chemokines for lymphocytes, monocytes, DC, and granulocytes (CXCL9, CXCL10, CXCL11, CXCL1, CXCL6, CXCL8 or IL-8, CCL2, CCL5, CCL7, CCL8, and CCL13) in human AT-MSC [85]. These cells also enhanced their ability to suppress PHA-induced PBMC proliferation after exposure to pro-inflammatory cytokines [85]. Exposure to other combination of pro-inflammatory cytokines (TNF-α, IFNγ, IFNα, and IL-1β) also induced HLA-DR expression, along with CD40, in human BM, AT, and UCM-derived MSC [32]; and upregulates the expression of the adhesion molecules CD54 and CD58 [32,59], and the immunosuppressive molecules, HLA-G, CD274, and PGE2, in MSC from the three sources, as well as HGF secreted by UCM-MSC and galectin-1 secreted by BM-MSC [32,59].

Functionally, priming with pro-inflammatory cytokines is generally associated to the improvement of immunosuppressive capacity of MSC [39], which is in accordance with the observed upregulation of the molecular mechanisms that promote a close interaction between MSC and immune cells – namely, increased expression of chemokines and their receptors, and

adhesion molecules – and the augmented expression of immunosuppressive molecules that allow MSC to modulate immune cell function.

1.4.2. Toll-like receptors' stimulation

Conversely to what is found for MSC exposure to pro-inflammatory cytokines, which increase MSC ability to modulate the immune response, stimulation of Toll-like receptors (TLR) from human MSC may maintain or impair their immunosuppressive potential. It is hypothesized that the presence of TLR ligands is indicative of a dangerous situation, wherein the organism is exposed to potentially pathogenic agents; in these cases MSC assume a pro-inflammatory role and downregulate the immune suppressive machinery. In turn, depending on the ligand concentration, timing, kinetics of activation, and type of MSC [63,86], MSC may assume an immunosuppressive function as well. For instance, the presence of pro-inflammatory cytokines in the absence (or low level) of TLR stimulation may be perceived as a situation where the pathogenic agent has already been eliminated and, therefore, inflammation resolution should take place; or as a chronic inflammation, wherein immune cell activation and effector function most probably have a deleterious rather than beneficial effect for the organism.

The use of the TLR2 ligand zymosan resulted in the increased expression of galectin-9 mRNA in human BM-MSC [74]. In mice, BM-MSC stimulation through TLR2 impair their capacity to induce the generation of Treg cells [86].

Human BM-MSC stimulated with polyinosinic–polycytidylic acid (poly(I:C)), the TLR3 ligand, display increased expression of the chemokines CCL5 (or regulated upon activation, normal T cell expressed and secreted, RANTES) , CXCL10 (IP-10); the cytokines IL-4, IL-10, IL-6, IL-8, IL-12p35, IL-23p19, IL-27p28, and interleukin-1 receptor antagonist (IL-1Ra); adhesion molecules CD54 and CD58; immunosuppressive molecules IDO, galectin-9, and PGE2, while downregulate TGF-β, Jagged-1, and galectin-1 expression [59,74,86-91]. It is worth to mention that human BM-MSC stimulated through TLR3 maintain the suppressive activity over CD3/CD28-induced T cell proliferation [87], improve their inhibitory effect over T cell proliferation in MLR [88], and become more resistant to IL-2-activated NK cell killing [92]. Of note, TLR3 stimulation increases human BM-MSC ability to enhance neutrophil survival [93].

Human BM-MSC treatment with lipopolysaccharide (LPS), the TLR4 agonist, increases the expression of the cytokines IL-1β, IL-6, IL-8, IL-23p19, IL-27p28; CCL5 and CXCL4; the adhesion molecules CD54 and CD58; the immunosuppressive molecules and growth factors

PGE2, IDO, CD73, galectin-9 (measure at mRNA level), Jagged-1, VEGF, FGF2, HGF, and IGF-1 expression, while slightly reduce TGF-β and galectin-1 expression [31,59,73,74,79,86-92]. LPS exposure also increase the secretion of PGE2 and IL-8 by human AT-MSC [31]; and of IDO, cyclooxygenase (COX)-2, IL-1α, IL-1β, IL-6, and IL-8 in UCM-MSC, and of the two latter cytokines in UCB-MSC [94,95]. Remarkably, human BM-MSC stimulated through TLR4 lose their suppressive activity over CD3/CD28-induced T cell proliferation [87], whereas increase their inhibitory effect over T cell proliferation in MLR [88]. In turn, TLR4 activation of human UCB-MSC seem to have no effect on their suppressive ability [86,95]. Of note, TLR4 stimulation increases human BM-MSC ability to enhance neutrophil survival [93].

TLR5 activation with flagellin of human UCB-MSC results in the increase of IL-8 secretion and seems to have no effect on their suppressive ability [86,95].

The stimulation of human BM-MSC with a combination of IFNγ and LPS upregulated TNF-α, IL-1β, IL-6, IL-8, IDO, and inducible T-cell co-stimulator ligand (ICOSL) mRNA expression [79], and significantly increased neutrophil migration towards MSC, an effect mediated by IL-8 secretion by MSC [89]. This increased ability to induce immune cell migration was confirmed *in vivo* by infusing mouse MSC pre-treated with IFNγ and LPS; treated MSC attract a higher number of granulocytes, T cells, NK cells, and DC compared to their non-treated counterparts [89]. Notwithstanding, there are contradictory data in the literature concerning the influence of TLR activation on the function of human MSC [7].

Interestingly, and taking into account the ability that MSC seem to have to hold in check their immunosuppressive activity when danger signals are sensed, it was demonstrated that human BM-MSC did not inhibit the T cell responses specific for either Epstein-Barr virus or cytomegalovirus, including proliferation, IFNγ secretion, and cytolytic activity against infected cells by cytotoxic T cells specific for the virus [96].

1.5. Mesenchymal stromal/stem cells' immunogenicyty

1.5.1. Low immunogenicity of mesenchymal stromal/stem cells

One of the great advantages of the use of MSC in cell therapy is their low immunogenicity [97]. This characteristic enables the use of allogeneic MSC for cell therapy with a low risk of immune recognition and rejection by the recipient, increasing their window of action inside the host. The expression of low surface levels of MHC-I molecules contributes

to MSC hypoimmunogenicity and protects them from NK cell-mediated lysis; the absence of MHC-II and costimulatory molecules, as CD40, CD80, and CD86, prevents MSC recognition by $CD4^+$ T cells [7]. Under the influence of IFNγ, the expression of MHC-I and MHC-II is upregulated and, in the presence of an inflammatory environment, the expression CD40 is induced in MSC as well [7]. In these circumstances, there are studies reporting that MSC still not elicit an immune response due to the absence of other costimulatory molecules and to the expression of proteins with a strong inhibitory effect over T cells, many of which are upregulated under inflammatory conditions [7]; while other studies describe that IFNγ enable human BM-MSC to become non-professional antigen-presenting cells [45]. In addition, MSC secrete Factor H, and express CD46, CD55, and CD59, thus being protected from the complement system action [98,99].

However, these concepts of low immunogenicity and inability to mediate T cell activation are being challenged by an increasing number of studies showing that MSC can, indeed, be recognized and destroyed by the recipient's immune system.

1.5.2. Mesenchymal stromal/stem cells as antigen-presenting cells

As referred above, a pro-inflammatory environment (or the influence of isolated pro-inflammatory cytokines, such as IFNγ or TNF-α) enhances MHC-I and induces MHC-II expression in human MSC which, under those conditions, may behave as non-professional antigen-presenting cells, acquiring the ability to present exogenous antigens to $CD4^+$ T cells and to cross-present exogenous antigens to $CD8^+$ T cells as well, inducing T cell activation [45]. Remarkably, in human BM-MSC, the induction and maintenance of MHC-II expression, as well as the antigen-presenting cell function, occur when they are exposed to low levels of IFNγ, and an increase of this cytokine's levels results in the downregulation of MHC-II expression and loss of antigen presentation ability by MSC [81].

Human BM-MSC either pulsed or infected with viral peptides, and pre-treated with IFNγ, were able to elicit MHC-I restricted $CD8^+$ T cells response, though to a lesser extent that other antigen-presenting cells [83]; noteworthy, contradictory results had been published concerning this subject [97]. However, while pulsed human BM-MSC were vulnerable to $CD8^+$ cytotoxic T cells (CTL) lysis, infected MSC were resistant due to the secretion of soluble HLA-G [83], showing that, under some conditions, the immunosuppressive molecules produced by MSC may overcome the increased expression of MHC-I, MHC-II, and costimulatory molecules induced by a pro-inflammatory environment.

Cross-presentation of exogenous antigen was demonstrated both *in vitro* and *in vivo* in IFNγ-primed murine BM-MSC [100]. Of note, IFNγ also enhanced the expression of transporter associated with antigen processing-1 (TAP1) and three proteasomal subunits, in MSC: low molecular mass polypeptides (LMP)2, LMP7, and multicatalytic endopeptidase complex subunit (MECL) [100]; conversely, there are some clues that this does not occur in human MSC which, in fact, was present some deficiencies in the MHC-I-related antigen-processing machinery components, even after IFNγ stimulation [83].

1.5.3. Allogeneic mesenchymal stromal/stem cells can be recognized and destroyed by the recipients' immune system

As extensively described, MSC can be target of cytolytic activity by activated (but not resting) NK cells [43,76,92,98,101-103] and by $\delta 2^+$ γδ T cells [104]. It is recognized that human BM-MSC express ligands for NK activating receptors: poliovirus receptor (PVR) and Nectin-2 (ligands of DNAM-1), ULBP and MICA (ligands of NKG2D); and NK cell-mediated MSC lysis is mainly attributed to NKp30, DNAM-1, and NKG2D activating receptors [76,92,101,103]. Interestingly, prior exposure of MSC to IFNγ make them escape to NK cell cytotoxic activity, because this cytokine enhance the expression of classical MHC-I molecules and HLA-E in MSC surface, which bind to the inhibitory receptors on NK cells and override the activating signals received by NK cells, resulting in the non-destruction of MSC [76,101]. IFNγ also decreases levels of ULBP in MSC surface, avoiding the activation of NKG2D activating receptors [76]. Likewise, TLR3 or TLR4 stimulation decreases MICA levels on MSC surface, augmenting their resistance to NK cell lysis [92]. In turn, human AT-MSC were shown to be more resistant to NK cell-mediated lysis than BM-MSC, which may be a reflection of their lower expression of CD112 (Nectin-2) and CD155 (PVR), ligands for DNAM-1 [33].

Noteworthy, it was demonstrated *in vivo* (in horse and rat, among other animals) that the infusion of allogeneic MSC was able to elicit production of antibodies against MSC after a single or multiple infusions [105,106]; however, the humoral response display a high inter-individual variability [105], and other studies describe that animals do not develop antibodies against the MSC infused [107]. Allogeneic MSC infusion in rhesus macaques induced the expansion of B cells, $CD8^+$ T cells, NK cells and NKT cells, as well as the production of antibodies against MSC upon a second infusion of MSC; remarkably, the extension of the immune response was found to be correlated with the haplotype differences between the donor and the recipient [108]. In the same line, a recent study showed that human allogeneic BM-MSC and AT-MSC can induce MHC-I-specific lysis by $CD8^+$ T cells, *in vitro* [48]. Noteworthy, the

immunogenicity of AT-MSC was lower than that of BM-MSC [48]. Thus, similarly to what was observed for NK cell [33], AT-MSC were more resistant to $CD8^+$ T cell-mediated cytotoxicity than BM-MSC [48].

In conclusion, the studies concerning the immunogenicity of MSC (*in vitro* and *in vivo*) yielded different, and sometimes contradictory, results. But, in fact, given the high variability of the experimental procedures among the studies, it is hard to compare them and to integrate their data. Notwithstanding, it seems clear that immune cells can recognized allogeneic MSC. However, the encounter between immune cells and allogeneic MSC may result in rejection, in suppression of the immune response, or in the induction of immune tolerance. But the factors that influence the fate of immune cells after the encounter with allogeneic MSC are still elusive. According to Griffin and colleagues [109], the analysis of *in vivo* studies points the route of MSC administration as a possible factor. Pre-treatment with IFNγ and *in vivo* MSC differentiation can potentially alter the immunogenic properties of allogeneic MSC [109], but their contribute for immune recognition of allogeneic MSC *in vivo* needs to be further elucidated.

2. THERAPEUTIC POTENTIAL OF MESENCHYMAL STROMAL/STEM CELLS

In the last decade, MSC have emerged as a potential therapeutic approach for diseases involving tissue damage and exacerbated immune responses. The natural ability to home to injured tissue and inflammation sites, undergo differentiation into different cellular components of the tissues engrafted, regulate the immune response, adapt their function to the microenvironmental conditions, and influence the behavior of the neighbor cells, endows MSC with potential unique advantages in cell-based therapies. Besides, MSC are easily isolated, expanded and cryopreserved, and display low immunogenicity. These singular features have been explored in the recent years and propelled clinical trials in a broad spectrum of disorders diverse in etiology and pathophysiology, such as osteogenesis imperfecta, spinal cord injury, nonhealing wounds, amyotrophic lateral sclerosis, multiple sclerosis, myocardial infarction, liver cirrhosis, diabetes mellitus, systemic lupus erythematosus, and GvHD, among others [110-112].

The recognition of the potential of MSC to differentiate into several different tissue types urged the interest in using these cells to treat conditions characterized by mesenchymal tissue lesion or loss, wherein engrafted MSC could act as a source of new cells capable to replace the damaged or lost ones. With this regard, an important number of investigations were carried out in the field of regenerative medicine. Remarkably, the benefits achieved by MSC-based therapies in tissue regeneration were found to be more related to the release of growth factors by MSC, or MSC-mediated modulation of the local inflammatory response – both of which actively contribute to stop tissue injury, enhance tissue regeneration, and decrease scar formation – than to the replacement of injured cells by progenitors arising from the infused MSC.

2.1. OSTEOGENESIS IMPERFECTA

Osteogenesis imperfecta is a genetic disease characterized by the production of defective type I collagen with consequent retardation of bone growth, bony deformities and increased risk of fractures [110,113], thus MSC were seen as a suitable therapeutic approach for this pathological condition. In fact, the initial clinical trials in children with osteogenesis imperfecta achieved promising results in the few months following bone marrow transplantation [113] or BM-derived MSC infusion [114], being observed that MSC migrated to bone where they differentiate into osteoblasts [113]. The improvements reported were

subsequently explained by the demonstration that osteocytes and osteoblasts synergistically stimulate the proliferation of mouse bone marrow MSC and their differentiation into osteoblasts [115]; in the same line, conditioned medium from osteocytes also induce osteogenesis by mouse MSC, but to a lesser extent [116]. However, the improvement in osteogenesis is not sustained with time after transplantation and it is hypothesized that the osteogenic differentiation ability of the transplanted stem cells is compromised by the micro-environmental conditions present in osteogenesis imperfecta patients [117].

2.2. SPINAL CORD INJURY

Likewise, the discovery of MSC ability to differentiate into neural and glial cell lineages [118] encouraged the investigation of the therapeutic use of MSC in disorders affecting the nervous system. Indeed, MSC have the ability to replace the lost neural cells, secrete neurotrophic factors that prevent apoptosis and promote neural regeneration and ameliorate inflammation [119], which make them a suitable therapeutic approach to spinal cord injury, amyotrophic lateral sclerosis and multiple sclerosis.

The loss of neural functions in spinal cord injury is due to direct mechanical insult to the spinal cord, responsible for the physical disruption of neural cells, and to the subsequent secondary delayed injury, triggered by the necrotic cells and inflammation [119]. Current treatment consists of administration of high dose steroids and surgical intervention to minimize cord edema and the secondary delayed injury, but their clinic benefits are limited. Animal models of spinal cord injury demonstrated that transplanted BM-derived MSC engraft the injured tissue and contribute to axonal growth and function recovery, being able to differentiate into neurons, oligodendrocytes and astrocytes *in vivo*, while inhibiting the inflammatory response and apoptosis of neurons and oligodendrocytes [56,118-120]. These encouraging results obtained in rat, pig and rhesus monkey models were followed by clinical trials in which patients experienced sensory and motor improvements after transplantation with autologous BM-MSC in the injury site, namely in tactile sensitivity, pain, lower limbs motor function, urinary and bowel function and somatosensory evoked potentials [121-124]. Although autologous BM cells transplantation in conjunction with the administration of granulocyte macrophage-colony stimulating factor (GM-CSF) had also shown clinical benefits in the past, in patients with spinal cord injury [121,125], the functional improvement achieved

with BM-MSC transplantation was more pronounced than that with BM mononuclear cells in rat spinal cord injury model [125].

2.3. Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis is a neurodegenerative disorder characterized by progressive degeneration of motor neurons in the cortex, brainstem and spinal cord, leading to progressive a lethal paralysis [126]. Pre-clinical trials in mice demonstrated the benefits of BM-MSC transplantation, namely in delaying the loss of motor function and increasing the lifespan of the animals [126]. However, after autologous BM-MSC infusion, via intrathecal or intravenous injection, no functional deterioration nor improvement was detected during 6 months in patients with amyotrophic lateral sclerosis, but an increased percentage of peripheral blood $CD4^+$ Treg cells and a decreased frequency of myeloid dendritic cells expressing maturation markers (CD40, CD83, and CD86) was observed immediately (4 hours and 24 hours) after MSC transplantation [127]. Likewise, Manzinni et al. [128] did not find a slower disease progression among patients who received autologous BM-MSC and those who did not in 9-years follow up. At the present, other clinical trials are in course, using BM-MSC differentiated into specialized neuron-supporting cells (to stably secrete neurotrophic factors) and using fetal spinal cord-derived neural stem cells [126].

2.4. Multiple sclerosis

Multiple sclerosis is an inflammatory, demyelinating disease of the central nervous system and, at this moment, there is no treatment capable of promoting the repair of the neural lesions conducing to the progressive disability of the patients. An extensive preclinical research has been carried out in the last years in experimental autoimmune encephalitis (EAE), an animal model of multiple sclerosis, in which intravenous administration of MSC in mice with this condition resulted in reduced disease severity, reduction of demyelination (both in brain and spinal cord), decrease of oligodendrocyte apoptosis and of T cells and macrophage infiltrate in the central nervous system, associated to preservation of the axons [129-135]. These processes were largely mediated by soluble factors – being demonstrated that conditioned medium from human MSC reduces EAE severity and promotes neural cell development and remyelination [136] – and primarily ascribed to the immunomodulatory

abilities of MSC and their soluble trophic factors, namely HGF and brain-derived neurotrophic factor (BDNF). In fact, infusion of MSC in EAE mice compromised the ability of T cells to undergo activation and produce IFNγ and TNFα and, noteworthy, intravenously injected MSC were detected in mice lymphoid organs [129-131,136,137].

Recently, clinical trials using autologous MSC have been reported in the literature. Intrathecal or intravenous infusion of autologous BM-MSC in multiple sclerosis patients led to functional improvement detected in a 6 months follow-up, along with an increased percentage of peripheral blood $CD4^+$ Treg cells and a decreased frequency of myeloid dendritic cells expressing maturation markers (CD40, CD83 and CD86) observed immediately (4 hours and 24 hours) after MSC transplantation [127]. In another clinical trial involving 10 patients with secondary progressive multiple sclerosis with clinical evidence of damage of the visual pathways, intravenous infusion of autologous BM-MSC improved the patients visual acuity and increased the optic nerve area, in a 10 months follow-up [138].

2.5. Cardiac Diseases

The recognized ability of MSC to undergo differentiation into cardiomyocytes, promote angiogenesis, reverse cardiac remodeling, and blunt exacerbated pro-inflammatory responses, make them a suitable cell therapy for distinct heart diseases, such as acute myocardial infarction, in which their anti-inflammatory properties would be of great value, and chronic infarction, wherein clinical benefits would be mainly achieved by MSC's regenerative properties and anti-fibrotic potential.

Animal models of cardiac diseases showed an overall improvement of the cardiac function after MSC transplantation, namely improvement of ventricular function and myocardial perfusion, and reduction of myocardial scar, infarct size, and ventricular remodeling [139-143]. In a swine model of chronic ischemic cardiomyopathy, transfused BM-MSC were shown to engraft into the infarcted tissue and border zone and subsequently differentiate into cardiomyocytes, vascular smooth muscle and endothelial cells *in vivo*, albeit a significant proportion of MSC remained undifferentiated. MSC-treated animals showed improved global and regional cardiac function, increased myocardial blood flow, reduction of the infarct size, and reverse remodeling. Of note, MSC-derived vascular smooth muscle and endothelial cells were incorporated into new blood vessels, contributing directly to the recovery of cardiac perfusion, and MSC engraftment was correlated with functional recovery in

contractility observed in BM-MSC-treated animals [141]. In acute myocardial infarction animal model, BM-MSC transplant was shown to prevent cardiomyocyte apoptosis, restore cardiac function, and reverse ventricular remodeling [143]. Shabbir et al. [144] highlighted the importance of trophic factors by demonstrating that BM-MSC, or MSC-conditioned medium, injected in skeletal muscle of a hamster heart failure model, were able to improve cardiac function, reduce cardiomyocyte apoptosis and fibrosis, and increase the expression of HGF, IGF-2, and VEGF in the myocardium tissue. These trophic factors are known to improve cardiac function, prevent apoptosis of cardiomyocytes, and have pro-angiogenic function [144-146].

In a rat model of experimental autoimmune myocarditis, intravenous administration of MSC was associated to clinical improvement and, from the immunologic point of view, resulted in a decreased percentage of peripheral blood $CD4^+$ T cells producing IFNγ or IL-17, as well as decreased number $CD4^+IL\text{-}17^+$ T cells in the myocardium [147].

The moderate benefits of autologous BM cells or BM-MNC administration in patients with myocardial infarction and ischemic cardiomyopathy had been described since 2001 [139]. Recently, data published by Heldman et al. [148] demonstrated that, in ischemic cardiomyopathy patients, autologous BM-MSC delivered by transendocardial stem cell injection were associated to better clinical outcome than autologous BM cells. Indeed, patients treated with autologous or allogeneic BM-MSC presented an improved cardiac function, decreased scar size, attenuated cardiac remodeling, and increased viable myocardial mass, suggesting myocardial regeneration [148-151]. Clinical benefits of locally administrated autologous BM-MSC and UC-MSC were also found in patients with coronary artery disease [152,153].

2.6. Acute liver failure and cirrhosis

The potential of human MSC to differentiate into hepatocytes *in vitro* [154,155], along with their immunomodulatory properties, raised the interest in using these cells for the treatment of liver diseases, particularly advanced liver cirrhosis and acute liver failure, two conditions involving loss of functional liver cells, wherein organ infiltration by inflammatory cells plays an important deleterious effect, and the only effective treatment available is liver transplantation [154]. A preclinical study with animal model of concavalin-induced liver injury in mice, reported that intravenous BM-MSC infusion ameliorates liver injury and inflammation, both at liver and systemically. Indeed, animals treated with MSC displayed reduced

inflammation and lymphocyte proliferation, along with decreased numbers of activated NKT cells, and levels of TNF-α, IFNγ, and IL-4, in both the liver and periphery [156]. Of note, BM-MSC were detected in the liver 24 hours after the infusion, but were undetectable after 1 month, which supports the hypothesis that the protective effect of MSC is mainly systemic [156]. Similar results were obtained after infusion of BM-MSC conditioned medium in liver failure animal models: reduction of hepatic cell death, liver leukocyte infiltration and serum levels of pro-inflammatory cytokines; increased serum levels of IL-10 and hepatic cell proliferation [157,158]. MSC-derived IL-6 seems to play a pivotal role in liver regeneration by inducing the expression of fibroblast-like protein 1 gene in hepatocytes, a critical gene for liver regeneration, and upregulating the expression of anti-apoptotic proteins [154,159]. In liver cirrhosis animal models, similar encouraging results were obtained: BM-MSC infusion induced regeneration of the hepatic tissues and reduced fibrosis [160]; whereas *in vitro* studies showed that MSC have the ability to modulate the function, induce apoptosis, and reduce the collagen type I production by liver stellate cells [161]. Results from liver cirrhosis clinical trials reported so far, using autologous BM-MSC, almost consistently show improvement of liver function in a significant percentage of patients, as revised by Volarevic et al. [154].

2.7. DIABETES MELLITUS

Diabetes mellitus comprises a heterogeneous group of metabolic disorders, resultant from impaired insulin production and/or peripheral insulin resistance, characterized by hyperglycemia associated to defective metabolism of carbohydrate, fat and proteins [162,163]. Chronic hyperglycemia ultimately causes blood vessel damage, leading to the secondary complications of diabetes, grouped into “macrovascular complications” and “microvascular complications”. Macrovascular complications occur as consequence of arteries damage and include accelerated cardiovascular disease, increasing the risk of coronary artery disease and myocardial infarction among these patients, as well as of cerebrovascular disease (strokes), peripheral arterial disease, and limb amputation. Microvascular complications result from the damage of small blood vessels in the retina (resulting in retinopathy that can lead to blindness), renal glomerulus (nephropathy, which can culminate in end-stage renal failure), and peripheral nerve (neuropathy, manifesting as pain, foot deformity, neuropathic ulceration, which may lead to the need of amputation). Foot ulceration, impaired bone healing, diabetic cardiomyopathy (defined as ventricular dysfunction in the absence of coronary artery disease,

valvular heart disease, or hypertension), erectile dysfunction, dementia, and depression are other secondary complications associated to diabetes [162,163].

Recent preclinical animal models demonstrated that systemic infusion of MSC reverts hyperglycemia in mice. In fact, human BM-MSC can differentiated into insulin-producing cells *in vitro*, expressing genes related to the development and function of pancreatic beta cells and secreting insulin in a glucose-dependent manner. When these cells were transferred to a type 1 diabetes mice experimental model, the diabetic symptoms were ameliorated [164]. Intravenous administration of undifferentiated BM-MSC also normalizes glycemia in type 1 and type 2 diabetes animal models and promote regeneration of pancreatic islands [163,165-168]. The beneficial effects observed in type 1 diabetes animal models are likely to be the result of the immunomodulatory properties of MSC, which were found to engraft lymphoid organs and induced systemic and local reduction of auto-immune T cells, along with an increase of regulatory T cells, and a shift from pro-inflammatory to anti-inflammatory cytokine profile in the pancreatic microenvironment. Of note, MSC infusion regenerated the local expression of epidermal growth factor (EGF) – a pancreatic trophic factor – in the pancreas and increased their levels in the plasma [166].

Beyond controlling hyperglycemia, MSC also act directly on the secondary complications of diabetic patients. For instance, in animal models of retinopathy, MSC derived from the BM, AT and placenta (intravenously, subretinally or intravitreally transfused) home to the damaged areas of the retina and differentiate into retinal cells, endothelial cells and astrocytes; they preserve the integrity of blood-retinal barrier and the retinal and the visual functions, while preventing retinal cell death and abnormal neovascularization, at least in part by secreting neuroprotective factors, such as basic fibroblast growth factor, nerve growth factor, brain-derived neurotrophic factor, ciliary-derived neurotrophic factor and glial-derived neurotrophic factor [163,169]. In the same line, diabetes mellitus animal models also proved that human AT, UC and BM-MSC prevented or repaired renal damage, an effect independent of the glycemic control. Systemically transferred MSC engrafted into the kidneys, wherein they are able to undergo differentiation into endothelial cells and secrete EGF and HGF; both factors prevent kidney injury and the latter downregulates CCL2 (or monocyte chemotactic protein 1, MCP-1) expression, leading to the reduction of macrophage recruitment and expression of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 in the renal tissue [163,165,170]. MSC's ability to produce pro-angiogenic factors (e.g. VEGF-A) surpasses the peripheral nerve's blood vessels damage on the basis of diabetic neuropathy which, accompanied by the production of anti-inflammatory and neurotrophic factors (FGF, nerve growth factor (NGF), neurotrophin-3),

leads to the improvement of peripheral nerve's blood supply and creates an appropriated microenvironment to neural cell recovery, as verified in animal models of diabetic neuropathy [163,171]. Concerning diabetic cardiomyopathy – a pathological condition caused by hyperglycemia and resulting in myocardial hypertrophy, abnormal matrix deposition, cardiomyocyte apoptosis and microvascular defects – preclinical studies with diabetic mouse showed that BM-MSC improved the cardiac function by differentiating into cardiomyocytes, decreasing cardiac cell apoptosis, and improving angiogenesis and cardiac remodeling; paracrine mediators released by MSC, such as IGF-1, HGF, CXCL12 (or stromal cell-derived factor 1, SDF-1), and FGF-2, possibly play an important function in these processes [163]. To date, the effect of MSC transplantation on diabetic retinopathy, nephropathy, neuropathy and cardiomyopathy has not been investigated in human.

In turn, topically administrated BM-MSC are currently being used in the treatment of diabetic wounds (and non-healing wounds of other etiologies) in humans, presenting remarkable results. The value of MSC had been previously demonstrated in preclinical studies, where their local or systemic administration resulted in the improvement of wound healing by MSC differentiation into epidermal and dermal cells, promotion of angiogenesis and collagen production, and secretion of growth factors (EGF, platelet-derived growth factor (PDGF), and VEGF); likewise, topically administrated BM-MSC reduced the ulcer's size and pain in diabetic patients, and also improved vascular regeneration and dermal matrix synthesis [111,163,172].

2.8. Systemic lupus erythematosus

Systemic lupus erythematosus is an autoimmune disease, characterized by chronic inflammation and production of auto-antibodies and associated with multiorgan injury, including the renal, cardiovascular, neural, musculoskeletal, cutaneous, and hematopoietic systems. The organ involvement and severity of the disease are highly variable among patients, nevertheless lupus nephritis and cytopenias are common manifestations and constitute important causes of morbidity and mortality [173,174].

In a mouse model of systemic lupus erythematosus, intravenous infusion of mouse BM-MSC resulted in the reduction in glomerular immune complex deposition, lymphocytic infiltration, and glomerular proliferation, pointing to improvements in what concerns to the renal function [175]. However, no effect was observed in the IgG anti-dsDNA serum levels, proteinuria, or survival [175].

In human, the advantages of the immunomodulatory properties of MSC are currently being tested in clinical trials, especially in treatment-refractory patients. Allogeneic MSC derived from BM and UCM, infused intravenously and in combination with immunosuppressive drugs, were demonstrated to induce stable remission, decrease disease activity and the serum levels of auto-antibodies and complement, improve renal function (including in patients with active and refractory lupus nephritis) and blood cell count [173,174,176-179]. Interestingly, MSC led to an increased frequency of peripheral blood Treg cells and decreased percentage of Th17 cells, which was associated to the decline of disease activity and to the improvement of anemia, leukopenia, and thrombocytopenia [174].

2.9. RHEUMATOID ARTHRITIS

Rheumatoid arthritis is an autoimmune disease triggered by an immune response against self collagen-rich components of the joints. This autoimmune response, mediated by Th1 and Th17 cells, leads to the chronic inflammation of the joints, with subsequent cartilage destruction and bone erosion. Pro-inflammatory cytokines, free radicals, and extracellular matrix-degrading enzymes, produced by inflammatory cells infiltrating the joints (Th1 and Th17 cells, macrophages, and neutrophils) play a critical role in joint damage [180].

In vitro assays show that human BM-MSC have the ability to inhibit the proliferation, activation, and production of TNF-α and IFNγ by peripheral blood $CD4^+$ and $CD8^+$ T cells from arthritis rheumatoid patients, stimulated with type II collagen, while increased levels of IL-4 and IL-10 are found in the co-culture supernatant [181]. These effects were shown to be partially mediated by TGF-β [181].

In collagen-induced arthritis (CIA), an experimental model of rheumatoid arthritis, intravenous administration of BM-MSC resulted in the delay of the disease onset, reduction of the paw swelling, and diminution of the infiltration of immune cells into the joints [182]. Apart from the improvement of the clinical signs of arthritis, a reduction in IL-6 serum levels, along with a decreased IFNγ and IL-17, and increased IL-5, IL-10, and IL-13 expression in cells isolated from the draining lymph nodes, were also detected among MSC-treated mice [182]. Noteworthy, MSC's administration schedule was shown to be of utmost importance in CIA [182]. In the same line of evidence, human AT-MSC treatment of CIA mice resulted in the abolishment of the arthritis progression or in the amelioration of CIA severity, when MSC were administrated at the disease onset or after CIA establishment, respectively [180]. This was

accompanied by the reduction of pro-inflammatory cytokines levels in the synovium (TNF-α, IL-1β, IL-6, IL-12, IL-17, and IFNγ) and serum (TNF-α and IFNγ), and by the augment of IL-10 in the synovium; these data were further confirmed in *in vitro* co-cultures of human AT-MSC with synovial cells, or draining lymph node cells, stimulated with type II collagen, wherein IL-10 and PGE2 were shown to play an important role in MSC-derived inhibition of TNF-α and IFNγ production. Remarkably, type II collagen stimulation of T cells from the draining lymph node of AT-MSC treated mice displayed a reduced number of $CD4^+TNF\text{-}\alpha^+$ T cells, whereas the number of $CD4^+IL\text{-}10^+$ T cells was increased, as well as the levels of TGF-β in the culture supernatant; these data are consistent with the finding that treatment with AT-MSC induced an increased percentage of $CD4^+CD25^{++}FoxP3^+$ Treg cells *in vivo*, in both synovium and draining lymph nodes [180].

To the best of our knowledge, only one clinical trial in rheumatoid arthritis published its results: this study enrolled 4 patients with refractory and persistently active rheumatoid arthritis and showed encouraging results for allogeneic MSC transplantation in this pathology [183].

2.10. CANCER

The observation that tumors recruit MSC, from both local (tissue-resident MSC) and distant sources, as well as intravenously administrated *ex vivo* cultured MSC [184-188], opened new possibilities for their use in cancer therapy as delivery vehicles of therapeutic agents. Although the mechanisms of MSC mobilization to tumors still largely unknown, they are likely to overlap with those described for inflammation sites, as a great proportion of tumors is associated to inflammation and recent data point out the importance of cytokines and chemokines (specially CXCR4/CXCL12) for MSC homing to both tumors and sites of inflammation [15,188-192]. After recruitment, MSC integrate the tumor stroma and undergo differentiation into various cellular components of the stroma (osteoblasts, adipocytes, pericytes, and fibroblasts, among others), subsequently interacting with malignant cells. Once inside the tumor, and given the complexity of the tumor microenvironment and of MSC, the end result of the interaction between MSC and tumor can be either pro or anti-tumorigenic. On the one hand, MSC were shown to have the ability of promoting tumor growth, epithelial-mesenchymal transition, angiogenesis, and metastasis, while modulating the immune cells'

function; but on the other hand, MSC have also the ability to downregulate Wnt and Akt signaling pathways, having an anti-tumorogenic function [188,189,193-195].

Despite this duality in MSC behavior regarding the tumors, an increasing number of studies have demonstrated that MSC engineered with specific anti-tumor genes can be an effective anti-tumor therapy [189]. In the same line, as MSC are easily expanded *in vitro*, poorly immunogenic, and have the ability to home to and engraft tumors, the research of the use of these cells as vehicles for delivery of drugs specifically to tumors has gained an increasing interest in recent years. In experimental glioma rat models, the intracranial administration of unmodified MSC, gene-modified MSC expressing IL-2, or gene-modified neural stem cells expressing IL-4, IL-12, or tumor necrosis factor-related apoptosis inducing ligand (TRAIL) was shown to have an anti-tumoral action [184]. Both IL-2-gene-modified and unmodified MSC retard tumor growth and prolong rat survival [184].

Other studies in carcinoma animal models support the efficiency of the use of engineered MSC, including IFN-α on melanoma, IFN-β on pancreatic cancer, IFNγ on chronic myeloid leukemia, IL-12 on melanoma and hepatocellular carcinoma, and TRAIL on breast cancer and lung cancer [189].

2.11. GRAFT *VERSUS* HOST DISEASE

Graft *versus* host disease (GvHD) is an important complication of allogeneic BM transplantation that arises from minor human leukocyte antigen (HLA) mismatch and is potentially lethal. Acute GvHD occurs 21 to 28 days after BM transplantation and is triggered by the donor's T cells which recognize and react against the recipient's cells, actively homing to and attacking target tissues, usually skin, liver and gastrointestinal tract. In turn, chronic GvHD presents some features of autoimmune disease and develops later, either as an extension of acute GvHD or in the absence of previous acute GvHD; it affects a variable number and type of organs leading to organ fibrosis and dysfunction [196,197].

The primary therapy for acute GvHD consists on immunosuppression with corticosteroids, but only 35% of the patients display a durable response to the treatment, and the prognosis for those refractory to therapy is very poor [196]; thus, most of the clinical trials using MSC has been performed in steroid-refractory patients.

After the encouraging results obtained by Le Blanc et al. [198] with the infusion of haploidentical MSC in one patient with treatment-resistant grade IV acute GvHD, clinical trials emerged and, despite the variations observed among the studies conducted by different research groups, a significant percentage of acute GvHD patients responding to treatment and a better overall survival rate was consistently reported [199-205]. Of note, the high response rate to MSC treatment was observed either in patients with only skin involvement, only gastrointestinal tract involvement, or multiorgan involvement [202]; for MSC derived from BM, MSC derived from UC, or for a premanufactured universal donor commercial preparation of BM-derived MSC (named "Prochymal") [205-207]; and for either BM-derived MSC from HLA-identical sibling donors, haploidentical donors, or third-party HLA-mismatched donors [200]. However, the variations in the number of MSC infused, number of infusions and their timing, which had been shown to be of utmost importance in animal models [208,209], limit comparisons between the abovementioned studies. It is noteworthy that von Bahr and colleagues [210] reported that the use of early-passage MSC (from passage 1-2) improved 1-year survival of therapy-resistant acute GvHD patients, compared with late-passage MSC (from passage 3-4).

In the same line, allogeneic BM-MSC transfusion in chronic GvHD steroid-refractory patients yielded promising results, being observed a complete or partial response in the majority of the patients, as well as an increased 2-year survival rate [211]. Of note, MSC seemed to be more effective in the lesions of the oral mucosa, gastrointestinal tract, and skin, not showing clinical benefits in chronic GvHD with lung involvement; furthermore, as expected, a low response was obtained in patients with advanced chronic GvHD because of the extensive organ fibrosis [196,211,212]. Importantly, MSC ameliorated sclerodermatous lesions (in all the 4 patients enrolled in the clinical trial conducted by Zhou et al.) [213] and dry eye syndrome (in an important percentage of patients) [214], which are two common pathological manifestations of chronic GvHD. As this condition is mainly mediated by Th2 cytokines, it is worth to mention that the infusion of MSC increases IFNγ and IL-2, and decreases IL-4 and IL-10, measured both at intracellular and plasma level; also, these changes were correlated with an increased percentage of $CD8^{+}CD28^{-}$ T cells [213,214].

Taken together, data published so far support that MSC therapy has clinical benefits in patients submitted to allogeneic hematopoietic stem cell (HSC) transplantation in what concerns to the prevention and treatment of acute and chronic GvHD [197]; nevertheless, despite da Silva et al. demonstrated that the use of human BM-stromal layers is efficient for

the *ex vivo* expansion/maintenance of HSC from BM and UCB [215,216], it remains unclear whether MSC enhance HSC engraftment *in vivo* [197].

2.12. TRANSPLANT REJECTION

In the last 5 years a number of clinical trials, most of them in small cohorts, has been giving clues about the potential benefit of MSC infusion to prevent or treat transplant kidney rejection. Autologous BM-MSC infused intravenously at the time or few days after kidney transplant allowed the reduction of the immunosuppressive drugs administration to patients; improved renal function; and showed a tendency to induce the decrease of peripheral blood $CD8^+$ T cells with memory phenotype ($CD8^+CD45RO^+CD45RA^-$), increase the frequency of $CD4^+CD25^{++}FoxP3^+CD127^-$ Treg cells, and reduce $CD8^+$ T cell-mediated immune response against donor antigens in *in vitro* assays [217-221]. Moreover, intravenous autologous BM-MSC administration in kidney transplanted recipients with subclinical rejection and/or an increase in interstitial fibrosis/tubular atrophy, showed clinical benefit, along with the inhibition of PBMC proliferation and expression of IFNγ and chemokines, in response to donor or third party antigens [222].

2.13. ISSUES REGARDING THE THERAPEUTIC USE OF MESENCHYMAL STROMAL/STEM CELLS

Although it is mandatory to conduct more clinical trials and study larger cohorts, published data foresee promising benefits of MSC therapy in a broad spectrum of disorders. This issue becomes particularly relevant as MSC-based therapies are being tested in untreatable and life-threatening diseases, and whose impact in the quality of life of the patients is devastating. However, some crucial questions need to be answered before MSC therapies enter the routine clinical practice.

The first concern is the long-term safety of MSC-based therapy, including the potential risk of malignant transformation or ectopic tissue formation. Despite malignant transformation of MSC hadn't been described *in vivo* so far, some studies suggest that MSC may favor survival and metastasis of pre-existing tumors [188,193,194]. In turn, the formation of intramyocardial calcification and ossification occurred in myocardial infarction murine models [139].

Other important question is whether autologous or allogeneic MSC should be used. Theoretically, the use of autologous MSC allows to circumvent problems related to the risk of transmission of infectious diseases or possible rejection of the transplanted MSC; in fact, the "immune-privilege" status of MSC is being challenged by an increasing body of evidence describing that allogeneic MSC trigger immune responses against donor antigens mediated by T cells and B cells [109]. But, on the other hand, the use of autologous MSC collected under pathological conditions may not be innocuous. For instance, in the treatment of GvHD, BM-MSC from the patient may be part of the malignant hematopoietic clone, or may be damaged during the chemotherapy conditioning regimen [196]. In fact, BM-MSC isolated from B-cell chronic lymphocytic leukemia patients were shown to produce altered levels of cytokines that enable them to promote the survival of the leukemic cells [223], and there are recent clues pointing in the same way for myelodysplastic syndromes [224]. Importantly, MSC isolated from patients with myelodysplastic syndrome were shown to carry the same genetic alterations than hematopoietic precursor cells (HPC) [225]; whereas in some chronic lymphocytic leukemia and acute lymphoblastic leukemia patients, it was detected BM-MSC aneuploidy [226]. In turn, concerning other disorders, it is uncertain if the functional abilities of MSC are fully preserved, being described a decreased immunosuppressive capability of MSC isolated from individuals with atherosclerosis [227], type 2 diabetes mellitus [227], systemic lupus erythematosus [228], and systemic sclerosis [110], though contradictory data had been published [110]. MSC from donors age above 65 years old also display decreased immunossupressive function [227]. Finally, other concern is the time spent to expand autologous MSC to a significant number of cells for therapeutic application, and this issue can preclude the use of autologous MSC under certain pathological condition, such as in situations of acute organ failure, where a ready-to-use source of allogeneic MSC would be more advantageous.

The best source of MSC to be applied in each disease is other pertinent question that is far from being solved. In fact, an increasing body of evidence is revealing differences among MSC arisen from different tissues that ultimately may impact their clinical outcome.

The preparation techniques, route of administration, optimal timing of administration, cell dose and number of infusions are other issues that can influence the end result of MSC-based therapy and need to be study in detail. In particular, the importance of timing of MSC administration had already been demonstrated in EAE and cirrhosis animal models [129,160]. Further investigation will be needed to determine the optimal protocol for each disorder that can benefit from MSC-based therapy.

3. IMMUNOSUPPRESSIVE ABILITIES OF MESENCHYMAL STROMAL/STEM CELLS

Although the enthusiasm for the therapeutic benefits of MSC arose firstly from the expectations on their ability to regenerate damaged tissue, by undergoing differentiation and substitute injured cells, the main clinical achievements came from MSC's ability to change the microenvironment surrounding the degenerating tissues, either by producing trophic factors or modulating inflammation, allowing a faster and efficient recovery of the damaged cells and tissues. Likewise, the immunomodulatory properties of MSC are of utmost importance in controlling the undesirable or exacerbated immune response underlying auto-immune diseases, inflammatory diseases, graft rejection, and GvHD.

In the last fifteen years, an increasing number of *in vitro* assays clearly demonstrated the immunomodulatory effect of MSC over immune cells and definitly contributed to unravel the mechanisms underlying MSC immunosuppressive abilities. The advantages of *in vitro* studies rely on the possibility to manipulate the variables in order to better understand MSC's function and mechanisms. For instance, co-culture of MSC with purified immune cells is a common tactic used to determine if the MSC's effects over those cell populations are direct or not; neutralization of specific molecules, by blocking antibodies or RNA interference techniques, are simple approaches to understand the contribution of the molecule for the MSC-driven immune suppression.

Nevertheless, *in vitro* assays cannot mimic all the complexity found *in vivo*. An this fact assume more relevance for cell populations highly sensitive to the microenvironment, as MSC are. As described previously in this chapter (section 1.4), MSC function is influenced by the presence of other cells, by the cytokine present in the milieu, and by the presence of pathogen-associated molecular pattern (PAMP). These factors are different in a healthy *versus* non-healthy tissue and, besides, they are not static during the course of an infection or tissue damage. Thus, the predictive power of *in vitro* assays is expected to be limited. In this setting, the importance of *in vivo* animal models becomes clear.

In this section, the characteristics, subpopulations, and functions of the different immune cell populations are briefly described, followed by a literature review disclosing the most relevant findings concerning how MSC modulate the immune cell population, assessed by *in vitro* and *in vivo* studies.

3.1. T CELLS

3.1.1. T cells' characteristics, subpopulations, and functions

The pivotal role of T cells in host defense becomes clear with the poor prognosis associated to the immunodeficiencies affecting this hematopoietic cell population. Beyond the important specific cytotoxic function against cells infected by viruses or intracellular bacteria and malignant cells, carried out by cytotoxic T lymphocytes (CTL), the production of cytokines by helper T cells (Th) is vital to the regulation of the effector functions of the innate and acquired immunity. After T cell receptor binding to specific MHC-II-antigen complex expressed by antigen-presenting cells, and depending on the microenvironmental conditions (type of cytokines in the milieu, concentration of antigens, costimulatory molecules and types of antigen-presenting cells) [229], $CD4^+$ T cells undergo activation, clonal expansion and differentiation into distinct effector phenotypes, namely Th1, Th2 and Th17, and into memory $CD4^+$ T cells. Each one of the effector Th cell subsets is characterized by the presence of distinctive transcription factors with properties of master transcriptional regulators and a particular expression pattern of cytokines. The cytokines produced will determine the recruitment and activity of the phagocytes and other immune cells, the antigen processing and presentation, the class of immunoglobulins produced by B cells, and will bias the immune system toward an inflammatory and cell-mediated response or an antibody-mediated immune response [230].

Th1 differentiation occurs in the presence of IFNγ, IL-12 and IL-27, and IFNγ induce the expression of the master transcriptional regulator T-box transcription factor (T-bet). The cytokine expression pattern associated to Th1 cells comprises IFNγ, IL-2, TNF-α and TNF-β. These cells promote an inflammatory immune response against intracellular bacteria or virus, malignant cells or grafts, which is characterized by recruitment and activation of macrophages and other phagocytic cells, activation of NK cells, induction of cytolytic phenotype in $CD8^+$ T cells and of the expression of IgG2a by B cells [229,231]. Th2 cells are responsible for the immune response against extracellular parasites and differentiate under the influence of IL-4 and IL-2. These effector T cells are characterized by the expression the master regulator GATA binding protein 3 (GATA3), together with the following cytokines: IL-4, IL-5, IL-9, IL-13, IL-10, IL-25, and amphiregulin. Th2 effector response includes induction of IgE expression by B cells and involves activation of basophils, mast cells, and eosinophils [229,231]. Polarization towards Th17 cells occurs in the presence of TGF-β and IL-6, and is characterized by the expression of the master transcriptional regulator retinoic acid receptor-related orphan receptor C2

(RORC2), the human correspondent to mouse retinoic acid receptor-related orphan receptor gamma-T (RORγt) [232]. IL-6 binding to its receptor on mouse T cells activates the signal transducer and activator of transcription (STAT)3, which induces the expression of RORγt [233], being demonstrated that STAT3 is also implicated in the differentiation of Th17 cells in human [232]. Th17 cells are involved in the immune response against extracellular bacteria and fungi. Their effector cytokines comprise IL-17A, IL-17F, IL-21, IL-22, IFNγ, and TNF-α, among others; in fact, the cytokines produced by Th17 cells depend on the cytokines present in the milieu where their differentiation takes place. The target-cells for IL-17 are distributed among a great variety of tissues throughout the body (namely, hematopoietic cells, osteoblasts, fibroblasts, endothelial cells, and epithelial cells) and IL-17 ligation induces the production of inflammatory cytokines by these cells. Therefore, Th17 effector response results in inflammation, by the induction of inflammatory cytokines in the IL-17 target-cells and recruitment of inflammatory cells to the site of inflammation [229,231,232]. Conversely, TGF-β in the absence of IL-6 promotes the differentiation of induced $CD4^+$ Treg cells, which specifically express the master regulator forkhead box P3 (FoxP3). These cells negatively regulate the immune response by the production of IL-10, IL-35, and TGF-β [229,231].

3.1.2. Mesenchymal stromal/stem cells' modulation of T cell functions

Given the important role of Th cells in orchestrating and regulating the immune response, unsurprisingly, the majority of studies conducted to assess the immunosuppression abilities of MSC focused on T cells. Remarkably, the immunosuppressive effect of MSC over $CD4^+$ T cells and CTL had been consistently described in the literature [231]. T cell suppression may be achieved indirectly, by regulating the function and activity of antigen-presenting cells (by modulating their cytokine expression or their ability to capture, process, and present antigens), which will be exploited later in this chapter (sections 3.5 and 3.6); or directly, through the interaction of membrane proteins from MSC and T cells (cell-to-cell contact) or through the release of soluble factors by MSC. However, there are important topics that need to be further investigated, such as to determine the effect of MSC on specific effector subsets or functional compartments of T cells, understand how specific pathological conditions will affect MSC immune-modulating functions, or perceive which source of MSC could maximize their clinical benefit for each specific pathological condition.

3.1.2.1. Mesenchymal stromal/stem cells inhibit T cell activation, proliferation, and function

In the last 15 years, *in vitro* assays extensively proved that MSC inhibit $CD4^+$ and $CD8^+$ T cells. Indeed, MSC have the ability to inhibit $CD4^+$ and $CD8^+$ T cell activation (evaluated by the expression of T cell activator markers, namely CD69, CD25, HLA-DR, CD38) and proliferation, upon stimulation with phytohemagglutinin (PHA) [26,31,38,46,58,234-237], IL-2 [235], anti-CD3/CD28 [30,38,58], concavalin A [129], or in mixed lymphocyte reactions (MLR) [26,31,38,75,234,235,237-239]. The activation and proliferation of $CD4^+$ and $CD8^+$ T cells are also impaired in transwell systems and with conditioned medium from lymphocytes and MSC co-culture, indicating that the suppressive effect mediated by MSC is achieved in part through the action of soluble factors [75,234,235,237,240]. However, the degree of suppression is lower than that observed when cell contact is allowed, demonstrating that cell-cell contact mechanisms also participate in the immunomodulation [235,240]. It is noteworthy that the decrease in the proliferation rate of T cells was not due to T cell apoptosis [45,235,241]; in fact, it was demonstrated that MSC can prolong survival of quiescent T cells and decrease the expression of Fas and Fas ligand (FasL) in activated T cells, thus inhibiting the activation induced cell death pathway [241]. Also, the inhibition of mitogen-mediated T cell proliferation is a process dependent of IL-2, as the addition of exogenous IL-2 to the co-culture system reverts the effects of MSC, as showed with mouse and baboons MSC [129,242]. Of note, the presence of MSC in PHA-stimulated PBMC or lymphocytes cultures decreases IL-2 mRNA expression in purified $CD4^+$ and $CD8^+$ T cells and the soluble levels of IL-2 detected in the culture supernatant [46,237,243]; similarly, the percentage of $CD4^+$ and $CD8^+$ T cells producing IL-2 protein, from phorbol 12-myristate 13-acetate (PMA) plus ionomycin-stimulated PBMC, is also reduced in the presence of MSC [244]. Conversely, in MLR assays, MSC do not decrease soluble levels of IL-2 and the addition of exogenous IL-2 do not restore T cell proliferation in human and mouse [243,245]; instead, an augment of soluble IL-10 is detected in the culture supernatant and, interestingly, blocking IL-10 further enhances the suppressive effect of MSC on T cell proliferation in human MLR, while no effect is observed after IL-10 blocking in PHA-stimulated lymphocytes [243]. However, in mouse MLR, inhibition of IL-10 or IDO restores T cell proliferation [246]. In the same line, human BM-MSC-derived IDO also suppresses T cell proliferation in human MLR [247].

Other soluble mediators produced by MSC are able to suppress T cell proliferation. It has been demonstrated that PGE2 plays an important role in this process, as blocking its synthesis partially restores T cell proliferation in PHA stimulation assays [31,236,243,248,249], though its

influence over the inhibition of alloantigen-activated T cells is controversial [26,243,248]; similarly, TGF-β, hepatocyte growth factor (HGF) [235], HLA-G1 and HLA-G5 [30,67,239] were also found to mediate the inhibition of T cell proliferation.

In fact, MSC were shown to arrest T cells in G0/G1 phase of the cell cycle by increasing the protein expression of $p27^{KIP}$ and decreasing phospho-Rb, cyclin D2 and cyclin A, in both mouse [245] and human [30], an effect mediated by HLA-G1 [30].

Overall, the published data demonstrate that MSC effectively inhibit T cell activation and subsequent clonal expansion, but the effect of MSC on T cells is dependent on the T cell stimuli and, depending on the environmental conditions, MSC make use of different inhibitory mechanisms; noteworthy, the degree of T cell suppression obtained is also dependent of the proportion of MSC in the cell culture [61,234,235,238,243].

Besides interfering with T cell activation and proliferation, MSC further inhibit T cell function and differentiation into effector cells. In general, co-culture of MSC with mononuclear cells (MNC) or purified $CD4^+$ T cells results in the decreased expression of cytokines implicated in the inflammatory response and characteristic of a Th1/Th17-mediated immune response, such as IL-1α, IL-1β, IL-2, IL-12, IL-17, IFNγ, and TNF-α, and upregulation of Th2 cytokines, namely IL-4, IL-5, IL-10, and IL-13 [31,39,78,182,231,236,244,250-253].

3.1.2.2. Effect of mesenchymal stromal/stem cells over Th2 cells

Human BM-MSC were shown to increase IL-4 expression in purified naive T cells ($CD45RA^+$) cultured under Th2 inducing conditions [236]; though the inhibition of IL-4, IL-10, and GATA3 mRNA expression is reported for PBMC stimulated with PHA in the presence of human BM-MSC [46,254], while no effect is observed for the percentage of $CD4^+GATA3^+$ T cells, in MLR, in the presence of conditioned medium from human amniotic MSC [255].

Interestingly, it seems that when MSC are in the presence of a pathological condition mediated by Th2 cells, they not only decrease IL-4, IL-5, and IL-13 production, but also increase IFNγ, IL-2, (Th1 cytokines), TGF-β, and IL-10 (Treg cytokines), and expand $CD4^+CD25^+FoxP3^+$ Treg cells, both *in vitro* and *in vivo* [213,231,249,256,257], a process mediated by PGE2 [249].

Similarly, when administrated *in vivo*, in mouse models of allergic airways inflammation (a Th2/Th17-mediated disease, characterized by increased levels of IL-4 and IL-17), BM-MSC increase IFNγ and IL-10 expression, while decrease IL-4, IL-13, and IL-17 [258-260]. In the same line of evidence, in a mouse model of ragweed induced-asthma (a Th2-mediated disease), BM-

MSC decreased IL-4, IL-5, IL-13, IgA1, and IgE protein levels and eosinophil infiltration in the lung, all of which are typically increased in asthma; this inhibitory effect over the Th2-mediated immune response is attributed to TGF-β [261]. On the other hand, in Th1/Th17-mediated disorders like the CIA animal model, MSC treatment increases the expression of IL-5, IL-10, and IL-13 in lymph node cells and splenocytes, and increases GATA3 mRNA expression in the latter cell subset [182], together with the clinical amelioration of the arthritis.

3.1.2.3. Effect of mesenchymal stromal/stem cells over Th1 cells

Concerning the MSC effect over Th1-mediated immune response, published data consistently describe an inhibitory effect over both Th1 differentiation and function (in human and mouse), associated to a decreased expression of IFNγ and TNF-α by fully-differentiated Th1 cells [129,231,236,251,262,263]. Nevertheless, all these studies inferred that MSC inhibit Th1 differentiation and function based on the decreased IFNγ levels in the culture supernatant or on the decreased percentage of IFNγ-producing $CD4^+$ T cells. Accordingly, it was recently described that conditioned medium derived from human amniotic MSC decreased the percentage of $CD4^+$T-bet^+ T cells in MLR [255]. However, when considering only the activated compartments of T cells, an increase of T-bet mRNA levels is observed upon co-culture with human MSC derived from BM, AT, or UCM [46].

Remarkably, recent data demonstrated that MSC upregulate IL-10 expression in IFNγ-producing $CD4^+$ T cells, endowing these Th1 cells with the ability to suppress T cell proliferation in an IL-10-dependent manner [264,265]; these cells do not express FoxP3 and, accordingly to their $CD4^+IL\text{-}10^+IFN\gamma^+$ phenotype, are designated as regulatory T type 1 (Tr1) cells (of note, Tr1 cells had been classically described as $IL\text{-}10^+TGF\text{-}\beta^+IL\text{-}4^-IL\text{-}2^-IFN\gamma^{+/-}$) [266,267]. The induction of CTLA-4 expression is also observed on T cells co-cultured with MSC [44,238].

Regarding the $CD8^+$ T cells and CTL, which are activated in the course of a Th1 immune response, MSC have the ability to inhibit CTL-mediated alloantigen-specific cytolytic activity [62,195,238] and reduce the secretion of granzyme B, TNF-α and IFNγ [195,231,241,244,268], being also observed a lower activity of the tyrosines activated downstream T cell receptor(TCR) signaling in the presence of MSC [97]. In a recent study in which skin-homing $CD8^+$ T cells isolated from vitiligo patients were co-cultured with autologous dermal MSC, it was detected decreased levels of IL-1α, IL-12, IL-13, IL-15, MIP-1δ, among others, comparatively to skin-homing $CD8^+$ T cell monocultures [269]. Similarly to what is reported for $CD4^+$ T cells, MSC can induce a regulatory phenotype in $CD8^+$ T cells, which acquire the ability to inhibit T cell proliferation and to downregulate the expression of costimulatory molecules (CD80/CD86) in

monocytes, by mechanisms independent of IL-10, PGE2 and TGF-β [231,268,270]. Conditioned medium derived from human amniotic MSC was decribed to increase the percentage of $CD8^+CD28^-$ T cells in MLR, which are considered to have a suppressor/regulatory activity [255]. Remarkably, the effect of MSC over effector $CD8^+$ T cells seems to be less efficient [58,62,231,244], being described in the literature that, despite of inhibiting $CD8^+$ T cell-mediated cytotoxicity when added at the day 0 of a 6 days MLR, if added at the day 3, or after, human BM-MSC were unable to inhibit CTL-mediated lysis [62]; moreover, effector $CD8^+$ T cells are more resistant to MSC-mediated suppression of TNF-α production, compared to naive and central memory $CD8^+$ T cells [244].

Accordingly, *in vivo* animal models of inflammatory diseases treated with MSC show a decreased protein expression of TNF-α, IFNγ, IL-1β, IL-2, IL-6, IL-12, IL-17, T-bet, and RORγT, while the protein expression of IL-4, IL-10, TGF-β, GATA3, and FoxP3 is increased [180,271-274]. The immunomodulation mediated by MSC was observed both in the periphery and in the damaged tissues [180,271,272].

3.1.2.4. Effect of mesenchymal stromal/stem cells over Th17 cells

Similarly to Th1 cells, most of the studies performed so far demonstrate that mouse and human BM-MSC impair *in vitro* differentiation of purified naive and memory $CD4^+$ T toward Th17 effector phenotype [9,251,263,275-278], an effect shared by human UC-MSC co-cultured with PBMC [279], and conditioned medium derived from human amniotic MSC in MLR [255]. However, other studies report the opposite showing that human BM-MSC induce $CD4^+$ T cells, especially memory $CD45RO^+CD4^+$ T cells, to differentiate into Th17 cells [252,254,280]. Likewise, there are contradictory data regarding the influence of MSC in the function of fully differentiated Th17 cells, being possible that, under different conditions, MSC can either enhance [251,262] or impair Th17 function, including the production of IL-17A, IL-17F, IL-22, TNF-α, and IFNγ [9,244,263,275,278,281]. This hypothesis was elegantly demonstrated by Svobodova and colleagues [282]: in their study they show that, when untreated or pre-treated with TGF-β, mouse BM-MSC favor the differentiation of splenocytes into $CD4^+FoxP3^+$ Treg cells, because of the high amounts of TGF-β secreted by MSC; in turn, if pre-treated with IL-1α, BM-MSC are induced to produce TGF-β and IL-6, promoting $CD4^+ROR\gamma t^+$ Th17 cell differentiation and IL-17 production.

Human and mouse BM-MSC-driven inhibition of Th17 differentiation and reduction of IL-17A and IL-22 expression in Th17 cells have been ascribed to PGE2 [9,275], as well as the induction of FoxP3 and IL-10 expression, converting fully differentiated human Th17 cells in

functional regulatory T cells, which have suppressive effects over T cell proliferation [9]. Of note, IL-10 produced by mouse BM-MSC mediates the inhibition of Th17 differentiation and reduces the expression of RORγt, IL-17A, and IL-17F in mouse T cells [278]. Likewise, PD-L1 expressed on mouse BM-MSC plasmatic membrane was found to have an essential role in MSC-derived inhibition of proliferation, RORγt and IL-17 protein expression by fully differentiated Th17 [263].

In vivo, infusion of AT-MSC was shown to inhibit Th17-mediated immune response in different mouse models of inflammatory diseases. AT-MSC reduced the percentage of Th17 cells in the lymph nodes of EAE animals [283] and in a murine model of systemic lupus erythematosus [274], the levels of IL-17 mRNA in the skin of mice with skin allograft [277], and the percentage of Th17 cells and IL-17 protein levels in the synovium of mice with CIA [180].

3.1.2.5. Effect of mesenchymal stromal/stem cells over Treg cells

Recently, it has been described that co-culture of human UCM-MSC or BM-MSC with putified $CD3^+CD4^+CD25^-$ T cells, stimulated with anti-CD3/CD28 plus IL-2, induces the generation of $CD3^+CD4^+CD25^+FoxP3^+$ Treg cells [37]. Indeed, a great number of studies using *in vitro* assays of MSC co-cultured with activated PBMC or purified $CD4^+$ T cells demonstrated that MSC favor the differentiation of $CD4^+CD25^+FoxP3^+$ Treg cells and enhance their activity [98,195,231,236,238,239,249,250,270,283-289], a process mediated by cell-to-cell contact [98,249,286,287] and by the soluble mediators PGE-2 [98,286,287], TGF-β [98,195,285-287], heme oxygenase-1 (HO-1) [285], soluble HLA-G5 [98,239,287], and LIF [98]. In the same line of evidence, the culture of purified $CD4^+$ T cells or PBMC with conditioned medium from human AT-MSC or BM-MSC, respectively, resulted in the augment of $CD4^+CD25^+FoxP3^+$ T cells associated to an increased protein expression of IL-10 in the supernatant, wherein the induction of Treg differentiation was mediated by TGF-β [285,290]. Likewise, the presence of conditioned medium derived from human amniotic MSC in MLR increased the percentage of $CD4^+FoxP3^+$ T cells, which were also induced to express higher levels of the immune suppressive molecules CD39, CD73, TGF-β, CD357 (AITR/GITR), and Helios [255].

Remarkably, human BM-MSC are also able to induce IL-10 expression in naive $CD4^+$ T cells under Th17 differentiating conditions and in fully differentiated Th17 cells (a process partially mediated by PGE2), in parallel with the upregulation of FoxP3 and downregulation of RORc [9]. Though the expansion of the Treg subset is an efficient mechanism for MSC to inhibit the proliferation and the effector functions of T cells, in a recent study the allogeneic stimulation of $CD4^+$ T cells or PBMC, under the influence of human UCB-MSC, did not induce

the increase of Treg subpopulation. Nevertheless, an effective suppression of T cell function was observed, mediated by the induction of IL-10 expression in IFNγ-producing T cells. In this particular study, it was observed that UCB-MSC increase the percentage of $CD4^+IFN\gamma^+IL\text{-}10^+$ T cells without changing the frequency of $CD4^+CD25^{++}FoxP3^+$ T cells, demonstrating that MSC may employ alternative mechanisms to negatively regulate T cell activity [264].

Besides, MSC can induce regulatory properties in human and mouse $CD8^+$ T cells, upregulating the expression of IL-10, TGF-β, and FoxP3 in this cell population [193,270]. And, finally, Treg cells can also be indirectly induced as result of MSC-derived inhibition of DC maturation and induction of IL-10 expression [98], as described in detail in subsection 3.5.

In *in vivo* animal models, AT-MSC induced an increased percentage of $CD25^+FoxP3^+$ T cells [283] and upregulated the expression of FoxP3 and IL-10 mRNA [277] detected in the draining lymph nodes from both mouse EAE and skin allograft models, respectively. In a CIA mouse model, AT-MSC treatment was shown to induce *de novo* generation of $CD4^+CD25^+FoxP3^+$ Treg cells, which was translated as an increased number of Treg and increased IL-10 and TGF-β protein expression, in both synovium and draining lymph nodes [180]. In the same line of evidence, in mouse model of allergen-driven airway inflammation, BM-MSC treatment increases the percentage of $CD4^+FoxP3^+$ Treg cells, accompanied by increased expression of IL-10, in both lungs and spleen [260]; and in a murine model of systemic lupus erythematosus administration of human AT-MSC increase the number of $CD4^+FoxP3^+$ Treg cells in the spleen [274].

3.1.2.6. Effect of mesenchymal stromal/stem cells over the distinct functional compartments of T cells

In recent years, there has been an increasing effort to understand how the effect of MSC on specific T cell effector subsets varied; however, there are few data comparing the immune-modulating effect mediated by MSC among the distinct T cell functional compartments: naive, central memory (CM), effector memory (EM) and effector compartments. In our previous work, we demonstrated that the capability of human BM-MSC to decrease the percentage of $CD4^+$ and $CD8^+$ T cells expressing TNF-α, IFNγ, and IL-2 is verified among all the naturally occurring functional compartments of T cells (naive, CM, EM and effector compartments), and at different extents. Of note, only a small percentage of naive CD4+ and CD8+ T cells expressed the abovementioned cytokines. Also, the reduction of TNF-α and IFNγ secretion is achieved not only by reducing the percentage of T cells expressing these cytokines, but also by diminishing the amount of cytokine produced *per* cell [244]. Interestingly, BM-MSC-mediated inhibition of

TNF-α expression was less effective among the effector and EM compartments, for both $CD4^+$ and $CD8^+$ T cells, and the same subsets were also the most resistant to MSC-induced inhibition of IFNγ expression in $CD4^+$ T cells, and the most inhibited for $CD8^+$ T cells producing IFNγ [244]. Mouse BM-MSC also exert a stronger inhibitory effect over IFNγ-producing memory $CD8^+$ T cells than naive $CD8^+$ T cells [291]. Under the influence of human BM-MSC, the inhibition of IL-2 and IFNγ expression was similar among all $CD4^+$ T cell functional compartments [244].

Consistently to what was observed for TNF-α, $CD4^+CD45RO^+$ and $CD8^+CD45RO^+$ T cells were shown to be less susceptible to proliferation's suppression mediated by human AT-MSC than their $CD45RO^-$ counterparts; interestingly, memory $CD4^+$ and $CD8^+$ T cells were also shown to be more resistant to the inhibitory effects of Treg cells over MLR-induced proliferation [292]. In the same line, a recent study showed that conditioned medium derived from human amniotic MSC inhibit MLR-induced proliferation of $CD4^+$ and $CD8^+$ T cells included in the EM, CM, and effector functional compartments. However, MLR didn't induced naive T cell proliferation, therefore it is not possible to compare this compartment with the remaing. Interestingly, after 6 days of MLR, the presence of conditioned medium derived from human amniotic MSC decreased the percentage of cells within the CM compartment and increased the percentage of naive T cells, for both $CD4^+$ and $CD8^+$ T cells [255].

Finally, in the studies describing that human BM-MSC were able to induce IL-17 expression by $CD4^+$ T cells, it was found that effect was exerted on memory $CD4^+CD45RO^+$ T cells, rather than in $CD4^+CD45RA^+$ [280] or $CD4^+CD45RO^-$ [254] T cells, isolated from healthy individuals or rheumatoid arthritis and osteoarthritis patients, respectively. In opposition, mouse BM-MSC were shown to inhibit IL-17 cytokine, which affected naive and memory $CD4^+$ T cells at similar level [275].

3.2. Other T cell subsets: γδ T cells and invariant Natural Killer T cells

3.2.1. γδ T cells

3.2.1.1 Characteristics and function of γδ T cells

γδ T cells belong to the non-conventional or innate lymphocyte family. Their T cell receptor (TCR) is composed of a γ and a δ chain with a restricted TCR region repertoire wherein, in human, only three Vδ genes and six Vγ genes are expressed. In human peripheral

blood, γδ T cells correspond to 1%-5% of total $CD3^+$ T cells, and $V\gamma9^+V\delta2^+$ is the most represented subset [104,293]. One important difference to the conventional αβ TCR T lymphocytes is that γδ T cells are not MHC-restricted, being able to recognize small peptides, soluble or membrane proteins, and small non-peptidic antigens, such as phospholipids, pyrophosphates (including the microbe-derived metabolites of the isoprenoid biosynthesis pathway), phosphoantigens produced by malignant cells, sulfatides, and alkylamines [104,293,294]. Also, because antigen recognition by Vγ9+Vδ2+ T cells does not require prior antigen processing or presentation by classical MHC or non-classical antigen-presenting molecules (CD1a, CD1b, CD1c, or CD1d), these cells are promptly activated during the early phase of the immune response. Similar to NK cells, γδ T cells are activated by ligands induced in a stress (DNA damage, heat stress) and/or infection context, namely MHC class I-related chain (MIC) A and B (MICA and MICB) and UL16 binding protein (ULBP) family, through the binding to the activating natural killer group 2, member D receptor (NKG2D), or direct recognition by γδ TCR [293]. Finally, human γδ T cells also express pattern recognition receptors (PRR), such as TLR [293].

The functional plasticity of γδ T cells is now beginning to be unraveled. Despite the first reports concerning the function of γδ T cells claim they exhibit cytotoxic activity against infected and transformed cells, together with the ability to produce TNF-α and IFNγ, it is now recognized that, besides this Th1-like phenotype, and depending on the microenvironment (cytokines and type of γδ TCR stimulus), γδ T cells can also acquire Th2, Th17, follicular helper T cells (Tfh), or Treg-like characteristics and functions, being also reported that activated Vδ2 T cells can act as antigen-presenting cells for $CD4^+$ T cell-mediated responses [104,293].

3.2.1.2. Effect of mesenchymal stromal/stem cells over γδ T cells

It was recently reported that human BM-MSC were able to inhibit proliferation of $V\gamma9^+V\delta2^+$ or $V\delta2^+$ T cells, induced by stimulation with IL-2 plus zoledronate [295] or bromohydrin pyrophosphate (BrHPP) [294] for the former γδ T cell subset, or by isopentenylpyrophosphate (IPP) [104] for the latter. The suppression of γδ T cell proliferation is mediated by PGE2 signaling through EP2 and EP4 receptors (which are highly expressed at their cell surface), whereas IDO, TGF-β1, IL-10, and HGF seem not to participate [104,294]. Concerning the effect of MSC over the cytokine production and cytolytic activity of γδ T cells, there are conflicting data in the literature: it was reported by Prigione and colleagues [104] that MSC did not inhibit TNF-α nor IFNγ production by $V\gamma9^+V\delta2^+$ T cells stimulated with IPP, whereas the expression of both cytokines were found to be suppressed when these cell subset

was stimulated with BrHPP [294]; likewise, MSC didn't alter the cytotoxic activity of $V\gamma9^+V\delta2^+$ T cells over multiple myeloma nor melanoma cell lines [295], but hamper their ability to lysis Daudi Burkitt's lymphoma cell line [294]. Concerning to the antigen-presenting cell functions of $V\delta2^+$ T cells, MSC affect neither the ability of $V\delta2^+$ T cells pulsed with *Candida albicans* bodies to stimulate the proliferation of autologous $CD4^+$ T cells, nor their expression of CD80 and CD86 costimulatory molecules [104].

3.2.2. Natural killer T cells

3.2.2.1.Characteristics and function of natural killer T cells

Natural killer T (NKT) cells represent a unique subset of T lymphocytes, which accounts for approximately 0.2% of peripheral blood T cells and is characterized by the simultaneous surface expression of αβ TCR with a restricted repertoire and NK cell markers, namely CD161 (or NKR-P1) and CD94 [296]. In human, there is a subset of NKT cells that express an invariant TCR α chain rearrangement (Vα24-Jα18) paired with the TCR β11 chain, designated as invariant NKT (iNKT) cells [230,296]. iNKT cells recognize glycolipid antigens (both foreign and self-antigens) presented in the context of CD1d, a non-classical antigen-presenting molecule associated to β2-microglobulin [104,230,296]. After TCR priming, these cells can produce either Th1 (IFNγ, TNF-α, and IL-12) or Th2 (IL-4, IL-5, and IL-13) cytokines, also possessing the ability to produce Th1 and Th2 cytokines simultaneously; IL-17 and IL-10 may be produced by iNKT cells as well [296,297]. Nevertheless, NKT cells can also act as cytotoxic cells, being able to kill target cells, mainly through Fas/FasL interaction [230]. The factors and mechanisms that govern the effector functions assumed by iNKT cells are still not fully elucidated, though is evident that the type of antigen, the nature of the cell that presents the antigen, the costimulatory signals, and the cytokines in the milieu influence the functional response of iNKT cells [296,297].

3.2.2.2. Effect of mesenchymal stromal/stem cells over natural killer T cells

Human BM-MSC were shown to efficiently suppress the proliferation of iNKT cells upon α-galactosylceramide stimulation, a process mediated by PGE2 and independent of IDO and TGF-β1 [104]. Additionally, human BM-MSC inhibit the secretion of IFNγ by $V\alpha24^+V\beta11^+$ iNKT cells, without altering the expression of TNF-α or their cytotoxic activity [104]. In an experimental mouse model of metastatic breast cancer, the administration of human peripheral blood-derived MSC decreased the number of $CD3^+NKp46^+$ NKT-like cells in spleen, besides favoring tumor growth and metastasis [298].

3.3. B CELLS

3.3.1. B cells' characteristics, subsets, and function

Humoral adaptive immunity relies on B cells' ability to expand and differentiate into specialized antibody-secreting cells, the plasma cells, in response to a specific antigen (Ag). The secreted antibodies have the ability to specifically bind to the Ag expressed by the pathogen agents and, depending on the antibody isotype, they can impede cell infection by binding and blocking yhe receptors used by the pathogen to enter the host cell; they can opsonize the pathogen, recruiting phagocytic cells and facilitating the phagocytic process; they can activate the complement cascade, resulting in the pathogen lysis; and they can promote antibody-dependent cell-mediated cytotoxicity, through binding to CD16 (or FcγRIII). After exposure to a foreign Ag, B cell activation may occur in a T cell-independent or T cell-dependent manner, and the latter usually presuppose the formation of germinal centers and the generation of both memory B cells and plasma cells [230].

After completing their differentiation process, transitional (immature) and naive (mature) B cells leave the BM and migrate to the lymphoid follicles, following the CXCL13 gradient produced by follicular DC, which binds to CXCR5 expressed on B cell surface; then, B cells follow a pattern of recirculation between blood and lymphatic systems, and lymphoid follicles, to maximize the chance to encounter specific foreign antigens [230]. During their lifespan, naive B cells are dependent of survival signals given by B-cell activation factor (BAFF) – which is expressed by DC, monocytes, macrophages, neutrophils, and follicular DC – binding to the corresponding receptors on B cells [230].

T cell-independent antigens activate B cells by crosslinking membrane immunoglobulin (Ig) receptors (which, together with CD79a and CD79b, forms the B cell receptor, BCR), or by binding to innate immune receptors, such as pattern recognition receptors (PRR), e.g. TLR [230]. Indeed, human B cell activation and differentiation into IgM-producing CD138+ plasma cells can be achieved through TLR9 stimulation in the absence of BCR crosslinking, which was verified for transitional (the most immature B cell compartment in peripheral blood), naive (mature B cells), and memory B cell compartments [299]. Likewise, signaling through TLR7 also activates and induces human naive B cell differentiation into IgG-secreting plasma cells, in the absence of BCR crosslinking and CD40 binding [299].

In a T cell-dependent response, B cells encounter and bind to the specific Ag through BCR; the Ag is subsequently internalized and processed, being ready to be presented in context

of a MHC-II molecule to a cognate activated $CD4^+$ T cell [230]. After Ag recognition, B cells upregulate CCR7 expression, which leads to their migration toward T cell zones of the secondary lymphoid organs, where CCR7 ligands, CCL19 and CCL21, are produced by stromal cells [230,300]. There, B cell presents the Ag to a cognate activated (by previous contact with DC) CD4+ T cell; CD40 (expressed on B cells) binds to CD40L (or CD154, expressed on T cells), and the costimulatory molecules CD80 and CD86 (on B cells) bind to CD28 (expressed on T cells) [230]. The signals that trigger B cell activation are mediated by (1) Ag crosslinking to BCR, (2) CD40 signaling, and (3) cytokines secreted by the cognate CD4+ T cell, whose expression is induced by CD40L and CD28 stimulation [230]. Then, activated B cells proliferate and downregulate CCR7, which allows them to leave the T-cell zone of the secondary lymphoid organ [230,300].

Apart from that, the initial activation of CD4+ T cells by DC expressing ICOSL induces B-cell lymphoma 6 (Bcl-6) expression on T cells, which is necessary for follicular T cells (Tfh) development. Bcl-6 downregulates CCR7 and P-selectin glycoprotein ligand 1 (PSGL-1) expression – allowing the exit of nascent Tfh from T cell zone of the secondary lymphoid organ – while upregulates CXCR5 expression. In turn, ICOSL expressed by activated B cells may give an important contribute to maintain Bcl-6 expression on the nascent Tfh cells along the B cell differentiation process [301].

At that point, both activated B cells and activated T cells enter B cell follicles, following CXCL13 gradient produced by follicular DC, which binds to CXCR5 on B cell and T cell surface. Once in the B cell follicle, B cells still need to interact with T cells (via CD40/CD40L) and go through further cycles of cell division, giving rise to the germinal center. Subsequently, B cells undergo somatic hipermutation and Ig class switching – a process regulated by the cytokines released by Tfh – and only those B cells which recognize Ag presented by follicular DC with high affinity and receive CD40L signaling through Tfh cells, are positively selected to undergo differentiation into memory B cells or plasma cells (this process is called affinity maturation process) [230,299].

The mechanisms that determine the differentiation of germinal center B cells into memory B cells or plasma cells are still elusive. There is the hypothesis that the differential activation of STAT5 or STAT3, for memory or plasma cell differentiation, respectively, has an important role in B cell fate [299]. Nevertheless, the transcription factors orchestrating memory B cell differentiation are still unknown. In fact, naive and memory B cell transcriptomes are very similar [302].

According to the current knowledge, Bcl-6 and paired box gene 5 (PAX-5) need to be downregulated to occur plasma cell differentiation [299,300]. Bcl-6 is highly expressed by germinal center B cells and has an essential role in promoting survival and proliferation of these cells [299]; PAX-5 is essential for B cell lineage commitment and maintenance of B cell identity, it is also vital for B cell development, being involved in VH to D Ig heavy chain recombination and in maintaining Bcl-6 expression in B cells [230,299,300,303]. However, both PAX-5 and Bcl-6 repress the expression of B-lymphocyte-induced maturation protein 1 (BLIMP-1), an essential and sufficient transcription factor for plasma cell differentiation [230,299,300,303]. Recent data point the interferon regulatory factor 4 (IRF-4) as the first transcription factor in the induction of B cells differentiation into plasma cells, and its expression is induced by CD40-mediated NF-kB activation [230,303]. In fact, IRF-4 downregulates PAX-5 and Bcl-6 [230] and induces the expression of BLIMP-1 [230,303]. Likewise, STAT3 activation on B cells by Tfh-derived IL-21, also induces BLIMP-1 expression and plasma cell differentiation (interestingly, Bcl-6 represses STAT3 transcription) [299,300]. BLIMP-1, in turn, represses the expression of a great number of genes involved in B cell proliferation, somatic hipermutation and Ig class switching, class II transactivator (CIITA, resulting in the suppression MHC-II expression), and Bcl-6, among others; while upregulates genes implicated in Ig production and secretion, such as X-box binding protein 1 (XBP1), which induces the transcription of genes that regulate endoplasmic reticulum stress response, to face the enormous protein production that characterize plasma cells [230,303]. Also, during the plasma cell differentiation process, BLIMP-1 downregulates CXCR5 expression (allowing the cells to leave the germinal center), while upregulates CXCR4 and Very late antigen 4 (VLA-4) expression, permitting the exit of plasma cells from the lymph node and their migration to peripheral tissues and specialized niches of the BM, where BM-MSC produce CXCL12 (or SDF-1, the CXCR4 ligand) which is essential for plasma cells entering in the BM; CXCL12 also gives survival signals to long-lived BM plasma cells [230,300,303].

Finally, it was recently described a new B cell subset, B10 cells. They are not confined to any specific phenotypically characterized B cell compartment and are identified by IL-10 expression, which endows them with regulatory functions. This cell subset comprises less than 1% of total human peripheral blood B cells [304]. B10 cells development depends on antigen binding to BCR and their effector function (IL-10 production) occurs in response to its specific antigen and with T cell help through IL-21. These factors endow B10 cells with antigen-specific regulatory function. Functionally, human B10 cells were demonstrated to inhibit pro-

inflammatory cytokine production by T cells and monocytes, *in vitro*; and animal model assays show that B10 cells have a suppressive effect over autoimmune inflammation, *in vivo* [304].

3.3.2. Effect of mesenchymal stromal/stems cell over B cells

The immunosuppressive abilities of MSC include the inhibition of B cell proliferation, activation, function, and differentiation into plasma cells. And, despite not being as widely study as T cells, the mechanisms underlying B cell suppression by MSC are now becoming unveiled. Noteworthy, and accordingly to what is described for other immune cell populations, the effect of MSC on B cells can be antagonistic, depending on external conditions and on B cells and MSC intrinsic factors. Among the factors known to influence the outcome of MSC over B cells, it can be enumerated the immune cell populations present in the culture system, the type of stimuli used for B cell activation and/or differentiation, the strength of the B cell response to the stimulus, and the MSC:B cells ratio.

It also worth mentioning that the immunomodulatory effect of MSC over B cell immune function is due to their direct effect over B cells, as demonstrated by studies using purified B cells in co-culture with MSC [305-308], but also to MSC action over other immune cell populations. In fact, MSC-mediated suppression of T cell function will indirectly hamper B cells, which is supported by the finding that the addition of CD40 agonist to MLR can reverse the suppressive effect of MSC over Ig production by B cells [55], and by the recent reports showing that, under some experimental conditions, MSC are only able to inhibit B cells when T cells are present in the culture system [309,310]; it is also worth to mention the MSC-derived downregulation of BAFF expression by DC [311]. Moreover, the suppressive effect of MSC over B cell is maintained in transwell, but is more pronounced when cell contact is allowed, pointing to both soluble factors and cell-to-cell contact-dependent inhibitory mechanisms [55,306].

3.3.2.1. Effect of mesenchymal stromal/stem cells on B cell proliferation

Inhibition of proliferation is one of the suppression mechanisms that MSC exert over B cells. Indeed, human BM-MSC were shown to inhibit B cell proliferation induced by PHA alone [46] or with CD40L [312], LPS (TLR4 agonist) [313], CpG (TLR9 agonist) alone [314] or in conjugation with CD40L, anti-Ig, IL-2, IL-4, and IL-10 [305,306], and phorbol ester (12-otetradodecanoyl-phorbol-13-acetate, TPA) [315]. This feature is shared by human AT-MSC, which have the ability of inhibit the proliferation of PHA-activated B cells [46]; and by human periodontal ligament stem cells, able to impair B cell proliferation upon CpG, CD40L, anti-Ig, IL-2, and IL-4 stimulation [306].

Interestingly, and conversely to what is observed for human BM- and AT-MSC, UCM-MSC co-cultured with PBMC activated with PHA have no effect over the proliferation of B cells [46], underlining the functional differences among MSC derived from different sources. However, one study carried out in mouse purified B cells, stimulated with CpG, CD40L, anti-IgM, and IL-4, reports that human UCM-MSC inhibits mouse B cell proliferation [308], highlighting the importance of the differences among animal species, and cell culture conditions (e.g. cell types present in the assay and cell stimuli used).

The suppression of B cell proliferation by human MSC is not attributed to apoptosis [305,306]. Instead, BM-MSC were shown to induce cell cycle arrest in G0/G1 phases [305,315]. Interestingly, despite of inhibiting B cell proliferation, there are evidences that human BM-MSC also promote B cell survival [315]. In fact, the upregulation of IL-6 production by human BM-MSC co-cultured with purified B cells [306], also increased in the supernatant of MLR with human BM-MSC [55], was demonstrated to protect B cells from apoptosis [306]. The role of soluble factors in human BM-MSC-driven inhibition of B cell proliferation was evidenced by transwell assays [305,306], and by the recent report that their derived membrane vesicles, which constitute a pathway of intercellular communication, actively participate in the suppression of B proliferation and function [314]. Notwithstanding, it was demonstrated that the membrane-bound proteins programmed death-ligand 1 (PD-L1) and PD-L2 [306], as well as galectin-9 [312] are involved in the suppression of B cell proliferation by human BM-MSC. Likewise, PD-L1 and PD-L2 mediate the same effect for mouse BM-MSC [175,316].

Remarkably, and similarly to what was found for T cells [241], human BM-MSC promote proliferation and survival of naive and transitional B cells in the absence of stimuli [307]. But this effect is also observed (again for naive and transitional compartments) when B cells are stimulated with CpG 2006, IL-2, anti-Ig, plus CD40L, as well as for naive, transitional and memory B cells after CpG 2006 plus IL-2 stimulation, in the absence of BCR stimulation and T cell help [307]. Irradiated human UCM-MSC also promote proliferation of purified B cells stimulated with CpG 2006, IL-2, anti-Ig, and CD40L [317]. In turn, mouse BM-MSC do not interfere with proliferation of B cells isolated from mouse spleen stimulated with CpG plus IL-2, but display a strong inhibitory effect upon CpG plus IL-2, anti-Ig, and CD40L stimulation [175].

3.3.2.2. Effect of mesenchymal stromal/stem cells on B cell activation and function

Besides interfering with B cell proliferation, human BM-, UCM-, and AT-MSC also hamper B cell activation, as demonstrated by the suppression of the acquisition of activating markers (CD69 and CD25) by B cells stimulated with PHA [46].

Therefore, if MSC induce a defective B cell activation, it is expected that B cell function will be impaired as well. This is confirmed by several studies reporting an inhibitory effect of MSC over Ig production. In fact, Ig secretion by mitogen-stimulated B cells was shown to be suppressed by both human BM- and AT-MSC, wherein AT-MSC display a higher degree of inhibition [318]. At least under the influence of human BM-MSC, this effect is transversal for IgM, IgG, and IgA immunoglobulin isotypes, concerning the amount of Ig production and/or the number of antibody-secreting B cells. More specifically, the inhibition of the secretion of these three Ig classes was consistently found after stimulation with CpG 2006 alone [314], or plus CD40L, anti-Ig, IL-2, IL-4, and IL-10 [305,306] (with similar results for human periodontal ligament stem cells [306]), in MLR [55], upon LPS stimulation [319], cytomegalovirus (CMV) [319], or varicella zoster virus (VZV) [312,319]. Interestingly, BM-MSC display a more pronounced inhibitory effect for IgM, followed by IgG, whereas IgA is less affected, in MLR assays [55]. MSC-driven suppression of Ig production is also reported in mouse [175,308,320].

Remarkably, depending on the intensity of the response obtained to LPS, CMV, or VZV stimulation (which displays inter-individual variation), human BM-MSC can yield opposing effects, either suppressing or promoting IgG production, for a strong *versus* weak degree of stimulation, respectively [319]. In line with this, it was reported that human BM-MSC promote an increased secretion of IgM by naive and transition B cells, and of IgM, IgG, and IgA by memory B cells, after stimulation with CpG 2006 and IL-2 (in the absence of anti-Ig and T cell help); as well as an increased IgG and IgA production by transitional B cells stimulated with CpG 2006, IL-2, anti-Ig, plus CD40L [307]. Under the influence of this latter stimuli plus irradiated human UCM-MSC, purified B cells also show increased IgM, IgG, and IgA, in culture supernatants [317].

There are few reports concerning whether MSC influence the antigen-presenting cell function of B cells, being described that human BM-MSC does not alter HLA-DR, CD40, CD80 and CD86 expression by B cells, after activation with LPS [313], or CpG, CD40L, anti-Ig, IL-2, IL-4, and IL-10 [305,306]. Interestingly, human BM-MSC also interfere with B cell migration to secondary lymphoid organs and entering into germinal centers by decreasing the percentage of B cells expressing the chemokine receptors CCR7, CXCR4, and CXCR5, after stimulation with

CpG, CD40L, anti-Ig, IL-2, and IL-4, with [305] or without [306] IL-10. These results in the reduction of B cell migration toward CCL19, CXCL12, and CXCL13, respectively [305,306]. Of note, the abovementioned chemokines receptors are constitutively expressed by B cells and are responsible for their homing to lymphoid organs [305].

Finally, human BM-MSC also reduce the percentage of B cells producing TNF-α and IFNγ, having no effect on IL-4 and IL-10 expression, upon stimulation with CpG 2006 plus CD40L, anti-Ig, IL-2, IL-4, and IL-10 [305]. Conversely, human AT-MSC were reported to expand the IL-10-producing B cell population (known to have regulatory functions), when non-stimulated or, to a greater extent, after LPS exposure [274,310].

3.3.2.3. Effect of mesenchymal stromal/stem cells on B cell differentiation into plasma cells

The demonstration of the immunosuppressive activity of MSC over B cell's Ig production in response to stimulation is highly suggestive of an inhibitory effect on the differentiation process from B cells to plasma cells. In this sense, and attending to the phenotypic characteristics of the different maturation stages of B cells, Tabera and colleagues [315] elegantly analyzed the influence of human BM-MSC on peripheral blood B cell differentiation into plasma cells. Plasma cell differentiation was induced by B cell stimulation with CpG, anti-Ig, plus pDC, and the phenotypic analyses allowed the identification and quantification of the following B cell maturation stages: immature B cell ($CD38^-$), mature B cell ($CD38^{-/+}$), lymphoplasmocytes ($CD38^+$) and plasma cell ($CD38^{++}$), based on the expression of CD38, CD138, CD19, CCR7 and the surface *versus* cytoplasmic expression of Ig. When BM-MSC where present in the culture system, the number of plasma cells ($CD38^{++}$ $CD138^+$) generated decreased significantly, confirming that BM-MSC inhibit B cell differentiation into plasma cell [315]. Overlapping results were achieved with purified B cells stimulated with CpG, CD40L, anti-Ig, IL-4, and IL-10 co-cultured with human BM-MSC or periodontal ligament stem cells [306,309]; and by adding human BM-MSC derived membrane vesicles to CpG-stimulated PBMC cultures [314].

Accordingly, it was reported that mouse BM-MSC [321] and human UCM-MSC [308] suppress terminal mouse B cell differentiation into plasma cells. This inhibitory effect is mediated by soluble factors [308,321], in which matrix metalloproteinase (MMP)-processed CCL2 may have a prominent role [320]. Despite inducing proliferation of plasmablast, MMP-processed CCL2 was also shown to mediate the MSC-driven inhibition of STAT3 activation in mouse plasma cells; the activation of STAT3 is crucial for plasma cell differentiation, because it

induces the expression of BLIMP-1 which, in turn, suppresses the expression of PAX-5 to allow the production of Ig [320]. Remarkably, MSC induce decreased BLIMP-1 mRNA levels and increased PAX-5 mRNA and protein expression [308,320,321]; and the increased of PAX-5 protein expression is also mediated by MMP-processed CCL2 derived from MSC [320]. Besides, in mouse, MSC also suppress p38 and Akt phosphorylation [308], which have an important role in B cell proliferation and differentiation; however, in human, it was reported that MSC are able to either promote or inhibit p38 phosphorylation, depending on the type or strength of B cell stimulation [315]. Notwithstanding, a recent report describes that irradiated human UCM-MSC promote plasma cell differentiation when co-cultured with purified B cells stimulated with CpG 2006, IL-2, anti-Ig, and CD40L [317].

3.3.2.4. In vivo studies

In vivo, mouse immunization with either T-dependent or T-independent antigens resulted in the decreased serum levels of antigen-specific IgM and IgG1, upon administration of conditioned medium from BM-MSC [321]. In mouse models of systemic lupus erythematosus, the administration of human AT-MSC [274], human BM-MSC combined with cyclophosphamide [107], or murine BM-MSC [311], yielded promising results, increasing survival and decreasing the levels of circulating anti-dsDNA IgG. MSC treatment also reduced the amount of activated B cells and plasma cells [274,311], while increased the percentage of IL-10-producing B cells [274], *in vivo*. However, other study using the same model, and mouse MSC, resulted in increased levels of circulating anti-dsDNA IgG and of IgG deposition in glomeruli, compared with animal subjected to cyclophosphamide treatment [107]. In turn, in mouse models of transplantation, MSC infusion prolong the allograft survival and reduce the serum levels of allospecific Ig, as well as IgG-allospecific intragraft deposits [107]. Likewise, in hemophilic mice (induced by FVIII immunization), MSC treatment also decreased anti-hFVIII IgG titer as well as the number of anti-hFVIII Ig-secreting cells [312,320].

3.3.2.5. Concluding remarks

Analyzing the published data altogether, there are an increasing body of evidence supporting that factors such the MSC tissue source, animal species, and ratio, type and strength of stimuli, and the presence of other immune cells, determine whether MSC will have a suppressive or activating action over B cell functions and differentiation. In fact, this aspect had been clearly demonstrated for B cell proliferation.

3.4. NATURAL KILLER CELLS

3.4.1. Natural killer cells' characteristics, subpopulations, and function

Natural killer (NK) cells belong to the innate arm of the immune system and have an important role in given a prompt response against virus, bacteria, and neoplastic cells by their ability to kill self altered cells without prior stimulation. Though they also possess the ability to produce chemokines and cytokines, thus influencing other immune cells, such as DC and T cells, the main function of NK cells is, in fact, to recognize infected or tumoral cells and destroy them [322]. And to distinguish among abnormal (infected or malignant) and normal cells, NK cells rely on the signals given by their inhibitory receptors – killer immunoglobulin-like inhibitory receptors (KIR), C-lectin-type-inhibitory receptors (CLIR), and leukocyte associated Ig-like receptor 1 (LAIR1 or CD305) – and activating receptors – NKp30, NKp44, and NKp46 (known as the natural cytotoxicity receptors, NCR), NKp80, NKG2D (CD314), CD69, and DNAM1/CD96 [322,323]. Nevertheless, there are other receptors, such as 2B4 (CD244) that can either have activating or inhibiting function, depending on the adaptor proteins to which they are associated [323]. It is the final balance between activating and inhibiting signals that will dictate the NK cell decision of kill or not to kill the target cell.

Concerning the inhibitory receptors, both KIR and CLIR bind to and recognize HLA-I molecules on the target cells, and the result of this interaction gives inhibitory signals to NK cells, because a normal level of MHC-I expression is typical of a self non-altered cell, as malignant transformation or viral infection are often associated to decreased expression of these molecules [322]. Though KIR generally give inhibiting signals to NK cells after binding to and recognizing HLA-B and HLA-C molecules, there are a few KIR receptors, such as KIR2DS4, able to interact with non-HLA proteins in tumor cells and give activating signals [230]. Finally, LAIR1 binding to its ligand – the Gly-Prol-Hyp common motif of collagens type I, II, III, XIII, XVII, and XXIII – leads to the recruitment of SHIP1 to its cytoplasmic tail, making this phosphatase available to dephosphorylate the immunoreceptor tyrosine-based activation motifs (ITAM) of activating receptors (such as CD16) and, therefore, hampering their function [322].

Regarding the NK cell activating receptors, CD16 is responsible for antibody-dependent cellular-mediated cytotoxicity [230]. CD16 binds to the fragment crystallizable (Fc) region of IgG, therefore it will enable the binding of innate immune cells bearing CD16 (NK cells, monocytes, macrophages, neutrophils, and eosinophils) to target cells whose Ag had previously been specifically recognized by cells from the acquired immune system. Thus, this

cytolytic mechanism endows cells from the innate arm of the immune system with antigen specificity. After binding to the target cell through CD16, NK cells release the content of their cytolytic granules in the cell contact interface, leading to target cell apoptosis [230]. Likewise, NKp30 (CD337), NKp44 (CD336), and NKp46 (CD335) receptors, which belong to the immunoglobulin superfamily, are thought to be only able to give activating signals, thus inducing the killing of the target cells [230,324]. NKp30 is constitutively expressed on NK cells and binds to B7-H6 molecules on tumoral cells; NKp46 is expressed by resting and activated NK cells and is thought to recognize ligands on malignant cells, which haven't been identified yet; finally, NKp44, which is only present on activated NK cells, can recognize both viral and tumor-associated ligands [324]. In human, the majority of NKG2D receptors (lectin-like type 2 receptors) gives activating signals upon binding to the nonpolymorphic MHC class I chain-related protein A (MICA) and B (MICB), or to UL16 binding proteins (ULBP members), which are induced by cellular stress, such as infection or DNA damage [230,324]; however, CD94/NKG2A binding to HLA-E (a nonclassic MHC-I molecule, whose levels of expression are directly proportional to MHC-I surface density) results in an inhibitory signal [101,230]. DNAM-1 is an activating receptor that binds to poliovirus receptor (PVR) and Nectin-2 [101]. Finally, the receptor 2B4 (CD244) can either trigger activating or inhibitory signals, though there are evidences that in human NK cells it acts mainly as an activating receptor [322,323]. 2B4 binds to CD48, but it can also bind to MHC-I, which results in the inhibition of 2B4-mediated killing [323]. Accordingly, to avoid NK cell aggression, self cells express high levels of MHC-I molecules (which will give inhibitory signals to NK cells), and low levels of ligands to NK cell activating receptors [322].

Notwithstanding, NK cells can also be stimulated by factors secreted by other immune or non-immune cells, such as type I IFN, IL-12, IL-15, and IL-18 [325]. In fact, IL-12 produced by antigen-presenting cells induces NK cell proliferation and IFNγ production; in turn, IFNγ and TNF-α produced by NK cells induce DC maturation and contribute to the assembly of a Th1 response [322,325].

Concerning the effector functions, NK cells induce apoptosis on target cells by FasL/Fas interaction, by the releasing of granzyme which enters the target cell trough the pores built by perforin molecules, or by antibody-dependent cell-mediated cytotoxicity, through the action of CD16 molecule [230]. NK cells can also have a regulatory function. In fact, NK cells are able to secrete TGF-β and IL-10, being described that the former cytokine is induced after KIR interaction with soluble HLA-I molecules, which are known to be upregulated in autoimmune

diseases [322]. Moreover, NK cells can secrete soluble FasL, which can bind to and induce the apoptosis of T cells, thus controlling the immune response [322].

Importantly, NK cells are far from constitute a homogeneous cell population. It is accepted that $CD56^{dim}$ NK cells (≈90% of peripheral blood NK cells) possess a more pronounced cytotoxic function, while the main function $CD56^{brigth}$ NK cells is cytokine production [322,325]. Nevertheless, despite representing only ≈ 10% of NK cells in the peripheral blood, $CD56^{bright}$ NK cells are the most represented NK subpopulation on secondary lymphoid tissues, and $CD56^{bright}$ CD94/$NKG2A^{+}$ NK cell subset is thought to give rise to $CD56^{dim}$ NK cells [324]. NK cell heterogeneity is also patent in the type of cytokine they express and, in this line, distinct subpopulations of NK cells have been identified based on their cytokine expression pattern, as the case of NK2, NK3, NKr, and NK22: NK2 cells are characterized by producing IL-4, IL-5, and IL-13; NK3 cells express IL-10; NKr cells produce TGF-β and are involved in maternal-fetal immune tolerance; finally, NK22 cells produce IL-22 which enable these cells to limit inflammation and protect gut mucosal integrity [322].

3.4.2. Effect of mesenchymal stromal/stem cells on NK cells

In recent years, an increasing number of studies had been made with the aim to understand the influence of MSC over NK cell function and the respective underlying mechanisms. Indeed, MSC have the ability to inhibit cytokine-induced NK cell proliferation, as well as NK cell effector functions.

3.4.2.1. Mesenchymal stromal/stem cells inhibit NK cell proliferation and activation

The inhibition of NK cell proliferation in MLR plus IL-2 [75,238] or after cytokine exposure [101,102,326,327] was observed in the presence of human BM-MSC, and was shown to be mediated by both soluble factors and cell-contact mechanisms [45,327]. While in IL-15-stimulated NK cells TGF-β and PGE2 were identified as interveners in this process [327], the inhibition of proliferation by human BM-MSC over IL-2-stimulated NK cells was the result of the synergistic action of PGE2 and IDO, in which TGF-β did not participate [326]. The inhibition of proliferation is dose-dependent and more significant for $CD56^{bright}$ NK cells [327]. Of note, the inhibition of PGE2 synthesis alone does not suppress the IL-2-induced NK cell proliferation, whereas IDO inhibition show a partial effect, but significantly lower than the effect observed when both PGE2 and IDO are inhibited simultaneously [326]. It is possible that several factors released by MSC contribute to the inhibition of NK cell proliferation, and the absence of one factor could be compensated by other factors present in the cell culture; notwithstanding, it is

described that PGE2 induces IDO expression [266], but whether the induction of IDO is necessary for the inhibitory effect of PGE2 over NK cell proliferation is still elusive.

In addition, human BM-, AT-, and UCM-MSC impair PHA-induced activation of both $CD56^{dim}$ and $CD56^{bright}$ NK cells (assessed by the expression of the activator markers CD69 – considered an early activator marker – and CD25), except for UCM-MSC that are unable to inhibit the activation of $CD56^{bright}$ NK cells subset [46]. However, there are conflicting results regarding the inhibitory effect of MSC over CD69 expression under the influence of BM- and AT-MSC, although is worth mentioning that there are important differences in the culture conditions of those who detected MSC-driven CD69 decrease [46,102,328] and those who did not [33,326].

3.4.2.2. Mesenchymal stromal/stem cells inhibit NK cell function

Accordingly with the impairment of NK cell proliferation and activation, human BM-MSC also display an inhibitory effect over NK cell production of TNF-α, IFNγ, and IL-10 (more pronounced for $CD56^{bright}$ than $CD56^{dim}$ NK cells) [75,76,102,236,326,327]; over granzyme B protein [102] and perforin mRNA expression [46]; over the expression of the activating receptors NKp30, NKp44, NKG2D, DNAM-1, 2B4, CD69, and CD16 (despite some controversy regarding MSC effect on NKG2D expression [33]), as well as IL-2Rγ chain (CD132, the common γ-chain of IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 receptors) [33,46,102,326,327]. Likewise, human UCM-MSC also inhibit IFNγ production by NK cells activated with IL-12 and IL-18, alone or in combination [329]. Conversely, no changes were detected on the NK cell expression of NKp46, KIR, and CD94/NKG2A upon co-culture with human BM-MSC [33,326].

Of note, the inhibition of IFNγ production was found to be mediated by HLA-G5 in allogeneic stimulated NK cells [239]; by activin A and PGE2, in IL-12 plus IL-18-activated NK cells [329]; and by PGE2 and IDO, in IL-2 plus IL-15-activated NK cells [76,329]. Interestingly, UCM-MSC also induced the inhibition of STAT4 and NF-kB activation, as well as a decrease in T-bet expression and DNA-binding activity (T-bet is the master regulator of IFNγ production in T cells and NK cells) [329]. On the contrary, it was demonstrated that neither PGE2 nor TGF-β are involved in the downregulation of cytokines, NKG2D, and 2B4 expression in IL-15-stimulated NK cells [76,327].

Functional assays demonstrated that both human AT- and BM-MSC decrease the degranulation capability of NK cells (measured by CD107b expression at NK cell surface) [33,239], while human BM-MSC also inhibit NK cell cytotoxicity induced by allogeneic antigens

from KIR-ligand incompatible target cells [238], as well as against tumor cell lines (after stimulation with either IL-2 or IL-15) [43,75,102,326,327]; this latter process is mediated by cell-cell contact, PGE2, and IDO, and independent of TGF-β [326,327]. HLA-G5 also participates in the impairment of NK cell cytotoxic activity mediated by MSC [239]. In turn, NK cell-mediated lysis of HLA-class I negative cell lines was not affected by MSC [62,327].

Remarkably, human UCM-MSC induce the expression of CD73 on NK cells. As NK cells constitutively express CD39, the induction of CD73 expression enable NK cells to produce adenosine. In fact, increased levels of adenosine are found in purified NK cells and human UCM-MSC co-cultures. Adenosine is known to reduce TNF-α and IFNγ production by NK cells and to prevent NK cell degranulation; it also has important suppressive functions over other immune cell populations (detailed in section 4.4 of this chapter) [330].

3.5. Dendritic cells

3.5.1. Characteristics, subpopulations, and function of dendritic cells

Dendritic cells (DC) comprise a heterogeneous population of immune cells, whose main function is antigen presentation to naive T cells and to influence their differentiation towards distinct types of effector T cells. Despite this essential function to trigger the adaptive immune response, DC also activate/regulate the function of other immune cells, such as B cells, monocytes, NK, and NKT cells.

Different DC subpopulations can be identified based on their immunophenotype and localization. In the peripheral blood it is possible to distinguish myeloid DC (mDC) and plasmacytoid DC (pDC). Both subsets are negative for CD3, CD19, CD56, and CD14 markers. pDC present high expression of CD123, is positive for CD4 and CD303 (or blood dendritic cell antigen-2, BDCA-2) and negative for CD33 and CD11c; these cells have the ability to produce copious amounts of type I IFN, assuming an important role in controlling viral infections and, besides peripheral blood, pDC can also be found in lymphoid tissues [230,331,332]. In turn, peripheral blood mDC are $CD33^{+}CD13^{+}CD11c^{+}CD123^{-}CD4^{-}$, and can migrate to peripheral tissues. Peripheral blood mDC have recently been suggested to be subdived on two subpopulations: CD1c (BDCA-1)$^{+}$ DC and CD141 (BDCA-3)$^{+}$ DC [332]. Another DC subpopulation identified in the peripheral blood corresponds to 6-sulfo $LacNAc^{+}$ (slan) DC, formerly known as M-$DC8^{+}$ DC [333,334]. Despite being considered a pro-inflammatory subpopulation of DC, due

to their ability to secrete large amounts of TNF-α and IL-12 [333,334], slanDC efficiently present antigen to naive T cells, can stimulate autologous T cells in MLR (which is considered a characteristic property of DC), induce alloantigen-specific cytotoxic $CD8^+$ T cells and tumor-specific cytotoxic T cells [334,335]. These cells are phenotypically characterized as positive for 6-sulfo LacNAc, CD16, CD33, CD4, CD32, CD11b, CD11c, CD13, CD45RA, and C5aR (CD88), and negative for CD14, CD64, and CLA [333-335]. However, despite previously published studies demonstrating these cells clearly possess DC functions, slanDC were shown to be included in the peripheral blood myeloid $CD14^{-/low}CD16^+$ cell population (comprising approximately one third of them) [336] recently denominated as "non-classical monocytes" by a panel of immunology experts (chapter 1, section 3.6) [332].

In the peripheral tissues, it is possible to identify Langerhans cells, which express CD1a (important for the presentation of non-peptide antigens) and langerin, and can be isolated from the basal and suprabasal layers of epidermis [331,337]; interestingly, the classification of Langerhans cells as DC are now being challenged by their transcriptional profiling and other characteristics that suggest these cells constitute a population of tissue-resident macrophages [338]. Interstitial DC are also found in peripheral tissues, specifically in the dermis, and express DC-SIGN [337]; finally, follicular DC are localized on B cell follicles, where they secrete survival factors for B cells within the secondary lymphoid organs and provide the contact of B cells with antigens, necessary for maturation of affinity and selection processes during their differentiation into memory B cells or plasma cells [230].

Overall, after DC differentiation in the BM, the cells enter peripheral blood and migrate into peripheral tissues. At this stage, they are considered immature DC, their function is to monitor the body for signs of threat and capture foreign antigens, therefore they are highly specialized in antigen capture and possess low ability to stimulate T cells. Accordingly, the immature DC present a high antigen-capturing activity, being able to uptake antigens through phagocytosis, receptor-mediated endocytosis (using C-type lectin receptors, Fc receptors, and complement receptor CR3), and pinocytosis [230,331,339]. Moreover, they express a pattern of chemokines receptors optimized to guide them to sites of inflammation [340,341]. After the encounter with antigens in the presence of dangerous signals – such as pathogen invasion, inflammation, or cell damage – DC undergo activation and maturation. This process can be triggered by the signals received through DC's PRR (which are activated by the binding of bacterial and viral products, or components released by damaged cells), Fc receptors (which bind to antibody-opsonized pathogens), or pro-inflammatory cytokines (TNF-α, IFNγ, and IL-1β) and prostaglandins [230,340]. During the maturation process DC diminish their phagocytic

and endocytic activity (which is associated to the downregulation of endocytic and phagocytic receptors), as well as the expression of chemokine receptors for inflammatory sites, while increase the expression of proteins needed for antigen presentation: class I and class II MHC molecules, costimulatory molecules (CD40, CD80, and CD86), and cell adhesion molecules. Besides, the expression of CD83 (considered as an activation marker for DC) is induced during the maturation process, similarly to CCR7, which follow the same pattern [331,339,340]. Importantly, the induction of this chemokine receptor enable the mature DC to migrate into the T cell areas of secondary lymphoid organs, where they can present the MHC-bound antigens to T cells, being capable to prime naive T cells and trigger the adaptive immune response [331,340,341].

Upon activation, depending on the signals given by the pathogen (as different pathogenic agents will activate distinct PPR), DC will be conditioned to produce a specific cytokine pattern that ultimately determines the sense of T cell differentiation. Roughly, gram-negative bacterial and viral products will stimulate TLR4/5 and TLR3/7/9, respectively, inducing DC to produce IL-12, which contributes to a Th1 immune response; the binding of fungi products to Dectin-1, stimulates the secretion of TNF-α, IL-6, and IL-23 by DC, leading to a Th17 response; in turn, the activation of TLR2/6 by these same pathogens, induces IL-10, TGF-β, and retinoic acid expression by DC, conducting to Treg differentiation; finally, TLR2/1 activation by helminthes will stimulate IL-10 expression by DC, while induces IL-4 production by mast cells, which will promote naive T cell differentiation into Th2 effector cells [230,342]. As pDC do not express TLR2, TLR3, TLR4 and TLR5, they are unable to respond to bacteria the same way mDC do [341]. In fact, the more recognized function of pDC is the production of great amounts of type I IFN in response to virus, which occurs through TLR7/9-dependent and independent pathways [343]. Of note, pDC also secrete TNF-α and IL-6 in response to virus; this latter, together with IFN-α, contributes to B cell differentiation into plasma cells [343]. Notwithstanding, the production of IFN-α can further activate pDC, mDC (including the promotion of IL-12 expression by mDC) and other antigen-presenting cells (monocytes and B cells), can activate NK (improving their cytolytic activity), NKT, and $CD8^+$ T cells, and bias T cell differentiation toward a Th1 phenotype [341].

Remarkably, the internalized and processed antigens can be presented by DC to $CD8^+$ T cells, in the context of MHC-I molecules (this process is called cross-presentation), allowing an effective activation of naive $CD8^+$ T cells and subsequent differentiation in CTL [230,331].

Conversely, specific conditions can also dictate the development of tolerogenic DC, which are considered to have an immature or semi-mature phenotype, being able to present antigens, but lacking the expression of costimulatory molecules or cytokines needed for the development of a T cell-mediated immune response. In fact, these tolerogenic DC have the ability to delete auto-reactive T cells, render T cells anergic, or induce their differentiation into regulatory T cells, including $CD4^+$ Treg and IL-10-producing suppressive $CD8^+$ T cells. This process is of utmost importance for maintaining central and peripheral tolerance, and both mDC and pDC participate in it [337,341].

The low frequency of DC in peripheral blood has definitely contributed to the common use of *in vitro* generated DC in research, and the studies on the effect of MSC on DC are not the exception. Caux et al. (1992) [344] and Romani et al. (1994) [345] published the first papers describing the protocols for *ex vivo* generation and expansion of human DC able to yield a reasonable amount of cells. In these studies, DC were generated from $CD34^+$ HPC from UCB or from mobilized peripheral blood, in culture with GM-CSF alone or in combination with TNF-α; or from peripheral blood $CD34^+$ HPC or $CD34^-$ cells cultured with GM-CSF plus IL-4 [344,345]. Since then, other protocols for DC generation and expansion have been developed (as thoroughly reviewed by Zou et al. [339]), using different precursor cells and distinct cytokines for either DC generation or the induction of DC maturation. Nevertheless, though DC can be generated from different types of hematopoietic cells, isolated from several tissues, and upon exposure to a vast array of cytokines, the majority of the studies uses peripheral blood monocytes or BM $CD34^+$ HPC, with GM-CSF in combination with either TNF-α or IL-4, a protocol which generates immature DC [339]. *In vitro* induction of DC maturation may be achieved by several pro-inflammatory cytokines, namely TNF-α, IL-1β, as well as by CD40L or bacterial products, such as LPS [331]. However, the great diversity of techniques for *ex vivo* DC generation and expansion is associated to distinct cellular responses, which have complicated the comprehension of the biology of DC in the past, and might hinder the understanding of the influence of MSC on DC activation, maturation, and function, in the present. For this reason, the use of DC directly isolated from human tissues is a valuable approach. Even though, the investigation of the effect of MSC on naturally occurring human peripheral blood DC was carried out in a small number studies [79,236,333].

3.5.2. Effect of mesenchymal stromal/stem cells on dendritic cells

The suppressive effect of MSC over DC is evident at different stages of DC ontogeny. In fact, according to the majority of published data, MSC have the ability to impair both

monocyte and CD34+ HPC differentiation into DC; to prevent DC maturation and, as consequence, DC effectiveness to prime T cells; and, finally, MSC induce a tolerogenic phenotype on DC, which become efficient suppressors of the T cell activation and function, or induce T cells to have a regulatory function.

3.5.2.1. Effect of mesenchymal stromal/stem cells on dendritic cell differentiation

When *in vitro* generation of DC from either BM precursors [346,347], UCB-derived $CD34^+$ HPC [348], or peripheral blood monocytes [25,47,53,348-351] is concerned, different studies demonstrated that human MSC prevent this differentiation process. Specifically regarding the *in vitro* differentiation of peripheral blood monocytes into DC under the influence of GM-CSF plus IL-4, human BM-MSC [25,47,53,348,349,352], AT-MSC [47], UCM-MSC [25], and amniotic mesenchymal tissue cells [351] are potent inhibitors of this process; conversely, UCB-MSC do not share this feature [353]. In fact, the presence of MSC prevents the downregulation of CD14, upregulation of CD1a, monocyte proliferation and acquisition of a typical DC morphology, expected during monocyte differentiation into DC. Moreover, BM-MSC, UCM-MSC, and UCB-MSC impair the endocytic activity of immature DC [25,350,353]. Similarly, human BM-MSC also prevent PBMC differentiation into mDC upon allogeneic stimulation in MLR [238]. Evidences point to the involvement of IL-6 [348], macrophage colony-stimulating factor (M-CSF) [348], PGE2 [53], and Notch signaling [347] in the inhibition of DC differentiation driven by human BM-MSC, but contradictory results have been published regarding this subject.

As expected, BM $CD34^+$ HPC and monocyte-derived DC generated in the presence of either human BM-MSC [47,53,347-349], AT-MSC [47], or amniotic mesenchymal tissue cells [351] failed to undergo maturation triggered by LPS, CD40L, or TNF-α. In fact, after stimulation of DC differentiated in the presence of MSC, there were decreased expression of HLA-DR, CD80, CD86, CD83, and IL-12 in DC, along with a reduced ability to stimulate T cell allogeneic activation in MLR, namely decreased T cell proliferation, expression of activation markers, and production of IFNγ. Interestingly, the presence of BM-MSC or AT-MSC also induces the upregulation of IL-10 expression by DC [47,347]. It is worth mentioning that monocyte-derived DC, differentiated and maturated in the presence of human periapical lesions-derived MSC, express higher levels of IDO, immunoglobulin-like transcript (ILT)3 and ILT4, a feature shared with tolerogenic DC [354]. Besides, these DC were shown to induce either T cell anergy, or suppressive T cells, demonstrated by the increased percentage of $CD4^+CD25^+FoxP3^+$ Treg cells, as well as of IL-10-producing $CD4^+$ T cells (Tr1) [347,354]. The induction of suppressive T cells is

mainly attributed to IDO [354] and Notch signaling [347], but ILT3 and ILT4 participate as well [354].

3.5.2.2. Effect of mesenchymal stromal/stem cells on dendritic cell maturation

To better understand whether MSC interfere specifically with DC maturation, a few working groups had investigated the influence of MSC over immature monocyte-derived DC, generated by GM-CSF plus IL-4 in the absence of MSC, and matured by LPS in the presence of MSC.

Co-culture of BM-MSC [25,31], AT-MSC [31], or UCM-MSC [25] with human peripheral blood monocyte-derived mDC (generated by GM-CSF plus IL-4) and pDC (generated by IL-3), stimulated with LPS (as a maturation-driving factor), results in the reduction of expression of markers associated to DC maturation: CD80, CD86, CD83, and HLA-DR. In the same line, BM-MSC, AT-MSC, and amniotic mesenchymal tissue cells also suppress TNF-α, IFNγ, and IL-12 production by mDC upon LPS-stimulation [31,351], while increase IL-10 secretion by pDC, and this latter effect is mediated by PGE2 [31]. Likewise, for human peripheral blood monocyte-derived mDC (generated by GM-CSF plus IL-4), in co-culture with T cells and stimulated with the superantigen staphylococcal enterotoxin B (SEB), the presence of human BM-MSC also reduces HLA-DR, TNF-α, and IL-12 expression, while have no effect neither on IL-1β nor CD86 expression [355]. Remarkably, co-culture of human BM-MSC with mature monocyte-derived DC also results in the downregulation of HLA-DR, CD1a, CD80, CD86, and CD83 [349].

3.5.2.3. Effect of mesenchymal stromal/stem cells on dendritic cell function

Importantly, it is consistently found that DC which undergo maturation under the influence of MSC display an impaired ability to present antigen and to induced allogeneic T cell proliferation, as well as a diminished capability to promote IFNγ expression by T cells, which is valid for both DC generated in the presence [348,349] and in the absence [11,31] of MSC. This is in accordance with the lower expression of MHC-II, costimulatory molecules and cytokines (such as TNF-α and IL-12) with an important function in T cell activation. Remarkably, after contact with MSC-treated DC, T cells become non-responsive to a second round of activation [11,354]. Furthermore, the effects exerted by human BM-MSC on DC (as well as on monocytes), endows these antigen-presenting cells with the ability to inhibit T cell response to the superantigen SEB, detected by the impairment of both T cell proliferation and IFNγ production [355]. This effect is partially dependent on IL-10 [98,355]. In fact, by the

maintenance of DC in an immature or semi-mature state, MSC may contribute to the induction of Treg cells [98,99].

A recent work found that the presence of human BM-MSC during monocyte-derived DC maturation by antigen loading, impairs DC to establish active immune synapses with T cells, thus hindering T cell priming [11]. This inability results from the alteration of actin distribution in DC, which is mediated by MSC-driven inactivation of Rac-1, a protein which participates in actin cytoskeleton changes and is required for a normal interaction between DC and T cells [11]. Similar data were previously published in murine [356].

Accompanying this tendency of impaired ability of DC to prime T cells, MSC also reduces DC ability to migrate towards CCL21, the CCR7 ligand which guides mature DC toward T cell areas of the secondary lymphoid organs [346]. The human BM-MSC-derived reduction of BAFF secretion by DC can also compromise B cell survival and function [311].

Similar results were found for rat and murine, wherein MSC inhibit TNF-α or LPS-induced maturation of DC, by preventing the upregulation of CD80, CD86, and MHC-II, and inhibiting IL-12, TNF-α, and IL-10 secretion [356-360]. Moreover, there are direct evidences that murine BM-MSC actually interfere with immature DC ability to process the antigen and present it in the context of a MHC-II molecule, and also impair antigen cross-presentation by DC, therefore, reducing antigen-specific proliferation of both $CD4^+$ and $CD8^+$ T cells [358,360]. In turn, mouse mature DC co-culture with BM-MSC also results in the decrease of CD40, CD80, CD86, and IL-12 expression, and increased expression of TGF-β, IL-10, and Jagged-2 by DC [361,362]. MSC-treated DC not only were unable to stimulate allogeneic T cell proliferation [361,362], but also had the ability to suppress T cell proliferation, IFNγ, and IL-2 production in MLR (with lymphocytes activated by non-MSC-treated mature DC), or by concavalin A-stimulated lymphocytes, demonstrating that mouse BM-MSC are able to induce immunoregulatory functions in mature DC [361]. The immunoregulatory properties of MSC-treated mature DC were mediated by Jagged-2, and were demonstrated to be functional in *in vivo* assays, in mice [361]. In addition, CCR7 surface expression and DC ability to migrate towards CCL19 is decreased [358]; accordingly, *in vivo* studies demonstrated that MSC administration reduces the migration of antigen-loaded DC to secondary lymphoid organs [356,360].

In opposition, a few number of studies support the hypothesis that once differentiated *in vitro* without the influence of MSC, immature DC would undergo normal maturation regardless the presence of MSC. Spaggiari and colleagues [53] described there were no differences among LPS-induced maturation of immature monocyte-derived DC (generated by

GM-CSF plus IL-4) in the presence or absence of human BM-MSC, concerning the upregulation of CD80, CD86, and CD83, as well as IL-12 production and the ability to stimulate T cells in a MLR. Analogous results were obtained by Li and colleagues (for DC derived from BM $CD34^+$ HPC cultured with GM-CSF, Flt3-L, and TNF-α) [347] and Aldinucci et al. [11], except that in this latter study MSC impaired DC ability to stimulate T cells. In turn, human UCB-MSC were shown to promote monocyte-derived DC maturation, even in the absence of a stimulator agent, and do not interfere with LPS-induced DC maturation, except for MSC ability to further increase IL-12 secretion by DC [353]. UCB-MSC also increase CCR7 expression by LPS-activated DC and increase their migration towards CCL21 [353]. Similar observations were made for human periapical lesions-derived MSC, which actually slightly increased the DC expression of HLA-DR, CD40, CD86, CD83, CCR7, IL-23, and IL-27 (while reduced IL-12 production), as well as DC allostimulatory potential, upon treatment with a pro-inflammatory cytokine cocktail (TNF-α, IL-6, IL-1β, and PGE2) [354].

3.5.2.4. Effect of mesenchymal stromal/stem cells on naturally occuring peripheral blood dendritic cells

Concerning the studies performed in naturally occurring human peripheral blood DC, an overall inhibition of DC pro-inflammatory functions was observed. All the studies described subsequently were carried out in freshly isolated peripheral blood mDC [79,236], pDC [236], and slanDC (or M-$DC8^+$ DC) [333], co-cultured with human BM-MSC.

Co-culture with human BM-MSC, in the absence of stimulating agents, was found to increase the percentage of $CD83^+$ mDC and upregulate HLA-DR expression by this cell population [79], while decreased the percentage of $CD83^+$ cells and the expression of HLA-DR in slanDC [333]. Under the same conditions, this latter DC subpopulation also displayed a reduced expression of CD86 and ICAM-1, while the expression of the inhibitory molecules ILT3 and ILT4 augmented [333]. Interestingly, when activated with LPS plus IFNγ, BM-MSC downregulate HLA-DR expression in mDC [79].

A consistent MSC-induced reduction of TNF-α expression was found for LPS-stimulated slanDC [333] and LPS or LPS plus IFNγ-activated mDC [79,236], an effect mediated by PGE2 [236,333]. In the same line, MSC impair the LPS-induced upregulation of the protein expression of CCL4 (MIP-1β), IL-6, and IL-12, and the mRNA levels of IL-1β in mDC, along with an increased secretion of IL-10 by pDC and mDC [79,236,333]. Again, PGE2 produced by MSC mediates the inhibition of IL-12 production [333].

Finally, MSC-treated slanDC (without stimulation) were found to be ineffective in stimulating allogeneic T cell proliferation, whereas MSC contact with LPS-stimulated slanDC abrogated their ability to trigger a Th1 immune response [333].

3.5.2.5. In vivo studies

In vivo, MSC infusion was shown to inhibit DC endocytosis, maturation, and IL-12 production, in a pancreatic islet transplantation mouse model [363]; to decrease the expression of CD40 and MHC-II in spleen DC from EAE mice [129]; to induce a tolerogenic phenotype in DC from a rat heart transplantation model [364]; and to reduce the migration of activated DC to the lymph nodes [360].

3.6. Monocytes and macrophages

3.6.1. Characterization, subsets, and function of monocytes

Monocytes comprise 5% to 10% of peripheral blood leukocytes. These immune cells migrate into tissues where they differentiate into potent phagocytic cells, macrophages. Once in the tissue, macrophages may integrate the tissue-resident mononuclear phagocytic cell population (which comprises both macrophages and DC), as is the case of osteoclasts, histiocytes, microglial cells, alveolar macrophages, or liver Kupffer cells [230,365]. Importantly, recent data strongly suggest that not all tissue-resident macrophages derive from peripheral blood monocytes; in fact, an important proportion of these cells has embryonic origin [338], though this proportion varies for different tissue types; for instance, fetal-derived macrophages are more represented in microglia and less represented in the gastrointestinal tract, in steady-state conditions [338]. Beyond the great phagocytic activity of monocytes/macrophages and their immune sentinel function, these cells also play other important roles in the immune response – as the production of cytokines (pro-inflammatory or anti-inflammatory) and antigen presentation – as well as in the clearance of interstitial environment (including the phagocytosis of cell debris, apoptotic cells, damaged and old erythrocytes, erythroid cells' expelled nuclei, among others) and in tissue repair [230,332,365,366].

3.6.1.1. Identification of peripheral blood monocyte subsets

Recently, a group of experts from the International Union of Immunological Societies (IUIS), World Health Organization (WHO), Dendritic Cells for Novel Immunotherapies (DCTHERA) European Network of Excellence, and European Macrophage and Dendritic Cell Society achieved a consensus for the nomenclature of monocyte and DC populations in human peripheral blood [332]. This consensus is particularly important for DC subpopulations because their identification based on distinct markers confuses the integration of the results obtained by different research groups. Another cell population that benefited from this consensus was $CD14^{-/low}CD16^{+}HLA\text{-}DR^{+}$ cells (designated by the consensus as non-classical monocytes), whose nature of monocyte *versus* DC had been discussed in the last decade. In short, the consensus include three subpopulations of monocytes, two subpopulations of mDC and a single population of pDC [332]. Peripheral blood monocyte subpopulations can be distinguished by their distinct phenotypic signature: classical monocytes present a strong expression of CD14, CD33 and HLA-DR, in the absence of CD16; intermediate monocytes express high levels of CD14 as well, but display an increasing expression of CD16 and a slight decrease of CD33 expression, compared to classical monocytes; non-classical monocytes express CD16 together with a dim to negative expression of CD14, they present the highest expression of CD45 and the lowest expression of CD33 among the three monocyte subpopulations [332]. All the three monocyte subpopulations express CD300e (or IREM-2), a marker that appears at later stages of monocyte differentiation and is not present in mDC [79,367].

3.6.1.2. Phenotypic and functional characteristics of monocytes

Non-classical monocytes have been subject of study from two decades now and were primarily ascribed to the monocytic lineage due to their ability to produce reactive oxygen species (ROS) and to the expression of cytoplasmic esterase [368]. Though this cell population seems to arise from monocytes, its nature of monocyte *versus* DC has been extensively discussed. The debate was further intensified with the discovery that about one third of the $CD14^{low/-}CD16^{+}$ non-classical monocytes correspond to a cell population previously identified as peripheral blood M-DC8+ DC [335,336,368], now designated as 6-sulfo LacNac+ DC, or slanDC (detailed in the section 3.5 of this chapter) [333,334], which possess the ability to present antigens to naive T cells and are excellent stimulators of autologous MLR, features attributed to DC [335]. Nevertheless, recent studies on expression profiling and hierarchical clustering have supported the close relationship among classical and non-classical monocytes [332], which was further sustained by the existence of a cell subpopulation with intermediate

characteristics between classical and non-classical monocytes, the intermediate monocytes, and which describe a clear phenotypic continuum between the loss of CD14 expression and the acquisition of CD16 [332]. Interestingly, this phenotypic continuum is also visible by analyzing other markers, such as CD64, CD115, CD163, CCR2, and CX3CR [369], and additionally supported by the continuous increase of CD45 intensity, accompanied by the decrease of CD33 expression and SSC light dispersion properties, sequentially from classical to intermediate and to non-classical monocytes [79]. Moreover, in the course of an infection or after *in vitro* stimulation with macrophage colony-stimulating factor (M-CSF), it is verified an increase of intermediate monocytes proportion, followed by an increase of non-classical monocytes [332]. These data and the fact that non-classical monocytes can give rise to both DC and macrophages, made the panel of experts agreed that this myeloid $CD14^{low/-}CD16^{+}$ cell population would be best called monocytes instead of DC [332]. Interestingly, macrophages derived from non-classical monocytes display higher phagocytic capability and a different protein expression pattern, in relation to classical monocytes-derived macrophages [369]. Non-classical monocytes are also more prone to differentiate into DC than the remaining monocyte subpopulations [369].

Non-classical monocytes comprise approximately 0.7% of peripheral blood leukocytes from healthy individuals, being less represented in normal BM (≈0.2%) [368]. Phenotypically they are characterized as $CD14^{-/low}CD16^{+}$HLA-DR^{+}; being also positive for CD4, like the remaining peripheral blood monocyte subpopulations. Non-classical monocytes display a higher expression of HLA-DR and CD86, and lower expression of CD14, CD11b, CD33, and CD38, compared to classical monocytes [332,368]; and in opposition to classical monocytes, non-classical monocytes express CD16 and lack CD64, myeloperoxidase, lysozyme and α-naphthyl acetate esterase expression [368]. Of note, these enzymes, with an important immune function in monocytes, are present from early stages of monocytic differentiation to macrophages [368].

Interestingly, the morphological analysis reveals the presence of cytoplasmic veils and pinocytic vesicles in non-classical monocytes, a feature shared with DC and not found in classical monocytes [368]. Functionally, the Ig/complement-mediated phagocytic activity and oxidative burst activity against *E. coli* of non-classical monocytes is lower compared to classical monocytes, and higher compared to both mDC and pDC [368]. Similarly to classical monocytes and mDC, non-classical monocytes have the ability to produce IL-1β, IL-6, IL-12, and IL-8 in response to LPS plus IFNγ, however the percentage of cells producing cytokines is lower compared to classical monocytes and mDC, also, the amount of cytokine produced *per* cell is

lower for non-classical than classical monocytes [368]. In contrast, in our hands, non-classical monocytes demonstrated to be more efficient TNF-α producers than classical monocytes, displaying equal percentage of producing cells but a higher amount of cytokine produced in a *per* cell basis, after the exposure to LPS plus IFNγ [79], as previously described by others [332,334].

Compared to peripheral blood mDC, non-classical monocytes express higher levels of CD86 and CD45, along with lower levels of HLA-DR, CD33, and lower antigen-presenting cell abilities [79,368]. A very complete and interesting study, with an extensive functional, morphologic, and phenotypical characterization of peripheral blood classical monocytes, non-classical monocytes, and DC – comprising the expression levels of adhesion molecules, costimulatory molecules, Ig Fc receptors, cytokine receptors, complement receptors, and complement regulatory proteins – and showing the differences among these cell populations, was published by Almeida and colleagues in 2001 [368].

In turn, the few studies concerning the function of intermediate monocytes demonstrated this population produce the highest levels of IL-10 upon LPS or zymosan exposure (which stimulate TLR4 and TLR2, respectively), possess the highest ability to present antigen to T cell, induce the highest levels of antigen-specific production of IFNγ by T cells, as well as the strongest T cell proliferative response to alloantigen, compared to the remaining monocyte subpopulations [369]. Intermediate monocytes also have an important role in angiogenesis [369].

3.6.1.3. Phenotypic and functional characteristics of macrophages

As previously referred, once monocytes achieve the peripheral tissues and differentiate into macrophages, they can assume distinct phenotypes and functions, depending on the current tissue needs. In this sense, in an injured tissue (in the sequence of trauma, infection, toxicity, ischemia, or autoimmune disease), the presence of a microenvironment rich in damage-associated molecular patterns (DAMP), or pathogen-associated molecular patterns (PAMP), will promote a pro-inflammatory response by immune and non-immune cells, through their pattern recognition receptors (PRR), which include TLR, C-type lectin receptors (CLR), retinoic acid-inducible gene (RIG)-I-like receptors (RLR), scavenger receptors, and NOD-like receptors (NLR) [342,365,370]. These conditions (which stimulate IFNγ secretion by NK cells and TNF-α production by macrophages) promote macrophage polarization to classically activated macrophages (M1 macrophages), which are pro-inflammatory macrophages that support Th1 or Th17 immune responses and display high antimicrobial potential. M1

macrophages (which can be generated *in vitro* by exposure to IFNγ or GM-CSF) are characterized by the production of IL-1, IL-6, IL-12, IL-23, TNF-α, ROS, and reactive nitrogen species (RNS), expression of inducible nitric-oxide synthase (iNOS) and IL1-R, together with high surface levels of MHC-II [365,366,370,371]. Upon activation, M1 macrophages (along with other tissue resident cells: stroma cells, DC, and mast cells) secrete chemokines that attract neutrophils and monocytes to the site of injury [338]. When the factors underlying the tissue injury are controlled, the resolution of inflammation takes place, which is accompanied by the decrease of PAMP and DAMP and increase of apoptotic cells (to which contributes the neutrophils formerly recruited to the injury site that, at this stage, are undergoing apoptosis). Macrophage clearance of apoptotic neutrophils induces the expression of anti-inflammatory molecules (TGF-β and IL-10) and growth factors (TGF-β and PDGF), contributing to inflammation resolution and tissue repair, respectively [365,370]. These anti-inflammatory tissue macrophages are likely to correspond to the alternatively activated macrophages (M2c macrophages) obtained upon *in vitro* stimulation with IL-10 and TGF-β, among other factors [365,371]. Of note, M2 macrophages both recruit and induce Th2 and Treg cells, which further supports their anti-inflammatory role [370]. However, in the cases where complete tissue repair is compromised, there is a predominance of profibrotic cytokines in the tissue microenvironment, which further induces the expression of profibrotic cytokines by macrophages as well, accompanied by the release of fibronectin and other extracellular matrix components, and by the expression of mannose and scavenger receptors, IL-1R11, FIZZ, and YM-1, characteristic of M2a macrophages [365,366]. M2a macrophages are obtained *in vitro* after exposure to IL-4 and IL-13, but whether M2a and M2c represent two distinct populations *in vivo* remains elusive [365]. Conversely, tissue macrophages have also the ability to limit and reverse fibrosis through the expression of MMP and CXCL10 (this latter can inhibit fibroblast proliferation in bleomycin-induced pulmonary fibrosis), however, surface markers able to identify these MMP-secreting fibrolytic macrophages are still not identified [365].

Notably, M2 macrophages display and increased expression and activity of CD39 and CD73, generating extracellular adenosine, which possess anti-inflammatory properties [372]; moreover, they secrete great amounts of IL-10 and have the ability to induce *de novo* $CD4^+CD25^+FoxP3^+$ Treg cells [373]. Besides M2 macrophages, other macrophage subsets with immunosuppressive function have been reported: regulatory macrophages (which produce high levels of IL-10), myeloid-derived suppressor cells, and tumor-associated macrophages [370]. Tumor-associated macrophages contribute to the maintenance of an immunosuppressive microenvironment in tumor surroundings, they express TGF-β, IDO, B7-

H4, and ILT3, actively recruit Treg and Th2 cells, and produce growth factors, angiogenic factors, and MMP9, promoting tumor cell proliferation and metastasis [374]. These cells derive from circulating monocytes recruited into tumor which, under the conditions of tumor microenvironment – containing LIF, IL-6, oncostatin M (OSM), and granulocyte colony stimulating factor (G-CSF) – differentiate into macrophages with immunosuppressive function and, despite the similarities with M2a and M2c macrophages, constitute a distinct cell subset [370,374].

3.6.2. Effect of mesenchymal stromal/stem cells on monocytes

The majority of published data regarding the effect of MSC on monocytes is more focused on the pro-inflammatory function of these cells, being unequivocal that MSC impede monocyte pro-inflammatory activity at different levels. Interestingly, MSC were also shown to increase monocyte survival (associated to downregulation of mRNA levels of apoptosis-related genes), and to augment the percentage of $CD14^+CD16^+$ monocytes [285].

3.6.2.1. Mesenchymal stromal/stem cells inhibit in vitro monocyte differentiation into dendritic cells

As previous mentioned in "Dendritic cells" section (section 3.5.2.1), human MSC impair *in vitro* monocyte differentiation into DC [25,47,53,348-351,375], giving rise to $CD14^+$ cells with impaired ability to stimulate allogeneic T cells [45,53,351,375]. MSC-derived PGE2 [53] and IL-6 [375] contribute for these effects.

3.6.2.2. Mesenchymal stromal/stem cells inhibit pro-inflammatory cytokine production by monocytes

Clearly, human BM-MSC have a negative impact over monocytes' ability to produce pro-inflammatory cytokines. Specifically, human BM-MSC decreased TNF-α and CCL4 (MIP-1β) production by LPS-activated monocytes (observed for classical, intermediate, and non-classical monocytes) [79,236], and reduce TNF-α and IL-12 secretion (with no effect on IL-1β) by monocytes in co-culture with T cells stimulated with the superantigen staphylococcal enterotoxin B (SEB) [355]. Remarkably, human BM-MSC also inhibit CCL3, CCL5, CXCL9, and CXCL10 mRNA expression in monocytes [79]. Likewise, human MSC suppress the production of IL-1β and ROS by macrophages stimulated with LPS or ATP [376].

3.6.2.3. Mesenchymal stromal/stem cells interfer with monocyte's allostimulatory ability

Human UCM-MSC also impair monocyte ability to induce proliferation in allogeneic T cells, which is partially due to the action of PGE2 [248]. Interestingly, mouse BM-MSC-derived PGE2 was reported to induce IL-10 expression by monocytes and macrophages [377], a cytokine with important suppressive functions over T cells. According to the impaired allostimulatory abilities, it was demonstrated that human BM-MSC, AT-MSC, UCM-MSC, and UCB-MSC hamper HLA-DR upregulation in monocytes [248,268,355], whereas human AT-MSC also decreased the expression of CD80 and CD86 [268].

3.6.2.4. Mesenchymal stromal/stem cells induce a regulatory function in monocytes and macrophages

Besides the downregulation of pro-inflammatory mediators, MHC-II molecules, and costimulatory molecules, human MSC enhance the expression of immune regulatory factors. AT-MSC upregulate ILT3 and ILT4 expression by monocytes [268]; and human BM-MSC co-cultured with either purified monocytes or PBMC induce the expression of IL-10 in monocytes [52,285,375], a process mediated by IDO [52] and IL-6 [375]. IL-10 induction is accompanied by the upregulation of CD206, CD163, and CCL18 [52,285]; these are typical markers of M2 macrophages and, remarkably, CCL18 enables monocytes to induce Treg cells [285]. Besides BM-MSC, human UCM-MSC and UCB-MSC also induced CD206 upregulation in peripheral blood monocytes [248].

Accordingly, human BM-MSC were shown to have a direct effect in skewing the polarization of monocytes towards $CD163^{+}CD206^{+}$ M2 macrophages [285,378,379]. All these effects are mediated by MSC-derived M-CSF, though other soluble factors are involved in the process [285]. Likewise, the presence of human placental MSC during granulocyte-macrophage colony-stimulating factor (GM-CSF)-induced monocyte differentiation into M1 macrophage, results in a shift to M2 macrophages differentiation [371]. In the same line, concerning macrophage functions, MSC reduced the expression of pro-inflammatory cytokines (IL-1α, IL-1β, IL-12p70, IL-17, CCL3, TNF-α, and IFNγ) and costimulatory molecules (CD40, CD80, and CD86), while increased the expression of IL-10, IL-6, VEGF, and co-inhibitory molecules (CD273, CD274, and B7-H4), and enhanced macrophage phagocytic activity over apoptotic cells [371,378,379]. Thus, accrding to the published data, MSC imprint a M2-like functional profile in macrophages.

Similar results were obtained in animals: in murine macrophages (RAW264.7) the zymosan-induced expression of TNF-α and IL-1α was hampered by TNF-α stimulated gene/protein 6 (TSG-6) produced by human MSC [380]; likewise, in mouse peritoneal macrophages stimulated with LPS, the presence of mouse BM-MSC impairs the production of the pro-inflammatory cytokines TNF-α, IFNγ, IL-6, and IL-12p70, and the upregulation of CD86 and MHC-II, while increases IL-12p40 and IL-10 expression, as well as macrophages ability to phagocyte apoptotic cells [381]. Accordingly, the ability to activate antigen-specific T cells was also reduced in macrophages co-cultured with MSC [381]. Of note, the downregulation of TNF-α, IL-6, and IL-12p70, and the promotion of IL-10 expression in LPS-stimulated mouse macrophages was mediated by PGE2 derived from MSC [381]. The low expression of pro-inflammatory cytokines, associated to high expression of IL-10 and enhanced ability to phagocyte apoptotic cells, are features that resemble the M2 macrophages involved in the resolution of inflammation. And, in the same line, similar results demonstrating that BM-MSC favor M2 macrophage differentiation were obtained in mouse BM-derived macrophages [382].

3.6.2.5. In vivo studies

Importantly, the MSC-induced decrease of TNF-α, IL-6, and IL-12p70, upregulation of IL-10 [377,381,383], and polarization towards M2 phenotype [382,384] was confirmed *in vivo*. In a mouse model of acute myocardial infarction, infusion of either human BM-MSC or UCM-MSC increased the proportion of M2 macrophages in peripheral blood and infiltrating the myocardium, accompanied by increased IL-10 levels and decreased IL-1β and IL-6 in the infarcted area [383]. Accordingly, a rat model of myocardial infarction showed that M2 macrophages were preferentially localized near MSC infiltrating the heart [382]. Moreover, human [384] and mouse [377] BM-MSC were shown to reduce the mortality in sepsis mouse models. The improved outcome was due to the increased phagocytic activity of macrophages against bacteria [384], and to increased IL-10 secretion by monocytes and macrophages [377] induced by MSC. IL-10 upregulation is mediated by PGE2 secreted by MSC, and IL-10 secretion by tissue macrophages seems to reduce neutrophil infiltration and organ oxidative damage [377].

3.6.2.6. Crosstalk between Mesenchymal stromal/stem cells and monocytes

Remarkably, under certain experimental conditions, the crosstalk between human MSC and monocytes enhances or is essential to the inhibition of T cell responses [52,248,268,355,385]. If, in the one hand, it was demonstrated that IL-1β produced by monocytes induces the expression of mediators with immunosuppressive function (e.g. PGE2

and IL-6) by UCM-MSC, and PGE2 is necessary to inhibit CD3/CD28-induced $CD4^+$ and $CD8^+$ T cell proliferation and IFNγ production [385,386]; on the other hand, MSC induce IL-10 expression by monocytes (via IL-6 and IDO) [52,285,375] which exerts suppressive effects over T cells [52]. Importantly, the dependence on monocytes/macrophages was also demonstrated *in vivo*, in a mouse model of sepsis, in which it was reported that depletion of monocytes and macrophages in the mouse made BM-MSC lose their beneficial effect on survival [377].

3.7. NEUTROPHILS

3.7.1. Neutrophils' characteristics and function

Neutrophils are the most represented leukocyte population in the peripheral blood (≈50-70% of total white cells) and act as the first line of defense against pathogenic agents. They are the first cells to arrive to the site of injury, where they have an important role in phagocytosis, pathogen killing (through the production of ROS and anti-bacterial proteins), and secretion of chemotactic factors able to recruit monocytes/macrophages [230,325]. Neutrophils are also highly versatile cells and, depending on the microenvironment, they can functionally behave as pro-inflammatory cells (producing the pro-inflammatory cytokines TNF-α, IL-1β, and IL-12p70), as immune suppressive and anti-inflammatory cells (associated to the production of IL-1Ra and TGF-β), can induce B cell class switching, or assume pro-angiogenic or pro-fibrogenic functions [325,387]. Remarkably, after exposure to GM-CSF, mouse BM neutrophils acquire morphological, phenotypic and functional properties of DC (such as the expression of CD11c, MHC-II, CD80, CD86, and IL-12, along with the ability to present antigen to naive CD4+ T cells), but retain neutrophil features (as the expression of Ly6G, CXCR2, and 7/4, and anti-microbial properties, including neutrophil extracellular traps (NET) formation) [388].

Despite being recognized the existence of functional heterogeneity in neutrophils, the information on the differentiation-promoting factors, kinetics among the different neutrophil subsets, and assays to clearly assess the mechanisms underlying their different functions, are still scarce. An efficient panel of markers for the phenotypic identification of the abovementioned distinct functional neutrophil subsets is also still underway, namely for the recently described granulocyte myeloid-derived suppressor cells. This cell population is constituted by neutrophils with the ability to suppress the adaptive immune response. The mechanisms that have been suggested to be employed by this neutrophil subset are the

increased arginase activity (which hampers T cell proliferation and activation by depleting L-arginine, and is also involved in the host defense against fungi), and the production of ROS (able to suppress T cell activation and proliferation, and also implicated in bacterial killing and immune cell chemotaxis) [387].

3.7.2. Mesenchymal stromal/stem cells regulate neutrophil function

3.7.2.1. In vitro studies

Attending to the effect of MSC on neutrophils, it is interesting to note that human BM-MSC actively recruit neutrophils, and this chemotactic effect is further enhanced in LPS-activated MSC [389]. Human BM-MSC and UCM-MSC inhibit apoptosis of resting and activated neutrophils [390,391]. Apoptosis inhibition by BM-MSC is associated to increased expression of the anti-apoptotic protein myeloid cell leukemia 1 (Mcl-1) and decreased levels of the pro-apoptotic protein Bax, while no change was observed in CD95 expression [390]. Neutrophil protection from apoptosis is mediated by IL-6 in BM-MSC [93,390] (but not in UCM-MSC [391]), and improved by previous BM-MSC activation through TLR3 or TLR4 [93]. To the enhanced neutrophil survival observed in TLR3-stimulated MSC, besides IL-6, also contribute GM-CSF and IFNβ, whereas in TLR4-activated MSC is GM-CSF which mediates the protection from apoptosis [93].

Concerning neutrophil function, human BM-MSC hamper N-formyl-L-methionin-L-leucyl-L-phenylalanine (f-MLP)-induced respiratory burst [390], which is consistent with the downregulation of NADPH oxidase-1 (an enzyme involved in the production of the superoxide anion) mRNA expression induced by human UCM-MSC (in a IL-6 and ICAM-1-dependent manner) [391], and the increased mRNA levels of the anti-oxidant enzyme catalase [391]. Conversely, poly(I:C), a specific ligand for TLR3) or LPS-activated human BM-MSC (but not non-stimulated MSC) increase respiratory burst in response to *E. coli* [93,389]. Likewise, neutrophil phagocytosis of bacteria is also increased by BM-MSC and further enhanced after the activation of this latter cell population with LPS [389]. Interestingly, non-activated human BM-MSC do not alter the expression of CD11b or CD62L (which participates in the adhesion of neutrophils to endothelium) in neutrophils stimulated with f-MLP [390], but after TLR3 or TLR4 stimulation of MSC, it is observed an increase of CD11b expression in neutrophil [93]. Neutrophil chemotaxis to f-MLP, IL-8, or C5a also remains unchanged after co-culture with non-stimulated human BM-MSC [390]. In turn, LPS-activated human BM-MSC upregulate CCL4 (MIP-1β) expression by neutrophils, an effect not shared with non-activated BM-MSC [389]; whereas non-activated UCM-MSC decreased CCL4 and IL-8 expression in neutrophils [391].

Finally, human UCM-MSC upregulate COX-2, while downregulate HO-1 expression in neutrophils [391].

Similar results were found in mouse: MSC-derived from compact bone increase neutrophil phagocytosis of bacteria [392]; and the addition of neutrophils, previously co-cultured with TNF-α-primed BM-MSC, to CD3-stimulated T cells has the ability to decrease T cell proliferation and activation (an effect mediated by nitric oxide, NO), whereas only a slight effect was observed for freshly isolated neutrophils (that did not contact with MSC) or neutrophils co-cultured with non-primed MSC [393]. Interestingly, freshly isolated neutrophils from tumor-bearing mice also inhibit T cell proliferation and activation, demonstrating that tumor environment conditions neutrophils to acquire an immunosuppressive phenotype [393]. Accordingly, an increased arginase activity, along with increased mRNA expression of iNOS, anti-inflammatory cytokines and their receptors (IL-10, IL-6, IL-4R, IL-10R), and a multitude of chemokines, occurred after neutrophil co-culture with mouse BM-MSC [393]. Remarkably, it was demonstrated *in vivo* that infusion of MSC-treated neutrophils promotes tumor progression [393].

3.7.2.2. In vivo studies

In vivo, it is described that MSC treatment reduces neutrophil organ infiltration in animal models of sepsis [377,392], ventilator-induced lung injury [394], and global cerebral ischemia [395], among others, which protects the tissues from ROS-induced damage. Consistent with the reduced neutrophil infiltration, are the decreased protein levels of myeloperoxidase, decreased ROS production, and reduced oxidative organ damage in MSC-treated animals [377,394]; notwithstanding, in MSC-treated septic mouse, the phagocytic activity of neutrophils was enhanced, improving bacterial clearance [392]. Although the mechanisms underlying MSC effects are not well elucidated, it is reported that adenosine (a suppressive molecule produced by MSC and detailed in the section 4.4 of this chapter) decrease neutrophil chemotactic activity and adhesion to the vascular endothelium [372], making plausible the involvement of this mediator in the inhibitory effects of MSC over neutrophils. Likewise, IL-10 has also been described to inhibit the rolling, adhesion and trans-epithelial migration of neutrophils [377].

3.8. EOSINOPHILS, MAST CELLS, AND BASOPHILS

Eosinophils, mast cells, and basophils are important players in allergic inflammation. In this condition, a Th2 immune response is triggered by an allergen, and the high levels of IL-4 and IL-13 secreted promote immunoglobulin class switch to IgE. IgE molecules bind to high affinity receptors (FcεRI) on eosinophil, basophil, or mast cell surface, and subsequent IgE crosslinking by allergen induce cell degranulation (and release of inflammatory mediators contained inside them), production of cytokines (including IL-4, IL-5, IL-8, IL-13, GM-CSF, and TNF-α), leukotrienes, and prostaglandins [230]. The inflammatory mediators released by mast cells degranulation increase vascular permeability and attract eosinophils and neutrophils to the site of inflammation; IL-4 and IL-13 stimulate Th2 immune response, reinforcing IgE production; IL-4 also plays an important role in mast cell priming and increases the expression of FcεRI in these cells; IL-5 recruits and activates eosinophils; IL-8 attracts neutrophils and T cells; whereas GM-CSF activates myeloid cells [40,230,396]. The consequent accumulation and degranulation of eosinophils and neutrophils largely contribute to tissue damage [230].

3.8.1. Eosinophils

Eosinophils comprise approximately 3% of peripheral blood leukocytes. Their main function is to protect the organism from parasites, which eosinophils attack by releasing the content of their granules. These cells also have phagocytic activity and participate in asthma and allergy [230].

The published data regarding how MSC affect eosinophil function came from *in vivo* studies using animal models of asthma/airway inflammation that consistently show clinical improvement and reduced eosinophil infiltration in the airways, after treatment with mouse MSC [260,261,396,397]. Asthma is a chronic airway inflammation, mediated by Th2 cells and characterized by increased levels of IL-4, IL-13, and IL-5 in the airways [396]. In mouse models of ovalbumin and ragweed-induced asthma, the effects of mouse BM-MSC or AT-MSC infusion include the reduction of inflammatory cells and eosinophil count, IL-4, IL-5, and IL-13 expression, and increase IL-10 and TGF-β levels in bronchoalveolar lavage fluid and lung draining lymph nodes. MSC also increase the numbers of $CD4^+FoxP3^+$ Treg cells in lung tissue, lung draining lymph nodes, and spleen; and decrease antigen-specific IgG1 and IgE levels, along with increased PGE2 levels, in the serum [260,261,396]. Interestingly, the modulation of cytokine expression and IgE levels by MSC was dependent on TGF-β production and Treg

induction, while the reduction of eosinophilia in the bronchoalveolar lavage fluid was mediated by TGF-β produced by MSC [260,261].

3.8.2. Mast cells

After their differentiation in the BM, wherein they represent <0.03% of BM hematopoietic nucleated cells, mast cells enter the blood circulation to reach different tissues. Once within the tissue, mast cells finish their maturation process acquiring a phenotype that is determined by the tissue they had integrated [230].

In 2011, Brown and colleagues [398] investigated the effect of mouse BM-MSC in the major functions of mouse mast cells. *In vitro* co-cultures of mouse BM-MSC with mast cells sensitized with IgE revealed an inhibitory effect over TNF-α secretion (mediated by PGE2) and mast cell degranulation; the inhibition of FcεRI-mediated mast cell degranulation was also confirmed *in vivo* [398]. Besides, mouse BM-MSC present the ability to impair mast cell migration to stem cell factor (SCF) as well [398]. *In vitro* studies made similar findings in human, demonstrating that gingiva-derived MSC and BM-MSC reduce TNF-α, IL-6, and IL-4 production by mast cells stimulated with PMA and calcium ionophore, in a PGE2-dependent manner [399]. Likewise, human UCB-MSC exert a strong inhibition of mast cell degranulation, in a TGF-β1 and PGE2-dependent manner; TGF-β1 also mediates the downregulation of FcεRI expression in mast cells observed in the presence UCB-MSC [40].

Atopic dermatitis is characterized by increased IgE serum levels against environment allergens; the consequent IgE-mediate mast cell degranulation results in the recruitment of neutrophils, eosinophils, and lymphocytes to the dermis, as explained above [40]. *In vivo*, in a murine model of atopic dermatitis, treatment with human UCB-MSC resulted in clinical improvement, associated to the decreased mast cell (as well as eosinophil and lymphocyte) infiltration in the skin, decreased mast cell degranulation, along with reduced IgG1 and IgE serum levels [40]. Likewise, infusion of human gingiva-derived MSC in a mouse model of contact hypersensitivity showed clinical benefits [399]. Remarkably, besides the immune suppressive actions of MSC over DC and T cells, it was also shown a reduced number of mast cell infiltration. Interestingly, the infused MSC localized near mast cells and inhibit their degranulation through PGE2 [399].

3.8.3. Basophils

Basophils are non-phagocytic granulocytes which constitute a less-represented leukocyte population in the peripheral blood (≈0.7%). Their main function is the defense against parasites, particularly helminthes. Like eosinophils, basophils attack the parasites by releasing the bioactive factors contained in their granules, among them histamine, which augment blood vessel permeability and smooth muscle contractility [230]. Basophil degranulation occurs in response to antibody binding to FcεRI surface receptors, and these cells are also associated to the development of allergies [230]. No data concerning the effect of MSC over basophils was published until the present moment.

3.8.4. Concluding remarks

Despite the small number published concerning the effect of MSC on the function of these innate immune cells with a critical role in the development of allergic inflammation, the data consistently show clinical benefits in the use of MSC-based therapies in animal models. Also, *in vitro* studies demonstrated MSC exert an inhibitory effect over eosinophil and mast cell functions. In the same line, and attending to the relevance of IL-9 in allergic inflammation, our group recently described that human BM-MSC decrease the percentage of Th9 and Tc9 cells [244], which was confirmed by a recent study showing that human conditioned medium from amniotic MSC co-cultured with T cells reduced IL-9 in the culture supernatant [255].

4. IMMUNOSUPPRESSIVE MECHANISMS OF MESENCHYMAL STROMAL/STEM CELLS

As detailed in section 1.1.2 of this chapter, the surface membrane expression of chemokine receptors allows MSC to be attracted to and migrate into sites of tissue injury or inflammation. In addition, the expression of adhesion (chapter 1, section 1.1.3) molecules enables a stable binding and close interaction between MSC and the target immune cells.

To inhibit the immune response, MSC rely on both soluble mediators and cell-to-cell contact mechanisms. For human MSC, it has been demonstrated that the modulation of the immune response may be accomplished by the soluble factors TGF-β, PGE2, IDO, LIF, HGF, IGF, HLA-G5, galectin-1, galectin-9, Jagged-1, adenosine, semaphorin-3A, IL-6, IL-10, HO-1, and TNF-α stimulated gene/protein 6 (TSG-6) [7,45,240,281,400]; or by the plasmatic membrane proteins HLA-G1, PD-L1, CD200, and B7-H4 [107,401] upon interaction with the correspondent ligand expressed at the plasmatic membrane of the target immune cell.

Importantly, MSC from different species may employ different immunosuppressive molecules, as the case of nitric oxide (NO) produced by mouse MSC and which seems to constitute an important immune suppressive factor employed by MSC of this specie, but not by human MSC. Likewise, there are evidences that, in human, distinct immunomodulatory factors may have a differential relevance for the immune regulatory function of MSC arising from different sources. This is inferred because there are important differences in the expression levels of immunomodulatory molecules among MSC derived from different tissues, and also because the inhibition of those immunomodulatory molecules in *in vitro* assays, where only the type of MSC used varies, lead to different outcomes. Thus, if the inhibition of the same molecule results in the abolishment of a given suppressive function for one type of MSC, but has no effect for another MSC type, it is likely that the latter type of MSC possesses other mechanisms that can surpass the deficiency of the neutralized factor, in opposition to the former MSC type.

It is also worth mentioning that our group [46] and others [26,37,39,43,44,47] demonstrated that human MSC isolated from different tissues, and tested under the same conditions, present differential immunosuppressive abilities, which is described in the section 1.2.3 of this chapter.

Remarkably, the exposure to inflammatory cytokines increases the expression of immunomodulatory molecules and adhesion molecules in MSC (chapter 1, section 1.4), demonstrating these cells are highly sensitive to microenvironment and physiologic state of

the organism, and also proving their versatility to adapt their functions to the current needs of the individual.

4.1. TRANSFORMING GROWTH FACTOR-β

4.1.1. The effect of transforming growth factor-β on immune cells

Transforming growth factor (TGF)-β is a regulatory cytokine able to influence the function of a broad range of cell lineages and involved in numerous physiologic processes, such as embryogenesis, carcinogenesis, and immune response [402]. Three isoforms of this cytokine (TGF-β1, TGF-β2, and TGF-β3) were identified in mammals, in which the isofrom TGF-β1 is predominantly expressed in the immune system [402,403]. Focusing on the role of TGF-β in the immune system, an increasing body of evidence supports its importance in the early T cell development, in the survival of naive T cells, in the generation of natural Treg cells in the thymus, and the induction of Treg cells in the periphery [402-404]; indeed, TGF-β has the ability to induce and maintain FoxP3 expression in T cells [229,287,402,404].

Besides the generation of Treg cells, this cytokine has an immunosuppressive effect over T cells, NK cells, DC, macrophages, mast cells and granulocytes [403]. TGF-β inhibits IL-2 expression by activated T cells, through inhibition of IL-2 promoter activity; it also interferes with T cell proliferation and differentiation into effector Th1, Th2, or Th17 cells [402,403]. The ability to impair IL-2 production will consequently condition T cell proliferation; notwithstanding, TGF-β also arrest T cell cycle by downregulating the expression of c-myc, cyclin D2, cyclin-dependent kinase 2 (CDK2), and cyclin E, while upregulates the expression of the cyclin-dependent kinase inhibitors p15, p21 and p27 [403]. The suppression of naive T cell differentiation into Th1, Th2, and Th17 is achieved by downregulating the expression of T-bet, GATA3, and RORγt (in the absence of IL-6 and with high concentration of TGF-β), respectively [229,402,403,405]. In fully differentiated Th1 cells, TGF-β inhibits IFNγ expression and impairs IL-12 signaling, while induces IL-10 expression [403,406]; whereas in $CD8^+$ T cells, it hampers cell activation and effector functions, specifically by decreasing the expression of granzyme B, inhibiting granule exocytosis, and cytotoxic activity [402,403]. On B cells, TGF-β inhibits expression of IgM and IgG, while induces IgA [407]. Moreover, TGF-β reduces IFNγ and NKG2D expression and cytolytic activity in NK cells [408-411].

In Lagerhans cells, TGF-β, though essential to their development, blocks their phenotypic maturation and phagocytic activity, and impairs the production of IL-12 [412]; similarly, it inhibits CD80 upregulation in human peripheral blood DC and monocytes [413]; impairs DC maturation, LPS-induced upregulation of CD80, CD86, CD40, and IL-12 expression, as well as their allostimulatory activity in MLR, in murine [414]; and inhibits mouse plasmacytoid DC expression of IFNα, IL-12, and TNF-α [415]. In addition, TGF-β was also shown to inhibit the induction of inducible nitric-oxide synthase (iNOS) and the production of TNF-α and MMP-12 in macrophages [403,416], and to have an important role in the Treg-mediated induction of M2 macrophages *in vivo*, in mouse [417].

4.1.2. Role of transforming growth factor-β in mesenchymal stromal/stem cells immune suppressive function

TGF-β is constitutively expressed by human BM-MSC and secreted at immunosuppressive concentrations; it is also upregulated after exposure to TNF-α or IFNγ [240,287]. Nevertheless, despite of the consistent description of the immunosuppressive functions of TGF-β in the literature, there are conflicting data in what concerns to its role in MSC-mediated immunosuppression. Using neutralizing antibodies directed against TGF-β1, Le Blanc and colleagues [234] demonstrated that the inhibition of PHA-stimulated T cell proliferation, observed in the presence of human BM-MSC, was not mediated by TGF-β. Conversely, the use of neutralizing anti-TGF-β antibodies restored human BM-MSC-derived inhibition of T cell proliferation in MLR [235]; and TGF-β derived from human BM-MSC has also been implicated in the downregulation of CD25, CD38, and CD69 expression by $CD4^+$ and $CD8^+$ T cells [84,181]. In the same line of evidence, and using the same strategy in peripheral blood or synovium $CD4^+$ and $CD8^+$ T cells from rheumatoid arthritis patients, stimulated with type II collagen, TGF-β was revealed to have an essential role in human BM-MSC-mediated inhibition of antigen-specific T cell proliferation and production of IFNγ and TNF-α, and in the induction of IL-4 production in T cells [181]. On the other hand, the inhibition of IFNγ and TNF-α production by T cells from mouse with CIA, and stimulated with type II collagen, in co-culture with human AT-MSC, was shown to be a process dependent of PGE2 and IL-10, rather than TGF-β [180].

In ragweed-induced asthma mouse model, intravenous infusion of BM-MSC alleviated asthma symptoms through the increased production of TGF-β, being reported that, under a Th2 environment, IL-4 and IL-13 upregulate TGF-β expression in MSC by activating STAT6 signal pathway [261]. Not surprisingly, it has been demonstrated that TGF-β is involved in the

induction of Treg cells by MSC, as this cytokine is known to have an essential role in FoxP3 induction and maintenance [98,195,229,285-287,402].

Of all the inhibitory effects of human BM-MSC over NK cells (inhibition of NK cell proliferation, downregulation cytokines and activating receptors expression, and impairment of NK cell cytotoxicity), only the inhibition of cell proliferation induced by IL-15 is ascribed to TGF-β [327]; in turn, under IL-2 stimulation, TGF-β seems not to participate in the inhibition of proliferation [326]. Regardless, culture of peripheral blood NK cells with TGF-β1 results in the downregulation of the activating receptors NKG2D and 2B4, as well as in cell cycle arrest by the induction of the expression of p15INK4b [418]. As the abovementioned data on MSC/NK cell co-cultures was obtained using neutralizing antibodies against TGF-β, we may postulate that MSC have redundant mechanisms in what concerns to immune cell suppression and, therefore, the inhibition of one of the mechanisms may not necessarily conduct to the abolishment or mitigation of the immunoregulatory effect.

Finally, TGF-β upregulates the expression of CD73 in $CD4^+$ and $CD8^+$ T cells, macrophages and DC [372,419]. CD73 is an ecto-enzyme that participates in the generation of adenosine, an immunosuppressive nucleoside, whose functions are explained in section 4.4 of this chapter.

4.2. Prostaglandin E2 (PGE2)

4.2.1. The effect of prostaglandin E2 on immune cells

Prostaglandin (PG) E2 is an arachidonic acid-derived eicosanoid produced by cyclooxygenase (COX)-1 and COX-2 (whose activity yield PGH2), followed by the enzymatic action of PGE synthases. COX-1 is constitutively expressed in several cell types, whereas COX-2 expression is inductive and rapidly upregulated under inflammatory conditions (such as in the presence of LPS, IFNγ, TNF-α, IL-1α, or IL-1β), where the conjugated action of both enzymes leads to the secretion of large amounts of PG, although is mainly the activity of COX-2 which regulates the amount of PGE2 produced. Though PGE2 can be produced by all cell types, in the context of an immune response, immune cells are the main source of this immune mediator, still epithelial cells and fibroblasts give an important contribute [420-423]. COX-2 expression is inhibited by the anti-inflammatory cytokines IL-4, IL-13, and IL-10; thus, these cytokines can regulate the synthesis of PG as well as of other arachidonic acid-derived lipid mediators (including leukotriene B_4 and platelet-activating factor) [420].

So far, four PGE2 receptors had been identified, the E prostanoid receptors (EP_{1-4}), which differ in what concerns to PGE2 affinity – wherein EP_3 and EP_4 are high affinity receptors, while EP_1 and EP_2 display a lower affinity to the ligand – and trigger distinct signaling pathways – EP_1 activates phospholipase C (PLC); EP_2 and EP_4 are Gs-coupled receptors, which trigger adenyl cyclase activity and cAMP/PKA/CREB pathway; whereas the study of EP_3 signaling is more complex because it possesses multiple isoforms generated by alternative splicing and can be coupled with either Gs or Gi, or regulate cell calcium levels. The four receptors also differ in terms of susceptibility to desensitization and duration of the signal, and EP_4 was shown to be able to activate PI3K-dependent extracellular signal-regulated kinase (ERK)1/2 pathway as well [420-424]. Roughly, one can say that EP_2 and EP_4 receptors are functionally involved in the anti-inflammatory properties ascribed to PGE2 [421]; nevertheless, PGE2 ability to activate different signaling pathways depending on its own concentration and on the environmental conditions, helps to explain the complexity and, sometimes, apparent contradictions of PGE2 biological functions.

In fact, in the early phases of the inflammatory response, PGE2 induces vasodilatation and promotes the local infiltration and activation of neutrophils, macrophages, and mast cells (also promoting mast cell degranulation through EP_1/EP_3 receptors), whereas in later stages of inflammation, PGE2 inhibits the production of pro-inflammatory cytokines and induces IL-10 expression in myeloid cells [423]. Concerning neutrophils, PGE2 is known to inhibit their activation and migration through EP_2 signaling [421]; and to suppress the expression of TNF-α (via EP_2 and EP_4 receptors) and enhance IL-6 production (mediated by EP_2 signaling) in mouse neutrophils [425]. Interestingly, despite being a chemoattractant to macrophages, PGE2 ligation to EP_2 at macrophage cell surface impairs its activation and phagocytosis [421,423]; PGE2 also decreases the production of ROS and induces the production of IL-10 by macrophages [421]. In mouse peritoneal macrophages, signaling through EP_2 and EP_4 reduces TNF-α and IL-12 expression in response to LPS (for both cytokines) or LPS plus IFNγ (for TNF-α) [426,427]. In human, EP_2 and EP_4 binding to PGE2 also decreases the pro-inflammatory cytokine production (namely TNF-α and IL-12) by monocytes and macrophages, and downregulates MHC-II expression [422,423,428]. Moreover, PGE2 is associated to the induction of IL-10 expression on tissue macrophages [423]. Noteworthy, this prostanoid has been shown to play an essential role in the development of tumor-associated suppressive macrophages and myeloid-derived suppressor cells [423].

Concerning NK cells, PGE2 suppresses NK cell activity in response to either IL-12 or IL-15, impairs the production of IFNγ, reduces the expression of receptors with an important role in

NK cell functions (CD94/NKG2C, DNAM-1, NKp80, 2B4 (CD244), and CD161), and decreases NK cytolytic activity against rat leukemic cells [421,423]. With respect to B cells, PGE2 was shown to inhibit both their proliferation and immunoglobulin production [429,430], or to induce Ig class switching and B cell differentiation into IgE-secreting plasma cells [422,424].

COX-2 and, subsequently, PGE2 expression is known to be upregulated by LPS and TNF-α in DC; and the DC-derived PGE2 can bind to EP_2 and EP_4 receptors on DC plasmatic membrane and autocrinally regulate their cellular functions [420]. Depending on the microenvironment and maturation stage, PGE2 may have opposite effects on DC function. Several studies pointed out that PGE2 impairs DC maturation, their MHC-II expression, and antigen presentation skills, while it upregulates IL-10 expression, through EP_2 and EP_4 ligation (although there are conflicting data concerning the IL-10 upregulation [431,432]); PGE2 treatment also results in the downregulation of IL-6, TNF-α, and IL-12 expression by DC [420,422,431,433-436], which is further enhanced and mediated by IL-10 [420,433,436] (the detailed anti-inflammatory properties of IL-10 are described later in this chapter, in section 4.6). In fact, PGE-driven IL-10 production by DC amplifies the regulation of the inflammatory response [420]. Of note, when IL-10 action is blocked, PGE2 promotes IL-6 production; however, as PGE2 induces the expression of IL-10, which downregulates IL-6 expression, the direct effect of PGE2 on IL-6 expression is masked by IL-10 action [420,433]. Interestingly, IL-10 prevents COX-2 expression by DC and monocytes (through the inhibition of NF-kB transcriptional activity and by accelerating the degradation of COX-2 mRNA), therefore, stopping the immune suppressive amplification loop [420]. Remarkably, these IL-10-producing DC have the ability to bias T cell differentiation toward Th2 phenotype and to suppress CTL response [421,423]. Besides IL-10, PGE2 is also described to upregulate the expression of the immune regulatory factors thrombospondin-1 and IDO in DC [423,437]. Conversely, when combined with TNF-α, PGE2 stimulates DC and promotes IL-12 secretion [420,434].

Interestingly, monocyte treatment with PGE2 inhibits the differentiation into DC [53,437], IL-12 production, and the ability to induce T cell proliferation in MLR [53]; while upregulates the expression of IDO, IL-10, NOS2, and IL-4Rα, generating a phenotype which overlaps with that of myeloid-derived suppressor cells [437]. These cells also express the suppressive factors ILT2, ILT3, ILT4, and PDL-1, and have the capability to inhibit $CD8^+$ T cell proliferation and granzyme B expression [437]. Notwithstanding, the action of PGE2 through EP_2 and EP_4 was also shown to induce maturation in human monocyte-derived DC and to enhance the expression of CD80, CD86, CD83, MHC-I, HLA-DR, CD40, and adhesion molecules (CD44, CD54, and CD58), principally when in the presence of pro-inflammatory cytokines (the

conditions tested correspond to TNF-α; TNF-α and IFNα; and TNF-α, IL-1β, and IL-6) [423,431,432,434,435]. Moreover, the presence of PGE2 during the maturation process of monocyte-derived DC was shown to reduce their secretion of IL-12p70, IL-6, and IL-10, and to condition DC to bias T cell response towards a Th2 profile [423,431,432,435].

PGE2 treatment, beyond increasing CCR7 expression, was also found to endow DC with the ability to migrate to secondary lymphoid organs (however, there are clues that this specific function of PGE2 can be also ensured by other factors *in vivo* [423]), in response to CCL19 and CCL21 (which are CCR7 ligands), and to CXCL12 (consistent with the observed PGE2-induced CXCR4 increase in DC); on the other hand, PGE2 decreases DC expression of CCL19, reducing the chance of naive T cell (which are $CCR7^{+}$) to migrate into secondary lymphoid organs [421-423,432,435,438-442]. Interestingly, once inside the lymph node, exposure of DC to PGE2 decreases their activation status and ability to prime T cells [422]. In the same line, this prostanoid decreases CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES) production by DC, reducing the immune cell infiltrate [421,422,436,442,443], as well as DC's CCR5 expression [435,442]; whereas increases the expression of CCL17 (or thymus and activation-regulated chemokine, TARC) and CCL22 (or macrophage-derived chemokine, MDC) by human monocyte-derived DC and peripheral blood myeloid DC (in an EP_2/EP_4 dependent-manner), which specifically attract Th2 memory T cells [442,444]. PGE2-derived upregulation of CCR7 expression is also observed on human monocytes, whereas during monocyte differentiation into macrophages, PGE2 cannot prevent the naturally occurring CCR7 downregulation [445].

In the context of acute inflammation, bacterial products or tissue damage signals, complement split products, PGE2 (e.g. secreted upon tissue mast cell degranulation in response to complement anaphylatoxins), among others, will attract neutrophils, and these cells will release chemotactic factors that attract macrophages to the site of injury [230]. The presence of PGE2 in the early phase of the inflammatory response will, *per se*, attract new mast cells and stimulate their production of CCL2 (MCP-1) [423], increasing the number of infiltrating macrophages, and induce IL-8 secretion [423], which enable the recruitment of more neutrophils to the site of inflammation, overall amplifying the PGE2 and histamine release, vasodilatation, and infiltration of immune cells in the inflammatory site. Reported data suggest the ability of PGE2 to recruit macrophages is limited in time, as this prostanoid also induces the downregulation of CCR5 and Mac-1 on monocytes and macrophages (reducing their ability to migrate), as well as the reduction of CCL5 (RANTES) and of all three CXCR3 ligands – CXCL9 (MIG), CXCL10 (IP10), and CXCL11 (ITAC) – limiting the recruitment of the

effector cells of a Th1 immune response: Th1 cells, CTL, NK cells (all of them described as $CCR5^+CXCR3^+$), and pro-inflammatory macrophages [423,444,446]. In turn, by promoting the expression of CCL17 (TARC) and CCL22 (MDC), PGE2 contribute to the recruitment of Th2 and Treg cells [423,444], and by upregulating CXCL12 (SDF-1) expression, it attracts myeloid-derived suppressor cells, supporting tissue repair and the termination of the inflammatory response [423,437].

PGE2 inhibits T cell proliferation [422,424], dampens IL-2 production by T cells and IL-2 signaling [423]; it also skews T cells towards a Th2 phenotype, inhibiting IFNγ expression by $CD4^+$ T cells [423,424], along with Ig class switching in B cells to IgE, the immunoglobulin class typically associated to a Th2 immune response [424]. The fostering of Th2 differentiation is further supported by the effect of PGE2 in inhibiting IL-12 production by DC and monocytes and, therefore, preventing the development of a Th1 immune response. In the same line, PGE2 also inhibits the cytotoxic functions of CTL [423]. Interestingly, it was reported that the treatment of purified $CD4^+CD25^-$ or $CD4^+CD25^+$ T cells with PGE2, induced the *de novo* generation of $CD4^+FoxP3^+$ Treg cells, for the former cell population, while increased the regulatory properties and upregulates FoxP3 expression in the latter [7,287,447]. Regardless, there are contradictory data published reporting that EP_4 stimulation on T cells or DC supports Th1 differentiation, while EP_2 and EP_4 signaling promotes Th17 differentiation [423,439,448,449]. Furthermore, the addition of exogenous PGE2 to *in vitro* activated γ9δ2 T cells prevents their proliferation, production of TNF-α and IFNγ, and impairs their cytolytic activity [294].

Not surprisingly, several bacteria [421] and tumor cells [423,437] make use of PGE2 immunosuppressive properties, by stimulating its production and/or inhibiting its degradation (by suppressing the activity of the PGE2 degrading enzyme 15-hydroxyprostaglandin dehydrogenase, 15-PGDH) in order to escape from immune surveillance.

4.2.2. Role of prostaglandin E2 in mesenchymal stromal/stem cells immune suppressive function

Human BM-MSC constitutively express both COX-1 and COX-2, and constitutively produce PGE2, whose secretion augments upon PBMC, monocyte, mature myeloid DC, or T cell co-culture, after exposure to TNF-α and/or IFNγ, or in the presence of LPS [9,26,31,53,236,240,286,287,294]; as well as after MSC co-culture with NK cells, independently of IFN-γ secretion [326]. Likewise, human AT-MSC and UCM-MSC constitutively express PGE2

as well [26,31,248], and the former upregulate its expression in response to LPS, PBMC, or mature myeloid DC co-culture [31], while UCM-MSC augment PGE2 secretion upon PBMC co-culture [26,248] or after IL-1β treatment [45,385].

The impairment of monocyte allostimulatory ability by human UCM-MSC is partially due to PGE2 action [248]. Indeed, it was reported that PGE2 secreted by human BM-MSC impairs monocyte differentiation into DC, suppresses IL-12 production and their ability to elicit T cell proliferation in MLR assays [53]; whereas mouse BM-MSC-derived PGE2 upregulates the expression of IL-10 by macrophages [286,287,377]. Similarly, in human, PGE2 was shown to mediate the BM-MSC-driven suppression of TNF-α secretion by DC [236], as well as the BM- and AT-MSC-derived induction of IL-10 production by pDC [31]. In the same line, synovial cells from mice with CIA stimulated *in vitro* with type II collagen, display a reduction of IFNγ and TNF-α protein expression when co-cultured with human AT-MSC, a process mediated by PGE2 and IL-10 [180].

Furthermore, PGE2 was demonstrated to mediate the human BM-MSC-driven suppression of $V\delta2^+$ γδ T cells [104,294] and $V\alpha24^+V\beta11^+$ iNKT cells [104] proliferation through EP_2 and EP_4 receptors (which are highly expressed at the γ9δ2 T cell surface) [104,294]. PGE2 is also involved in human BM-MSC-mediated inhibition of NK cell proliferation and cytotoxicity upon stimulation with IL-15 [327] or IL-2 [326]; in the latter condition, PGE2 and IDO have a synergistic suppressive effect over NK cell proliferation and cytotoxic abilities [326].

PGE2 secreted by human BM-MSC [26,31,236,240,243], AT-MSC [26,31], UC-MSC [26,248], and induced pluripotent stem cells (iPSC) [249] is required for modulation of cell proliferation in MLR and PHA-stimulated PBMC; likewise, PGE2 secreted by mouse BM-MSC [182] also mediates the inhibition of T cell proliferation. Accordingly, PGE2 was shown to reduce mRNA levels of CDK2 and other genes involved in cell proliferation [31]. PGE2 synthesis inhibitor reverses human BM-MSC-mediated suppression of IFNγ production by T cells [236], a mechanism shared with human UCM-MSC [385], although there are contradictory data concerning the influence of PGE2 over IFNγ suppression [31]. Remarkably, PGE2 was shown to be one of the factors underlying human BM- and AT-MSC induction of IL-10 expression on T cells [31] and also on fully differentiated Th17 cells [9]. In the same line of evidence, porcine BM-MSC was shown to induce $CD4^+IL\text{-}10^+IFN\gamma^+$ T cells in MLR assays, in an IDO- and PGE2-dependent manner; and those MSC-induced $CD4^+IL\text{-}10^+IFN\gamma^+$ T cells were demonstrated to possess the ability to suppress PBMC proliferation in MLR assays through IL-10 secretion [266].

Regarding the role of PGE2 in the influence of MSC over T cell differentiation, it is described that both human BM- and AT-MSC can suppress the expression T-bet, STAT1, and STAT4 (involved in Th1 differentiation), and GATA-3, STAT6, and c-Maf (implicated in Th2 differentiation), at mRNA level [31]. Interestingly, Yañez and colleagues [31] demonstrated that, for AT-MSC, PGE2 mediates this effect; conversely, inhibition of PGE2 synthesis by BM-MSC did not restore the mRNA expression of the abovementioned factors. Concerning the effect of MSC-derived PGE2 over the differentiation towards Th17 phenotype, the studies carried out apparently yield conflicting data, because the distinct protocols used may explain the differences in the results obtained. In the one hand, the inhibition of PGE2 synthesis was shown not to reverse RORc downregulation mediated by either human BM- or AT-MSC co-cultured with PBMC activated with PHA [31]. On the other hand, PGE2 derived from BM-MSC was demonstrated to inhibit Th17 differentiation from mouse [275,276] and human naive T cells (identified as $CD4^+CD45RA^+$) [9], activated by CD3/CD28 under Th17 differentiation conditions; as well as the expression of IL-17A, IL-22, IFNγ, and TNF-α by fully differentiated mouse [275] and human [9] Th17 cells. Finally, English and colleagues [286] demonstrated that PGE2 is also involved in the generation of Treg cells by human BM-MSC.

Of note, porcine BM-MSC-derived PGE2 induces the expression of IDO in PBMC [266], whose immunosuppressive is detailed in the next section (section 4.3 of this chapter).

4.3. Indoleamine 2,3-dioxygenase

4.3.1. The effect of indoleamine 2,3-dioxygenase on immune cells

Indoleamine 2,3-dioxygenase (IDO) is an intracellular tryptophan degrading enzyme, ubiquitously expressed in mammalian tissues; of note, the immune system express preferentially IDO1, in relation to IDO2. IDO catalyzes the rate-limiting step of tryptophan catabolism: the conversion of tryptophan in N-formylkynurenine. N-formylkynurenine is further degraded in kynurenine which is ultimately catabolized to the terminal metabolites picolinic acid or quinolinic acid. Thus, the action of IDO leads to tryptophan starvation and to the production of tryptophan metabolites [450,451]. The ability to degrade the essential amino acid tryptophan acts not only as a defense mechanism against viruses and intracellular pathogens, but also as an immunoregulatory mechanism to prevent overreactive inflammatory responses [451]. IDO expression on DC is upregulated by CD80/CD86 binding to CTLA-4, type I IFN, IFNγ and other cytokines, as well as TLR4, TLR7/8, and TLR9 ligands, which makes sense in

the context of the immunoregulatory function of IDO in limiting the duration and magnitude of the inflammatory response [451-453]. Conversely, IL-6 promotes IDO inhibition through proteosomal degradation, in a suppressor of cytokine synthesis (SOCS)3 dependent-manner, thus preventing the establishment of immune tolerance whenever dangerous signals are received [451].

The immunosuppressant effect of IDO relies not only on tryptophan depletion, but also on the increasing concentration of tryptophan metabolites with immunomodulatory functions. Noteworthy, IDO activity can hamper T cell responses directly, by affecting T cell activation/differentiation, or indirectly, by modulating the function of antigen-presenting cells. Indeed, in the presence of TGF-β, IDO is phosphorylated and assumes functions of a signaling molecule which induces a tolerogenic phenotype in pDC [451]; in the same line, the intermediate tryptophan metabolite 3-hydroxyanthranilic acid (3-HAA) impairs IL-12, IL-6, and TNF-α production by BM-derived DC stimulated with LPS, as well as the upregulation of CD40, CD80, CD86, and I-A, decreasing the ability of DC to induce T cell activation both *in vitro* and *in vivo* [454]. On the other hand, the inhibition of IDO activity in human monocyte-derived DC hampers the upregulation of CD80, CD86, HLA-DR, CD83, and CD40, induced by LPS, TNF-α, or CD40L treatment, and decreases the expression of CXCR4 [455].

Concerning the direct effects of IDO activity on T cells, the tryptophan metabolites kynurenine, picolinic acid, and quinolinic acid were shown to suppress the proliferation of human $CD4^+$ T cells, $CD8^+$ T cells, and NK cells, upon PHA activation or in MLR [240,450]. Likewise, IDO activity in both pDC and monocyte-derived DC was proven to induce the generation and/or expansion of $CD4^+FoxP3^+$ Treg cells by a mechanism dependent of IDO and CD80/CD86 binding to $CD4^+$ T cells [456,457]. In fact, both tryptophan depletion and kynurenine-mediated activation of aryl hydrocarbon receptor, a transcription factor expressed by naive T cells, bias T cells towards a $FoxP3^+$ Treg phenotype [451]. Of note, there are other enzymes beyond IDO that catabolyze other essential amino acids and whose action leads not only to the inhibition of T cell proliferation by amino acid starvation, but also to the expansion of Treg cell population [451].

In vivo, IDO is implicated in materno-fetal tolerance and in tumor escape from the immune system. Of note, increased IDO expression is associated to a poor clinical outcome for both solid tumors and hematological malignancies [451,452,456,458].

4.3.2. Role of indoleamine 2,3-dioxygenase in mesenchymal stromal/stem cells immune suppressive function

Although not constitutively expressed, upon IFNγ, TLR3, or TLR4 stimulation, IDO1 and IDO2 are induced in human BM-MSC [45,88,240,247]. Accordingly, it is described that, in human, the IDO gene promoter possesses response elements for IFNγ [240].

In fact, IDO enzymatic activity in human BM-MSC prevents T cell proliferation upon PBMC stimulation with anti-CD3/CD28 [52]. Likewise, either the inhibition of IDO activity, by using the chemical antagonist 1 methyl l-tryptophan [75,240], or the addition of tryptophan [247] to human BM-MSC/MLR co-cultures reverse the human BM-MSC-mediated inhibition of T cell proliferation in MLR. Interestingly, the same result is achieved by the addition of kynurenine to MLR which, together with the observation that kynurenine levels are augmented in MLR in co-culture with MSC, supports that, regardless of tryptophan depletion, kynurenine accumulation participates in the inhibition of T cell proliferation mediated by MSC [240]. Similarly, impairment of T cell proliferation by human AT-MSC is also mediated by IDO [459]. Conversely, IDO does not participate in the human BM-MSC-driven inhibition of IL-17, IL-22, TNF-α, and IFNγ production by Th17 [9], but contributes to the inhibition of Th17 differentiation mediated by mouse MSC [276].

In addition, it was demonstrated that IDO and PGE2 derived from human BM-MSC have a synergistic effect over the inhibition of IL-2-stimulated NK cell proliferation and cytotoxicity [326]; as well as that IDO participates in the induction of IL-10 expression by monocytes, driven by human BM-MSC [52]. Likewise, porcine BM-MSC was shown to induce $CD4^+IL\text{-}10^+IFN\gamma^+$ T cells in MLR assays, which was mediated by PGE2 and IDO. These $CD4^+IL\text{-}10^+IFN\gamma^+$ T cells are capable of suppressing PBMC proliferation in MLR assays, in an IL-10-dependent manner [266].

In a mouse renal allograft model, MSC treatment was shown to augment the serum levels kynurenine, indicative of an increased IDO enzymatic activity, which were correlated to higher percentages of $CD4^+CD25^{++}FoxP3^+$ Treg cells in both the spleen and the allograft. Moreover, the use of IDO-knockout MSC or IDO inhibitor leaded to the loss of graft tolerance [287].

4.4. ADENOSINE/CD39-CD73

4.4.1. The effect of adenosine on immune cells

The extracellular nucleotides and nucleosides comprise a group of bioactive molecules whose levels are tightly regulated by ecto-enzymes. Extracellular adenosine triphosphate (ATP) has the ability to induce a pro-inflammatory response by monocytes and lymphocytes, while its derivative nucleoside, adenosine, possesses an immunosuppressive function mainly mediated by the A_{2A} adenosine receptor (ADORA2A) and A_3 adenosine receptor [372,460,461]. Adenosine is generated after the hydrolysis of extracellular ATP or ADP into AMP, by the ecto-nucleotidase CD39, and sequential dephosphorylation by ecto-5'-nucleotidase (CD73); the extracellular levels (and function) of adenosine are, in turn, regulated by adenosine deaminase (ADA), which converts adenosine into inosine [460,461].

Though CD39 was firstly described as a B cell activation marker, it was later shown to be also expressed on subsets of activated T cells, $CD4^+CD25^{++}FoxP3^+$ Treg cells, NK cells, NKT cells, monocytes, macrophages, DC, and neutrophils [372,460]. In fact, $CD4^+CD25^{++}FoxP3^+$ Treg cells express both CD39 (at the plasmatic membrane) and CD73 (mostly located in the cell cytoplasm) and use adenosine to suppress T cell responses [372,419,461]. Furthermore, CD73 expression in $CD4^+$ and $CD8^+$ T cells, macrophages, and DC is upregulated by TGF-β [372,419], and the expression of ADORA2A is upregulated in T cells upon activation, which increases their susceptibility to adenosine-mediated regulation [461,462]. Previous studies demonstrated that adenosine inhibits proliferation, decreases the expression of CD25 and CD28, and the production of IL-2, IFNγ, granzyme B, perforin, FasL, and TRAIL by mouse T cells [461-464]; treatment with ADORA2A agonist inhibits fully-differentiated mouse Th1 cells' proliferation and IFNγ production, with no effect on Th2 cell proliferation [460]. In human, adenosine was found to inhibit $CD4^+$ T cell proliferation and the expression of IL-2, TNF-α, IFNγ, and several chemokines in $CD8^+$ T cells and $CD4^+$ Th1 cells, as well as the priming and cytotoxic activity of CTL [465,466], and to impair the adhesion of activated T cells to tumor cells [461]. These suppressive effects over T cell function are achieved by interfering with T cell receptor (TCR)/CD3 signaling, specifically by inhibiting the phosphorylation, and subsequent activation, of Lck and zeta-chain-associated protein kinase of 70 kDa (ZAP-70) tyrosine kinases; and by impairing the signal transduction mediated by IL-2, as a result of the inhibition STAT5 activity [461].

Beyond this massive inhibitory effect over T cells, wherein adenosine interferes with the three activation signals of T cells (provided by TCR/CD3, CD28 and IL-2), this immunosuppressive molecule also modulates the function of antigen-presenting cells. Specifically, it impairs the oxidative burst in monocytes and macrophages, decreasing the production of nitric oxide and superoxide; and inhibits monocyte maturation and differentiation into macrophages [467,468]. In the same line, adenosine downregulates the expression of MIP-1α, IL-12 and TNF-α and induces the expression of IL-10 in monocytes and macrophages, shifting macrophage pro-inflammatory phenotype to anti-inflammatory phenotype [461,467-469]; while in human DC, adenosine impairs the production of IL-12 and TNF-α, and induces the secretion of IL-10 which, altogether, will impair the development of a Th1 immune response [461,469,470]. Macrophage's expression of A2A receptors is upregulated by HO-1 (section 4.12 of this chapter) [468]. Besides, a decreased expression of the costimulatory molecules CD80, CD86, and CD40 is also observed in mouse B cells under the influence of adenosine [461].

Concerning NK cells, adenosine was shown to diminish their proliferation rate, reduce the expression of TNF-α and IFNγ, induce defective granule exocytosis, and impair cytotoxic activity, in both mouse [461,471] and human [330,461,465]. Similarly, NKT cells produce lower levels of IFNγ under the influence of adenosine [469]. In neutrophils, the activation of A2A receptors suppresses the production of ROS and of the pro-inflammatory cytokines TNF-α, CCL3 (MIP-1α), CCL4 (MIP-1β), CXCL2 (MIP-2α), and CCL20 (MIP-3α); adenosine also downregulates the expression of adhesion molecules, interfering with neutrophil ability to adhere to the vascular endothelium [469]. Conversely, activation of A1 receptor activation promote neutrophil migration and adhesion to the endothelium [469].

4.4.2. Role of adenosine in mesenchymal stromal/stem cells immune suppressive function

A proportion of human BM-MSC co-expresses CD39 and CD73, however, though the percentage of BM-MSC expressing CD73 is consensual (≈90%), the frequency of CD39+ human BM-MSC is not, being described to be ≈15-35% [400] and ≈80% [281]. The presence of activated T cells was shown to induce an increased production of adenosine by BM-MSC, which is accompanied by an increased percentage of BM-MSC expressing CD39 on their plasmatic membrane and of $CD73^+$ T cells [400]. Interestingly, a remarkable increase of the percentage of $CD39^+CD73^+$ Treg cells and $CD39^+CD73^+$ BM-MSC [400], as well as an increased CD39 and CD73 mRNA levels in Th17 cells [281], is also observed in co-cultures of BM-MSC

with activated T cells, further increasing the enzymatic machinery responsible for adenosine production and ATP degradation. In addition, under these conditions, it is observed the upregulation of ADORA2A mRNA and the decreased expression of ADA mRNA in T cells [400], contributing to enhance the immunosuppressive effect mediated by adenosine. Similarly, an increased CD73 expression is verified in NK cells in co-culture with UC-MSC [329].

The important role of adenosine in the context of MSC-mediated T cell inhibition was established by demonstrating that the blockage of ADORA2A signaling reverts the inhibitory effect of human BM-MSC over T cell proliferation, in co-cultures of BM-MSC with T cells [400]. Besides, anti-CD39 treatment of human-BM-MSC and Th17 cells co-cultures, abrogates MSC-mediated inhibition of Th17 proliferation and reduction of IL-17 and IFNγ production by Th17 cells [281]. Adenosine was also demonstrated to mediate the murine BM-MSC-derived inhibition of T cells proliferation [472].

4.5. Semaphorin-3A, galectin-1, galectin-3, and galectin-9

4.5.1. Semaphorin-3A and galectin-1

Neuropilin-1 is a receptor, present on T cells and DC, with the ability to bind to semaphorin-3A, galectin-1, and neuropilin-1 (homotypic interaction), among others. While the homotypic interaction plays an important role in intercellular interactions (e.g. contributes to T cell/DC interaction, or facilitates the cluster of T cells and tumor cells, in the context of an immune response), binding to semaphorin-3A or galectin-1 delivers inhibitory signals to T cells [473,474].

Catalano and colleagues [473] demonstrated that the soluble molecule semaphorin-3A inhibits T cell-mediated immune response, namely T cell proliferation, cytokine production, and cytotoxicity. The inhibition of proliferation of T cells stimulated with anti-CD3/CD28 (though no inhibitory effect was observed over PMA-stimulated T cells) was associated with an increase of $p27^{KIP1}$ expression and to the decreased secretion of IL-2 by T cells [473]. In turn, the impairment of IL-2, IL-4, IL-10, and IFNγ production is attributed to semaphorin-3A blockage of CD3/CD28-mediated activation of c-Jun, c-Fos, nuclear factor of activated T-cells (NF-AT), NF-kB, and ERK1/2, which disable IL-2 induction. Of note, semaphorin-3A increases the expression of Rap-1, which sequestrates Raf-1, impairing the activation of MEK and the downstream ERK1/2 [473]. Remarkably, semaphorin-3A is also used as a mechanism of tumor

evasion from the immune system, which is achieved either by the direct inhibitory effect over T cells or by the blockage of the homotypic interaction among neuropilin-1 expressed on T cells and tumor cells (as semaphorin-3A will compete for neuropilin-1 ligation) [473].

In turn, galectins, an evolutionarily conserved family of β-galactoside-binding proteins, have been intensively studied in the recent years due to their role in materno-fetal tolerance [475]. They are widely express in human, being present in both lymphoid and non-lymphoid tissues; moreover their expression is upregulated in activated T cells, B cells, Treg cells, and inflammatory macrophages [475]. Galectin-1 has an inhibitory effect over T cell activation, which is mediated by binding to CD45, CD43, and CD7, or neuropilin-1, and includes impairment of proliferation, induction of apoptosis on Th1 cells, and bias of the immune response from Th1 towards a Th2 profile; notwithstanding, it promotes the survival of naive T cells and induces tolerogenic DC [474-476]. This inhibitory molecule is also involved in the suppressive mechanisms of Treg cells and in the malignant cells escape from the immune system [475,476].

Interestingly, human BM-MSC express both semaphorin-3A [474] and galectin-1 [59,475,477], being reported that these factors contribute to MSC-mediated inhibition of T cell proliferation. Of note, galectin-1 is constitutively expressed by human BM-MSC and its expression is upregulated under inflammatory conditions [59,477]; it can be detected in human BM-MSC intracellularly, at the cell surface, and also secreted as a soluble molecule to the extracellular milieu, though membrane-bound galectin-1 is more effective than its soluble form [475-477]. Besides the anti-proliferative effect over both $CD4^+$ and $CD8^+$ T cells, galectin-1 is also responsible for the reduction of TNF-α, IFNγ, IL-5, and IL-12 production by PBMC stimulated with either IL-2/OKT3 or CD28/OKT3, whereas its regulatory effect over IL-4 and IL-10 depends on the type of stimulus given [475].

4.5.2. Galectin-3 and galectin-9

Galectin-3 is also a negative regulator of T cells, able to induce T cell apoptosis through binding to CD7 and CD29, and which contributes to cancer immune escape [477-479]. Similarly to galectin-1, galectin-3 is also constitutively expressed on human BM-MSC and human UC-MSC; it localizes intracellularly, at the cell surface, or is secreted into the extracellular environment; and its expression is upregulated upon TLR2 stimulation [477,478,480]. Galectin-3 was also shown to be involved in the suppressive activity of human BM- and UC-MSC over T cell proliferation [477,478,480].

Concerning galectin-9, it is expressed by immune cells, endothelial cells, and fibroblasts [312]. It can bind to different receptors, including the glycoreceptor TIM-3 (T-cell immunoglobulin domain and mucin domain-3), expressed on terminally differentiated Th1 cells, Th17 cells, and CTL, being reported that galectin-9/TIM-3 binding induces death of effector Th1 and Th17 cells, and impairs cytokine production by the former cell population [74,479]. Moreover, galectin-9 induces Treg cells from naive $CD4^+$ T cells and inhibits Th17 differentiation by mechanisms either dependent or independent of TIM-3 [74]. This inhibitory molecule is also expressed by tumor cells [479]. In human BM-MSC, the expression of galectin-9 is induced by pro-inflammatory conditions – e.g. upon co-culture with activated PBMC, T cells, or B cells; and/or after treatment with IFNγ, TNFα, IL-1α, IL-1β, PGE2; or by TLR2, TLR3, or TLR4 stimulation [74,312] – and it plays an active role in MSC-mediated suppression of T cell proliferation [74,312], B cell proliferation, and IgG secretion by B cells [312].

4.6. Interleukin-10

4.6.1. The effect of interleukin-10 on immune cells

Interleukin-10 (IL-10) cytokine family includes IL-19, IL-20, IL-22, IL-24, and IL-26 cytokines [481,482]. IL-10, originally described as the cytokine synthesis inhibitory factor (CSIF), is a pleiotropic cytokine with a predominant anti-inflammatory function though, depending on the microenvironmental conditions, it can display immunostimulatory activity as well; IL-10 is mainly produced by macrophages, but monocytes, specific T cell subsets, and specific B cell subsets can also secrete this cytokine [304,420,481,483]. Interestingly, IL-10 expression is induced in monocytes/macrophages upon LPS stimulation, which suggest this cytokine acts to control the inflammatory response triggered by LPS; notwithstanding, it is also induced by PGE2 (chapter 1, section 4.2), in monocytes, DC, and T cells, wherein IL-10 mediates and improves PGE2's immunosuppressive effect [420,433]. In turn, IL-10 will subsequently inhibit COX-2 (and consequently PGE2) expression in DC and monocytes/macrophages [420], preventing the perpetuation of an immune suppressive environment.

IL-10 receptor is a heterodimer constituted by an α (IL-10Rα) and a β chain, and its engagement with IL-10 in monocytes leads to the phosphorylation of JAK1 and Tyk2, with subsequent activation of STAT3 (mainly), but also STAT1 and STAT5, depending on the microenvironment; and to the induction of *de novo* synthesis of SOCS3. Roughly, STAT3

activation mediates the anti-inflammatory effects of IL-10 in myeloid cells; interestingly, in macrophages pre-exposed to IFNγ, IL-10 is modulated to preferentially activate STAT1, instead of STAT3, which ultimately results in a pro-inflammatory effect [483].

The biological effects of IL-10 comprise the reduction of the expression of pro-inflammatory cytokines (TNF-α and IL-1β) and chemokines in neutrophils, presenting similar effects on eosinophils and mast cells [481]. In monocytes/macrophages, IL-10 also inhibits the production of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, IL-12, G-CSF, GM-CSF, and TNF-α) induced by IFN-γ or endotoxin, a process associated with the induction of SOCS3 expression. Notwithstanding, IL-10 upregulates the expression of anti-inflammatory mediators, such as IL-1 receptor antagonist (IL-1Ra) and soluble TNF-α receptors, and decreases the expression of MHC-II, CD86, and cell adhesion molecules, hampering antigen-presentation capability of monocytes/macrophages. Finally, it inhibits monocyte differentiation into DC, and downregulates the expression of CD86, IL-6, IL-12, TNF-α, and leukotriene B_4 by DC [413,420,433,481,482,484]. IL-10 promotes increased phagocytic activity in monocytes/macrophages, associated to the increased expression of IgG-Fc receptors (CD16, CD32, and CD64) and scavenger receptors (CD163 and CD14) [481]. In macrophages, IL-10 signaling through its receptor not only inhibits NF-kB p65/p50 translocation to the nucleus (by inhibiting IKK activity), but also blocks the DNA-binding of NF-kB already located in the cell nucleus; it also drives to the accumulation of the suppressive p50/p50 NF-kB homodimer in the nucleus, thus impeding the transcription of pro-inflammatory cytokines [481]. Besides, IL-10 inhibits STAT1 phosphorylation induced by IFNα and IFNγ, decreasing the expression of IFN-induced genes [481,483].

Due to the decrease of pro-inflammatory cytokines produced by monocytes and macrophages, particularly IL-12, IL-10 indirectly hampers Th1 immune response. Nevertheless, this anti-inflammatory cytokine also exerts direct effects on T cells, leading to the reduction of IL-2, IFNγ, IL-4 and IL-5 expression and preventing T cell proliferation, albeit these effects are more pronounced in naive T cells than in activated or memory T cells, probably because IL-10Rα is downregulated upon T cell activation [481]. Noteworthy, the presence of IL-10 in the course of T cell activation induces a regulatory phenotype, characterized by the production of IL-10 by T cells [481]. On the contrary, no inhibitory effect over $CD8^+$ T cells has been described so far for IL-10 [481].

Conversely, on NK cells, IL-10 presents a stimulatory effect, enhancing their proliferation and cytokine production (IFNγ, TNF-α, and GM-CSF), as well as the proliferation of $CD56^{bright}$ NK

cell subpopulation, induced by IL-2 [481]. Likewise, a discreet stimulatory effect on B cells is also observed: IL-10 prevents apoptosis, and enhances proliferation and differentiation into plasma cells [481].

Animal models underscore the biological importance of IL-10 in controlling exacerbated inflammatory states *in vivo*, such as those found in autoimmune diseases. Likewise, deregulation of IL-10 expression has been described in human autoimmune diseases, whereas IL-10 overexpression has been associated to several human malignant diseases and to a poorer outcome [481]. In the same line, viruses use IL-10 to escape from the immune system, either by stimulating IL-10 secretion by host immune cells, or by producing IL-10 homologs [481].

4.6.2. Role of interleukin-10 in mesenchymal stromal/stem cells immune suppressive function

Given the broad spectrum of immune cells whose function is regulated by IL-10, the role of this anti-inflammatory cytokine in the MSC-driven immunosuppression is being increasingly investigated. Interestingly, MSC avail themselves of IL-10 to regulate immune cells, not only by secreting the cytokine, but also by inducing its production in a wide range of immune cells [246,264,359,375].

Rat BM-MSC-derived IL-10 inhibits DC maturation and IL-12 secretion, while stimulates IL-10 production by DC [359]. These inhibitory effects were dependent on the activation of JAK1/STAT3 pathway induced by IL-10 in DC. In fact, JAK1/STAT3 activation was demonstrated to be necessary for IL-10 production by DC [359]. Likewise, mouse BM-MSC conditioned medium stimulates mouse splenocytes to produce IL-10, which will, in turn, prevent T cell proliferation in MLR [246]; mouse AT-MSC have the ability to impair the production of IFNγ and TNF-α by synovial cells from mice with CIA, stimulated with type II collagen, in a IL-10- and PGE2-dependent manner [180]. In turn, IL-10 secreted by mouse BM-MSC has the ability to impair Th17 cell differentiation, as well as IL-17A and IL-17F production, as demonstrated by silencing IL-10 gene in MSC by RNA interference technique [278]. In mouse T cells, IL-10 inhibits STAT3 activation (in opposition to what had been described for monocytes and DC [359,481,482]), a transcription factor activated by IL-6 or IL-23 during Th17 differentiation and responsible by inducing the expression of RORγt and IL-17 [232,233,278]. Interestingly, IL-10 activates STAT5 in T cells which, in turn, activates SOCS3, a suppressor of IL-6/STAT3 signaling pathway; in fact, IL-10 reduces STAT3 binding to promoter regions of RORγt, IL-17A, and IL-17F genes, disabling RORγt expression and Th17 differentiation [278].

Human BM-MSC constitutively express IL-10 at immunosuppressive concentrations [240] and can also induce human monocytes and T cells to produce IL-10 [375,485]. In human MLR, IL-10 mediates the inhibitory effect of BM-MSC on T cell proliferation [239], though it is not involved in the downregulation of CD25, CD38, and CD69 expression in both $CD4^+$ and $CD8^+$ T cells, driven by human BM-MSC [84].

Of note, IL-10 induces the expression of HO-1, an enzyme with anti-inflammatory properties (detailed in section 4.12 of this chapter), in human DC [486] and mouse macrophages [481]; IL-10 also upregulates the expression of HLA-G (chapter 1, section 4.15) in human BM-MSC and monocytes [239].

4.7. Interleukin-6 and leukemia inhibitory factor

4.7.1. The effect of interleukin-6 and leukemia inhibitory factor on immune cells

Leukemia inhibitory factor (LIF) and IL-6 belong to the same cytokine family. Beyond the sequence homology and structure similarity between these cytokines, they share a common receptor and signal transducer subunit, gp130, and display overlapping functions, such as the induction of acute phase proteins *in vivo* [487,488]. Under different conditions these cytokines can either promote or inhibit inflammation, albeit they possess other important functions not related to the immune system (such as embryo implantation, maintenance of the pluripotency of mice embryonic stem cells, differentiation/maturation and survival of neurons and oligodendrocytes, bone metabolism, proliferation of muscle satellite cells and cardiomyocytes, for LIF; and epithelial regeneration, regulation of fat and bone metabolism, as well as several neural functions, for IL-6) [487-490].

In what concerns to the immunologic function of IL-6, their anti-inflammatory properties have been attributed to signaling through membrane-bound IL-6 receptor (IL-6R), designated by classic signaling, whereas pro-inflammatory function is associated with signaling mediated by soluble IL-6R (sIL-6R), or trans-signaling [491]. Distinct lines of evidence highlight the role of IL-6 in the transition between the innate and acquired immune response, and between the acute inflammation and the inflammation resolution [488]. In an initial phase of an insult, IL-6, along with other pro-inflammatory factors, leads to neutrophil infiltration into the injury site. The proteolytic cleavage of IL-6R expressed on neutrophils will amplify IL-6 trans-signaling, leading to the expression of monocyte-attractive chemokines and upregulating adhesion

molecules expression on endothelial cells and lymphocytes, leading to the monocyte and lymphocyte infiltration in the site of injury, and enabling an acquired immune response [488]. IL-6 was shown to skew T cell differentiation towards Th17 or Th2, being recognized that it is necessary for Th17 differentiation and maintenance; also, it represses FoxP3 transcription, consequently suppressing Treg differentiation [488,492]. In contrast, LIF has the ability to inhibit Th17 differentiation, while supporting the expansion of Treg cell population *in vivo* [492-494]. Importantly, the role of LIF and IL-6 in cancer immune escape was proved by demonstrating that these cytokines induce monocyte polarization toward a M2 macrophage phenotype, both *in vitro* and in tumor-associated macrophages. These cells express high levels of IL-10 and low levels of IL-12, and can induce $FoxP3^+$ Treg cells; along with these anti-inflammatory properties, M2 macrophages also participate actively in tissue regeneration, contributing to the inflammation resolution [373,374].

4.7.2. Role of interleukin-6 and leukemia inhibitory factor in mesenchymal stromal/stem cells immune suppressive function

Recently, Melief and colleagues [375] demonstrated that IL-6, present in supernatant of human BM-MSC and monocytes co-cultures, skewed monocyte differentiation toward an IL-10-producing M2 macrophage phenotype and inhibited their differentiation into immature DC, resulting in a negative regulation of the inflammation and antigen presentation. IL-6 is secreted by both BM-MSC and monocytes, and is further upregulated when the cells are in co-culture; BM-MSC secrete higher amounts of the cytokine, compared to monocytes [375]. Of note, BM-MSC constitutively express IL-6 [79,375], a feature shared with human AT-MSC [292]. Interestingly, Najar and colleagues [60] reported that, at low doses, MSC support T cell proliferation, including Treg cell expansion, in a IL-6-dependent manner. In contrast, Melief and colleagues [285] demonstrated that IL-6 was not necessary for MSC-driven Treg cell expansion, perhaps due to the presence of other factors in the culture medium that were proven to possess the ability to induce Treg cells: TGF-β1 and HO-1 (produced by MSC), and CCL18 (derived from M2 monocytes). Finally, it was also reported that IL-6 produced by human fetal BM-derived MSC can increase the percentage of Th17 cells, with no effect in the number of Treg cells [252], demonstrating this cytokine can have opposite effects under different conditions.

Concerning to LIF, it is expressed by human AT- and UCM-MSC and upregulated in the presence of activated PBMC; it also mediates MSC-derived inhibition of T cell proliferation and induction/expansion of Treg cell population [38,495].

4.8. HEPATOCYTE GROWTH FACTOR

4.8.1. The effect of hepatocyte growth factor on immune cells

Hepatocyte growth factor (HGF) is a pleiotropic cytokine involved in liver regeneration, that plays an essential role in the regulation of cell proliferation, survival, and differentiation in a variety of organs, and also has a prominent role in the resolution of inflammation following an acute phase response, due to its anti-inflammatory activity [496-498]. As explained above (section 4.7), IL-6 is an acute phase cytokine produced after TLR4 stimulation (in an NF-kB-dependent manner), which intervenes both in the inflammatory phase and in the inflammation resolution of the acute phase response, through the induction of HGF expression and other mechanisms [496].

In fact, HGF acts as a switch from a pro-inflammatory to an anti-inflammatory response. The signaling cascade activated after HGF binding to its receptor, c-Met, leads to PI3K/Akt activation and subsequent phosphorylation and inactivation of glycogen synthase kinase-3 beta (GSK3β), a kinase with important functions in glycogen metabolism and in the regulation of cytokine expression [496,497]. GSK3β inactivation, in mouse macrophages [496] and human proximal tubular epithelial cells [497-499], is associated with an increased interaction between the co-activator protein (CBP) and phospho-CREB, making CBP/p300 less available to interact with NF-kB; also, in the presence of HGF, it is observed a reduced phosphorylation of the NF-kB p65; altogether leading to the decline of NF-kB transcriptional activity. Therefore, HGF switches off the transduction of NF-kB-dependent pro-inflammatory cytokines, such as IL-6 production by mouse macrophages [496], IL-6 and CCL5 expression by human proximal tubular epithelial cells [497,499], while induces the production of IL-10 by mouse macrophages [496].

In vivo, in mouse chronic renal injury models, HGF inhibits the renal infiltration of T cells and monocyte/macrophage, by suppressing the NF-kB-induced CCL5 and CCL2 expression, and blocks Th1 polarization [497,499,500]. In addition, HGF was also shown to ameliorate GvHD mouse model, by reducing donor T cell infiltration in the liver, consequently preventing hepatic lesions, and by decreasing the serum levels of IL-12, and IFNγ and TNF-α mRNA expression in the liver and small intestine [501]. Moreover, HGF reduces allergic airway inflammation and regulate eosinophil functions [502].

4.8.2. Role of hepatocyte growth factor in mesenchymal stromal/stem cells immune suppressive function

Human BM-, AT-, and UCM-MSC constitutively express HGF at immunosuppressive concentrations [26,240], which is further upregulated in BM-MSC, in the presence of IFNγ [240] or in co-culture with T cells [26]. Nevertheless, the demonstration that HGF is an important soluble factor that mediates the inhibition of T cell proliferation in MLR, by human BM-MSC, was revealed by the use of neutralizing antibodies directed against HGF [235]; likewise, HGF derived from human placental MSC also inhibit CD3/CD28-induced T cell proliferation [503]. In opposition, HGF seems not to be involved in the suppression of PHA-induced T cell proliferation by human BM-, UCM-, or AT-MSC [26,234]. As T cells do not express c-Met, the action of HGF on T cells is likely to be indirect and mediated by HGF-induced IL-10 upregulation in monocytes [503].

4.9. Insulin-like growth factor

Insulin-like growth factor (IGF)-1 and IGF-2 are pleiotropic growth factors, which can bind to either type 1 IGF receptor or insulin receptor, to promote cell proliferation and differentiation, also having an important role in the regulation of metabolism, behavior, and hematopoiesis [504]. Furthermore, there are six insulin-like growth factor-binding proteins (IGFBP), whose function ranges from blocking the biological effect of IGF, by impeding its interaction with the respective receptors, to acting as carrier proteins, facilitating IGF transport in the circulation and avoiding its proteolytic degradation [504,505].

From the immunologic point of view, both pro and anti-inflammatory actions had been ascribed to IGF [505,506]. Specifically, it has been reported the ability of IGF-2 to inhibit TNF-α-dependent activation of JNK in oligodendrocytes; in turn, IGF-1 was shown to enhance IkB dephosphorylation in astrocytes, consequently preventing NF-kB nuclear translocation, and to impair TNF-α-mediated NF-kB activation in human colonic adenocarcinoma cells; moreover, IGF-1 was also shown to induce IL-10 expression by T cells [504,505]. Interestingly, the expression of IGFBP2 and IGFBP3 by human BM-MSC was shown to be involved in the suppression of PBMC proliferation [507].

4.10. TNF-α STIMULATED GENE/PROTEIN 6

4.10.1. The effect of TNF-α stimulated gene/protein 6 on immune cells

Despite the anti-inflammatory properties of TNF-α stimulated gene/protein 6 (TSG-6) are recognized for a long time, only recently their modulatory actions over the cells of the innate and adaptive immune system have been demonstrated. In fact, TSG-6 has the ability to decrease human neutrophil and monocyte/macrophage infiltration at the site of injury, being demonstrated that TSG-6 binds to IL-8, impeding not only the chemokine transport across an endothelial monolayer but also its interaction with its receptor (CXCR2) expressed on neutrophils [380,508]. TSG-6 also inhibits TNF-α and IL-1α production by mouse macrophages [380]; to induce tolerogenic DC (with decreased expression of IL-12, CD40, CD80, and CD86, and higher production of IL-10 and TGF-β), capable of generating $CD4^{+}FoxP3^{+}$ Treg cells [509]; and to suppress T cell proliferation and IFNγ production, which was associated to TSG-6-dependent repression of ZAP-70, linker for activation of T cells (LAT), and p42 mitogen-activated protein kinase (MAPK) phosphorylation [509].

4.10.2. Role of TNF-α stimulated gene/protein 6 in mesenchymal stromal/stem cells immune suppressive function

The relevance of TSG-6 in MSC-mediated immunosuppression was demonstrated in several *in vivo* animal models (myocardial infarction, cornea injury, autoimmune diabetes, and peritonitis) [380,509-511]. In fact, human BM-MSC express TSG-6 [512] and its expression is upregulated after stimulation with TNF-α and other pro-inflammatory cytokines [380]. Human MSC-derived TSG-6 relies on different mechanisms to inhibit inflammation, and affects distinct immune cells.

It was demonstrated *in vivo* that human MSC-derived TSG-6 mediates the inhibition of neutrophil and monocyte/macrophage infiltration, in a rat cornea injury model and mouse peritonitis model, respectively [380,510,511]. Human BM-MSC-derived TSG-6 also suppresses TLR2-induced TNF-α and IL-1α expression by mouse macrophages. It was demonstrated, in mouse macrophages, that TSG-6 – bounded or not to hyaluronic acid – interacts with CD44 and, consequently, induces CD44/TLR2 dissociation, resulting in the inhibition of TLR2/NF-kB signaling pathway [380,511,513]. Similarly to human, TSG-6 produced by mouse BM-MSC inhibits DC maturation and function, apparently by interfering with MAPK and NF-kB signaling.

This inhibitory effect includes the suppression of CD80, CD86, MHC-II, and IL-12 expression, as well as the impairment of DC-induced T cell proliferation and IFNγ secretion in MLR [514]. Finally, human MSC-derived TSG-6 inhibits T cell proliferation and IFNγ production by mouse splenocytes, *in vitro* [509].

4.11. NOTCH SIGNALING

4.11.1. The effect of Notch signaling on immune cells

The Notch receptor family includes four trans-membrane proteins members (Notch-1-4). Their binding to their ligands (Delta-like 1, 3, 4, or Jagged 1, 2) triggers the cleavage of Notch intracellular domain (NICD), which translocates to the nucleus, where it activates gene transcription; this corresponds to the canonical Notch signaling pathway. The remaining modes of activation of Notch signaling are collectively designated as non-canonical Notch signaling and, interestingly, those are the ones which seem to be involved in pathological conditions, thus, a therapeutic intervention that specifically inhibits non-canonical signaling would allow to improve the pathological condition without interfering with the normal physiologic processes mediated by Notch [515].

The Notch signaling pathways are involved in morphogenesis, HSC self-renewal, cell growth, differentiation, and survival. In immune cells, Notch is involved in T cell development, regulation of T cell activity and effector cell differentiation, being demonstrated that Jagged 2 induces Th2 differentiation, but also that Notch signaling is important in Treg,Th1 and Th17 cell differentiation, as well as for CTL. Notch also mediates the differentiation of marginal zone B cells *versus* follicular B cell fate in the spleen; the differentiation of DC (induced by Notch-1 and Delta-like 1); and the development of megakaryocytes [515-517].

Regardless, Notch signaling is also involved in immune suppression, either directly, or indirectly, by its essential role in Treg cell generation and maintenance [518]. In macrophages, Notch-1 signaling inhibits the expression of TLR4-induced TNF-α and IL-6 (by suppressing the activating phosphorylation of ERK1/2 and, consequently, hampering NF-kB activity), while induces the production of IL-10 [519]; likewise, non-canonical activation of Notch in DC, results in PI3K activation and induction of IL-10 expression [515]. In what concerns to mouse T cells, Delta-like 1 and Jagged 1 induce the inhibition of the expression of early activation markers (CD69 and CD25) and of T cell proliferation, whereas Delta-like 4 enhances T cell activation and

proliferation [520]; similarly, Notch-1 agonist inhibits CD3/CD28-induced mouse CD4+ T cell activation, proliferation and production of IL-2, TNF-α, and IFNγ [521].

4.11.2. Role of Notch signaling in mesenchymal stromal/stem cells immune suppressive function

It is widely known that MSC induce tolerogenic DC, with low capability to stimulate T cell proliferation in MLR. In mouse, this attribute is partially dependent of Jagged 2 expression by DC, which is upregulated after contact with mouse BM-MSC [361].

As human BM-MSC express the Notch-1 ligands Jagged 1, Jagged 2, and Delta-Like 1, 3, and 4 [7,522], there is an active research to investigate whether human MSC suppress the immune system by activating Notch signaling pathways in immune cells. In fact, it is already proved that Jagged 1 expressed by human AT-MSC and BM-MSC mediates the inhibition of T cell proliferation, and the reduction of IL-2 and IFNγ production, which is associated to the inhibition of NF-kB activity [90,523]. Furthermore, the activation of Notch-1 on $CD4^+$ T cells by Jagged 1 expressed on human BM-MSC was proved to mediate the induction of Treg cells by MSC [522].

Interestingly, TLR3 and TLR4 stimulation of MSC induces the downregulation of Jagged 1 in these cells and, consequently, reduces MSC ability to suppress T cell proliferation [90].

4.12. HEME OXYGENASE-1

4.12.1. The effect of heme oxygenase-1 on immune cells

Heme oxygenase-1 (HO-1) is an ubiquitously expressed enzyme which catalyzes the heme degradation into biliverdin, carbon monoxide (CO), and free iron. Its expression is induced in most cell types under oxidative stress conditions, in which this enzyme displays important anti-oxidant and cytoprotective functions, but also under inflammatory environments, wherein HO-1 contributes to the regulation of the immune response. This enzyme plays also an important role in trophoblast survival and pregnancy success [267,524,525]. The HO-1-derived CO stimulates monocyte differentiation into macrophages, and an increased HO-1 expression is associated with the decreased expression of pro-inflammatory cytokines and increased production of anti-inflammatory cytokines in macrophages [524]; in the same line, HO-1 was recently shown to be involved in macrophage

polarization to the M2 phenotype [526]. Moreover, HO-1 and its metabolites are also involved in hampering the activation of basophils and the pro-inflammatory function of mast cells [524]; in preventing the production of pro-inflammatory cytokines (IL-12p70, IL-6, and TNF-α) and inducing a tolerogenic phenotype on DC [486,524,526]; in impairing T cell proliferation by preventing IL-2 expression [525]; and in the regulation of $CD4^+$ and $CD8^+$ Treg cell function [524]. In fact, human $CD4^+$ Treg cells constitutively express HO-1 and this enzyme was demonstrated to mediate Treg suppressive functions [525].

Regarding *in vivo* animal models, HO-1-knockout mice develop chronic inflammation and possess defective immune regulation mechanisms; additionally, HO-1 and CO were also shown to decrease EAE disease activity, to promote allograft survival and to reduce neutrophil and eosinophil infiltration to sites of inflammation [524].

4.12.2. Role of heme oxygenase-1 in mesenchymal stromal/stem cells immune suppressive function

Human BM-MSC express HO-1, but the protein expression levels of this enzyme display a great interindividual variability [267,527]. Noteworthy, rat BM-MSC were also shown to express HO-1, but its expression declines with the passage number [527]. HO-1 was shown to be involved in human BM-MSC-mediated inhibition of T cell proliferation in MLR [527]. In addition, human BM-MSC induce Treg cells and enhance their expression of IL-10 and TGF-β by mechanisms partially dependent on HO-1 [267,285,528]. The translation of the importance of HO-1 in MSC-based therapies was demonstrated by Chabannes and colleagues [527], in a rat model of cardiac allotransplantation, wherein the inhibition of HO-1 prevented the protective effect of rat BM-MSC infusion in graft rejection.

4.13. MATRIX METALLOPROTEINASE-1-CLEAVED CCL2

The recognition that some post-translational modifications naturally occurring in chemokines can alter their biologic activity and produce isoforms with antagonistic effects has been explored for many years, with the intent to exploit them as a therapeutic approach. This is the case of CCL2 (MCP-1), whose N-terminal proteolytic cleavage converts its agonist form into an antagonist form [529]. Remarkably, MSC-derived MMP-1 is able to cleave CCL2 yielding a CCL2 variant with antagonistic activity, which mediates the inhibitory effect over Ig production exerted by mouse BM-MSC [320]. In fact, MMP-1-processed CCL2 leads to Akt and

STAT3 inactivation in plasma cells, and the activity of STAT3 is essential for the induction of BLIMP-1 expression which, in turn, is necessary for Ig production (see section 3.3.1 of this chapter) [320]. Actually, the study carried out by Rafei and colleagues [320] suggests that MMP-1-cleaved CCL2 decreases Ig production by inducing the dedifferentiation of plasma cells into plasmablasts, which is supported by the decreased BLIMP-1 expression and increased levels of PAX5, as well as the increased proliferative activity, observed in plasma cells after treatment with MSC-conditioned medium or MMP-1-cleaved CCL2. Furthermore, MMP-1-cleaved CCL2 also upregulates the expression of PD-L1 (an immunosuppressive molecule whose function is detailed in chapter 1, section 4.17.2) and inhibits STAT3 phosphorylation (and activation) on $CD4^+$ T cells from EAE mice, thus inhibiting the secretion of IL-17 [530].

Likewise, *in vivo*, MSC also demonstrated the ability to reduce anti-hFVIII IgG titers in hemophilic mice [320], and to ameliorate the symptomatic neuroinflammation in EAE mice [530], through MMP-1-processed CCL2 binding to CCR2.

4.14. Erythropoietin-producing hepatocellular B2 and ephrin-B2

Erythropoietin-producing hepatocellular (Eph) receptors embrace a large family of membrane-bound receptors with tyrosine kinase activity which, together with their ligands, ephrins, is involved in a multitude of biological processes, comprising axon development, angiogenesis, regulation of MSC migration and differentiation, and regulation of the immune response, including T cell development and function [531]. Despite the controversy concerning the effect of Eph receptors and ephrins on T cells, ephrin-A1 was demonstrated to inhibit CD4+ T cell proliferation, suppress Th2 cell activation and Th2 cytokines production, and prevent activation-induced cell death; in turn, EphB6/ephrin-B2 interaction enhances T cell response to antigen, whereas ephrin-B2 and ephrin-B3 are involved in T cell co-stimulatory signaling. Remarkably, there are evidences that the final effect of Eph/ephrin is dose-dependent, which may explain the contradictory data [531].

Recently, Nguyen and colleagues [531] demonstrated that human BM-MSC express EphB2 and ephrin-B2, whose ligands, ephrin-B1 and EphB4, respectively, are highly expressed on human T cells. They also reported that both EphB2 and ephrin-B2 were implicated in MSC-induced inhibition of T cell proliferation in MLR. Moreover, EphB2 and ephrin-B2 hampered the mRNA expression of IL-2, IL-17, TNF-α, and IFNγ by T cells. Interestingly, EphB4 stimulation increases the expression of TGF-β1 by human BM-MSC [531].

4.15. Human leukocyte antigen G1 and human leukocyte antigen G5

4.15.1. The effect of human leukocyte antigen G on immune cells

The human leukocyte antigen (HLA)-G is a non-classical HLA class I molecule with important immune suppressive functions. It was firstly identified in first-trimester trophoblasts, in the fetal-maternal interface, where it was demonstrated to have a decisive contribution in materno-fetal tolerance. Alternative splicing of HLA-G primary transcript gives rise to seven different isoforms, four of which correspond to membrane-bound proteins (HLA-G1-4), and the remaining three to soluble proteins (HLA-G5-7); of note, a soluble HLA-G1 isoform is generated after the proteolytic cleavage of membrane HLA-G1 (shed HLA-G1) [239,532]. HLA-G binds to killer immunoglobulin-like receptor (KIR2DL4 or CD158d), leukocyte immunoglobulin-like receptor (ILT-2, LILRB1, or CD85j), and ILT-4 (also known as LILRB2, or CD85d). KIR2DL4 is expressed on NK cells, ILT-4 is specific from the myeloid lineage, whereas ILT-2 is expressed by monocytes, DC, T cells, B cells, and NK cells [239].

The immune regulatory functions of HLA-G include inhibition of TNF-α and IFNγ production by PBMC [533], and suppression of IFNγ secretion by γδ T cells [532]; induction of tolerogenic DC, with impaired ability to prime T cells and increased expression of IL-10 and IL-6 [239,532,534]; inhibition of B cell proliferation, differentiation, and Ig secretion [532]; reduction of the cytolytic activity of CTL, γδ T cells, and NK cells [239,532]; suppression of αβ T and γδ T cell proliferation [239] and reduction of the migratory abilities of activated CD4+ T cells, by reducing their expression of CCR2, CXCR3, and CXCR4 chemokine receptors [532]; and induction of Treg cells [534].

4.15.2. Role of human leukocyte antigen-G in mesenchymal stromal/stem cells immune suppressive function

In 2007, Nasef and colleagues [67] reported for the first time the expression HLA-G in adult MSC, specifically in human BM-MSC. Subsequent studies showed that human BM-MSC express HLA-G1 and HLA-G5 [30,239], however, the data published about the expressed isoforms are contradictory. Of note, the expression of HLA-G isoforms was also detected in human fetal liver-derived MSC [30].

Remarkably, HLA-G5 produced by human BM-MSC was shown to impair NK cell cytolytic activity and IFNγ production, to induce IL-10 secretion by peripheral blood lymphocytes, and to expand $CD4^{+}CD25^{+}FoxP3^{+}$ Treg cell population [239]. In turn, human MSC-derived HLA-G1 has

a role in MSC-induced decrease of IFNγ and increase IL-10 production by T cells [30]. Finally, MSC-driven suppression of T cell proliferation in MLR was shown to be mediated by both HLA-G1 and HLA-G5, being demonstrated that, at molecular level, HLA-G1 upregulates the expression of $p27^{KIP}$, while downregulates the expression of cyclin D1 and cyclin A, and decreases the levels of phosphorylated-Rb in T cells [30,239]. HLA-G1 and G5 expression by human BM-MSC is upregulated by cell contact with activated T cells, moreover, HLA-G5 secretion is also enhanced after exposure to IL-10 [30,239].

4.16. CD200

CD200 (or OX-2) is a membrane glycoprotein that belongs to the immunoglobulin superfamily. It has a broad distribution, including hematopoietic and non-hematopoietic cells (neurons, cardiomyocytes, endothelial cells, among others), and its expression is upregulated upon inflammation [41,401]; CD200 is also expressed on human BM-MSC [41,535], human UCM-MSC [41], but not detected on human UCB-MSC [42], though its expression was reported to be heterogeneous among the BM-MSC population and to display a great inter-individual variation [42]. In turn, CD200 receptor (CD200R) is restricted to the lymphoid (including T, B, NK, and NKT cells) and myeloid (DC, mast cells, eosinophils, basophils, neutrophils, and macrophages, especially regulatory M2 macrophages) lineages [42,401].

The use of knock-out mice lacking CD200 and blocking antibodies against CD200R, demonstrated the importance of CD200/CD200R axis in controlling autoimmunity and in graft survival, being also described that some viruses express CD200 homologues to escape from the immune system [401]. In fact, signal imparted by CD200/CD200R interaction inhibits Ras, MAPK, and NF-kB activity. As result, there is a decreased production of TNF-α and IL-6 by mouse macrophages activated with IFNγ or IL-17 (with no inhibitory effect over macrophages stimulated with LPS) [536], inhibition of IL-5, IL-13, IL-8, CXCL9, and CXCL10 protein expression by human monocytes [536], and decrease of mast cell degranulation, TNF-α and IL-13 production [401,536]. Besides the inhibitory effect of CD200R signaling over the synthesis of several pro-inflammatory cytokines (TNF-α, IFNγ, IL-1, IL-6, and IL-17), it is reported an induced expression of the anti-inflammatory IL-10 and TGF-β [401]. Moreover, there are evidences showing that differentiation of monocytes into DC in the presence of CD200R agonists leads to the induction of tolerogenic DC, able to induce Treg cells in a IL-10- and TGF-β-dependent manner [537]; CD200R agonists also induces Treg cells from thymocytes [537]; and, finally,

CD200 binding to CD200R expressed on T cells induce their differentiation towards either a Treg or Tr1 cells expressing IL-10 and TGF-β [401].

Human BM-MSC-derived CD200 was shown to mediate the inhibition of TNF-α production by macrophages stimulated with IFNγ [42].

4.17. B7 FAMILY: ICOS/ICOSL, PD-1/PD-L1 AND B7-H4

4.17.1. Inducible T-cell costimulator ligand (ICOSL) or CD275

The inducible T-cell costimulator (ICOS or CD278) molecule is promptly induced after T cell activation (in both $CD4^+$ and $CD8^+$ T cells) and markedly enhanced after CD28 ligation, being therefore absent in naive T cells [538]. This molecule delivers a strong second signal of T cell activation upon binding to its ligand (ICOSL, or CD275, or B7h), which is constitutively expressed on antigen-presenting cells and in several non-hematopoietic tissues [538,539]. ICOS signaling promotes the expression of CD40L (by stabilizing CD40L mRNA) and, in general, also promotes the production of IL-4, IL-5, IL-10, TNF-α, IFNγ, and GM-CSF [538,540,541]. Moreover, this costimulatory signal is involved in Th17 cell differentiation and IL-17 production, perhaps by inducing IL-21 expression by T cells [538,540,542]. Although, according to the reports publish so far, ICOS displays contradictory functions, which is suggestive of a complex role in the immune regulation, the final result of ICOS signaling depends on the microenvironment conditions. Nevertheless, it is reported that ICOS signaling preferentially favors Th2 polarization, function and humoral immune response (including the production of IL-4, IL-10, and IL-13), wherein the downstream activation of PI3K leads to the upregulation of NF-ATc1 which, in turn, enhances c-Maf expression and ultimately regulates IL-4 production [538-540].

In the same line of evidence, the crucial role of ICOS/ICOSL axis on B cell responses to T-dependent antigen, arose from the observation that ICOS-/- deficient mice were unable to form germinal centers and to undergo immunoglobulin isotype switching. In fact, it was demonstrated that stimulation of T cells via ICOS, by DC bearing ICOSL, within the T cell zone of secondary lymphoid organs, upregulates the expression of Bcl-6 and changes their expression pattern of adhesion molecules and chemokine receptors, including CXCR5 upregulation, that will ultimately drive T cell migration into the B cell follicles, following the CXCL13 gradient [301] (see section 3.3.1 of this chapter). Once within the B cell follicle, ICOSL

expressed on B cells will be essential for Tfh cells development within the germinal center (though the need of ICOS signaling seems to be overcome in high antigen concentration conditions) [301]. Then, fully differentiated Tfh cells provide stimuli to B cells – through CD40L, IL-21, and PD-1, among others – supporting B cell proliferation and affinity maturation within the germinal center; other cytokines produced by Tfh will control immunoglobulin isotype switching [301,540]. Of note, ICOS signaling enhances IL-21 expression in Tfh cells, a process mediated by the transcription factor c-Maf [301].

Besides, ICOS/ICOSL interaction is also essential for Treg cell expansion, maintenance, and function, though there is no direct evidence that ICOS signaling induce Treg differentiation [538,540,543]. ICOS is highly expressed in a subset of $CD4^+FoxP3^+$ Treg that produce IL-10; moreover, ICOS signaling induces production of IL-10 by $CD4^+$ effector T cells and regulatory $FoxP3^-$ Tr1 cells [540,543]. NK and NKT cells also express ICOS, but its function on these cells remains to be elucidated [538].

The active role of ICOS/ICOSL signaling in *in vivo* immunosuppression was evidenced in breast cancer, being demonstrated that tumor-associated pDC induce the expansion of Treg cells and the secretion of IL-10 by $CD4^+$ memory T cells within the tumor tissue, a process mediated by ICOSL. Further evidence of the ICOS/ICOSL immunosuppressive activity *in vivo* comes from the fact that a higher frequency of $ICOS^+$ cells in the tumor tissue correlates with a poorer prognosis in breast cancer [541].

Concerning MSC, ICOSL mRNA expression was recently detected by Yi and colleagues [544] and our group [79,244] in human BM-MSC, whose expression was enhanced in the presence of TNF-α, IFNγ [544], PMA plus ionomycin [244], and LPS plus IFNγ [79]. However, up to now, ICOSL expression in MSC at protein level has never been assessed, and the role of MSC-derived ICOSL in the modulation of the immune system hasn't yet been demonstrated.

4.17.2. Programmed death-ligand 1 (PD-L1) or B7-H1 or CD274

Programmed death-1 (PD-1, or CD279) receptor is expressed at low levels on resting T cells, B cells, NK cells, NKT cells, and myeloid cells; but its expression increases upon cell activation, and is further upregulated on T cells (a process mediated by NF-ATc1) under conditions of persistent antigen exposure and chronic inflammation, consequently being highly expressed on non-functional, exhausted T cells, that appear in the course of chronic viral infection [540,545,546]. PD-1 is a negative regulator of T cell activation, being involved in peripheral immune tolerance in both $CD4^+$ and $CD8^+$ T cells, upon binding to their ligands:

programmed death-ligand 1 (PD-L1, CD274, or B7-H1) or PD-L2 (or CD273, or B7-DC) [540]. In fact, it was demonstrated that binding of PD-L1 or PD-L2 to PD-1 inhibits TCR signaling and T cell activation, even in naive T cells, wherein PD-1 is modestly expressed [545]. PD-L1 is present in activated T cells, B cells, DC, monocytes/macrophages, BM-derived mast cells, as well as in a number of non-hematopoietic tissues; while PD-L2 expression can be induced on DC, macrophages, B cells, and mast cells from BM [539,546]. Both PD-L1 and PD-L2 expression is regulated by cytokines [546]. Of note, PD-L2 presents higher affinity for PD-1 as compared to PD-L1 but, interestingly, PD-L1 is also able to bind to CD80 [546,547].

PD-1/PD-L1 interaction results in the inhibition of the activating phosphorylation of CD3ζ chain and ZAP-70, which are activated downstream TCR binding to MHC/antigen complex by the kinases Fyn and Lck; it also hampers PI3K activation, that occurs in consequence of CD28 signaling in T cells [230,546,547]. The enzymatic activity of PI3K leads to the activation of the downstream signal molecule Akt, which has the ability to upregulate the expression of glucose transporters and the glycolytic enzyme activity, fuelling the proliferation of activated T cells [546,547]. Beyond glucose metabolism, Akt is also involved in other important metabolic processes, including protein synthesis, cell survival, and cell proliferation [548]. Interestingly, it is known that CTLA-4 activation results in the inhibition of Akt activity, interfering with the same signaling pathway as PD-L1, but at a downstream level; noteworthy, PD-L1-mediated T cell inhibition is more effective than that of CTLA-4 [547]. Likewise, PD-L1 binding to PD-1 expressed on B cells impedes the activating phosphorylation of Akt, p38, and ERK, involved in the three signaling pathways triggered after a productive BCR stimulation [175].

PD-L1 ligation to PD-1 hampers T cell proliferation, which is overcome by excessive anti-CD3/CD28 stimulation, and modulates cytokine production (namely, decreases IFNγ and increases IL-10 secretion) [539,549]. According to the data collected so far, it seems that, while in naive T cells PD-1 impedes T cell activation, in non-naive T cells PD-1 signaling leads to the loss of effector functions and to T cell exhaustion [547]. Importantly, PD-1 signaling also induces the generation of *de novo* $CD4^+FoxP3^+$ Treg cells from naive $CD4^+$ T cells (in the presence of TGF-β), in mice [546,550,551]. Treg induction by PD-1 is dependent from Akt/mTOR cascade inhibition and PTEN upregulation [546]. Besides, PD-1 augments the suppressive effect of pre-existent Treg cells [546]. In the same line, PD-1 signaling pathway also inhibits the macrophage and DC response to TLR stimuli, and decreases IL-6 and TNF-α expression in macrophages [546,549].

This inhibitory molecule was shown to be one of the mechanisms of tumor escape from the immune system: in a melanoma mouse model, PD-1/PD-L1 interaction in tumor infiltrating $CD4^+$ T cells contributes to their conversion in $CD4^+FoxP3^+$ Treg cells [550]; whereas in human, a higher expression of PD-L1 is associated to a poorer prognosis in both solid tumors (for instance pancreatic, esophageal, and ovarian cancer, melanoma, and renal cell carcinoma) and B-chronic lymphocytic leukemia [539,552]. Increased expression of PD-1 or PD-L1 has also been observed in other human B and T cell malignancies, in which the malignant cells showed to be resistant to specific cytolysis by T cells, *in vitro* [539].

In addition, PD-1 pathway was also shown to be involved in the immunosuppressive effect of MSC over T and B cells. Different studies demonstrated that co-culture of BM-MSC with immune cells induces the expression PD-L1 and PD-L2 in the former cell population [78,263,306,316], an effect shared with TNF-α, IFNγ, IL-6, or IL-17 [78,175,263]. PD-1 pathway is involved in human BM-MSC inhibition of B cell proliferation [306] and T cell production of IFNγ [78], whereas for human decidual stromal cells, it mediates the inhibition of TNF-α, IFNγ, and IL-2 production by $CD4^+$ T cells [553]. In mice, PD-1 signaling participates in BM-MSC-mediated inhibition of T and B cell proliferation [175,316], as well as in the suppression of the proliferation and IL-17 production by fully differentiated Th17 cells [263,530].

4.17.3. B7-H4

B7-H4 is an inhibitory member of the B7 family. In human, B7-H4 surface protein expression can be induced on T cells, B cells, monocytes, and DC, after *in vitro* stimulation; this molecule is also present in non-immune cells; and tumor tissues were shown to express this inhibitory protein constitutively, including tumor-associated macrophages [539,540,545,548]. Remarkably, Treg cells can also induce B7-H4 expression in antigen-presenting cells, in an IL-10-dependent manner [540]. However, the receptor for B7-H4 hasn't been identified yet [545].

In mice, B7-H4 has the capability to impair $CD4^+$ and $CD8^+$ T cell proliferation, and its inhibitory effect affects both naive and pre-activated T cells, however naive T cells are more sensitive to B7-H4. This inhibitory molecule also impairs IL-2 production by T cells, inhibits the upregulation of CD25 and CD69 induced by T cell activation with anti-CD3/CD28, and hampers $CD8^+$ T cell cytotoxic activity [540,548,554]. In humans, B7-H4 was shown to inhibit $CD4^+$ and $CD8^+$ T cell proliferation by inducing G0/G1 cell cycle arrest and apoptosis on activated T cells, along with the impairment of IFNγ expression by PBMC [548,555]. Wang and colleagues [548] have recently demonstrated that, on the basis of T cell inhibition by B7-H4, was the interference with the signaling pathways triggered by CD28, namely the inhibition of the

activation of Akt (and, consequently, also impairs the activity of the Akt's substrate GSK3), ERK1/2, p38, and JNK (and its substrate Jun) [548,554]. Whereas Akt is involved in the regulation of the cellular uptake and metabolism of glucose, cell survival, and cell proliferation, ERK1/2 and JNK have a pivotal role in inducing IL-2 expression [546,548]. Conversely, B7-H4 did not affect the activity status of neither Lck nor ZAP-70, upon T cell stimulation with anti-CD3/CD28, indicating that B7-H4 only acts over signal molecules localized at a downstream level of TCR and CD28 pathways [548]. Remarkably, the addition of exogenous IL-2 can overcome the inhibitory effect of PD-L1 and B7-H4, apparently because IL-2 leads to STAT5 activation which, in turn, is able to activate Akt [546-548].

It was recently demonstrated that human BM-MSC express B7-H4 [556]. This molecule was shown to mediate BM-MSC inhibition of T cell activation and proliferation, by inducing cell cycle arrest, and impede NF-kB translocation to the nucleus [556,557].

5. OBJECTIVES

Since the report of Le Blanc and colleagues [198], in 2004, with first evidence of the clinical benefit of MSC-based therapy in humans, in one patient with treatment-resistant grade IV acute GvHD, an increasing body of evidence further confirmed the utility of MSC in the treatment of a wide range of diseases. However, distinct outcomes are not rarely reported when using MSC in clinical trials, *in vivo* animal models, or even *in vitro* assays. To explain these different end results, scientists have postulated that MSC arisen from distinct sources may have distinct abilities, concerning both the immunosuppression and production of growth factors. In this line, a significant number of studies have recently showed important differences among MSC isolated from different human tissues using *in vitro* assays. Notwithstanding, it is recognized that the activation/maturation/differentiation state of immune cells is different for distinct pathologies and, considering the same disorder, different patients may display distinct features regarding the distribution and function of the immune cells comprised in the aforementioned compartments. Furthermore, it is recognized that the differences found in those immune cell compartments can be associated to a distinct clinical course or may have prognostic value. Nevertheless, despite the growing knowledge on the influence of human MSC over the main immune cell populations, the information on specific (and less represented) immune cell subpopulations/compartments is scarce. Noteworthy, the few studies published so far in this field consistently described that the suppressive effect of MSC is distinct among the subpopulations defined within the main immune cell populations (e.g. naive *versus* memory T cells; fully differentiated effector T cells *versus* T cells undergoing differentiation into effector cells).

Based on the above background, the **main objective** of this study aimed at gaining insight on the differences among human MSC derived from different tissues, concerning their immunosuppressive potential; and, as bone marrow-derived MSC are the most used in human immune-therapy protocols, we sought to investigate the influence of human BM-MSC in naturally occurring peripheral blood human monocyte subpopulations, mDC, and T cell functional compartments.

In order to accomplish this goal, we addressed the following **specific objectives**:

1. To investigate and compare the effect of human MSC derived from BM, AT, and UCM on PHA-induced activation and proliferation of peripheral blood lymphocyte subpopulations, and on the mRNA expression of molecules with an important immune function for T cells and NK cells.

2. To evaluate the influence of human BM-MSC on cell activation and cytokine production by peripheral blood monocyte subsets and mDC, upon stimulation with LPS plus IFNγ.

3. To investigate how human BM-MSC modulate PMA plus ionomycin-induced cytokine production by each naturally occurring functional compartment of peripheral blood $CD4^+$ and $CD8^+$ T cells.

4. In order to better understand how the stimulating agents used in our *in vitro* models for T cells (PMA plus ionomycin) and monocytes/DC (LPS plus IFNγ) stimulation influence human BM-MSC, we analyzed the phenotype and mRNA expression in non-stimulated BM-MSC and after stimulation with either PMA plus ionomycin, or LPS plus IFNγ.

The following chapters of this doctoral thesis thoroughly describe the studies carried on to accomplish the proposed specific objectives. The materials and methods used and the results obtained for each study are presented and discussed separately.

CHAPTER 2 |

MESENCHYMAL STEM CELLS FROM UMBILICAL CORD MATRIX, ADIPOSE TISSUE AND BONE MARROW EXHIBIT DIFFERENT CAPABILITY TO SUPPRESS PERIPHERAL BLOOD B, NATURAL KILLER AND T CELLS

1. ABSTRACT

Introduction: The ability to self-renew, be easily expanded *in vitro* and differentiate into different mesenchymal tissues, soon render mesenchymal stem cells (MSC) an attractive therapeutic method for degenerative diseases. The subsequently discovery of their immunosuppressive ability, encouraged clinical trials in graft-*versus*-host disease and auto-immune diseases. Despite of sharing several immunophenotypic characteristics and functional capabilities, the differences between MSC arising from different tissues still unclear and the published data are conflicting.

Methods: Here, we evaluate the influence of human MSC derived from umbilical cord matrix (UCM), bone marrow (BM) and adipose tissue (AT), co-cultured with PHA-stimulated peripheral blood MNC, on T, B and NK cell activation; T and B cells' ability to acquire lymphoblast characteristics; mRNA expression of IL-2, FoxP3, T-bet and GATA3, on purified T cells, and TNF-α, perforin and granzyme B on purified NK cells.

Results: MSC arisen from all the three tissues were able to prevent $CD4^+$ and $CD8^+$ T cell activation and acquisition of lymphoblast characteristics and $CD56^{dim}$ NK cell activation, wherein AT-MSC showed a stronger inhibitory effect. Moreover, AT-MSC blocked T cell activation process in an earlier phase than BM- or UCM-MSC, yielding a great proportion of T cells in non-activated state. Concerning B cells and $CD56^{brigh}$ NK cells, UCM-MSC did not influence neither their activation kinetics nor PHA-induced lymphoblast characteristics, conversely to BM- and AT-MSC which displayed an inhibitory effect. Besides, when co-cultured with PHA-stimulated MNC, MSC seem to promote Treg and Th1 polarization, estimated by the increased expression of FoxP3 and T-bet mRNA within purified activated T cells, and to reduce TNF-α and perforin production by activated NK cells.

Conclusions: Overall, UCM-, BM- and AT-derived MSC hamper T cell, B cell and NK cell mediated immune response by preventing their acquisition of lymphoblast characteristics, activation and changing the expression profile of proteins with an important role in immune function, except for UCM-MSC showing no inhibitory effect on B cells under these experimental conditions. Despite of the similarities between the three types of MSC evaluated, we detect important differences that should be taken into account when choosing the MSC source for research or therapeutic purposes.

2. INTRODUCTION

Mesenchymal stem cells (MSC) are multipotential non-hematopoietic stem cells that possess the ability to self-renew and to differentiate in response to chemical, hormonal or structural stimuli into different lineages of mesenchymal tissues, such as osteocytes, chondrocytes, neurocytes and adipocytes [192,558-563]. MSC can be isolated from adult tissues, such as bone marrow, adipose tissue, endometrial polyps, menstrual blood, *etc* [559], and from fetal tissues, such as placenta, umbilical cord blood and matrix [34,564]. Their ability to differentiate into different tissues is variable according to their tissue of origin [560]. Bone marrow is the traditional source of human MSC, however there, they represent a rare population of approximately 0.001% to 0.01% of total nucleated cells and their frequency tends to decline with increasing age [1,4,34,565]. Although adult MSC have the ability to expand in culture whilst retaining their growth and multilineage potential [566], compared with MSC from fetal sources, they undergo less cell divisions before they reach senescence [560].

All MSC seem to share a significant number of characteristics, even if isolated from different sources: they are plastic adherent, exhibit a fibroblast-like morphology, express certain cell-surface markers (CD90, CD73 and CD105) and are distinguished from HPC and leukocytes by lacking CD34, CD45, CD14 and HLA-DR expression [14,192,560,567]. MSC secrete several cytokines, growth factors and extracellular matrix molecules that play an important role in the regulation of hematopoiesis, angiogenesis and in immune and inflammatory response [564]. Other interesting characteristics are that MSC can migrate and home to tissues and organs in response to growth factors, cytokines, chemokines or adhesion molecules and, therein, mediate immunomodulatory actions [14,565,568-570]. Moreover, due to their multipotency, MSC are a very attractive choice for clinical applications in several immune disorders such as arthritis, encephalomyelitis, systemic lupus erythematosus and in regenerative diseases, including diabetes and skin grafting [564-566,568,571]. Their low immunogenicity, immunomodulatory capacity and ability to differentiate into cells that regenerate damaged tissues, had already allowed the use of MSC in clinical trials for cellular and gene therapy [14,565,566,572-574]. MSC are able to inhibit the proliferation and function of T, B and natural killer (NK) cells, the cytolytic effects of antigen-primed cytotoxic T cells (CTL) by the induction of regulatory T cells (Treg) [14,568,572,574]. The immune modulation by MSC seems to be mediated by secretion of soluble factors, creating an immunosuppressive microenvironment. This niche also protects MSC from environmental insults, including

cytotoxic chemotherapy and pathogenic immunity [192,575]. Beyond that, there are studies reporting that a separation of MSC and mononuclear cells (MNC) by a semi-permeable membrane does not abrogate the inhibition of lymphocyte proliferation [316,572,576-579].

Different studies affirm that different molecules expressed by MSC are responsible for, or could contribute to, suppression of lymphocyte proliferation [14,565,572,580-582]. MSC have also been demonstrated to interfere with dendritic cells (DC) differentiation, maturation and function, by soluble factors. Consequently, this interference can be involved in suppression of T cells proliferation, as well as in the induction of regulatory antigen-presenting cells [565,570,572,581,583,584]. Moreover, MSC seem to differently modulate the function of the various T cell subsets, what is explained in detail in the review of Duffy *et al* [231].

In this study, we performed co-cultures of PHA-stimulated MNC with MSC from different sources (umbilical cord matrix, adipose tissue and bone marrow), for four days, to evaluate the immunomodulatory effects of MSC on the acquisition of lymphoblast characteristics, by T and B lymphocytes, and on immune cell activation, which has been assessed by the expression of CD69, CD25 and HLA-DR on $CD4^+$ and $CD8^+$ T cells, B cells and NK cells. After cell sorting of the different compartments of activated T cells, we have measured transcripts for T-bet, GATA3 and FoxP3, to infer about T cell polarization to Th1, Th2 or Treg, respectively; the expression of mRNA for IL-2 was also quantified. In addition, the different compartments of activated NK cells were purified and mRNA expression of TNF-α, perforin and granzyme B was measured to evaluate the effect of MSC on these proteins with an important role in NK cell function.

This study shows that MSC derived from different tissues possess different immunosuppression capabilities and their action varies with the immune cell type.

3. METHODS

3.1. MSC isolation, purification and co-culture with peripheral blood mononuclear cells

Co-cultures were carried out in 6 well tissue culture plates (Falcon, Becton Dickinson Biosciences, BD, San Jose, CA, USA) with peripheral blood mononuclear cells (MNC) from healthy donors and allogeneic human MSC from the 3 different sources, bone marrow (BM), adipose tissue (AT) and umbilical cord matrix (UCM, also known as wharton's jelly MSC), in the presence or absence of phytohemagglutinin (PHA, Irvine Scientific, Santa Ana, CA, USA), as

mitogenic stimulus. Biological samples were obtained from healthy donors, with informed consent, and the study was approved by the Ethics Committees of Instituto Português de Oncologia de Lisboa Francisco Gentil (Laws n.º 97/95, nº 46/2004) and Maternidade de Bissaya Barreto (ref. 356/Sec). MSC were isolated from at least 2 different healthy donors for each cell source and appropriately cultured and purified to homogeneity, as previously described [65,585,586].

In short, MNC from BM aspirates were plated at a density of $2x10^5$ cells/cm^2 on T-175 flasks (Falcon BD) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, streptomycin (0.025 µg/ml), penicillin (0.025 U/ml) (Gibco, Life Technologies, Paisley, UK), at 37ºC and 5% CO_2 in a humidified atmosphere. Medium was changed twice a week. BM-MSC were isolated based on adherence to plastic, and near cell confluence (70-80%) exhausted medium was removed from the flasks, cells were washed with phosphate buffered saline (PBS, Gibco) and detached from the flask by adding Accutase solution (Sigma, St. Louis, MO, USA) for 7 minutes at 37ºC. Isolated BM-MSC expressed their characteristic immunophenotype being CD73, CD90 and CD105 positive and negative for CD31, CD34, CD45 and CD80. Cell number and viability were also determined using the Trypan Blue (Gibco) exclusion method [65].

For AT-MSC isolation, AT samples were collected into a conical tube containing PBS supplemented with 20 mg/mL of human serum albumine, washed extensively with PBS and then digested with a 0.1% (w/v) Collagenase Type II (Sigma) solution for 30 minutes at 37ºC. After neutralizing the enzymatic activity of Collagenase with DMEM 10% fetal bovine serum, 1% (v/v) penicillin (10000 U/mL)/streptomycin (10000 g/mL) (Gibco) and 0.1 % (v/v) Fungizone (Gibco), the adipose tissue was centrifuged at 2500 rpm for 10 minutes, in order to obtain a high density stromal vascular fraction. The contaminating red blood cells were lysed by ressuspending the pellet in 160 mM NH_4Cl solution for 10 min at room temperature. Once again, the reaction was stopped with DMEM 10% fetal bovine serum, followed by centrifugation. Then, the suspension was filtered though a nylon mesh of 100 µm to eliminate the cellular debris and a cell count was performed. After plating and incubating overnight at 37ºC, 5% CO_2, the cells were washed with PBS in order to remove non-adherent cells. The cells were maintained at 37ºC, 5% CO_2, in a fully humidified atmosphere in DMEM 10% fetal bovine serum and the medium changed every 3-4 days. When cells reached near 80% confluence, cells were harvested using Accutase and plated into new T-flasks (3000 cells/cm^2). The number of cells and cellular viability were also determined [586].

For UCM-MSC isolation, umbilical cords were rinsed with PBS (Gibco), arteries and veins were dissected and the remaining tissue fragments were digested with 0.1% collagenase type II (Sigma) for 4 hours at 37ºC. The solution was filtered and washed with Iscove's Modified Dulbecco's Media (IMDM, Gibco) supplemented with streptomycin (0.025 µg/mL) and penicillin (0.025 U/mL) (Gibco). Cell number was determined using the Trypan Blue (Gibco) exclusion method. Cells were plated in T-flasks at an initial density of 10 000 cells/cm^2 using DMEM supplemented with 10% fetal bovine serum, StemPRO® MSC Serum-Free Medium, or StemPRO® MSC SFM XenoFree (all from Gibco) culture media and kept at 37ºC and 5% CO_2 in a humidified atmosphere. When using StemPRO® MSC SFM/StemPRO® MSC SFM XenoFree cell culture, surfaces were pre-coated with CELLstartTM CTSTM (Invitrogen, Life Technologies, Paisley, UK) following the manufacturer's instructions. After 48 hours of culture, the nonadherent cells were removed and cells were maintained by renewing the medium every 3-4 days. Cultures were monitored by microscopy (Olympus CK40 optical microscope, Central Valley, PA, USA) in order to assess cell morphology and spreading [585].

All assays were performed using MSC between passage 3 and 5 and, prior to cell cultures, MSC identity was confirmed by immunophenotypic analysis: $5x10^5$ cells were harvested to perform flow cytometry analysis for the MSC markers CD105, CD73 and CD90. MNC were purified from heparinized peripheral blood by density-gradient centrifugation (Lymphoprep, Axis-Shield PoC AS, Oslo, Norway) at 1310 x g, for 20 minutes. Cell cultures were maintained in RPMI 1640 with GlutaMax medium (Invitrogen) supplemented with 10% fetal bovine serum (Gibco) and antibiotic-antimycotic (Gibco). Control cultures consisted on MNC in the absence of MSC, with or without PHA stimulation (17 replicates for each condition). With this strategy, different sets of co-cultures were generated: MNC+BM-MSC and MNC+PHA+BM-MSC (5 replicates for each condition); MNC+UCM-MSC, and MNC+PHA+UCM-MSC (7 replicates for each condition); MNC+AT-MSC and MNC+PHA+AT-MSC (5 replicates for each condition). A total of 300 000 MNC were added to each well and, in the conditions where the cells were activated by PHA, a concentration of 10 µg/mL of the mitogen was used; in each well where MSC were included, we added 30 000 cells, establishing a ratio of 10:1 (MNC:MSC). These plates were kept in culture for four days at 37°C, in 5% CO_2 and 90% humidity. After four days under each condition, the cultured cells were washed with PBS 1X, pH7.4 (Gibco) and centrifuged at 540 x g for 5 minutes.

3.2. Identification and quantification of the different compartments of activated T, B and NK cells

3.2.1. Immunofluorescent staining:

Each cell culture was used for two purposes: (1) phenotypic study by flow cytometry, to evaluate the acquisition of lymphoblast characteristics by T and B cells and the activation kinetic of lymphocytes and (2) cell sorting and purification of the compartments of activation of lymphocytes, for gene expression analysis on T and NK cells. In order to identify different lymphocyte subtypes, we used monoclonal antibodies (mAb) conjugated with the following fluorochromes: fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), phycoerythrin cyanin 7 (PE-Cy7 or PC7), allophycocyanin (APC), allophycocyanin hillite 7 (APC-H7), pacific blue (PB) and amcyan (AC). T cells were identified by CD3 PB (clone UCHT1, BD Pharmingen, San Diego, CA, USA) expression and, among this cell population, $CD4^+$ T and $CD8^+$ T cells were identified based on CD4 FITC (clone 13B8.2, Beckman Coulter, Brea, CA, USA) and CD8 AC (clone SK1, BD) expression, respectively; CD19 FITC (clone SJ25C1, BD) or CD19 PC7 (clone J4.119, Beckman Coulter) were used to identify B cells, and CD56 PC7 (clone N901, Beckman Coulter) positivity, in the absence of CD3 expression, for NK cells; finally, we used CD90 APC (clone 5E10, BD Pharmingen) to identify and exclude MSC. To define the different stages of lymphocyte activation, we used mAb against CD69 PE (clone TP1.56.3, Beckman Coulter), CD25 APC-H7 (clone M-A251, BD Pharmingen) and HLA-DR PerCP (clone G46-6, BD Pharmingen).

3.2.2. Flow cytometry data acquisition and analysis:

Cells were acquired on a FACS Canto^{TM} II (BD) using FACSDiva software (BD), and 100 000 events were analyzed using Infinicyt 1.5 software (Cytognos, Salamanca, Spain). Through multi-parametric flow cytometry analysis, we identified four different subpopulations of $CD4^+$ and $CD8^+$ T cells: non-activated $CD69^-CD25^-HLA\text{-}DR^-$; earlier activated $CD69^+CD25^-HLA\text{-}DR^-$; intermediate activated $CD69^+CD25^+HLA\text{-}DR^-$; and later activated $CD69^+CD25^+HLA\text{-}DR^+$. In B cells, $CD56^{dim}$ NK and $CD56^{bright}$ NK cells, only three subpopulations were quantified: non-activated $CD69^-CD25^-$, earlier activated $CD69^+CD25^-$ and activated $CD69^+CD25^+$.

3.3. Cell sorting of the activation compartments of T and NK cells

Cultured lymphocytes were purified by fluorescence-activated cell sorting, using FACSAria flow cytometer (BD). Each compartment of activated lymphocytes were sorted

according to their typical phenotype: $CD69^{-}CD25^{-}HLA\text{-}DR^{-}$, $CD69^{+}CD25^{-}HLA\text{-}DR^{-}$, $CD69^{+}CD25^{+}HLA\text{-}DR^{-}$ and $CD69^{+}CD25^{+}HLA\text{-}DR^{+}$ for T cells; and $CD69^{-}CD25^{-}$, $CD69^{+}CD25^{-}$ and $CD69^{+}CD25^{+}$ for NK cells.

3.4. Gene expression analysis

The cell sorted subsets were centrifuged for 5 minutes at 300 x g and the pellet resuspended in 350μL of RLT Lysis Buffer (Qiagen, Hilden, Germany) and total RNA extraction performed with the RNeasy Mini kit (Qiagen) according to the supplier's instructions. Total RNA was eluted in a 50μl volume of RNase-free water. In order to quantify the amount of total RNA extracted and to verify RNA integrity, samples were analyzed using a 6000 Nano Chip kit, in an Agilent 2100 bioanalyzer (Agilent Technologies, Walbronn, Germany) and 2100 expert software, according to the manufacturer's instructions. RNA was reverse transcribed with SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen), according to the manufacturer's instructions. Relative quantification of gene expression by real-time PCR was performed in the LightCycler 480 II (Roche Diagnostics, Rotkreuz, Switzerland). Real-time PCR reactions were carried out using 1× QuantiTect SYBR Green PCR Master Mix (Qiagen), 1 X QuantiTect Primer Assay (TNFα: QT01079561; FoxP3: QT00048286; IL2: QT00015435; Tbet: QT00042217; Gata3: QT00095501; Granzime B: QT01001875; Perforin: QT00199955) (Qiagen) and 20ng of cDNA sample, in a total volume of 10 μl. The reactions were performed using the following thermal profile: 15 min at 95°C, and 40 cycles of 15 sec at 94°C, 30 sec at 55°C and 30 sec at 72°C. All samples were run in duplicate. Melting point analysis was done to ensure the amplification of the specific product. Real-time PCR results were analyzed with the LightCycler software (Roche Diagnostics). GeNorm Reference Gene Selection kit (PrimerDesign Ltd., Southampton, England) in conjunction with the geNorm software (PrimerDesign Ltd.) were used to select the reference genes to normalize data. The reference genes used for gene expression analysis, in T cells, were the splicing factor 3a, subunit 1 (SF3A1) and the topoisomerase (DNA) I (TOP1) and, in NK cells, were the ubiquitin C (UBC) and the 18S rRNA. The normalized expression levels of the genes of interest were calculated by using the delta-Ct method.

3.5. Statistical analysis

To determine the statistic significance of the differences observed between different culture conditions, non-parametric Mann-Whitney test and Wilcoxon paired-sample test were performed, using Statistical Package for Social Sciences (IBM SPSS, version 17.0, Armonk, NY,

USA). Data were expressed as mean percentage ± standard deviation. Statistically significance differences were considered when *p* value was lower than 0.05.

4. RESULTS

4.1. MSC prevent T and B cells' acquisition of lymphoblast characteristics, except for UCM-MSC that are unable to inhibit this process in B

Lymphocytes undergo multiple rounds of clonal division after mitogenic stimulation. Consequently, in the presence of PHA, lymphocytes start to proliferate and the cells acquire a lymphoblast morphology, which can be observed by flow cytometry as an increased forward scatter (FSC, corresponding to an increase in cell size) and side scatter (SSC, corresponding to an increased granularity) light dispersion properties, as shown in figure 2. A prior analysis of FSC-Area vs FSC-Height dot plot allowed an easy identification and exclusion of doublets.

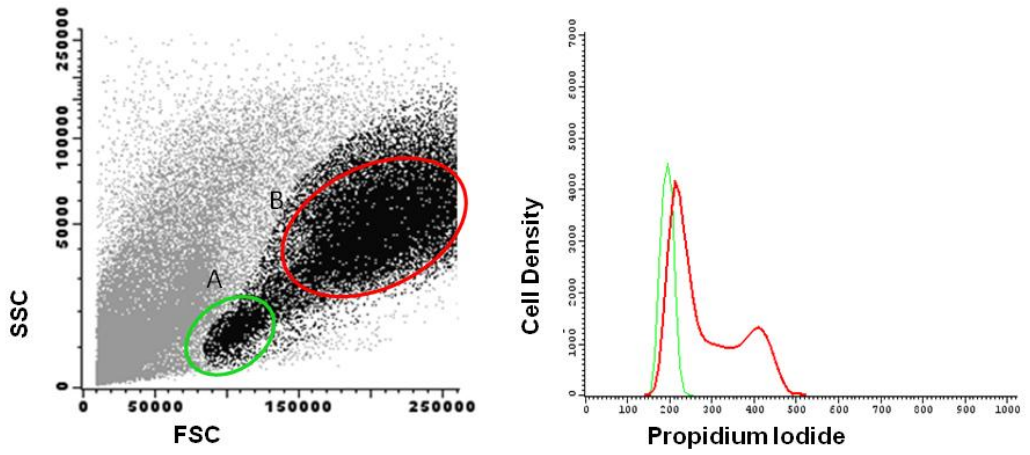

Figure 2. Cells displaying increased FSC and SSC light dispersion properties correspond to proliferating cells. Left: Bivariate dot plot histogram illustrating mononuclear cell (MNC) culture after PHA stimulation, displaying two distinct cell populations: (A) MNC with low forward scatter (FSC) and side scatter (SSC) light dispersion properties and (B) MNC with increased FSC and SSC properties. **Right**: Density histogram illustrating the DNA content of MNC from population A (green) and B (red), measured after propidium iodide staining, and proving that cells with increased FSC and SSC are actively proliferating.

MNC were co-cultured with BM-, UCM- and AT-MSC at the ratio of 10:1, in the presence of PHA. When compared with the MNC+PHA control assay, in the presence of MSC, the frequency of $CD4^+$ and $CD8^+$ T cells with increased FSC and SSC properties was lower, as shown in table 1 ($p < 0.05$ for BM-, UCM- and AT-MSC). Although only 18% of B cells acquired lymphoblast characteristics in control assay, a marked and statistically significant decrease of

the percentage of B cells with increased FSC and SSC properties occurred in the presence of BM- and AT-MSC, whereas UCM-MSC did not influence this process on B cells under these experimental conditions (Table 1).

Table 1. Percentage of cells displaying increased FSC and SSC light dispersion properties

% of cells with increased FSC and SSC	MNC + PHA	MNC + PHA + BM-MSC	MNC + PHA + UCM-MSC	MNC + PHA + AT-MSC
	n = 17	*n = 5*	*n = 7*	*n = 5*
Total mononuclear cells	51 ± 3	31 ± 4 [a; d]	41 ± 15[d]	21 ± 10 [a; c; d]
Total T cells	56 ± 2	31 ± 6 [a; d]	47 ± 19[d]	19 ± 13 [a; c; d]
$CD4^+$ T cells	54 ± 4	26 ± 3 [a; d]	47 ± 22[d]	15 ± 11 [a; c; d]
$CD8^+$ T cells	59 ± 4	38 ± 11 [a; d]	49 ± 16[d]	21 ± 7 [a; b; c; d]
B cells	18 ± 3	9 ± 1 [a]	18 ± 9 [b]	7 ± 4 [a; c; d]

Percentage (mean ± standard deviation) of cells with increased forward scatter and side scatter light dispersion characteristics after PHA stimulation, in the absence or in the presence of MSC from different sources, within the total MNC in culture and within the following cell populations: total T cells, $CD4^+$ T cells, $CD8^+$ T cells and B cells.

Results are expressed as mean percentage ± standard deviation. Statistically significant differences were considered when p <0.05 for Mann-Whitney test: [a]comparing with MNC+PHA; [b]comparing with MNC+PHA+BM-MSC; [c]comparing with MNC+PHA+UCM-MSC. Statistically significant differences were considered when p <0.05 for Wilcoxon paired-sample test: [d]comparing with MNC+PHA.

FSC, forward scatter ; SSC, side scatter; MNC, mononuclear cells; PHA, phytohemagglutinin; BM, bone marrow; UCM, umbilical cord matrix; AT, adipose tissue; MSC, mesenchymal stromal/stem cells.

4.2. MSC derived from different tissues influence differentially the distribution of T, B and NK lymphocytes among non-activated and activated compartments

To investigate the kinetics of lymphocyte activation, in the presence or absence of PHA and MSC arisen from different tissues, we analyzed four subpopulations of both $CD4^+$ and $CD8^+$ T cells, phenotypically defined as: $CD69^-CD25^-$HLA-DR^- (non-activated), $CD69^+CD25^-$HLA-DR^- (earlier activated), $CD69^+CD25^+$HLA-DR^- (intermediate activated) and $CD69^+CD25^+$HLA-DR^+ (later activated), in different culture conditions. Likewise, we identified three subpopulations of B cells, $CD56^{dim}$ and $CD56^{bright}$ NK cells: $CD69^-CD25^-$ (non-activated), $CD69^+CD25^-$ (earlier activated) and $CD69^+CD25^+$ (activated).

As expected, lymphocytes from MNC cultures undergo activation only after exposure to PHA (Table 2). In these culture conditions, we observe that the co-culture of MSC from different sources presents a different effect in the inhibition of the activation.

Analyzing $CD4^+$ and $CD8^+$ T cells from MNC culture, in the presence of PHA and absence of MSC, after 4 days of culture, we observe that the largest proportion of cells displays an intermediate activated phenotype (Table 2); the presence of either BM- or UCM-MSC in the

cell culture increases the frequency of earlier activated cells ($p < 0.05$ for both MSC types and for both T cell subpopulations); whereas the presence of AT-MSC is associated to a significant increase of cells within the non-activated compartment ($p < 0.05$) and, to a lesser extent, in earlier activated compartment ($p < 0.05$) and, consequently, is observed an important decrease in intermediate activated compartment ($p < 0.05$), for both $CD4^+$ and $CD8^+$ T cells (Table 2).

In what concerns to B cells, when stimulated with PHA in the absence of MSC, the earlier activated compartment is the most representative. As consequence of BM and AT-MSC, B cells are partially inhibited to proceed to the earlier activated stage, as a result, the frequency of cells in that compartment is diminished, and this decrease has statistic significance only for both MSC types (Table 2). Conversely, UCM-MSC seem to be unable to inhibit the development of B cell activated phenotype (Table 2).

Finally, co-cultures of MSC with MNC in the presence of PHA, present similar results for $CD56^{dim}$ NK cells, regardless the MSC nature and consisting in an augment of the non-activated compartment ($p < 0.05$ for all MSC, comparing with MNC+PHA), at the expenses of the earlier activated compartment (Table 2). However, a distinct behavior is observed in $CD56^{brigh}$ NK cells, whose presence of PHA has a significantly smaller effect, comparing to the other lymphocyte's populations considered in this study (Table 2). Despite this fact, we are able to observe a decreased percentage of $CD56^{bright}$ NK cells undergoing activation, when co-cultured with BM- or AT-MSC ($p < 0.05$ for both); conversely, UCM-MSC have no effect on the kinetic of $CD56^{bright}$ NK cells activation (Table 2).

4.3. MSC differentially influence mRNA expression of FoxP3, T-bet, Gata3 and IL-2 on T cells

The analysis of gene expression levels of transcription factors and IL-2, among the different T cell activation compartments, previously sorted and purified, was performed in order to better understand the immunomodulatory mechanisms underlying MSC.

In control assay (MNC+PHA), FoxP3 mRNA levels increase along with the progress of T cell activation; notably, the presence of AT-MSC strongly increases FoxP3 transcripts ($p < 0.05$; Figure 3A). Attending to T-bet, whose mRNA expression increases in the earlier activated stage of the control assay, an increase that is not maintained in intermediate and later activation stages, it is observed that MSC further increase and maintain its expression along the activation process ($p < 0.05$; Figure 3B). Conversely, GATA3 mRNA is not induced by PHA stimulation and the presence of MSC does not increase GATA3 mRNA levels (Figure 3C).

Table 2. Distribution of T, B and NK cells among the respective activation compartments

Cell population	MNC	MNC + BM-MSC	MNC + UCM-MSC	MNC + AT-MSC	MNC + PHA	MNC + PHA + BM-MSC	MNC + PHA + UCM-MSC	MNC + PHA + AT-MSC
	n = 17	*n = 5*	*n = 7*	*n = 5*	*n = 17*	*n = 5*	*n = 7*	*n = 5*
Total T cells	**82 ± 5**	**80 ± 11**	**80 ± 7**	**86 ± 5**	**86 ± 3**	**79 ± 8**	**71 ± 17**	**86 ± 5**
CD 4^+ T cells	**81 ± 5**	**81 ± 9**	**77 ± 9**	**84 ± 3**	**77 ± 3**	**84 ± 7**	**67 ± 8**	**76 ± 10**
$CD69^-CD25^-HLA\text{-}DR^-$	100 ± 0	100 ± 0	99 ± 2	100 ± 0	25 ± 7	23 ± 8	18 ± 9	43 ± 17 [a; b; c; d]
$CD69^+CD25^-HLA\text{-}DR^-$	0 ± 0	0 ± 0	1 ± 2	0 ± 0	6 ± 1	14 ± 4 [a; d]	15 ± 8 [a; d]	13 ± 1 [a; d]
$CD69^+CD25^+HLA\text{-}DR^-$	0 ± 0	0 ± 0	0 ± 0	0 ± 0	67 ± 7	58 ± 6 [a]	66 ± 17	43 ± 18 [a; d]
$CD69^+CD25^+HLA\text{-}DR^+$	0 ± 0	0 ± 0	0 ± 0	0 ± 0	3 ± 2	4 ± 2	2 ± 2	1 ± 0
CD 8^+ T cells	**19 ± 5**	**19 ± 9**	**23 ± 9**	**14 ± 3**	**23 ± 3**	**16 ± 7**	**33 ± 8**	**24 ± 10**
$CD69^-CD25^-HLA\text{-}DR^-$	99 ± 0	99 ± 2	98 ± 3	100 ± 1	18 ± 10	22 ± 3	12 ± 1 [b]	41 ± 12 [a; b; c; d]
$CD69^+CD25^-HLA\text{-}DR^-$	1 ± 0	1 ± 2	2 ± 2	0 ± 1	9 ± 1	16 ± 4 [a; d]	18 ± 9 [a; d]	14 ± 2 [a]
$CD69^+CD25^+HLA\text{-}DR^-$	0 ± 0	0 ± 0	0 ± 0	0 ± 0	68 ± 11	59 ± 6 [a]	67 ± 10	45 ± 14 [a; b; c; d]
$CD69^+CD25^+HLA\text{-}DR^+$	0 ± 0	0 ± 0	0 ± 0	0 ± 0	4 ± 2	2 ± 3 [d]	2 ± 2	1 ± 1
B cells	**2 ± 0**	**2 ± 1**	**4 ± 4**	**3 ± 2**	**3 ± 1**	**4 ± 1**	**4 ± 3**	**3 ± 2**
$CD69^-CD25^-$	98 ± 1	95 ± 4	97 ± 3	98 ± 1	40 ± 6	63 ± 23	37 ± 9	72 ± 15 [a; d]
$CD69^+CD25^-$	2 ± 1	5 ± 4	3 ± 3	2 ± 1	56 ± 7	32 ± 15 [d]	60 ± 6 [b]	27 ± 14 [a; d]
$CD69^+CD25^+$	0 ± 0	0 ± 0	0 ± 0	0 ± 0	4 ± 3	5 ± 10	4 ± 4	1 ± 1
Total NK cells	**16 ± 5**	**19 ± 10**	**16 ± 5**	**12 ± 5**	**12 ± 2**	**20 ± 9**	**20 ± 9**	**11 ± 5**
$CD56^{bright}$ NK cells	**3 ± 1**	**2 ± 1**	**3 ± 2**	**4 ± 3**	**8 ± 0**	**6 ± 3**	**6 ± 5**	**8 ± 4**
$CD69^-CD25^-$	99 ± 0	99 ± 1	99 ± 3	100 ± 1	82 ± 2	94 ± 2 [a; d]	80 ± 20	97 ± 1 [a; b; d]
$CD69^+CD25^-$	1 ± 0	1 ± 1	1 ± 3	0 ± 1	17 ± 2	6 ± 2 [a; d]	18 ± 17	3 ± 1 [a; b; d]
$CD69^+CD25^+$	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1 ± 0	1 ± 0	2 ± 3	1 ± 0
$CD56^{dim}$ NK cells	**97 ± 1**	**98 ± 1**	**97 ± 2**	**96 ± 3**	**92 ± 0**	**94 ± 3**	**94 ± 3**	**92 ± 4**
$CD69^-CD25^-$	97 ± 2	96 ± 7	97 ± 4	99 ± 1	29 ± 3	73 ± 18 [a; d]	74 ± 6 [a; d]	79 ± 19 [a; d]
$CD69^+CD25^-$	3 ± 2	4 ± 7	3 ± 4	1 ± 1	68 ± 2	25 ± 17 [a; d]	25 ± 6 [a; d]	20 ± 19 [a; d]
$CD69^+CD25^+$	0 ± 0	0 ± 0	0 ± 0	0 ± 0	3 ± 0	1 ± 1	1 ± 1	1 ± 0

Distribution (mean ± standard deviation) of total T cells, $CD4^+$ and $CD8^+$ T cells, B cells, total NK cells, and $CD56^{dim}$ and $CD56^{bright}$ NK cells in the MNC culture and among the following activation stages: non-activated $CD69^-CD25^-HLA\text{-}DR^-$, earlier activated $CD69^+CD25^-HLA\text{-}DR^-$, intermediate activated $CD69^+CD25^+HLA\text{-}DR^-$, and later activated $CD69^+CD25^+HLA\text{-}DR^+$, for T cell subpopulations; and non-activated $CD69^-CD25^-$, earlier activated $CD69^+CD25^-$ and activated $CD69^+CD25^+$, for B cells and NK cell subpopulations. MNC were subjected to different culture conditions: absence or presence of PHA and absence or presence of MSC from different sources.

Results are expressed as mean percentage ± standard deviation. Statistically significant differences were considered when p <0.05 for Mann-Whitney test: [a]comparing with MNC+PHA; [b]comparing with MNC+PHA+BM-MSC; [c]comparing with MNC+PHA+UCM-MSC. Statistically significant differences were considered when p <0.05 for Wilcoxon paired-sample test: [d]comparing with MNC+PHA.

MNC, mononuclear cells; PHA, phytohemagglutinin; BM, bone marrow; UCM, umbilical cord matrix; AT, adipose tissue; MSC, mesenchymal stromal/stem cells.

Finally, in control assays, the maximal expression of IL-2 mRNA is observed in earlier activated stage, wherein the presence of MSC of any source under study leads to a reduction, which is more notable for AT-MSC (p <0.05 for BM- and AT-MSC), as depicted in figure 3D.

4.4. MSC differentially influence mRNA expression of perforin, granzyme B and TNF-α on NK cells

Our data show that, on earlier activated and activated NK cells, both TNF-α and perforin mRNA expression is suppressed by all of the three types of MSC tested (Figure 4A and 4B, respectively). Concerning granzyme B mRNA expression, we observed that UCM-MSC induce an increased expression of this cytotoxic protein on activated NK cells, whereas BM- and AT-MSC slightly reduce it (Figure 4C).

5. DISCUSSION

To assess the influence of MSC from different sources in the induction of lymphoblast characteristics on T and B cells, ability of T, B and NK cells to progress along the activation process and mRNA expression of genes related to T cell polarization and NK cytotoxic activity, MNC cultures, in the absence/presence of PHA and in the absence/presence of MSC, were carried out.

PHA is a lectin with the ability to bind and crosslink different cell membrane glycoproteins, leading to the polyclonal activation of lymphocytes [587]. Although TCR, CD3 and CD2 crosslinking by PHA mimics the T cells' first signal of activation, leading to PKC activation and increasing the cytoplasmatic calcium levels, it is not sufficient to promote T cell activation, being required accessory signals given by antigen-presenting cells, which are also essential for IL-2 expression by T cells and, consequently, for their proliferation [587-590]. The early activation marker CD69 is expressed on NK, B and T cell surface 4 hours after activation and is implicated on the transcription of IL-2 and TNF-α; 12-24 hours after cell activation, the α subunit of IL-2 receptor (CD25) expression is upregulated, allowing the assembling of the high-affinity IL-2 receptor on T cell plasmatic membrane; finally, between 48-60 hours, T cells initiate HLA-DR expression [591-594].

According to our data, both BM- and AT-MSC, when co-cultured with PHA-stimulated MNC, inhibit B cell acquisition of lymphoblast characteristics and progression from non-activated to earlier activated stage, wherein AT-MSC display the strongest inhibitory capability, which corroborates with the results published by Bochev *et al* [318], who showed that AT-MSC had a stronger ability to inhibit immunoglobulin (Ig) production by B cells than BM-MSC.

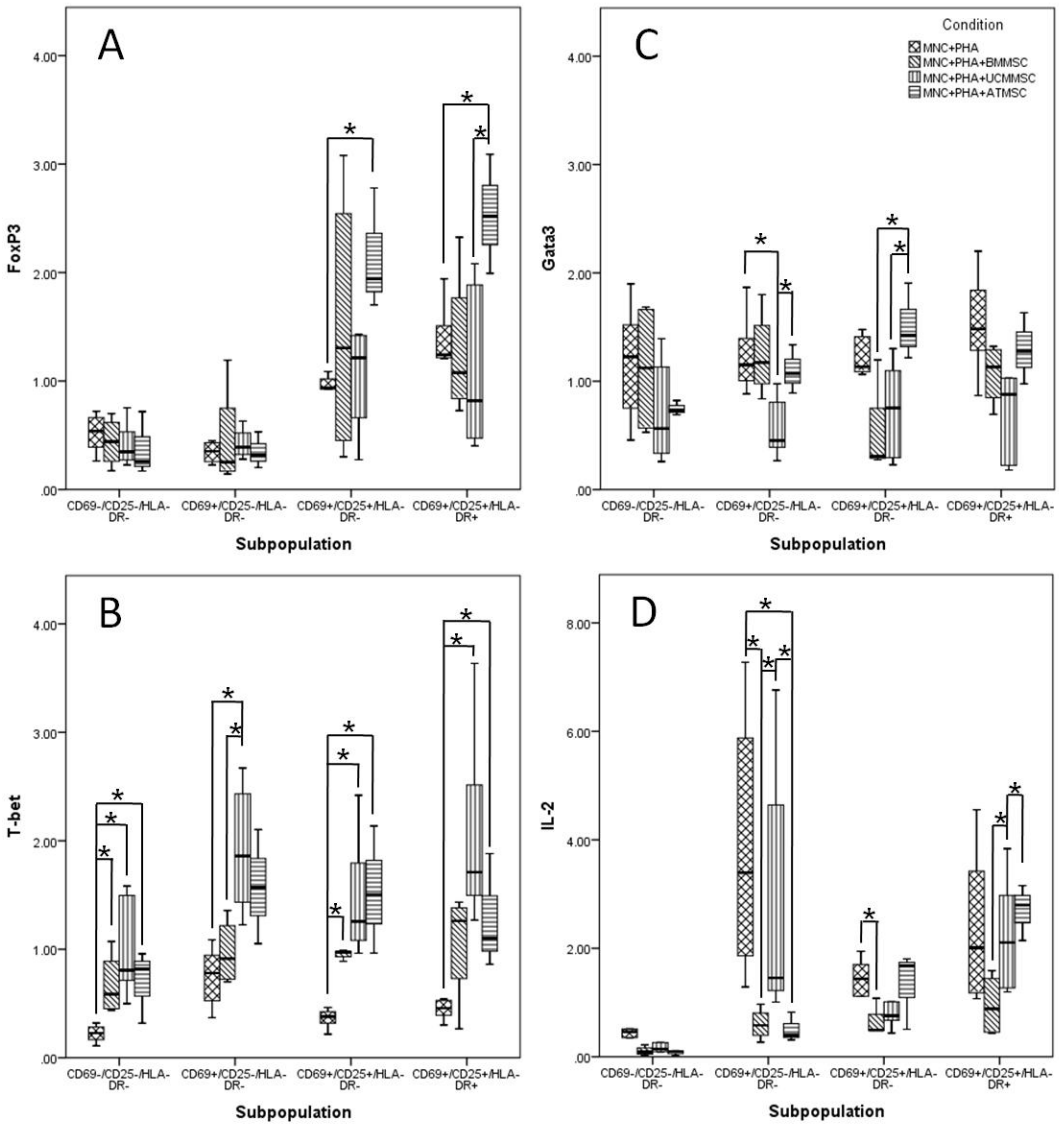

Figure 3. mRNA expression of FoxP3, Tbet, GATA3 and IL-2 among the different T cells' activation compartments. Semi-quantitative analysis of FoxP3 **(A)**, T-bet **(B)** GATA3 **(C)**, and IL-2 **(D)** mRNA expression for each activation stage phenotypically identified on T cells: non-activated ($CD69^-CD25^-$ $HLA\text{-}DR^-$), earlier activated ($CD69^+CD25^-HLA\text{-}DR^-$), intermediate activated ($CD69^+CD25^+HLA\text{-}DR^-$) and later activated ($CD69^+CD25^+HLA\text{-}DR^+$). *Differences statistically significant ($p < 0.05$, Mann-Whitney and Wilcoxon paired-sample test). MNC, mononuclear cells; PHA, phytohemagglutinin; BM, bone marrow; UCM, umbilical cord matrix; AT, adipose tissue; MSC, mesenchymal stromal/stem cells.

Conversely, UCM-MSC do not influence neither B cells' ability to acquire lymphoblast characteristics nor the distribution among the activation compartments.

Several studies describe the inhibitory effect of MSC on B cell proliferation, activation, ability to differentiate to plasma cells and/or produce Ig [55,305,315,316,318,321], demonstrating that human BM-MSC block stimulated B lymphocytes on G_0/G_1 phases of the cell cycle and interfere with the phosphorylation status of ERK1/2 and p38 MAPK [315].

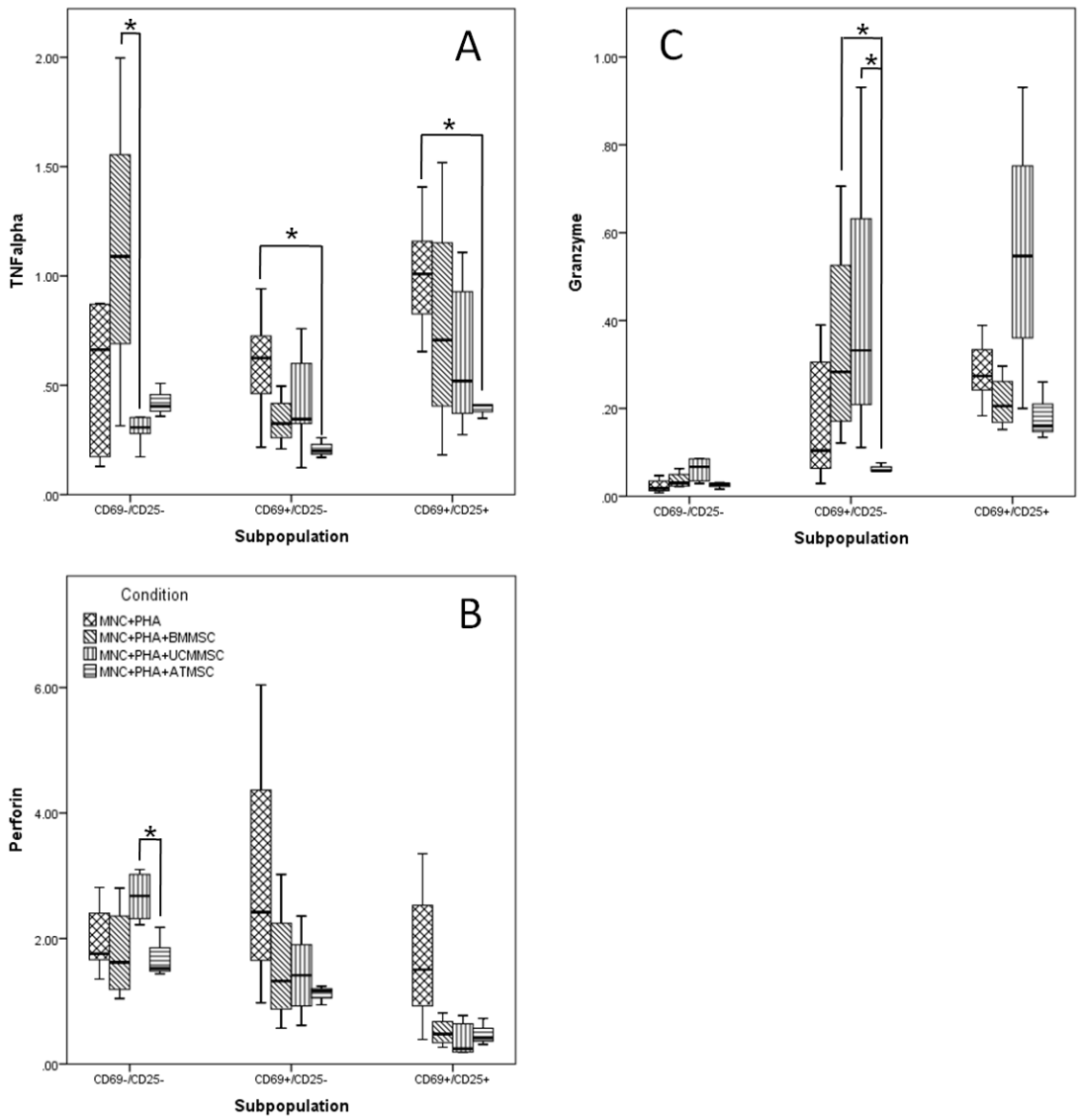

Figure 4. mRNA expression of perforin, granzyme B and TNF-α among the different NK cells' activation compartments. Semi-quantitative analysis of TNF-α **(A)**, perforin **(B)**, and granzyme B **(C)** gene expression for each cell activation stage phenotypically identified on NK cells: non-activated ($CD69^-CD25^-$), earlier activated ($CD69^+CD25^-$) and activated ($CD69^+CD25^+$). *Differences statistically significant (p <0.05, Mann-Whitney and Wilcoxon paired-sample test). MNC, mononuclear cells; PHA, phytohemagglutinin; BM, bone marrow; UCM, umbilical cord matrix; AT, adipose tissue; MSC, mesenchymal stromal/stem cells.

Conversely to T cells, where a number of studies have been done to unveil the mechanisms beneath MSC suppression of the immune response, there are few studies concerning this matter on B cells. Based on the results yielded by previous studies on T cells, it is hypothesized that programmed cell death 1 ligand 1 (PD-L1), alternatively cleaved CCL2, prostaglandin E2 (PGE2), transforming growth factor-β (TGF-β), indoleamine 2,3-dioxygenase (IDO) and hepatocyte growth factor (HGF), whose constitutive or induced expression on MSC had already been demonstrated, might be the effector molecules supporting B cell inhibition. On mouse and murine models, PD-L1 [107,175,316] and alternatively cleaved CCL2 [107,320]

were demonstrated to mediate MSC inhibition of B cells; in contrast, TGF-β and IDO were not involved in this process [175,321]. PGE2 is known to be capable of inhibit human B cell function [429,430], but its relevance on MSC-induced B cell inhibition is still unknown.

In our experimental design, MSC can act directly on B cells, but it should be also taken into account their suppressive effect on the other MNC in culture which, in turn, will condition B cell activation and proliferation. Indeed, by preventing T cell activation, cytokine production and downregulating BAFF expression on DC [595], MSC can indirectly and efficiently hamper B cell activation, proliferation and function. Likewise, there are growing evidences that the microenvironment conditions, such as type of stimulus, presence of other cell types and MSC:B cell ratio are determinant for the effect of MSC on B cells [98,175,305,315,319,596]. In fact, under appropriate conditions, MSC can enhance B cell proliferation, differentiation and Ig production [107,276,315,319].

Here we describe, for the first time, that the origin of MSC is essential in determining B cell fate, demonstrating that AT- and BM-MSC inhibit B cell activation and acquisition of lymphoblast characteristics, whereas UCM-MSC do not, under the same culture conditions.

All the three types of MSC tested, when co-cultured with MNC in the presence of PHA, inhibit the acquisition of lymphoblast characteristics by $CD4^+$ and $CD8^+$ T lymphocytes, more marked for AT-MSC, followed by BM-MSC and, finally, UCM-MSC. Similarly, all MSC from different tissues displayed the ability to suppress the activation of $CD4^+$ and $CD8^+$ T cells. Remarkably, whereas UCM- and BM-MSC block the cell activation after the upregulation of CD69 (at the earlier activated stage), AT-MSC strongly inhibit the passage from non-activated to earlier activated stage, yielding a great proportion of non-activated T cells after mitogenic stimulus. Again, the effect of UCM-MSC is milder, as the percentage of cells achieving the intermediate activated stage does not differ from that observed in the absence of MSC. Likewise, the three types of MSC inhibit IL-2 mRNA expression on T cells, more evident in earlier-activated stage, in which UCM-MSC display a more moderate effect. In sum, MSC suppress T cell immune response by reducing their proliferation and blocking their activation, what is in part consequence of IL-2 reduction, a cytokine essential for T cell proliferation and differentiation, being described that the addition of exogenous IL-2 partially reverts MSC-mediated inhibition [129].

MSC ability to inhibit T cell proliferation, activation and IL-2 expression is extensively described in the literature [39,45,58,61,234,248,596,597], as well as the capability to modulate the expression of other cytokines and diminish CTL cytolytic function. These capabilities are, at

least in part, mediated by soluble factors produced by MSC [45,400], namely TGF-β [286], PGE2 [26,31,424,596], IDO [52,596,598], leukemia inhibitory factor (LIF) [38], HLA-G5 [67,239,246,596], galectin [59,246,474,475,478], Jagged-1 [90], adenosine [461,465] and semaphorin-3A [473,474], and/or cellular contact and interaction between the MSC surface proteins HLA-G1 [30,67], PD-L1 [316], CD200 [41,401] and B7-H4 [548,556,557], and the respective receptors on T cells.

T cell proliferation is inhibited by LIF [38], TGF-β [286], Jagged-1 [90], PGE2 [26,31,424,596], IDO [52,596,598], galectin-1 and -3 [59,474,475,478], HLA-G5 and HLA-G1 [67,239,246,596], PD-L1 [316], B7-H4 [548,556,557], semaphorin-3A [45,90,473,474] and adenosine [461]. Both semaphorin-3A and adenosine prevent T cell proliferation by antagonizing signaling pathways downstream TCR and CD28; adenosine is also involved in the inhibition of IL-2, CD25, IFN-γ and TNFα expression and decreases cytolytic ability of CTL [461,465,473]. The negative costimulatory molecule B7-H4 prevents T cell activation, proliferation and production of IL-2 and other cytokines, by inhibiting NF-kB, ERK1/2 and JNK; B7-H4 also downregulates CD69 and CD25 expression on T cells [548,556,557].

Besides the effect exerted directly on T cells, indirect suppression also occurs by inhibition of DC maturation, as demonstrated for BM-MSC, consisting on the reduction of endocytosis and antigen processing capability, decreased expression of costimulatory molecules, chemokine receptors and cytokines essential for T cell activation (such as IL-12) and, consequently, diminishing DC capability to prime T cells, whereas stimulating IL-10 expression and, therefore, inducing tolerogenic DC able to expand $CD4^+$ Treg [31,45,98,107,238,360].

As previously described by our group [599] and others [600], MNC stimulation with PHA induces the expression of IFN-γ and IL-2, which activate antigen-presenting cells, contributing to the increased expression of IL-1, -6, -12 and TNF-α described on PHA-stimulated MNC [600-602], and creating the conditions for Th1 polarization (IFN-γ plus IL-12), characterized by T-bet expression. In fact, we observe an increased T-bet mRNA expression in all the three T cell activation compartments sorted and purified from MNC stimulated with PHA and the presence of MSC seems to potentiate and preserve T-bet expression along the activation stages. When FoxP3 mRNA expression is concerned, we observe an augment in intermediate and later activated T cells from PHA-stimulated MNC cultures, more pronounced in the presence of MSC, specially AT-MSC. Thus, our data show that, using this experimental design, MSC favor T cell differentiation toward Th1 and Treg, but we have to be aware that this effect is exerted on

a small number of cells, since, in the presence of MSC, the percentage of T cells undergoing activation is very limited.

In opposition to the results obtained for T-bet mRNA expression, MSC support to Treg ($CD4^+FoxP3^+$) induction and expansion is widely described in the literature, contributing for this occurrence PGE2, TGF-β, HLA-G5, IDO, LIF, IL-10 and CD200, whose expression increases after MSC exposure to proinflammatory factors [9,38,45,52,98,195,229,231,236,239,249,270,286, 287,401,596].

Although the increased T-bet expression on T cells may apparently be contradictory to previous works affirming that MSC inhibit Th1 polarization [182,231,236,251,252], it is worth to mention that, in the present work, it was measured T-bet mRNA expression within each one of the four purified T cells' activation compartments, while in the aforementioned studies the sense of T cell polarization was inferred by the pattern of cytokines expressed, specifically by the decreased IFN-γ levels. As our data clearly demonstrate, the presence of MSC reduce the number of T cells undergoing activation, which conditions an overall reduction of IFN-γ levels on culture supernatant, even assuming the polarization is skewed toward Th1. In fact, it hasn't already been described a direct mechanism by which MSC inhibit Th1 polarization, being hypothesized that it is an indirect effect of both the impairment of DC capability to prime naive T cells and the induction of Treg expansion [231]. Furthermore, MSC decrease IFN-γ expression in human mixed lymphocyte reaction [45], human MNC cultures [61,475], human T cell cultures [236] and mouse differentiated Th1 cells [251,263], which will bias the conclusions of those who use IFN-γ levels to infer about Th1 polarization. Of note, recent studies yielded contradictory data, showing that, under specific conditions, MSC may increase the levels of IFN-γ and IL-10 in the culture supernatants [61,249], which suggests that MSC influence on IFN-γ expression will depend on different factors, such as type of stimulation, type of hematopoietic cells present in cell culture and presence of cytokines on the milieu. Finally, a recently described $T\text{-}bet^+$ Th1 cell subpopulation co-expressing IFN-γ and IL-10 [265] was demonstrated to be induced and expanded by MSC [485]. These T cells arise from Th1 polarized cells that start producing IL-10, assuming regulatory activity [485]. Likewise, TGF-β, known to be produced by MSC, promotes IL-10 production by Th1 cells which, in turn, reduces IFN-γ expression by the same cells [406].

Thus, focusing our experimental design, MSC may act directly on T cells present in MNC fraction or, posteriorly, on PHA-mediated Th1-polarizing cells, since MSC immunomodulatory ability increases after their contact with proinflammatory cytokines, whose concentration in

culture medium raises after PHA-induced MNC activation. Namely, IFN-γ and/or TNF-α augment TGF-β expression by MSC [286,287], which induce IL-10 expression by Th1 cells and, consequently, reduces their IFN-γ production. Additionally, MSC downregulate IFN-γR expression on T cells surface [485], rendering them less prone to respond to IFN-γ. Also, we should not discard MSC effect on the DC present in the cell culture that may as well influence T cell function.

The increased mRNA expression of T-bet promoted by MSC, observed along all the T cell activation process and within each activation compartment, make us think that MSC are able to suppress T cell-mediated inflammatory response by reducing the proportion of T cells that undergo activation but, once initiated the activation process, MSC will not alter T cell differentiation sense in PHA-stimulated MNC, thus, T cells will continue to differentiate towards Th1. Nevertheless, as MSC also decrease IL-2 mRNA expression on T cells undergoing activation, we may expect a limited clonal expansion of the activated lymphocytes that will further reduce the final number of effector T cells. Finally, there are several evidences supporting that, after the conclusion of the differentiation process, MSC are able to inhibit Th1 effector functions. Altogether, MSC may inhibit Th1 immune response not by altering the sense of T cell polarization but, instead, by reducing the number of Th1 effector cells and by inhibiting the effector functions of differentiated Th1 cells.

In relation to GATA-3, although some studies confirm that MSC modulate cytokine production by T lymphocytes toward a Th2 pattern, there are no evidences pointing to Th2 differentiation mediated by MSC [182,231]; accordingly, our data show no influence of MSC on GATA-3 mRNA expression. This finding is in agreement with the MSC-mediated inhibition of B cell activation and acquisition of lymphoblast characteristics, observed in the present study.

In what concerns to NK cells, PHA-induced activation is prevented for all the three types of MSC tested, except for UCM-MSC that are unable to inhibit the activation of $CD56^{bright}$ NK cell subset. Once again, AT-MSC exhibit the most pronounced inhibitory effect.

In agreement with our results, previous studies described the downregulation of CD69 expression on NK cells under the influence of BM-MSC [102,328]. Indeed, all the effects of MSC already described in the literature are consistent with the inhibition of NK cell activation: MSC prevent NK cell proliferation [238,326,327,596]; induce downregulation of functional NK receptors [33,43,102,326,327,596]; inhibit the expression of proinflammatory cytokines (such as IFN-γ and TNF-α) [33,45,98,102,326,327,596] and granzyme A [33] and prevent NK cell degranulation [33]. As a result of all the effects aforementioned, MSC hamper NK cell cytolytic

activity [43,326,327,596]. Different evidences point to an important role of PGE2, TGF-β, IDO, HLA-G5, HLA-G1 and adenosine on NK cell inhibition, despite of some conflicting results on this matter [45,67,98,102,195,239,326,327,461,465,596]. Moreover, the differences between MSC from different sources still unclear, with studies yielding contradictory data for BM-MSC inhibitory ability [43,102,326].

According to our data, after PHA stimulation, NK cells express perforin and TNF-α mRNA, which is downregulated by all the three types of MSC. This fact is in agreement with previous studies concerning TNF-α [45,327], but diverges from the results recently published by DelaRosa *et al* showing that perforin production remains unchanged in the presence of either BM- and AT-MSC co-cultured with purified NK cells [33]. Similarly, we observed that AT- and BM-MSC induce a slight reduction of granzyme B mRNA expression in the later activated stage of NK cells, whereas DelaRosa *et al* show that BM- and AT-MSC had no effect on NK cells' granzyme B expression [33]. It is worth to remind that DelaRosa's approach differs from ours, as in our study MSC were co-cultured with MNC, and the presence of other immune cells in the culture may influence NK cell behavior. Finally, Yen *et al* concluded that human embryonic stem cell-derived mesenchymal progenitors present a stronger ability to inhibit NK cell function compared to BM-MSC, by reducing the expression of NK-activating receptors and NK cell cytotoxic activity [43]; here, we observe that both BM- and UCM-MSC present a similar ability to inhibit $CD56^{dim}$ NK cell activation and TNF-α and perforin mRNA expression.

In fact, it is difficult to compare results from studies concerning the MSC immunomodulatory ability because MSC are highly sensitive to the microenvironment and modulate their function according to the external conditions. Their function will vary depending on the ratio MSC:immune cells, the immune cells present in the cell culture, the immune cell activation status and the cytokines levels in the milieu [26,58-61,578], wherein proinflammatory cytokines, namely IFN-γ and TNF-α, increase MSC expression of IDO [39,45,52], galectin-1 [59], PGE2 [39,45,286,287], CD200 [41], TGF-β [286,287], IL-10 [286] and PD-L1 [263], although there are conflicting results [603]. On the whole, these evidences point to the improvement of immunomodulatory ability of MSC whenever they are in an environment resembling a chronic inflammation condition. Conversely, TLR3 or TLR4 stimulation, mimicking an acute inflammation state, will reduce MSC immunomodulatory ability and decrease Jagged-1 and galectin-1 expression on MSC, similarly to that observed on Treg [45,59,90,248]. Thus, MSC will adapt their function according to the actual conditions and requirements of the organism.

With respect to the differences observed among the different types of MSC in the present study, the inhibitory ability of AT-MSC is stronger than that of BM- and UCM-MSC, what is in agreement with other published studies [26,39]. Although a few studies attempted to unravel the functional differences among AT-, BM- and UCM-MSC [31,36,603], recent works shed light on this subject, showing that AT-MSC express an higher level of COX-1 and PGE2 [26] and UCM-MSC display a lower expression of PGE2 and IDO compared to BM-MSC [39].

6. CONCLUSION

Overall, UCM-, BM- and AT-MSC inhibit both acquisition of lymphoblast characteristics and activation of T cells, wherein AT-MSC display a more pronounced effect and UCM-MSC a milder effect. Of note, AT-MSC strongly impede T cells to proceed from non-activated to earlier activated stage, yielding a large proportion of T cells that remain non-activated, whereas with UCM-MSC, despite of the accumulation of T cells in earlier-activated stage, the proportion of T cells achieving the intermediate-activated state after 4 days of culture is equal to that observed in the absence of MSC. Nevertheless, MSC induce mRNA expression of the master transcriptional regulators of Treg and Th1 polarization on the T cells that proceed on the activation process. Thus, MSC may inhibit Th1 immune response not by altering the sense of T cell polarization but, instead, by reducing the final number of Th1 effector cells (both by preventing T cells to undergo activation and by hampering IL-2 production by activated T cells) and by inhibiting the effector functions of differentiated Th1 cells.

Concerning B cells, our work showed that AT- and BM-MSC are capable of inhibit their activation and acquisition of lymphoblast characteristics, whereas UCM-MSC are not. To the best of our knowledge, this is the first report showing that UCM-MSC are unable to prevent activation of B cells from MNC cultures stimulated with PHA and display a behavior completely different from their counterparts arising from other tissues, clearly showing that MSC from different tissues may imply different immunomodulatory properties.

Finally, all the three sources of MSC tested revealed a strong inhibitory capability over $CD56^{dim}$ NK cell activation, as well as over the production of TNF-α and perforin, whereas the activation of $CD56^{bright}$ NK cells was only inhibited by BM- and AT-MSC.

CHAPTER 3 |

HUMAN BONE MARROW-DERIVED MESENCHYMAL STROMAL CELLS DIFFERENTIALLY INHIBIT CYTOKINE PRODUCTION BY PERIPHERAL BLOOD MONOCYTE SUBPOPULATIONS AND MYELOID DENDRITIC CELLS

1. Abstract

Introduction: The immunosuppressive properties of mesenchymal stromal/stem cells (MSC) rendered them an attractive therapeutic approach for immune disorders and an increasing body of evidence demonstrated their clinical value. However, the influence of MSC on the function of specific immune cell populations, namely monocyte subpopulations, is not well elucidated.

Aim: Here, we investigated the influence of human bone marrow MSC on the cytokine and chemokine expression by peripheral blood classical, intermediate and non-classical monocytes and myeloid dendritic cells (mDC), stimulated with lipopolysaccharide plus interferon (IFN)γ.

Results: We found that MSC effectively inhibit tumor necrosis factor (TNF)-α and macrophage inflammatory protein (MIP)-1β protein expression in monocytes and mDC, without suppressing CCR7 and CD83 protein expression. Interestingly, mDC exhibited the highest degree of inhibition, for both TNF-α and MIP-1β, whereas the reduction of TNF-α expression was less marked for non-classical monocytes. Similarly, MSC decreased mRNA levels of interleukin (IL)-1β and IL-6 in classical monocytes, CCL3, CCL5, CXCL9 and CXCL10 in classical and non-classical monocytes and IL-1β and CXCL10 in mDC.

Conclusions: MSC do not impair the expression of maturation markers in monocytes and mDC under our experimental conditions, nevertheless they hamper the pro-inflammatory function of monocytes and mDC, which may impede the development of inflammatory immune responses.

2. Introduction

Mesenchymal stromal/stem cells (MSC) correspond to undifferentiated cells capable of self-renewal and to differentiate along different cell lineages [1]. The detailed study of their immunophenotypic characteristics facilitated MSC identification, quantification and isolation from different human adult tissues, such as bone marrow, adipose tissue, muscle, among others [1,13,34,46]. In turn, the discovery of their immunosuppressive potential, converted them into an attractive therapeutic approach for autoimmune diseases and pathological conditions where the activation of the immune system entails deleterious effects.

In the recent years, an increasing number of studies have reported the inhibitory effect of MSC over immune cells, wherein the majority of them focused on T lymphocytes [98,231]. However, even concerning T cells, only a limited number of studies analyzed and compared the influence of MSC over distinct functional T cell subsets, and demonstrated that functional T cells subsets are in fact differentially regulated by MSC [46,231,244,251,263,275,291]. In turn, as antigen-presenting cells have a pivotal role in T cell activation, differentiation and in directing their polarization [604], the study of MSC influence over monocytes and dendritic cells (DC) became an active field of research. Nevertheless, the number of studies performed in natural occurring DC is scarce [236,333], as the majority of them was carried out in monocyte-derived DC differentiated *in vitro* with GM-CSF and IL-4. Furthermore, to the best of our knowledge, no study investigated and compared the influence of MSC over the recently identified peripheral blood classical, intermediate and non-classical monocyte subpopulations [332].

In 2010, Ziegler and colleagues [332] identified three distinct subpopulations within peripheral blood monocytes, which are phenotypically and functionally characterized as follows: classical monocytes are phenotypically characterized as $CD14^{++}CD16^{-}$ [332,369]; intermediate monocytes, identified by $CD14^{++}CD16^{+}$ phenotype, display the highest expression of class II major histocompatibility complex (MHC), compared to the remaining peripheral blood monocyte subpopulations, together with an increased ability to present antigens to T cells and to induce antigen-specific secretion of interleukin (IL)-12 and interferon (IFN)γ, besides, when challenged with zymosan or lipopolysaccharide (LPS), this cell subset presents the highest expression level of IL-10 [332,369]; finally, non-classical monocytes, phenotypically characterized as $CD14^{+}CD16^{++}$, are the most predisposed to differentiate into DC and induce the highest rates of T cell proliferation, however, after stimulation with LPS and IFNγ, they are less efficient in producing IL-1β, IL-6, IL-12 and tumor necrosis factor (TNF)-α compared to classical monocytes and myeloid dendritic cells (mDC) [332,369,605,606]. Of note, macrophages derived from $CD16^{+}$ monocytes possess higher phagocytic activity than those generated from classical monocytes [369].

mDC correspond to a peripheral blood subset of DC, which are likely to be in transit from the bone marrow to tissues, where they will contact with foreign antigens and undergo maturation. Accordingly, peripheral blood mDC share some characteristics with immature DC, such as antigen uptake, processing and presentation activity, followed by T cell activation, the lack of CD83 and the production of IL-1β, IL-6, IL-12 and TNF-α after activation with LPS and IFNγ [332,604-609]. Of note, depending on the stimulus, mDC can acquire an anti-

inflammatory expression profile, reducing IL-12 while increasing IL-10 expression, thus inducing a Th2 immune response [604,608]. It was recently described that peripheral blood mDC can be phenotypically distinguished in two subpopulations, CD1c (BDCA-1)$^{+}$ and CD141 (BDCA-3)$^{+}$, with remarkable functional differences [332,608,609].

In the present study, we investigated influence of human bone marrow-derived MSC on peripheral blood monocyte subpopulations (classical, intermediate and non-classical monocytes) and mDC, stimulated with LPS and IFNγ, in a co-culture system. With this aim, we proceeded to the quantification, by flow cytometry, of TNF-α, CCL4 (macrophage inflammatory protein (MIP)-1β), CCR7, CD83 and HLA-DR in the abovementioned immune cell populations. mRNA expression of IL-1β, IL-6, CCL3 (MIP-1α), CCL5 (regulated on activation, normal T cell expressed and secreted; RANTES), CXCL9 (monokine induced by gamma interferon; MIG) and CXCL-10 (interferon-gamma-inducible protein (IP)-10) was evaluated in previously purified classical and non-classical monocytes, as well as IL-1β and CXCL10 in purified mDC by. Besides, we assessed protein and mRNA expression of immune mediators and adhesion molecules in non-stimulated MSC or after LPS and/or IFNγ treatment.

3. Material and Methods

3.1. Collection and isolation of peripheral blood mononuclear cells and bone marrow mesenchymal stromal cells

Peripheral blood samples from a total of six healthy donors (1 male and 5 females; mean age of 44±7 years, ranging from 22 to 51 years old), collected in heparin at the Blood and Transplantation Center of Coimbra (Portugal), and human BM samples from healthy donors (age ranging from 20 to 40 years old) were included in the present study. The use of these biological samples for research purpose was approved by Serviço de Transplantação de Progenitores Hematopoiéticos (UTM) do Instituto Português de Oncologia de Lisboa Francisco Gentil (laws 97/95 and 46/2004), and all participants gave written informed consent before entering in the study.

Peripheral blood mononuclear cells (MNC) were isolated by Lymphoprep (Stemcell Technologies, Vancouver, Canada) gradient density centrifugation and then washed twice in Hank's Balanced Salt Solution (HBSS, Gibco, Life Technologies, Paisley, UK). The MNC pellet

was resuspended in RPMI 1640 with GlutaMax medium (Invitrogen, Life Technologies) with antibiotic-antimycotic (Gibco), to the final concentration of 10^6 cells/500 µl.

Peripheral blood MNC were subsequently analyzed for protein and mRNA expression in the following experimental conditions: 1) 10^6 MNC + 500 µl RPMI (negative control); 2) 10^6 MNC + 0.5x10^6 MSC (negative control); 3) 10^6 MNC + LPS + IFNγ (positive control); 4) 10^6 MNC + 0.5x10^6 MSC + LPS + IFNγ; 5) 10^6 MNC + 0.5x10^6 MSC, followed by MSC depletion and, then, stimulation with LPS+IFNγ. All the aforementioned cell cultures were carried out for 20 hours at 37°C, in a sterile environment with 5% CO_2 and humidified atmosphere, plus an incubation period of 6 hours with the stimulator agents. The cell culture and stimulation protocols are detailed below, in "Immunophenotypic study of MSC and peripheral blood monocytes and mDC" section.

For the isolation of human BM-MSC, MNC were isolated from BM samples by using a Sepax S-100 system (Biosafe, Eysins, Switzerland) in accordance with the instructions of the manufacturer. Cell number and viability were determined using the Trypan Blue (Gibco) exclusion method.

BM MNC were plated at a density of 2x10^5 cells/cm^2 in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% qualified fetal bovine serum (FBS, Sigma, Spain). After a 3-day incubation at 37ºC in humidified atmosphere containing 5% CO_2, the non-adherent cell fraction was discarded, and the adherent culture was maintained with a complete medium renewal every 3-4 days. After reaching a 70-80% confluency cells were detached using TrypLE (Life Technologies) for 7 minutes and then replated at an initial density of 3000 cells/cm^2. For this study, MSC passages 3 and 5 were used.

MSC identity was confirmed by performing fluorescent morphological analysis, mesodermal differentiation assays (osteogenic, adipogenic and chondrogenic) and immunophenotype characterization as described by Dominici et al. [2].

Subsequently, MSC were resuspended in RPMI 1640 with GlutaMax medium (Invitrogen) with antibiotic-antimycotic (Gibco) to the final concentration of 0.5x10^6 cells/500 µl. The protein and mRNA expression of MSC were studied in the following experimental conditions: 1) 0.5x10^6 MSC + 500 µl RPMI (non-stimulated MSC); 2) 0.5x10^6 MSC + 500 µl RPMI + LPS; 3) 0.5x10^6 MSC + 500 µl RPMI + IFNγ; and 4) 0.5x10^6 MSC + 500 µl RPMI + LPS + IFNγ. All the aforementioned cell cultures were carried out for 20 hours at 37°C, in a sterile environment with 5% CO_2 and humidified atmosphere, plus an incubation period of 6 hours with the

stimulator agents. The cell culture and stimulation protocols are detailed below, in "Immunophenotypic study of MSC and peripheral blood monocytes and mDC" section.

3.2. Immunophenotypic study of MSC and peripheral blood monocytes and mDC

3.2.1. MSC stimulation with LPS, IFNγ and LPS plus IFNγ

For the immunophenotypic study of MSC, we plated in 8 wells of tissue culture plates (Falcon, Becton Dickinson Biosciences, BD, San Jose, USA) $0.5x10^6$ MSC in 1 ml of RPMI 1640 with GlutaMax medium (Invitrogen) with antibiotic-antimycotic (Gibco). MSC were cultured for 20 hours at 37°C, in a sterile environment with 5% CO_2 and humidified atmosphere (to be in the same experimental conditions than those MSC co-cultured with MNC). Then, MSC were stimulated with LPS (100 ng/ml) from *Escherichia coli* (serotype 055:B5, Sigma), or IFNγ (100 U/ml, Promega, Madison, USA), or LPS+IFNγ (100 ng/ml and 100 U/ml, respectively), in duplicate (to perform the immunophenotypic study and mRNA expression quantification), for 6 hours at 37ºC in humidified atmosphere containing 5% CO_2; in the remaining 2 wells, MSC were not stimulated.

3.2.2. Immunophenotypic study of MSC

For each experimental condition tested, cells were detached using TrypLE (Gibco); after incubating for 10 minutes at -20ºC, the content of each well was transferred to a 12x75 mm polystyrene cytometer tube, centrifuged for 5 minutes at 540xg and the supernatant was discarded. MSC immunophenotype was assessed using the 7-color monoclonal antibody (mAb) combination detailed in table 3 (tube 1). The cell pellet was incubated with the mAb for 10 minutes in the darkness and washed with phosphate buffered saline (PBS). Finally, cells were resuspended in 500 µl of PBS and immediately acquired in a FACSCanto II (BD) flow cytometer.

3.2.3. Co-culture of peripheral blood MNC and MSC and in vitro stimulation with LPS plus IFNγ

In 6 wells of tissue culture plates (Falcon) 10^6 MNC were plated in 1 ml of RPMI 1640 with GlutaMax medium (Invitrogen) with antibiotic-antimycotic (Gibco), and in 9 wells of tissue culture plates (Falcon) we plated 10^6 MNC + $0.5x10^6$ MSC in a final volume of 1 ml, establishing a ratio of 2:1 (MNC:MSC). Cells were cultured for 20 hours at 37°C, in a sterile environment with 5% CO_2 and humidified atmosphere. The content of 3 wells with co-cultured MNC+MSC was subjected to MSC depletion, using the EasySep Human CD271 Selection kit (Stemcell Technologies), according to the manufacturer's instructions.

Table 3. Panel of mAb reagents (with clones and commercial source) used for the immunophenotypic characterization of classical, intermediate and non-classical monocytes, mDC and MSC

	Fluorochrome							
Tube	**PacB**	**PacO**	**FITC**	**PE**	**PerCPCy5.5**	**PECy7**	**APC**	**APCH7**
1	CD44 clone IM7 Biolegend	CD45 HI30 Invitrogen	CD106 51-10C9 BD Pharmingen	CD73 AD2 BD Pharmingen	CD184 12G5 BD	CD13 Immu103.44 Beckman Coulter	CD90 5E10 BD Pharmingen	
2	CD16 3G8 BD Pharmingen	CD45 HI30 Invitrogen	CD83 HB15a Beckman Coulter	CCR7 3D12 BD Pharmingen	CD14 M5E2 BD Pharmingen	CD33 D3HL60.251 Beckman Coulter	IREM-2 UP-H2 Immunostep SL	HLA-DR L243 BD
3	CD16 3G8 BD Pharmingen	CD45 HI30 Invitrogen	cyTNF-α MP6-XT22 BD Pharmingen	cyMIP-1β D21-1351 BD Pharmingen	CD14 M5E2 BD Pharmingen	CD33 D3HL60.251 Beckman Coulter	IREM-2 UP-H2 Immunostep SL	HLA-DR L243 BD
4	CD16 3G8 BD Pharmingen			CD123 SSDCL Y107D2 Beckman Coulter	CD14 M5E2 BD Pharmingen	CD33 D3HL60.251 Beckman Coulter	IREM-2 UP-H2 Immunostep SL	HLA-DR L243 BD

Abbreviations: mAb, monoclonal antibody; mDC, myeloid dendritic cells; MSC, mesenchymal stromal/stem cells; PacB, pacific blue; PacO, pacific orange; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCPCy5.5, peridinin chlorophyll protein-cyanine 5.5; PECy7, phycoerythrin-cyanine 7; APC, allophycocyanin; APCH7, allophycocyanin-hilite 7. Commercial sources: Biolegend (San Diego, CA, USA); Invitrogen, Life Technologies (Carlsbad, CA, USA); BD Pharmingen (San Diego, CA, USA); BD (Becton Dickinson Biosciences, San Jose, CA, USA), Beckman Coulter (Miami, FL, USA); Immunostep S.L (Salamanca, Spain).

Then, LPS+IFNγ (100 ng/ml and 100 U/ml, respectively), were added to 3 wells with MNC, 3 wells with co-cultured MNC+MSC and to the 3 wells with MNC+MSC where MSC had been depleted; and the cells in the remaining wells (3 with MNC and 3 with MNC+MSC) were not stimulated. To one of the wells in each experimental condition (MNC, MNC+MSC, MNC+LPS+IFNγ, MNC+MSC+LPS+IFNγ, MNC+MSC+Depetion+LPS+IFNγ), we added brefeldin A (10 µg/ml) from *Penicillium brefeldiamun* (Sigma), to prevent the release of *de* novo produced cytokines outside the cells. The samples were incubated at 37 °C, in a sterile environment with 5% CO_2 humidified atmosphere, for 6 hours.

The samples with brefeldin A were used for the study of TNF-α and MIP-1β expression in monocytes and mDC, by flow cytometry, while the expression of CD83, CCR7 and HLA-DR, by flow cytometry, and the mRNA expression of cytokines, in monocytes and mDC, were performed in the samples without brefeldin A. All the aforementioned protein and mRNA expression studies were performed in all the different culture conditions: MNC, MNC+MSC, MNC+LPS+IFNγ, MNC+MSC+LPS+IFNγ, MNC+MSC+Depetion+LPS+IFNγ.

3.2.4. Immunophenotypic study of peripheral blood monocytes and mDC

For each experimental condition tested, cells were detached using TrypLE (Gibco); after incubating for 10 minutes at -20ºC, the content of each well was transferred to a 12x75 mm

polystyrene cytometer tube, centrifuged for 5 minutes at 540xg and the supernatant was discarded. Immunophenotypic analysis of peripheral blood monocytes and mDC, cultured in the presence/absence of LPS+IFNγ and in the presence/absence of MSC, was performed using 8-color mAb combinations, detailed in table 3. For the study of CD83, CCR7 and HLA-DR expression (tube 2), cultured cells were incubated with the mAb for 10 minutes in the darkness, washed with PBS and, finally, resuspended in 500 µl of PBS and immediately acquired in a FACSCanto II (BD) flow cytometer. To study TNF-α and MIP-1β expression (tube 3), cells were stained with the mAb for surface proteins antigens (CD16, CD45, CD33, CD14, IREM-2, HLA-DR) and, after an incubation period of 10 minutes in the darkness, washed with PBS. For intracellular staining, Fix&Perm (Caltag, Hamburg, Germany) reagent was used, according to the manufacturer's instructions and in parallel with the mAb for TNF-α and MIP-1β. After washing twice with PBS, the cell pellet was resuspended in 500 µl of PBS and immediately acquired.

3.2.5. Data acquisition and analysis

Data acquisition was performed in a FACSCanto II (BD) flow cytometer equipped with the FACSDiva software (v6.1.2; BD). For both MSC and MNC immunophenotypic studies, the whole sample from each tube was acquired and stored, corresponding to a number of events always above $0.1x10^6$ or $0.5x10^6$ events, respectively. For data analysis, the Infinicyt (version 1.7) software (Cytognos SL, Salamanca, Spain) was used.

3.2.6. Immunophenotypic identification of classical, intermediate and non-classical monocytes and mDC

Attending to the markers used to analyze the expression of CCR7, CD83, HLA-DR, TNF-α and MIP-1β in classical, intermediate and non-classical monocytes and mDC, we used the following gate strategy to identify these four cell populations (Figure 5): classical monocytes express high levels of CD14 in the absence of CD16, together with high expression of CD33 and HLA-DR and being also positive for IREM-2 (CD300e); intermediate monocytes express high levels of CD14 as well, but display an increasing expression of CD16, associated to a slight decrease of CD33 expression, compared to classical monocytes; in turn, non-classical monocytes show CD16 positivity with a decreasing expression of CD14, they present the highest expression of CD45 along with the lowest expression of CD33 among the three monocyte subpopulations, and HLA-DR and IREM-2 expression is between that of classical and intermediate monocytes; mDC have lower side-scatter light dispersion properties and CD45

expression than monocytes, they present high expression of CD33 and HLA-DR in the absence of CD14, CD16 and IREM-2.

3.3. Cell purification by fluorescence-activated cell sorting

Monocytes and mDC cell populations from the cell cultures were purified by FACS, using FACSAria II flow cytometer (BD), according to their typical phenotype. Thus, the 6-color mAb combination used (Table 3, tube 4) allowed the identification of classical monocytes (HLA-$DR^{+}CD33^{+}$IREM-$2^{+}CD14^{++}CD16^{-}$), non-classical monocytes (HLA-$DR^{+}CD33^{+}$IREM-$2^{+}CD14^{+}CD16^{+}$) and mDC (HLA-$DR^{++}CD33^{++}$IREM-$2^{-}CD14^{-}CD16^{-}$). The purified cell populations were subsequently used for the quantification of mRNA expression.

3.4. Analysis of mRNA expression in MSC and peripheral blood monocyte and mDC

The content of each well of cultured MSC under the different experimental condition tested, or the purified mDC and monocyte subpopulations, was transferred to a 1.5 ml eppendorf tube, centrifuged for 5 minutes at 300xg and the pellet resuspended in 350 µl of RLT Lysis Buffer (Qiagen, Hilden, Germany). Total RNA was extracted with the RNeasy Micro kit (Qiagen), according to the supplier's instructions. Then, total RNA was eluted in a 20 µl volume of RNase-free water RNA was reverse transcribed with Tetra cDNA Synthesis ® (Bioline, London, UK), according to the manufacturer's instructions. Relative quantification of gene expression by real-time PCR was performed in the LightCycler 480 II (Roche Diagnostics, Rotkreuz, Switzerland). Real-time PCR reactions were carried out using 1x QuantiTect SYBR Green PCR Master Mix (Qiagen), 1x QuantiTect Primer Assay (for MSC: TNF3: QT01079561; IL-10: QT00041685; IL-8: QT00000322; IL-6: QT00013461; ICOSL: QT00023660; IDO: QT00000504; TGFβ2: QT00025718; IL-1β: QT00021385; for purified classical and non-classical monocytes and mDC: IL-1β: QT00021385; IL-6: QT00083720; CCL3: QT01008063; CCL5: QT00090083; CXCL9: QT00013461; CXCL10: QT01003065) (Qiagen), in a final volume of 10 µl. The reactions were performed using the following thermal profile: 1 cycles of 10 min at 95°C, 50 cycles of 10 sec at 95°C, 20 sec at 55°C and 30 sec at 72°C, 1 cycle of 5 sec at 95°C, 1 min at 65°C and continuo at 97°C, and 1 cycle of 10 sec at 21°C. All samples were run in duplicate. Real-time PCR results were analyzed with the LightCycler software (Roche Diagnostics). GeNorm software (PrimerDesign Ltd., Southampton, England) was used to select the reference genes to normalize data. The reference genes used for gene expression analysis were: cytochrome c1 (CYC1) and splicing factor 3a subunit 1 (SF3A1) for MSC; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and topoisomerase DNA I (TOP1) for classical monocytes; and GAPDH

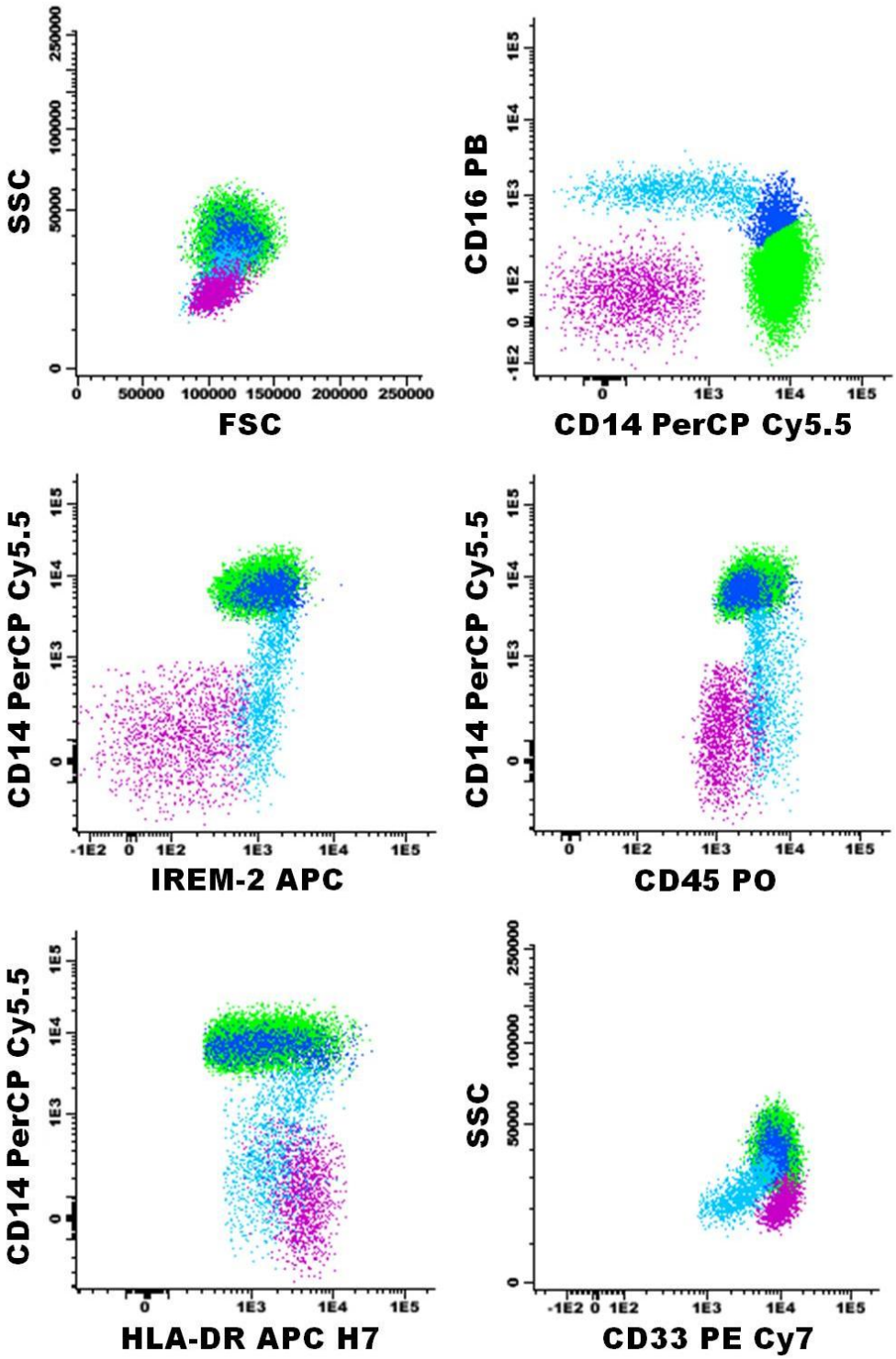

Figure 5. Phenotypic characteristics of peripheral blood classical, intermediate, and non-classical monocytes, and mDC. Bivariate dot plot histograms illustrating the phenotypic strategy for the identification of the different monocyte subpopulations and myeloid dendritic cells (mDC) from peripheral blood. Classical monocytes (green events) express CD14 in the absence of CD16, they also show high reactivity for CD45, CD33, IREM-2, and HLA-DR; intermediate monocytes (dark blue events) are characterized as CD14-positive displaying an increasing positivity to CD16, together with a high expression of IREM-2 and positivity for CD45, CD33 and HLA-DR; non-classical monocytes (light blue events) are CD16-positive with a decreasing expression of CD14, they are highly positive to IREM-2 and CD45, while presenting the lowest CD33 expression among monocytes subpopulations; mDC (pink events) are phenotypically characterized as negative for CD14, CD16, and IREM-2, they present lower expression of CD45 and SSC properties, and higher expression of HLA-DR and CD33, compared to monocytes.

and beta-2 microglobulin (B2M) for non-classical monocytes and mDC. The normalized expression levels of the genes of interest were calculated by using the delta-Ct method.

3.5. IDO detection by Western Blot

Indoleamine-2,3-Dioxygenase (IDO) protein levels expression were determined by western blot in MSC without stimulation cultured in the presence or absence of MNC and in MSC stimulated with LPS, IFNγ or LPS+IFNγ, in the presence or absence of MNC.

Cell lysates were prepared using RIPA buffer (Sigma) supplemented with complete protease inhibitor cocktail (Roche). After centrifugation at 10,000xg for 10 min at 4ºC, supernatants were collected. Seeblue Plus2 Prestained Protein Standard (Invitrogen) and Precision Plus Protein Standard (BioRad, Warszawa, Poland) were used as standard. SDS-polyacrylamide gel electrophoresis was performed for separation of the proteins, and western blot were subsequently performed. For protein specific detection, the membranes were incubated with the primary mAb anti-IDO (clone 10.1, Merck Millipore, Darmstadt , Germany) diluted 1:5000.

3.6. IL-6 production analysis

IL-6 production was determined by enzyme-linked immunosorbent assay (ELISA), using a commercially available ELISA kit (RayBiotech, Norcross, USA), in the supernatant of MSC cultured in the presence/absence of MNC, with or without stimulation by LPS, IFNγ or LPS+IFNγ. The cultured cells were pelleted and the media was recovered to be tested by ELISA, performed according to the manufacturer's instructions.

3.7. Statistical analyses

To determine the statistic significance of the differences observed between different culture conditions, non-parametric Friedman's paired-sample test was performed, using Statistical Package for Social Sciences (IBM SPSS, version 17.0, Armonk, NY, USA). Data were expressed as mean percentage ± standard deviation. Statistically significant differences were considered when p value was lower than 0.05.

4. RESULTS

In order to better understand how MSC regulate the immune function of the recently described monocyte subpopulations and mDC, we evaluated the expression of proteins involved in cell migration, activation/maturation, antigen presentation and the production of pro-inflammatory cytokines, in LPS plus IFNγ stimulated monocytes and mDC in the presence or absence of MSC.

4.1. MSC inhibit TNF-α and MIP-1β protein expression in monocytes and mDC

Considering TNF-α and MIP-1β expression, our results showed that MSC decreased both the percentage of cells producing TNF-α and MIP-1β ($p < 0.05$, for all cell populations) as well as the amount of protein produced *per* cell (measured as mean fluorescence intensity, MFI, $p < 0.05$ for TNF-α-producing non-classical monocytes and mDC, and for MIP-1β-producing classical and intermediate monocytes and mDC), wherein MSC depletion prior to LPS+IFNγ stimulation resulted in a less effective reduction of the percentage of TNF-α producing monocytes (Figure 6A). It is worth mentioning that MSC inhibition capability is different for distinct pro-inflammatory proteins and for the different cell populations addressed in this study: MSC are less effective in regulating MIP-1β than TNF-α, and the expression of TNF-α in non-classical monocytes and of MIP-1β in classical monocytes is inhibited to a lesser extent than that of the remaining cell populations (Figure 6B). Furthermore, mDC exhibited the highest percentage of inhibition among all cell populations under study (Figure 6B). We found a similar behavior for MSC+MNC co-cultures stimulated solely with LPS (data not shown). Transwell assays demonstrated that the regulatory effect of MSC over TNF-α and MIP-1β was partially due to soluble factors (data not shown).

4.2. MSC influence CCR7 and CD83 protein expression in monocytes and mDC

In peripheral blood, classical, intermediate and non-classical monocytes and mDC from healthy individuals do not express CCR7 or CD83, however, 24 hours-culture of MNC in RPMI induced CCR7 and CD83 expression in a small percentage of intermediate monocytes and in an important percentage of mDC, further increased when co-cultured with MSC (Figure 7A-B). For classical and non-classical monocyte subpopulations, CCR7 and CD83 were only expressed after LPS+IFNγ stimulus. Of note, the simultaneous presence of MSC and LPS+IFNγ generated an even higher percentage of $CCR7^+$ cells for classical and intermediate monocytes, and also of $CD83^+$ cells for latter cell population. Interestingly, if MSC were depleted prior to LPS+IFNγ

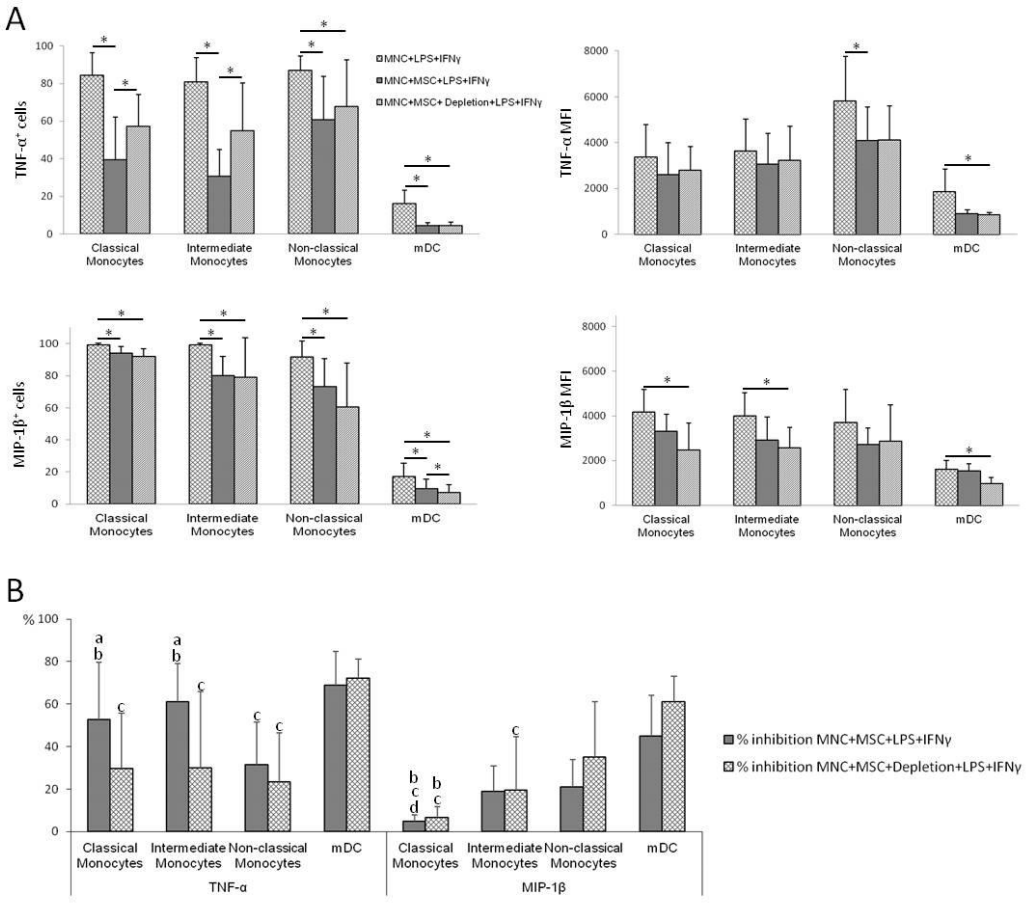

Figure 6. MSC inhibition of TNF-α and MIP-1β protein expression in monocytes and mDC. (A) Percentage (mean ± standard deviation) of cells producing TNF-α and MIP-1β and amount of protein expressed per cell, measured as mean fluorescence intensity (MFI, mean ± standard deviation), for classical, intermediate and non-classical monocytes and mDC, under the following culture conditions: upon MNC stimulation with LPS+IFNγ (MNC+LPS+IFNγ), MNC co-cultured with MSC and stimulated with LPS+IFNγ in the presence of MSC (MNC+MSC+LPS+IFNγ), MNC co-cultured with MSC and stimulated with LPS+IFNγ immediately after depletion of MSC from the culture system (MNC+MSC+Depletion+LPS+IFNγ). Statistically significant differences were considered when $p<0.05$ (*) for Friedman's paired-sample test. **(B)** Percentage (mean ± standard deviation) of inhibition by MSC (present in the culture system during LPS+IFNγ MNC activation, or depleted prior to activation) on the percentage of monocytes and mDC producing TNF-α and MIP-1β. Statistically significant differences were considered when $p<0.05$ for Friedman's paired-sample test: [a] vs the same cell population in MNC+MSC+Depletion+LPS+IFNγ condition; [b] vs non-classical monocytes in the same culture conditions; [c] vs mDC in the same culture conditions; [d] vs intermediate monocytes in the same culture conditions. MSC, mesenchymal stromal/stem cells; TNF-α, tumor necrosis factor-α; MIP-1β, macrophage inflammatory protein-1β; mDC, myeloid dendritic cells; LPS, lipopolysaccharide; IFNγ, interferon γ; MNC, mononuclear cells.

stimulation, the percentage of $CCR7^+$ cells was similar to that observed in MNC+LPS+IFNγ condition for both monocyte subpopulations (Figure 7A). We found no important alterations in the amount of CCR7 expressed *per* cell (MFI) among the different culture conditions, for all cell populations considered; however an increased CD83 MFI was observed in intermediate and non-classical monocytes and mDC in MNC+MSC+LPS+IFNγ culture condition (data not shown).

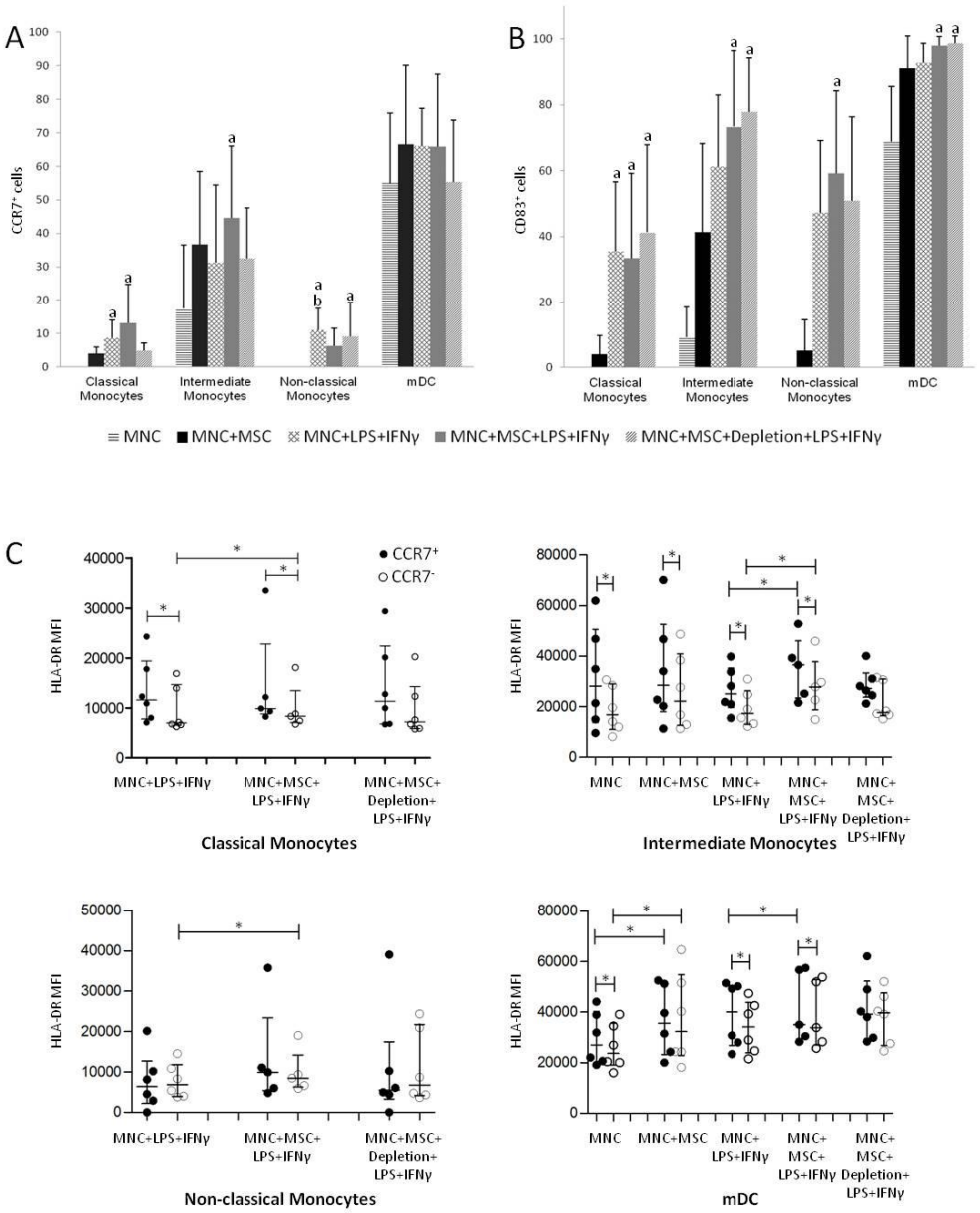

Figure 7. Influence of MSC on CCR7, CD83 and HLA-DR protein expression in monocytes and mDC. Percentage (mean ± standard deviation) of $CCR7^+$ **(A)** and $CD83^+$ **(B)** cells among classical, intermediate and non-classical monocytes and mDC, under the following culture conditions: non-stimulated MNC (MNC), non-stimulated MNC co-cultured with MSC (MNC+MSC), MNC stimulated with LPS+IFNγ (MNC+LPS+IFNγ), MNC co-cultured with MSC and stimulated with LPS+IFNγ in the presence of MSC (MNC+MSC+LPS+IFNγ), MNC co-cultured with MSC and stimulated with LPS+IFNγ immediately after depletion of MSC from the culture system (MNC+MSC+Depletion+LPS+IFNγ). Statistically significant differences were considered when $p<0.05$ for Friedman's paired-sample test: [a] vs MNC; [b] vs MNC+MSC. **(C)** Expression of HLA-DR (measured as mean fluorescence intensity) among $CCR7^+$ and $CCR7^-$ monocyte subpopulations and mDC. Statistically significant differences were considered when $p<0.05$ (*) for Friedman's paired-sample test. MSC, mesenchymal stromal/stem cells; mDC, myeloid dendritic cells; MNC, mononuclear cells; LPS, lipopolysaccharide; IFNγ, interferon γ.

To further confirm that $CCR7^+$ monocytes and mDC corresponded to cells undergoing maturation, we evaluated the amount of HLA-DR expressed *per* cell (MFI), because mature

monocytes and mDC express higher levels of HLA-DR. Accordingly, we found that HLA-DR expression (MFI) was higher for $CCR7^+$ vs $CCR7^-$ cells, for classical and intermediate monocytes subpopulations and mDC, reaching statistical significance (Figure 7C).

4.3. MSC differentially regulate cytokine and chemokine mRNA expression in mDC, classical and non-classical monocytes

The regulation of IL-1β, IL-6, CCL3, CCL5, CXCL9 and CXCL10 mRNA expression by monocytes and mDC was also evaluated. MSC showed a high ability to reduce mRNA expression of all the cytokines and chemokines under study in classical monocytes ($p < 0.05$ for CCL5 and CXCL10) (Figure 8); remarkably, the mRNA expression of IL-1β, IL-6, CCL3 and CCL5 was even lower for MSC+MNC co-culture in which MSC were depleted before MNC stimulation with LPS+IFNγ ($p < 0.05$ for CCL5), except for CXCL9 ($p < 0.05$). However, for non-classical monocytes, only the mRNA expression of chemokines was significantly downregulated ($p < 0.05$ for CCL5, Figure 8); whereas, in mDC, IL-1β mRNA expression was downregulated ($p < 0.05$) and CXCL10 remained unchanged under the influence of MSC (Figure 8). Remarkably, MSC depletion prior to LPS+IFNγ stimulation abrogated CCL3 and diminished CCL5 and CXCL10 mRNA regulation for non-classical monocytes, while decreasing CXCL10 mRNA expression in mDC (Figure 8).

4.4. LPS and IFNγ modulate mRNA and protein expression in MSC

To understand how the MNC stimulators used in this study affect MSC activity, we evaluated the expression of molecules with an important role in the immune function, at mRNA and protein level, in non-stimulated MSC and after LPS and/or IFNγ stimulation (Figure 9).

MSC showed constitutive expression of CD13, CD44, CD73, CD90, CD106, CD184 and IL-6 proteins and IL-6, inducible costimulatory ligand (ICOSL) and transforming growth factor β (TGF-β1) mRNA transcripts (Figure 9). The variation of the expression of the proteins assessed by flow cytometry is expressed here as the ratio of the (MFI of MSC+stimulator)/(MFI of non-stimulated MSC). The protein expression of CD73 and CD184 increased after LPS (ratio of 1.47±1.10 and 1.28±0.55, respectively), IFNγ (ratio of 1.24±0.74 and 1.23±0.35, respectively) and LPS+IFNγ (ratio of 1.34 and 1.49, respectively); CD90 increased after treatment with LPS or LPS+IFNγ (ratio of 1.64±1.16 and 1.49±0.70, respectively); whereas only the stimulation of MSC with LPS+IFNγ increased the protein expression of CD44 and CD106 (ratio of 1.22±0.24 and 1.30±0.47, respectively); finally, CD13 expression showed no alterations after treatment with the stimulator agents. None of the described results reached statistical significance.

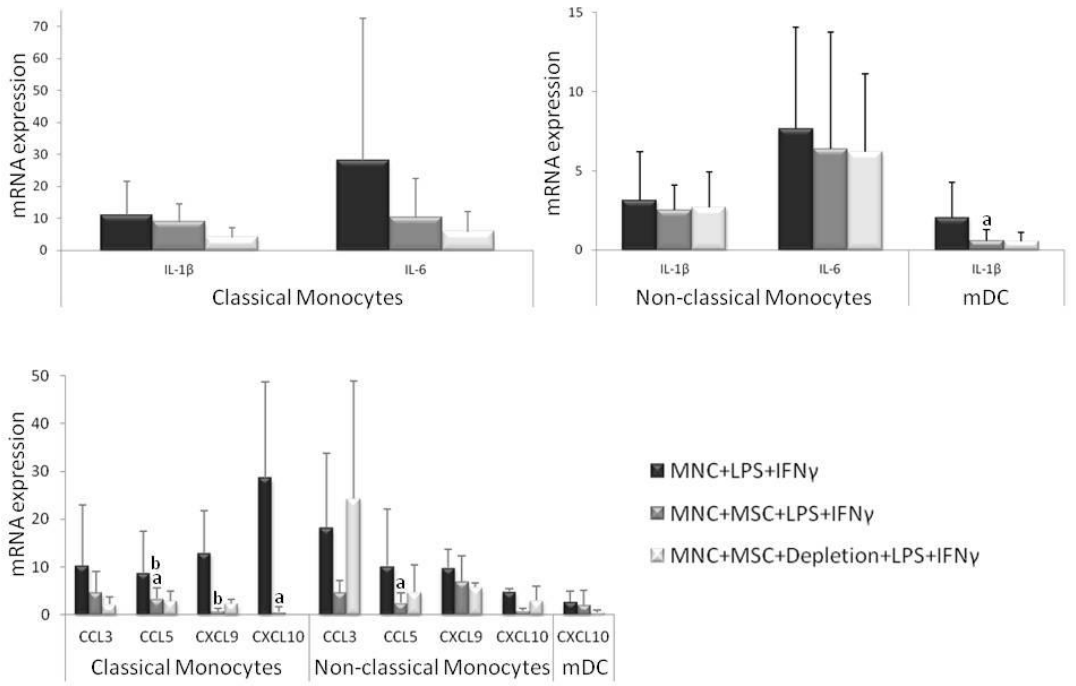

Figure 8. MSC effect on cytokines and chemokines mRNA expression in monocytes and mDC. Semi-quantitative analysis of IL-1β, IL-6, CCL3, CCL5, CXCL9 and CXCL10 mRNA expression in classical and non-classical monocytes and mDC, under the following culture conditions: : MNC stimulated with LPS+IFNγ (MNC+LPS+IFNγ), MNC co-cultured with MSC and stimulated with LPS+IFNγ in the presence of MSC (MNC+MSC+LPS+IFNγ), MNC co-cultured with MSC and stimulated with LPS+IFNγ immediately after depletion of MSC from the culture system (MNC+MSC+Depletion+LPS+IFNγ). Statistically significant differences were considered when $p<0.05$ for Friedman's paired-sample test: [a] vs MNC+LPS+IFNγ; [b] vs MNC+MSC+Depletion+LPS+IFNγ. MSC, mesenchymal stromal/stem cells; mDC, myeloid dendritic cells; IL, interleukin; MNC, mononuclear cells; LPS, lipopolysaccharide; IFNγ, interferon γ.

At mRNA level, we verified that LPS+IFNγ stimulation induced upregulation of TNF-α, IL-1β, IL-6, IL-8, IDO and ICOSL. Besides, MSC stimulation with LPS increased the mRNA expression of TNFα and IL-1β, while decreasing ICOSL; an upregulation of IL-8 mRNA was observed after stimulation with either LPS or IFNγ; and IDO mRNA levels increased in MSC stimulated with IFNγ (Figure 9). However, the analysis of the supernatant from MSC cultures by ELISA, showed that IL-6 was constitutively expressed by MSC and neither LPS nor IFNγ altered their expression at protein level. Additionally, IDO was not detected by western blot in MSC lysates (data not shown) and no alterations were found in TGF-β1 mRNA expression after MSC stimulation with LPS and/or IFNγ (Figure 9). None of the stimulators altered MSC morphology or differentiation capability; and though MSC stimulated with LPS showed a higher level of expansion, it occurred after the first passage and after culture day 10, thus not affecting our results (data not shown).

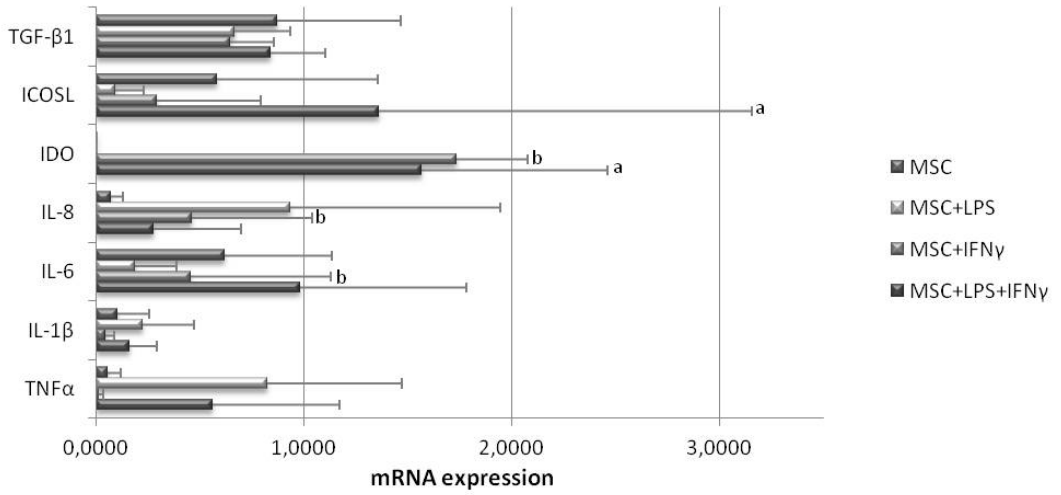

Figure 9. Modulation of mRNA expression of pro and anti-inflammatory proteins in MSC by LPS, IFNγ and LPS+IFNγ. Semi-quantitative analysis of TGF-β, ICOSL, IDO, IL-8, IL-6, IL-1β and TNF-α mRNA expression in MSC after stimulation with LPS, IFNγ, or LPS+IFNγ. Statistically significant differences were considered when $p<0.05$ for Friedman's paired-sample test: [a] vs MNC; [b] vs MNC+LPS. MSC, mesenchymal stromal/stem cells; LPS, lipopolysaccharide; IFNγ, interferon γ; TGF-β, transforming growth factor β; ICOSL, inducible costimulatory ligand; IDO, indoleamine-2,3-dioxygenase; IL, interleukin; TNF-α, tumor necrosis factor-α; MNC, mononuclear cells.

5. DISCUSSION

The aim of the present work was to gain a deeper insight on the immunosuppressive effect of human bone marrow-derived MSC on the recently described peripheral blood monocyte subpopulations (classical $CD14^{++}CD16^{-}$, intermediate $CD14^{++}CD16^{+}$ and non-classical $CD14^{+}CD16^{++}$ monocytes) [332,369] and mDC. To the best of our knowledge, this is the first study reporting the effect of MSC on these specific cell populations. To accomplish this goal, the expression of TNF-α, CCL4 (MIP-1β), CCR7 and CD83 was evaluated by flow cytometry and the mRNA expression of IL-1β, IL-6, CCL3 (MIP-1α), CCL5 (RANTES), CXCL9 (MIG) and CXCL10 (IP-10) was quantified in FACS-sorted and purified classical and non-classical monocytes, and IL-1β and CXCL10 mRNA levels were evaluated in mDC.

Finally, we evaluated protein and mRNA expression in non-stimulated MSC and after LPS and/or IFNγ treatment, in order to understand how their regulatory function could be affected. As previously described, we found a slight increase in the mRNA of the pro-inflammatory cytokines TNF-α and IL-6, along with a significant increase in the chemotactic IL-8 [36,86,87,89,90,94,610] and the immunosuppressor IDO, ICOSL and CD73 [52,88,94], upon

stimulation with LPS+IFNγ. Of note, an increased expression of other immunoregulatory molecules, such as fibroblast growth factor 2 (FGF2), hepatocyte growth factor (HGF), cyclooxygenase 2 (COX2), prostaglandin E2 (PGE2), upon TLR4 stimulation had also been described in the literature [73,90,92,94]. Moreover, LPS+IFNγ promoted an increased protein expression of adhesion molecules in MSC: CD106 (VCAM-1), CD184 (CXCR4), CD54 (ICAM-1), CD90 (Thy-1) and CD44. These adhesion molecules are involved in cell-to-cell and cell-extracellular matrix interaction and their upregulation had been reported to improve both MSC interaction with immune cells, namely T cells, and MSC migration [9,10,611,612]. Taken together these data suggest that, under an inflammatory microenvironment, MSC increase the expression of adhesion molecules that allow their close interaction with immune cells, contributing to increase the efficiency of paracrine mediators with immunosuppressive function produced by MSC.

Our data showed that MSC efficiently inhibit TNF-α and MIP-1β production, in all the three monocytes subpopulations and mDC, by reducing both the percentage of producing cells and the amount of cytokine produced *per* cell, albeit our results suggested that MSC exert a more pronounced inhibitory effect over TNF-α expression than over MIP-1β. The partial inhibition observed in transwell assays suggests that both soluble factors and cell contact mechanisms are involved in this process. Interestingly, MSC exerted a higher inhibition over TNF-α production by intermediate monocytes and mDC (compared to classical and non-classical monocytes), which correspond to the two cell populations more skilled for antigen presentation to T cells [369]. Furthermore, MSC impaired the mRNA expression of all the cytokines and chemokines under study in classical monocyte, as well as of the chemokines in non-classical monocytes and of IL-1β in mDC. Taken together, our data support that MSC inhibit the pro-inflammatory function of monocytes and mDC both by reducing the expression of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and also by downregulating chemotactic factors that attract T cells and monocytes (CCL3, CCL4, CCL5, CXCL9 and CXCL10) and whose expression is induced by LPS plus IFNγ.

In fact, LPS binding to TLR4 expressed on monocytes and mDC plasmatic membrane results in the downstream activation of MAPK (ERK1/2, JNK and p38), NF-kB and AP-1 [613], followed by the induction of the transcription of TNF-α, IL-1β, IL-6, MIP-1β (CCL4), CCL3, CCL5, CXCL9 and CXCL10 [614-621]. IFNγ signaling can also contribute to IL-1β, CXCL9 and CXCL10 mRNA expression in human monocytes, macrophages and DC [616,622-624]. Despite the overlapping signaling molecules participating on TNF-α and MIP-1β gene expression induction

[614], MSC showed more efficiency in inhibiting TNF-α, suggesting differences between these two signaling pathways.

Taking into account previous studies which investigated the mechanisms underlying the inhibition of cytokine and chemokine expression by MSC, we can point out the constitutively expressed IDO, Jagged-1, adenosine and/or PGE2 as possible candidates to the impairment of TNF-α production observed in the cells under study [90,236,420,422,428,454,519,625]; whereas PGE2 has also been described to reduce MIP-1β expression in monocyte-derived DC [439,626]. As, for the evaluation of protein expression by flow cytometry, we added brefeldin A to the cell culture together with LPS+IFNγ (which stopped the proteins synthesized *de novo* in the Golgi apparatus and prevented them to reach the extracellular medium), the inhibition of TNF-α and MIP-1β production can only be attributed to proteins constitutively expressed by MSC, or induced after their contact with peripheral blood MNC.

Conversely, the assays made to evaluate mRNA expression were conducted in the absence of brefeldin A, so, for the final inhibitory effect accounted proteins constitutively expressed by MSC or induced by the contact with MNC, stimulator agents, or cytokines produced by MNC in response to LPS plus IFNγ activation. In fact, stimulation of MSC with LPS and/or IFNγ induces the expression of several immunosuppressor factors, such as HGF, nitric oxide (NO) and insulin-like growth factor (IGF) and increase Jagged-1, PGE2 and IDO expression [73,87,88,92,94]. In line with this, previous studies demonstrated that IL-6 expression might be inhibited by Jagged-1 and HGF in macrophages [519,627], or by IDO in DC [454], though IDO presents the opposite effect in human cancer cells [628]. In turn, PGE2 has the ability to impair the expression of CCL3, CCL5 and CXCL10 in human DC, macrophages [444,626,629], microglial cells and astrocyes [630] and of CXCL9 and CXCL10 in human breast cancer cells [623]; HGF and NO were demonstrated to inhibit CCL5 expression in human renal tubular epithelial cells and in mouse keratinocytes, respectively [497,498,631], while CXCL10 expression in melanoma cells is downregulated by NO [632]. The decreased expression of genes induced by TLR4 signaling pathway was shown to result from ERK1/2 inhibition by Jagged-1, and NF-kB inhibition by PGE2, HGF, IDO and adenosine [420,454,498,519,625,627,633].

In the present study, neither CCR7 nor CD83 protein expression was inhibited by the presence of MSC in the cell culture; furthermore, $CCR7^+$ cells displayed a higher HLA-DR MFI than their $CCR7^-$ counterparts; altogether suggesting that MSC do not inhibit monocyte and mDC activation/maturation under our experimental conditions. Regarding the important role of CCR7 in monocyte and DC migration, it was demonstrated that solely the upregulation of

CCR7 expression does not necessarily corresponds to increased migration ability; however, it has been reported that PGE2 can upregulate the expression of CCR7, CD83 and HLA-DR in DC and of CCR7 in monocytes and, simultaneously, increase the cells' migratory response to the lymph node-derived CCR7 ligands, CCL19 and CCL21 [422,431,434,438-440,445]. IFNγ share the ability to increase CCR7 expression in monocyte-derived DC and, if administrated with TLR4 agonist, to potentiate their CCR7-driven migration; in turn, NO and IGF-I can induce CD83 expression in DC [506,624,634]. According to our data, MSC do not impede the expression of CD83 and CCR7 in LPS+IFNγ-activated monocyte and mDC, but impair their pro-inflammatory function by decreasing the expression of TNF-α, IL-1β and IL-6. In the same line, the reduction of chemokine expression induced by MSC may inhibit classical and non-classical monocytes' ability to recruit T cells and monocytes, consequently hampering the interaction of these antigen-presenting cells with T cells.

It was recently reported that macrophages and monocytes can assume immunoregulatory functions, induced by IL-6 and/or PGE2, and characterized by decreased TNF-α expression and increased IL-10 production [52,371,378,379,635], wherein MSC can actively participate [268,377]. Attending to the particular experimental conditions of the present study, as reported here and by others, MSC constitutively express IL-6 which is further upregulated upon stimulation with LPS [86,87,89,90,92,94] and this cytokine has the ability to downregulate TNF-α expression in monocytes [351,375]. Several studies reported MSC-derived inhibition of CD83, CD80, CD86, HLA-DR upregulation in DC differentiation assays [25,47,333,348-350,375] and impairment of their migratory ability, both *in vitro* and *in vivo*, toward CCL21 [346,636]. In opposition, van der Berk et al. reported that human UCB-MSC did not inhibit monocyte differentiation into immature DC and supported not only DC maturation but also the migration towards CCL21 [353]. It is worth to mention that we detected ICOSL mRNA in MSC, as recently described by Yii et al. [544], which was upregulated upon LPS+IFNγ stimulation. This finding opens new possibilities for the immunosupressive mechanisms of MSC, because ICOSL induces IL-10-producing regulatory T cells [604].

Here, we did not use a protocol to differentiate monocytes into DC, instead we analyzed the naturally occurring peripheral blood monocytes subpopulations and mDC (which corresponds to a more physiological condition) to assess the influence of MSC. Our results suggest that, upon MNC stimulation, MSC do not impede monocyte or mDC to express the activation/maturation markers CD83, CCR7 and HLA-DR. Conversely, MSC impair monocyte and mDC pro-inflammatory function, by decreasing the production of TNF-α, IL-1β and IL-6,

and reducing the monocytes' ability to recruit T cells and other monocytes, by diminishing MIP-1β, CCL3, CCL5, CXCL9 and CXCL10 expression.

As MSC are highly sensitive to the microenvironment, small differences in the experimental protocol may yield different results. In fact, concentration and time of exposure to LPS may conditioned MSC influence on immune cells [87,635,637]; also, the state of activation/maturation of DC will determine different responses to MSC regulatory factors [422]; all these variables difficult the comparison of the results obtained in different studies.

6. CONCLUSIONS

Altogether, our results showed that bone marrow-derived MSC inhibit TNF-α and MIP-1β protein expression in activated classical, intermediate and non-classical monocytes, as well as in mDC. Remarkably, the inhibition observed was more pronounced for TNF-α expression than for MIP-1β and, regarding TNF-α, non-classical monocytes were more resistant to MSC-induced suppression. Of note, mDC exhibited the highest degree of regulation for both TNF-α and MIP-1β. Similarly, MSC downregulate mRNA expression of pro-inflammatory cytokines and chemokines by monocytes and mDC. Conversely, the induction of activation/maturation markers (CD83, CCR7 and HLA-DR) by LPS plus IFNγ activation was not impaired by MSC. Thus, despite of not suppressing the activation/maturation of monocytes and mDC, MSC impair their pro-inflammatory function by reducing the expression of pro-inflammatory cytokines and chemotactic factors for monocytes and T cells, which may ultimately hamper the development of an inflammatory immune response.

CHAPTER 4 |

EFFECT OF HUMAN BONE MARROW MESENCHYMAL STROMAL CELLS ON CYTOKINE PRODUCTION BY PERIPHERAL BLOOD NAIVE, MEMORY AND EFFECTOR T CELLS

1. ABSTRACT

Introduction: The different distribution of T cells among activation/differentiation stages in immune disorders may condition the outcome of mesenchymal stromal cells (MSC)-based therapies. Indeed, the effect of MSC in the different functional compartments of T cells is not completely elucidated.

Methods: We investigated the effect of human bone marrow MSC on naturally occurring peripheral blood functional compartments of $CD4^+$ and $CD8^+$ T cells: naive, central memory, effector memory and effector compartments. For that, mononuclear cells (MNC) stimulated with phorbol myristate acetate (PMA) plus ionomycin were cultured in the absence/presence of MSC. The percentage of cells expressing tumor necrosis factor (TNF)-α, interferon (IFN)γ, interleukin (IL)-2, IL-17, IL-9 and IL-6, and the amount of cytokine produced, was assessed by flow cytometry. mRNA levels of IL-4, IL-10, transforming growth factor (TGF)-β and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) in purified $CD4^+$ and $CD8^+$ T cells, and phenotypic and mRNA expression changes induced by PMA+ionomycin stimulation in MSC, were also evaluated.

Results: MSC induced the reduction of the percentage of $CD4^+$ and $CD8^+$ T cells producing TNF-α, IFNγ and IL-2 in all functional compartments, except for naive $IFN\gamma^+CD4^+$ T cells. This inhibitory effect differentially affected $CD4^+$ and $CD8^+$ T cells, as well as the T cell functional compartments; remarkably, different cytokines showed distinct patterns of inhibition regarding both the percentage of producing cells and the amount of cytokine produced. Likewise, the percentage of $IL\text{-}17^+$, $IL\text{-}17^+TNF\text{-}\alpha^+$ and $IL\text{-}9^+$ within $CD4^+$ and $CD8^+$ T cells and of $IL\text{-}6^+CD4^+$ T cells was decreased in MNC-MSC co-cultures. MSC decreased IL-10 and increased IL-4 mRNA expression in stimulated $CD4^+$ and $CD8^+$ T cells, while TGF-β was reduced in $CD8^+$ and augmented in $CD4^+$ T cells; with no changes for CTLA-4. Finally, PMA+ionomycin stimulation didn't induce significant alterations on MSC phenotype, but increased indoleamine-2,3-dioxygenase (IDO), inducible costimulatory ligand (ICOSL), IL-1β, IL-8 and TNF-α mRNA expression.

Conclusions: Overall, our study showed that MSC differentially regulate the functional compartments of $CD4^+$ and $CD8^+$ T cells, which may differentially impact their therapeutic effect in immune disorders. Furthermore, the influence of MSC on IL-9 expression can open new possibilities for MSC-based therapy in allergic diseases.

2. INTRODUCTION

The discovery of the immunosuppressive potential of mesenchymal stromal cells (MSC) propelled a large number of studies in the past decade, mainly focusing on T cells. The suppressive effect of MSC over T cells comprises inhibition of T cell proliferation, activation, differentiation in effector cells and effector function, by altering their cytokine profile and impairing the cytolytic activity of cytotoxic T cells [231]. MSC-derived immunosuppression can be achieved by direct MSC-T cells interaction, through plasmatic membrane proteins or soluble factors produced by MSC, or indirectly by MSC-mediated suppression of antigen-presenting cells [45]. In fact, human bone marrow (BM) MSC impair dendritic cell maturation and decrease the expression of co-stimulatory molecules and interleukin (IL)-12, while increasing IL-10 expression, consequently hampering T cell activation [45,53,348,349,635]. A similar effect is observed in monocytes which, in the presence of human BM-MSC, develop an anti-inflammatory phenotype with increased IL-10 expression [355,375,379].

However, it is well established that the behavior of MSC depend on numerous factors, such as the source of MSC, 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 [46,59,61,62,263]. In turn, the information available on the effect of MSC over T cells at different stages of activation/differentiation is scarce, and the data concerning the influence of MSC on the naive-effector T cell differentiation process are contradictory. Most of the publications describe an inhibitory action over Th1 and Th17 differentiation, along with a decreased expression of the cytokines related to these effector phenotypes: interferon (IFN) γ, IL-2 and tumor necrosis factor (TNF)-α, for Th1; and IL-17A, IL-17F and IL-21, for Th17, both *in vitro* and *in vivo* [9,180,231,263,271,275,281]. Nevertheless, some studies reported that MSC promoted Th17 differentiation and IL-17A production [231,251,252,254,280].

Different disorders of the immune system, and different stages of each immune disease, are characterized by distinctive distribution of T cells among activation/differentiation compartments, which is a reflection of the T cell subsets with a predominant role in the disease etiology. This highlights the importance of knowing the effect of MSC on each individual functional compartment, in order to predict and understand the outcome of MSC cell therapy.

Here, we investigate the suppressive effect of human BM-MSC over peripheral blood $CD4^+$ and $CD8^+$ T cells distributed among the naturally occurring naive, central memory (CM),

effector memory (EM) and effector compartments. Peripheral blood mononuclear cells (MNC) were stimulated with phorbol myristate acetate (PMA) plus ionomycin in the absence/presence of MSC, and the expression of IL-2, TNF-α and IFNγ was evaluated by flow cytometry for $CD4^+$ and $CD8^+$ T cells distributed among the naive, CM, EM and effector compartments; the expression of IL-6, IL-9 and IL-17 was also evaluated in total $CD4^+$ and $CD8^+$ T cells. In addition, IL-4, IL-10, TGF-β and CTLA-4 mRNA levels were assessed in FACS-purified $CD4^+$ and $CD8^+$ T cells. Finally, the protein expression of CD13, CD44, CD73, CD90, CD106, CD184 (CXCR4) and mRNA transcripts of IDO, ICOSL, TGF-β1, IL-1β, IL-8 and TNF-α were evaluated in non-stimulated MSC and after stimulation with PMA plus ionomycin.

In this study, we report that the functional compartments of $CD4^+$ and $CD8^+$ T cells are differentially regulated by MSC, which may condition the outcome of MSC cell therapy in immune disorders with distinctive distribution of T cells among activation/differentiation compartments. Of note, MSC decrease the percentage of $CD4^+$ and $CD8^+$ T cells expressing TNF-α, IFNγ, IL-2, IL-6, IL-9 and IL-17, as well as the amount of TNF-α and IFNγ produced at a single-cell level. The influence of MSC on IL-9 can be a possible mechanism contributing to allergic inflammation amelioration by MSC.

3. METHODS

3.1. Collection and isolation of peripheral blood mononuclear cells and bone marrow MSC

Peripheral blood samples from a total of 8 healthy donors (1 male and 7 females; mean age of 41±11 years, ranging from 21 to 50 years old), collected in heparin at the Blood and Transplantation Center of Coimbra (Portugal), and human BM samples from healthy donors (age ranging from 20 to 40 years old), were included in the present study. The use of these biological samples for research purpose was approved by the Ethics Committee of Instituto Português de Oncologia de Lisboa Francisco Gentil (Laws nº 97/95, nº 46/2004) and all participants gave written informed consent prior entering in the study.

Peripheral blood mononuclear cells (MNC) were isolated by Lymphoprep (Stemcell Technologies, Vancouver, Canada) gradient density centrifugation and then washed twice in Hank's Balanced Salt Solution (HBSS, Gibco, Life Technologies, Paisley, UK). The MNC pellet

was resuspended in RPMI 1640 with GlutaMax medium (Invitrogen, Life Technologies) with antibiotic-antimycotic (Gibco), to the final concentration of 10^6 cells/ml.

Peripheral blood MNC were subsequently analyzed for protein and mRNA expression in the following experimental conditions: 1) $0.5x10^6$ MNC + 500 µl RPMI (negative control); 2) $0.5x10^6$ MNC + $0.25x10^6$ MSC (negative control); 3) $0.5x10^6$ MNC + 500 µl RPMI + PMA + ionomycin (positive control); 4) $0.5x10^6$ MNC + $0.25x10^6$ MSC + PMA + ionomycin. All the aforementioned cell cultures were carried out for 20 hours at 37°C, in a sterile environment with 5% CO_2 and humidified atmosphere, plus an incubation period of 4 hours with the stimulator agents. The cell culture and stimulation protocols are detailed below, in "Immunophenotypic study of MSC and peripheral blood T cells" section.

For the isolation of human BM-MSC, MNC were isolated from BM samples using Sepax S-100 system (Biosafe, Switzerland), according to manufacturer's instructions. Cell number and viability were determined using the Trypan Blue (Gibco) exclusion method.

BM MNC were plated at a density of $2x10^5$ cells/cm^2 in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% qualified fetal bovine serum (FBS, Sigma, Spain). After a 3-day incubation at 37ºC in humidified atmosphere containing 5% CO_2, the non-adherent cell fraction was discarded, and the adherent culture was maintained with a complete medium renewal every 3-4 days. After reaching a 70-80% confluency cells were detached using TrypLE (Life Technologies) for 7 minutes and then replated at an initial density of 3000 cells/cm^2. For this study, MSC passages 3 and 5 were used.

MSC identity was confirmed by performing fluorescent morphological analysis, mesodermal differentiation assays (osteogenic, adipogenic and chondrogenic) and immunophenotype characterization as described by Dominici et al. [2].

Subsequently, MSC were resuspended in RPMI 1640 with GlutaMax medium (Invitrogen) with antibiotic-antimycotic (Gibco) to the final concentration of $0.5x10^6$ cells/ml. The protein and mRNA expression of MSC was studied in the following experimental conditions: 1) $0.25x10^6$ MSC + 500 µl RPMI (non-stimulated MSC); 2) $0.25x10^6$ MSC + 500 µl RPMI + PMA + ionomycin. All the aforementioned cell cultures were carried out for 20 hours at 37°C, in a sterile environment with 5% CO_2 and humidified atmosphere, plus an incubation period of 4 hours with the stimulator agents. The cell culture and stimulation protocols are detailed below, in "Immunophenotypic study of MSC and peripheral blood T cells' section.

3.2. Immunophenotypic study of MSC and peripheral blood T cells

3.2.1. MSC stimulation with PMA plus ionomycin

For the immunophenotypic study of MSC, we plated in 4 wells of tissue culture plates (Falcon, Becton Dickinson Biosciences, BD, San Jose, USA) $0.25x10^6$ MSC in 1 ml of RPMI 1640 with GlutaMax medium (Invitrogen) with antibiotic-antimycotic (Gibco). MSC were cultured for 20 hours at 37°C, in a sterile environment with 5% CO_2 and humidified atmosphere (to be in the same experimental conditions than as those MSC co-cultured with MNC). Then, MSC from two of the four wells were stimulated with PMA (50ng/ml; Sigma) plus ionomycin (1µg/ml; Boehringer Mannheim, Germany), for 4 hours at 37ºC in humidified atmosphere containing 5% CO_2; in the remaining 2 wells, MSC were not stimulated. The immunophenotypic study and mRNA expression quantification were performed in non-stimulated and PMA+ionomycin-stimulated MSC.

3.2.2. Immunophenotypic study of MSC

For each experimental condition tested, cells were detached using TrypLE (Gibco); after incubating for 10 minutes at -20ºC, the content of each well was transferred to a 12x75 mm polystyrene cytometer tube, centrifuged for 5 minutes at 540xg and the supernatant was discarded. MSC immunophenotype was assessed using the 8-color monoclonal antibody (mAb) combination detailed in table 4, tube 1. The cell pellet was incubated with the mAb for 10 minutes in the darkness and washed with PBS. Finally, cells were resuspended in 500 µl of PBS and immediately acquired in a FACSCanto II (BD) flow cytometer.

3.2.3. Co-culture of peripheral blood MNC and MSC and in vitro stimulation with PMA plus ionomycin

In 4 wells of tissue culture plates (Falcon) $0.5x10^6$ MNC were plated in 1 ml of RPMI 1640 with GlutaMax medium (Invitrogen) with antibiotic-antimycotic (Gibco), and in other 4 wells of tissue culture plates (Falcon) we plated $0.5x10^6$ MNC + $0.25x10^6$ MSC in a final volume of 1 ml, establishing a ratio of 2:1 (MNC:MSC). Cells were cultured for 20 hours at 37°C, in a sterile environment with 5% CO_2 and humidified atmosphere.

After the incubation period, PMA+ionomycin (50ng/ml and 1µg/ml, respectively) were added to 2 wells with MNC and 2 wells with co-cultured MNC+MSC; the cells in the remaining wells (2 with MNC and 2 with MNC+MSC) were not stimulated. Brefeldin A (10 µg/ml), from *Penicillium brefeldiamun* (Sigma), was added to 1 well of each experimental condition – 1)

MNC, 2) MNC+MSC, 3) MNC+PMA+ionomycin, and 4) MNC+MSC+PMA+ionomycin – to prevent the release of *de novo* produced cytokines outside the cells. Then, we proceeded to an incubation at 37 °C, in a sterile environment with 5% CO_2 humidified atmosphere, for 4 hours.

The samples with brefeldin A were used for the study of cytokine expression in T cells by flow cytometry, while the mRNA expression was performed in the samples without brefeldin A. All the aforementioned protein and mRNA expression studies were performed in all the different culture conditions: MNC, MNC+MSC, MNC+PMA+ionomycin, MNC+MSC+ PMA+ionomycin.

3.2.4. Immunophenotypic study of peripheral blood T cells

For each experimental condition tested, cells were detached using TrypLE (Gibco); after incubating for 10 minutes at -20ºC, the content of each well was transferred to a 12x75 mm polystyrene cytometer tube, centrifuged for 5 minutes at 540xg and the supernatant was discarded. Immunophenotypic analysis of peripheral blood T cells, cultured in the presence/absence of PMA+ionomycin and in the presence/absence of MSC, was performed using 7-color mAb combinations, detailed in table 4. In short, cells were stained with the mAb for surface proteins antigens (CD3, CD27, CD4, CD45RA and CD8) and, after an incubation period of 10 minutes in the darkness at room temperature, washed with PBS. For intracellular staining, Fix&Perm (Caltag, Hamburg, Germany) reagent was used, according to the manufacturer's instructions and in parallel with the mAb for TNF-α and IL-17 (tube 2), IFNγ and IL-6 (tube 3), or IL-2 and IL-9 (tube 4). After washing twice with PBS, the cell pellet was resuspended in 500 µl of PBS and immediately acquired.

3.2.5. Data acquisition and analysis

Data acquisition was performed in a FACSCanto II (BD) flow cytometer equipped with the FACSDiva software (v6.1.2; BD). For both MSC and MNC immunophenotypic studies, the whole sample from each tube was acquired and stored, corresponding to a number of events always above $0.1x10^6$ or $0.5x10^6$ events, respectively. For data analysis, the Infinicyt (version 1.7) software (Cytognos SL, Salamanca, Spain) was used.

Table 4. Panel of mAb reagents (with clones and commercial source) used for the immunophenotypic characterization of MSC and peripheral blood T cells.

	Fluorochrome							
Tube	**PacB**	**PacO**	**FITC**	**PE**	**PerCPCy5.5 or PECy5**	**PECy7**	**APC**	**APCH7**
1	**CD44** clone IM7 Biolegend	**CD45** HI30 Invitrogen	**CD106** 51-10C9 BD Pharmingen	**CD73** AD2 BD Pharmingen	**CD184** 12G5 BD	**CD13** Immu103.44 Beckman Coulter	**CD90** 5E10 BD Pharmingen	
2	**CD3** UCHT1 BD Pharmingen		**cyTNF-α** MP6-XT22 BD Pharmingen	**cyIL-17** SCPL1362 BD Pharmingen	**CD27** 14CD27 Beckman Coulter	**CD4** SFCI12T4D11 Beckman Coulter	**CD45RA** HI100 BD	**CD8** SK1 BD
3	**CD3** UCHT1 BD Pharmingen		**cyIFNγ** 4S.B3 BD Pharmingen	**cyIL-6** MQ2-6A3 BD Pharmingen	**CD27** 14CD27 Beckman Coulter	**CD4** SFCI12T4D11 Beckman Coulter	**CD45RA** HI100 BD	**CD8** SK1 BD
4	**CD3** UCHT1 BD Pharmingen		**cyIL-2** MQ1-17H12 BD	**cyIL-9** MH9A3 BD Pharmingen	**CD27** 14CD27 Beckman Coulter	**CD4** SFCI12T4D11 Beckman Coulter	**CD45RA** HI100 BD	**CD8** SK1 BD
5	**CD4** RPA-T4 BD Pharmingen		**CD25** M-A251 BD Pharmingen	**CD127** R34.34 Beckman Coulter		**TCRγδ** 11F2 BD Pharmingen	**CD8** B9.11 Beckman Coulter	**CD3** SK7 BD Pharmingen

Abbreviations: mAb, monoclonal antibody; MSC, mesenchymal stroma/steml cells; PacB, pacific blue; PacO, pacific orange; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCPCy5.5, peridinin chlorophyll protein-cyanine 5.5; PECy5, phycoerythrin-cyanine 5; PECy7, phycoerythrin-cyanine 7; APC, allophycocyanin; APCH7, allophycocyanin-hilite 7.
Commercial sources: Biolegend (San Diego, CA, USA); Invitrogen, Life Technologies (Carlsbad, CA, USA); BD Pharmingen (San Diego, CA, USA); BD (Becton Dickinson Biosciences, San Jose, CA, USA), Beckman Coulter (Miami, FL, USA).

3.2.6. Immunophenotypic identification of T cells within the different functional compartments

The identification and quantification of the four T cell functional compartments, was carried out after the exclusion of cell debris (which corresponded to events with very low forward scatter (FSC) and heterogeneous side scatter (SSC) light dispersion properties) and doublets (identified by their FSC-area and FSC-height characteristics). Then, T cells were identified based on CD3 positivity and intermediate FSC and SSC properties (Figure 10). Within this cell population, the functional compartments of $CD4^+$ and $CD8^+$ T cells (phenotypically characterized as $CD3^+CD4^+CD8^-$ and $CD3^+CD4^-CD8^+$, respectively) were identified according to their differential expression of CD45RA and CD27, as follows: naive T cells are characterized by $CD45RA^+CD27^+$ expression, CM T cells are $CD45RA^-CD27^+$, EM and effector T cells display $CD45RA^-CD27^-$ and $CD45RA^+CD27^-$ immunophenotype, respectively (Figure 10).

3.3. Cell purification by fluorescence-activated cell sorting

$CD4^+$ and $CD8^+$ T cell populations from the cell cultures were purified by fluorescence-activated cell sorting (FACS), using FACSAria II flow cytometer (BD), according to their typical

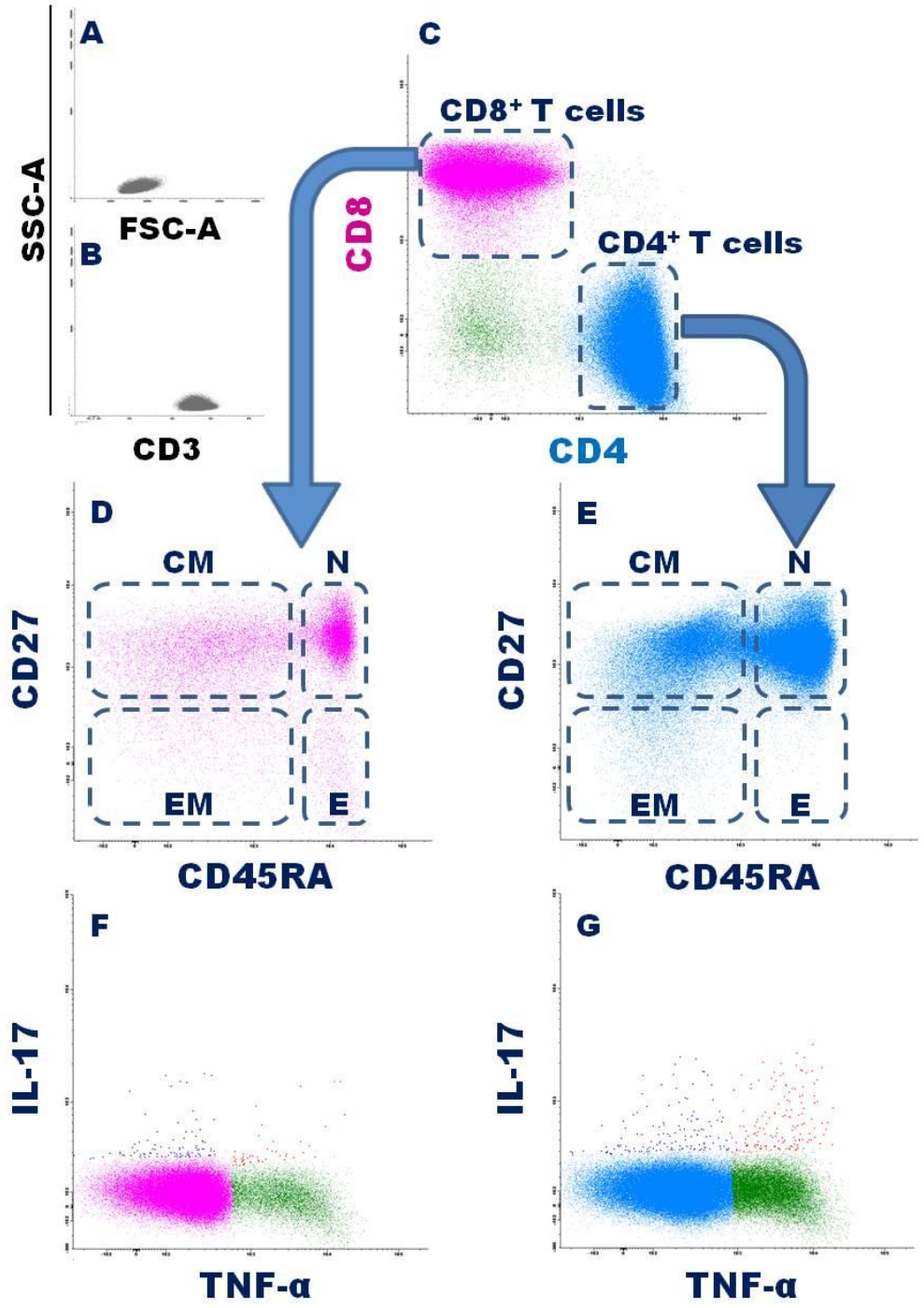

Figure 10. Gating strategy for identifying $CD4^+$ and $CD8^+$ T cells' functional compartments and cytokine expression among each T cell compartment. Bivariate dot plot histograms illustrating **(A-C)** overall T cells in the cell culture and **(C)** $CD4^+$ (blue events) and $CD8^+$ (pink events) T cells. The identification of T cells' functional compartments were made within $CD8^+$ **(D)** and $CD4^+$ **(E)** T cell subpopulations, based on CD45RA and CD27 expression, as follows: $CD45RA^+CD27^+$ phenotype corresponds to naive (N) T cells, $CD45RA^-CD27^+$ corresponds to central memory (CM), effector memory (EM) T cells are $CD45RA^-CD27^-$, and effector T cells display $CD45RA^+CD27^-$ phenotype. The expression of cytokines (illustrated in panel **(F)** for $CD8^+$ T cells and in **(G)** for $CD4^+$ T cells) was evaluated within each $CD4^+$ and $CD8^+$ T cell functional compartments.

phenotype. Thus, the 6-color mAb combination used (Table 4, tube 5) allowed the identification of $CD4^+$ T cells ($CD3^+CD4^+CD8^-TCR\gamma\delta^-$) and $CD8^+$ T cells ($CD3^+CD4^-CD8^+TCR\gamma\delta^-$). The remaining mAb found in the panel were used to exclude γδ T cells (which correspond to $CD3^+TCR\gamma\delta^+$ events) and regulatory T cells (which are $CD25^{++}CD127^{-/dim}$). The purified cell populations were subsequently used for the quantification of mRNA expression.

3.4. Analysis of mRNA expression in MSC and purified CD4+ and CD8+ T cells

The content of each well of cultured MSC under the different experimental condition tested, or the purified $CD4^+$ and $CD8^+$ T cell populations, were transferred to a 1.5 ml eppendorf tube, centrifuged for 5 minutes at 300xg and the pellet resuspended in 350 µl of RLT Lysis Buffer (Qiagen, Hilden, Germany). Total RNA was extracted with the RNeasy Micro kit (Qiagen), according to the supplier's instructions. Then, total RNA was eluted in a 20 µl volume of RNase-free water. RNA was reverse transcribed with Tetra cDNA Synthesis ® (Bioline, London, UK), according to the manufacturer's instructions. Relative quantification of gene expression by real-time PCR was performed in the LightCycler 480 II (Roche Diagnostics, Rotkreuz, Switzerland). Real-time PCR reactions were carried out using 1x QuantiTect SYBR Green PCR Master Mix (Qiagen), 1x QuantiTect Primer Assay (for MSC: TNF3: QT01079561; IL-8: QT00000322; IL-1β: QT00021385; TGF-β1: QT00025718; ICOSL: QT00023660; IDO: QT00000504; for purified CD4+ and CD8+ T cells: IL-10: QT00041685; IL-2: QT00015435; IL-4: QT00012565; TGF-β: QT00000728; CTLA-4: QT01670550) (Qiagen), in a final volume of 10 µl. The reactions were performed using the following thermal profile: 1 cycles of 10 min at 95°C, 50 cycles of 10 sec at 95°C, 20 sec at 55°C and 30 sec at 72°C, 1 cycle of 5 sec at 95°C, 1 min at 65°C and continuo at 97°C, and 1 cycle of 10 sec at 21°C. All samples were run in duplicate. Real-time PCR results were analyzed with the LightCycler software (Roche Diagnostics). GeNorm software (PrimerDesign Ltd., Southampton, England) was used to select the reference genes to normalize data. The reference genes used for gene expression analysis were: cytochrome c1 (CYC1) and splicing factor 3a subunit 1 (SF3A1) for MSC; beta-2 microglobulin (B2M) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (YWHAZ) for CD4+ and CD8+ T cells. The normalized expression levels of the genes of interest were calculated by using the delta-Ct method.

3.5. Statistical analysis

To determine the statistic significance of the differences observed between different culture conditions, non-parametric Wilcoxon and Friedman's paired-sample tests were

performed, using Statistical Package for Social Sciences (IBM SPSS, version 17.0, Armonk, NY, USA). Data were expressed as mean percentage ± standard deviation. Statistically significant differences were considered when p value was lower than 0.05.

4. RESULTS

To investigate MSC ability to regulate cytokine protein expression in $CD4^+$ and $CD8^+$ T cells and how the influence of MSC varies among the different T cell functional compartments – naive, central memory (CM), effector memory (EM) and effector compartment – non-stimulated or PMA+ionomycin-stimulated MNC were cultured in the absence or presence of MSC. We used 8-color flow cytometry to identify the four abovementioned compartments among $CD4^+$ and $CD8^+$ T cells and analyze the protein expression of TNF-α, IFNγ and IL-2 within each cell compartment, and of IL-17, IL-6 and IL-9 in total $CD4^+$ and/or $CD8^+$ T cells. Besides, mRNA expression of IL-4, IL-10, TGF-β and CTLA-4 in purified $CD4^+$ and $CD8^+$ T cells was also assessed. Finally, MSC were stimulated with PMA plus ionomycin and the protein expression of CD13, CD44, CD73, CD90, CD106, CD184 (CXCR4) and mRNA levels of IDO, ICOSL, TGF-β, IL-1β, IL-8 and TNF-α were evaluated and compared to that of non-stimulated MSC.

4.1. MSC decrease the frequency of T cells producing TNF-α, IFNγ and IL-2, as well as the protein expression at single cell level, and regulate differentially the distinct T cell functional compartments

Co-culture of MSC with PMA+ionomycin-stimulated MNC decreased the percentage of both $CD4^+$ and $CD8^+$ T cells expressing TNF-α ($p < 0.05$) – observed in all individuals enrolled in this study – as well as the amount of protein produced *per* cell ($p < 0.05$), measured as mean fluorescence intensity (MFI), in all cell compartments analyzed, except for TNF-α MFI in naive $CD4^+$ T cells, where MSC did not induce changes (Figure 11). Of note, the effect of MSC over the frequency of TNF-α^+ cells was more pronounced in $CD4^+$ T cells, compared to $CD8^+$ T cells, and in naive and CM compartments for both $CD4^+$ and $CD8^+$ T cells (Figure 11).

Concerning IFNγ and IL-2, we observed an overall reduction of the frequency of both $CD4^+$ and $CD8^+$ T cells producing these cytokines in MSC+MNC+PMA+ionomycin co-cultures ($p < 0.05$ for all functional compartments of $CD8^+$ T cells producing IFNγ and for effector $CD4^+$ T cells producing IL-2), as illustrated in figure 12 and 13. Of note, MSC decreased the percentage of IFNγ-producing $CD4^+$ and $CD8^+$ T cells in 63% and 88% of the individuals under study,

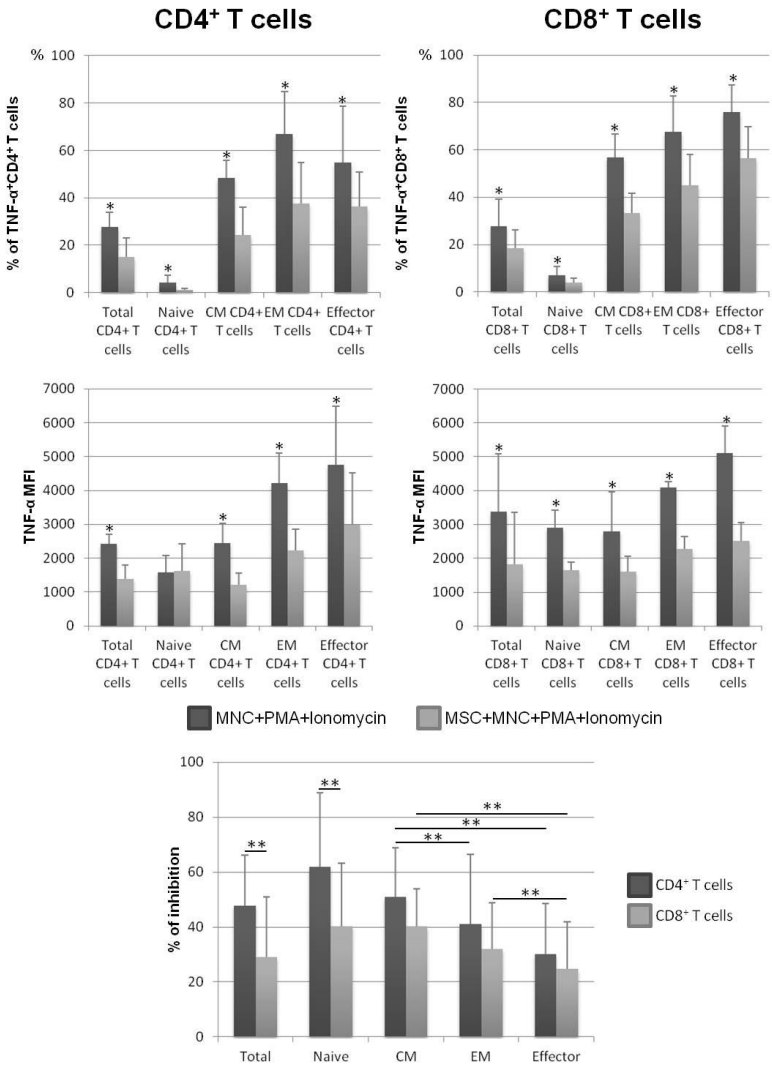

Figure 11. Percentage of CD4$^+$ and CD8$^+$ T cells expressing TNF-α. Upper panels: Percentage (mean ± standard deviation) of CD4$^+$ and CD8$^+$ T cells, distributed among their functional compartments, producing TNF-α (within the correspondent functional compartment of CD4$^+$ or CD8$^+$ T cells, respectively) and amount of protein expressed per cell, measured as mean fluorescence intensity (MFI, mean ± standard deviation), after MNC stimulation with PMA plus ionomycin in the absence of MSC (MNC+PMA+ionomycin) or in co-culture with MSC (MSC+MNC+PMA+ionomycin). The **lower panel** represents the percentage of inhibition induced by MSC on the percentage of CD4$^+$ and CD8$^+$ T cells expressing TNF-α. Statistically significant differences were considered when $p<0.05$ for Wilcoxon paired-sample test: * vs. MSC+MNC+PMA+ionomycin; ** $p<0.05$ for the groups indicated in the figure. TNF-α, tumor necrosis factor-α; MNC, mononuclear cells; PMA, phorbol myristate acetate; MSC, mesenchymal stromal/stem cells; CM, central memory; EM, effector memory.

respectively; and in 75% and 63% of the individuals for CD4$^+$ and CD8$^+$ T cells expressing IL-2, respectively. This decrease affected all T cell compartments, though it was less pronounced in naive T cells and not visible for naive CD4$^+$ T cells producing IFNγ (Figures 12 and 13). MSC diminished the amount of IFNγ produced by T cells (MFI), primarily in EM and effector

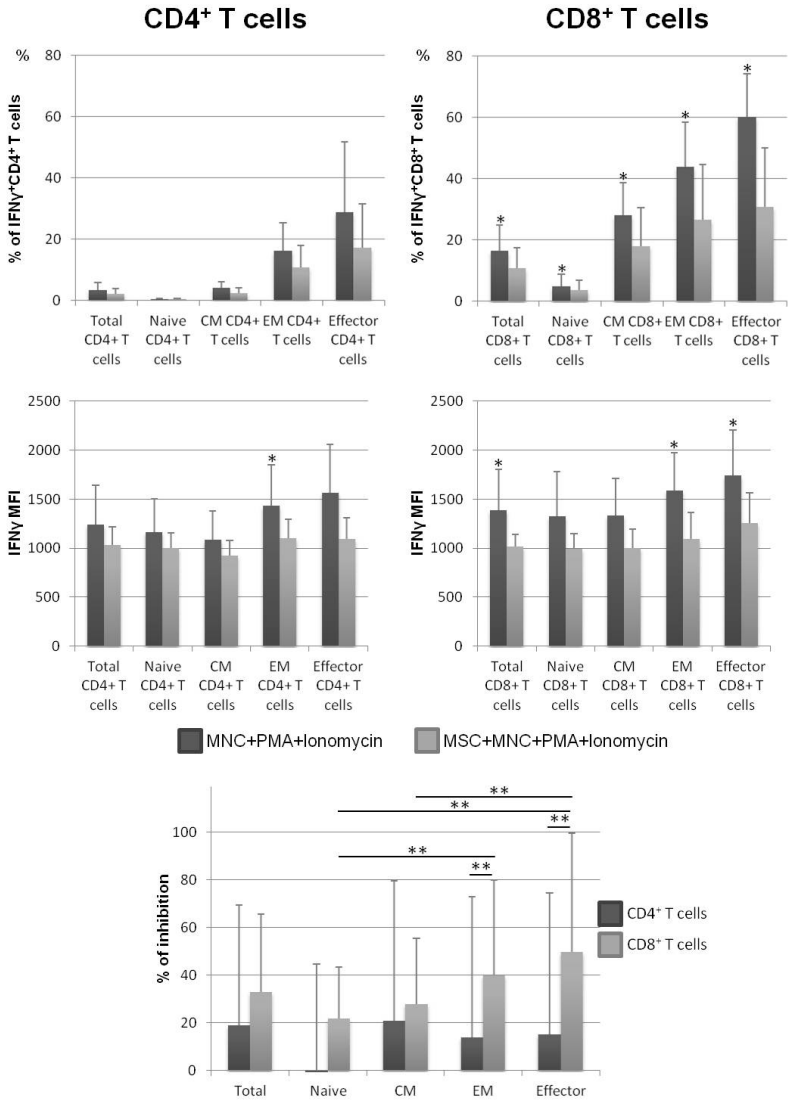

Figure 12. Percentage of CD4$^+$ and CD8$^+$ T cells expressing IFNγ. Upper panels: Percentage (mean ± standard deviation) of CD4$^+$ and CD8$^+$ T cells, distributed among their functional compartments, producing IFNγ (within the correspondent functional compartment of CD4$^+$ or CD8$^+$ T cells, respectively) and amount of protein expressed per cell, measured as mean fluorescence intensity (MFI, mean ± standard deviation), after MNC stimulation with PMA plus ionomycin in the absence of MSC (MNC+PMA+ionomycin) or in co-culture with MSC (MSC+MNC+PMA+ionomycin). The **lower panel** represents the percentage of inhibition induced by MSC on the percentage of CD4$^+$ and CD8$^+$ T cells expressing IFNγ. Statistically significant differences were considered when p<0.05 for Wilcoxon paired-sample test: * vs. MSC+MNC+PMA+ionomycin; ** p<0.05 for the groups indicated in the figure. IFNγ, interferon γ; MNC, mononuclear cells; PMA, phorbol myristate acetate; MSC, mesenchymal stromal/stem cells; CM, central memory; EM, effector memory.

compartments (p <0.05 for EM CD4$^+$ T cells, EM CD8$^+$ T cells and effector CD8$^+$ T cells); an effect more pronounced among CD8$^+$ than CD4$^+$ T cells (Figure 12).In turn, concerning the amount of IL-2 produced, effector CD4$^+$ T cells from MSC+MNC+PMA+ionomycin co-cultures displayed a lower IL-2 MFI compared to those from stimulated MNC cultures (p <0.05), whereas no differences were found in the remaining T cell compartments (Figure 13).

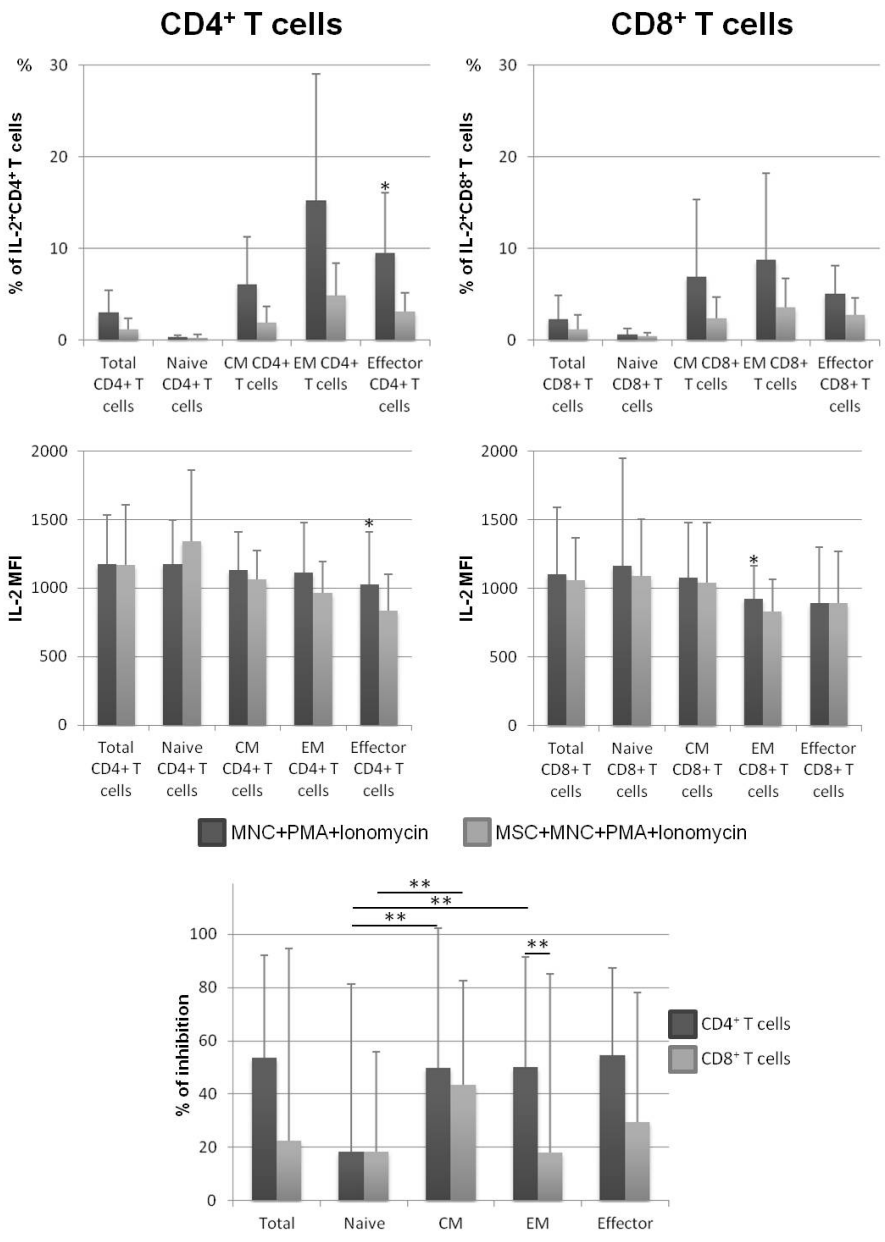

Figure 13. Percentage of CD4$^+$ and CD8$^+$ T cells expressing IL-2. Upper panels: Percentage (mean ± standard deviation) of CD4$^+$ and CD8$^+$ T cells, distributed among their functional compartments, producing IL-2 (within the correspondent functional compartment of CD4$^+$ or CD8$^+$ T cells, respectively) and amount of protein expressed per cell, measured as mean fluorescence intensity (MFI, mean ± standard deviation), after MNC stimulation with PMA plus ionomycin in the absence of MSC (MNC+PMA+ionomycin) or in co-culture with MSC (MSC+MNC+PMA+ionomycin). The **lower panel** represents the percentage of inhibition induced by MSC on the percentage of CD4$^+$ and CD8$^+$ T cells expressing IL-2. Statistically significant differences were considered when $p<0.05$ for Wilcoxon paired-sample test: * vs. MSC+MNC+PMA+ionomycin; ** $p<0.05$ for the groups indicated in the figure. IL-2, interleukin-2; MNC, mononuclear cells; PMA, phorbol myristate acetate; MSC, mesenchymal stromal/stem cells; CM, central memory; EM, effector memory.

We observed neither CD4$^+$ nor CD8$^+$ T cells produce cytokines in non-stimulated MNC and non-stimulated MNC+MSC cultures.

4.2. MSC decrease the frequency of T cells producing IL-17, IL-6 and IL-9

The presence of MSC in the stimulated-MNC culture resulted in a decreased percentage of both Th17 and Tc17 cells ($p < 0.05$), verified in all individuals studied, without alteration of the amount of IL-17 produced *per* cell, measured as MFI (Figure 4). Similarly, MSC reduced the frequency IL-17$^+$TNF-α$^+$CD4$^+$ and IL-17$^+$TNF-α$^+$CD8$^+$ T cells ($p < 0.05$), which was observed in all individuals under study. In these T cell populations, IL-17 MFI remained constant, while a lower TNF-α MFI was detected in both cell populations for MSC+MNC+PMA+ionomycin co-cultures ($p < 0.05$ for CD8$^+$ T cells; Figure 14).

The frequency of CD4$^+$ and CD8$^+$ T cells expressing IL-6 or IL-9 was lower in the presence of MSC ($p < 0.05$ for IL-9$^+$CD4$^+$ T cells and IL-9$^+$ CD8$^+$ T cells); 88% of individuals showed decreased percentage of CD4$^+$ T cells expressing IL-6, while in 75% and 100% of the individuals MSC reduced CD4$^+$ and CD8$^+$ T cells producing IL-9, respectively (Figure 15).

We observed no cytokine production by CD4$^+$ or CD8$^+$ T cells in non-stimulated MNC and non-stimulated MNC+MSC cultures.

4.3. mRNA expression of IL-4, IL-10, TGF-β and CTLA-4 in purified CD4+ and CD8+ T cells from MNC cultured in the absence/presence of MSC

Overall, MSC decreased IL-10 and increased IL-4 mRNA expression in stimulated CD4$^+$ and CD8$^+$ T cells; while TGF-β mRNA levels were reduced in CD8$^+$ and augmented in CD4$^+$ T cells, and CTLA-4 remained constant regardless of MSC presence in the MNC culture (Figure 16). Of note, a high inter-individual variability in mRNA levels of all molecules studied was observed.

4.4. Protein and mRNA expression in PMA plus ionomycin stimulated vs non-stimulated MSC

The analysis of the protein expression of CD13, CD44, CD73, CD90, CD106, CD184 (CXCR4) by flow cytometry showed no differences with biological significance for these molecules (data not shown), albeit there were significant statistical differences for CD106 (MFI of 2499±861 and 2713±869 for non-stimulated and stimulated MSC, respectively) and CD184 (MFI of 7103±2059 and 8116±2224 for non-stimulated and stimulated MSC, respectively). In turn, MSC stimulation with PMA plus ionomycin induced the increase of IDO, ICOSL, IL-1β, IL-8 and TNF-α mRNA levels ($p < 0.05$ for IL-1β, IL-8 and TNF-α) and showed a tendency to decrease TGF-β mRNA (not statistically significant), as illustrated in figure 17.

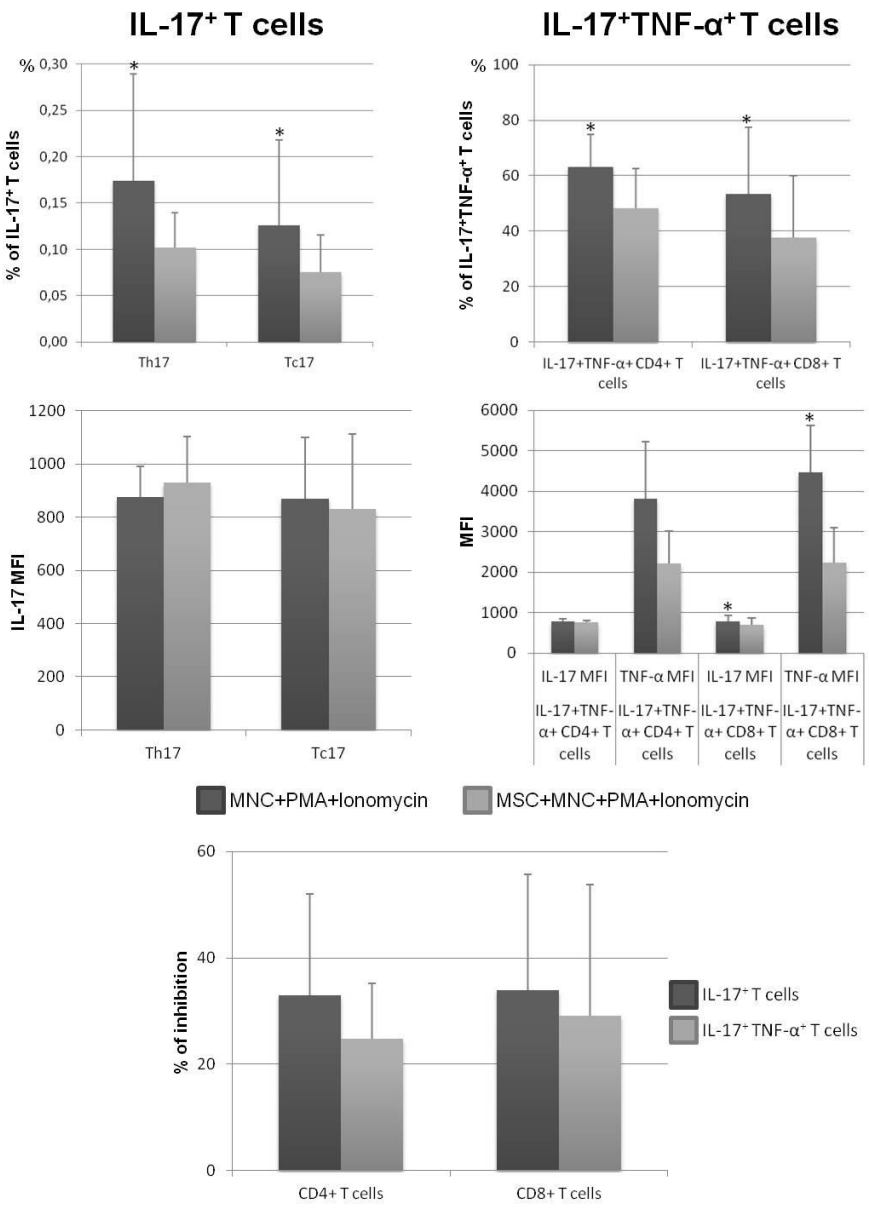

Figure 14. Percentage of $CD4^+$ and $CD8^+$ T cells expressing IL-17. Left upper panels: Percentage (mean ± standard deviation) of $CD4^+$ and $CD8^+$ T cells producing IL-17 (within total $CD4^+$ T cells and total $CD8^+$ T cells, respectively) and amount of protein expressed per cell, measured as mean fluorescence intensity (MFI, mean ± standard deviation), after MNC stimulation with PMA plus ionomycin in the absence of MSC (MNC+PMA+ionomycin) or in co-culture with MSC (MSC+MNC+PMA+ionomycin). **Right upper panels:** Percentage (mean ± standard deviation) of $CD4^+$ and $CD8^+$ T cells producing simultaneously IL-17 and TNF-α (within $IL\text{-}17^+CD4^+$ T cells and $IL\text{-}17^+CD8^+$ T cells, respectively) and amount of protein expressed per cell, measured as mean fluorescence intensity (MFI, mean ± standard deviation), after MNC stimulation with PMA plus ionomycin in the absence of MSC (MNC+PMA+ionomycin) or in co-culture with MSC (MSC+MNC+PMA+ionomycin). The **lower panel** represents the percentage of inhibition induced by MSC on the percentage of $CD4^+$ and $CD8^+$ T cells expressing IL-17 or IL-17 and TNF-α. Statistically significant differences were considered when $p<0.05$ for Wilcoxon paired-sample test: * vs. MSC+MNC+PMA+ionomycin. IL-17, interleukin-17; MNC, mononuclear cells; PMA, phorbol myristate acetate; MSC, mesenchymal stromal/stem cells; TNF-α, tumor necrosis factor-α; CM, central memory; EM, effector memory.

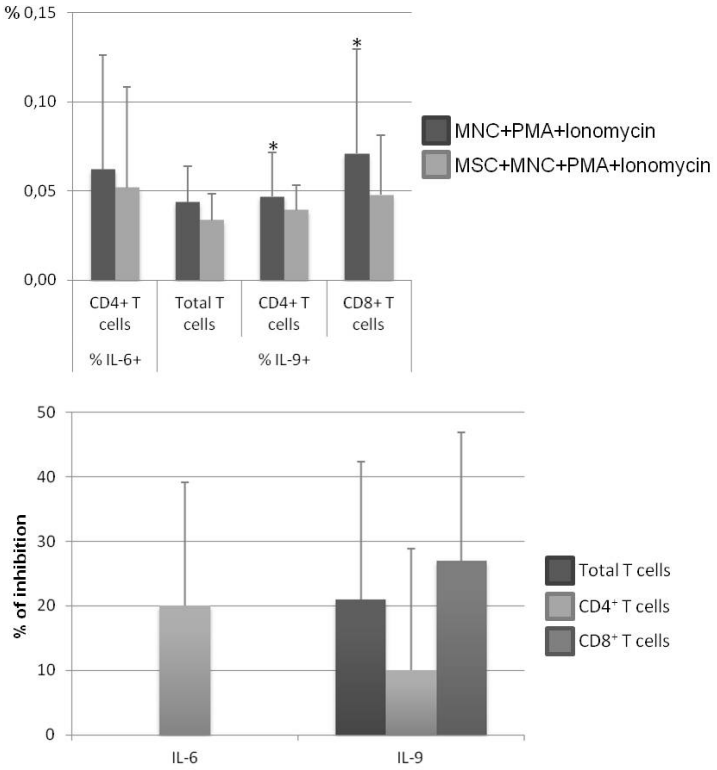

Figure 15. Percentage of $CD4^+$ and $CD8^+$ T cells expressing IL-6 or IL-9. Upper panel: Percentage (mean ± standard deviation) of $CD4^+$ T cells expressing IL-6 (within total $CD4^+$ T cells) and of total, $CD4^+$ and $CD8^+$ T cells producing IL-9 (within total T cells, $CD4^+$ T cells and total $CD8^+$ T cells, respectively), after MNC stimulation with PMA plus ionomycin in the absence of MSC (MNC+PMA+ionomycin) or in co-culture with MSC (MSC+MNC+PMA+ionomycin). **Lower panel:** Percentage of inhibition induced by MSC on the percentage of total, $CD4^+$ and/or $CD8^+$ T cells expressing IL-6 or IL-9. Statistically significant differences were considered when $p<0.05$ for Wilcoxon paired-sample test: * vs. MSC+MNC+PMA+ionomycin. IL-6, interleukin-6; IL-9, interleukin-9; MNC, mononuclear cells; PMA, phorbol myristate acetate; MSC, mesenchymal stromal/stem cells.

5. Discussion

Despite of the increasing number of studies on MSC-derived immunosuppression of T cells, those which investigated whether MSC action differed among T cell functional compartments are scarce. As the immune system disorders candidates to MSC cell therapy comprise a heterogeneous group concerning the distribution of T cells among their functional compartments and the effector T cells underlying the disease, it becomes urgent to understand how MSC regulate the distinct T cell functional compartments.

Thus, in the present study, the influence of MSC in the cytokine expression profile of naive, CM, EM and effector compartments of $CD4^+$ and $CD8^+$ T cells was evaluated directly in

these subsets naturally occurring in the peripheral blood of healthy individuals. The abovementioned functional compartments were identified within $CD4^+$ and $CD8^+$ T cell populations by flow cytometry, based on their differential expression of CD45RA and CD27. The production of IL-2, TNF-α and IFNγ was further evaluated within each cell compartment. To the best of our knowledge, this is the first study reporting the effect of MSC on the cytokine expression by T cells that analyzed simultaneously all the four functional compartments of $CD4^+$ and $CD8^+$ T cells naturally occurring in the peripheral blood.

Our results showed that, in a co-culture system with MNC stimulated with PMA+ionomycin, MSC downregulate TNF-α, IFNγ and IL-2 protein expression in both $CD4^+$ and $CD8^+$ T cells, which corroborates with previous studies reporting that MSC induce the decrease of this cytokines detected in the co-culture medium [129,181,236,237,250,252,475], at mRNA level [46,252,254], or the percentage of T cells producing cytokines [181,245,251,263,291] in *in vitro* experiments and *in vivo* animal experimental models [180,271,272].

The analysis of the expression of those pro-inflammatory cytokines within each T cell functional compartment revealed that MSC has the ability to modulate the function of T cells included in naive, CM, EM and effector compartments, however to a different extent. Interestingly, effector $CD8^+$ T cells, which present the highest percentage of TNF-α-producing cells among $CD4^+$ and $CD8^+$ T cell functional compartments, correspond to those which are less sensitive to MSC-mediated TNF-α downregulation. Therefore, as TNF-α constitute an important effector mechanism of $CD8^+$ T cell-mediate immune response, with a pivotal role in the pathophysiology of several autoimmune disorders and GvHD, we may postulate that MSC present a milder inhibition on cells with a higher degree of differentiation. Conversely, IL-2 expression is highly inhibited in CM, EM and effector $CD4^+$ T cells. MSC-mediated IL-2 downregulation had already been described for $CD4^+$ or total T cells and is correlated to MSC ability to impair T cell proliferation [46,129,254]. Similarly to Aggarwal et al. [236], that reported MSC-mediated inhibition of IFNγ in human $CD45RA^+$ T cells, we observed a decreased frequency of naive T cells producing IFNγ, but only among $CD8^+$ T cells, while no inhibitory effect was detected over naive $CD4^+$ T cells. Besides, a more pronounced inhibitory effect was observed among the CM, EM and effector compartments, compared to naive cells, for $CD4^+$ and $CD8^+$ T cells; similarly, Krampera et al. [291] found a stronger inhibitory effect over mouse memory T cells, compared to naive T cells. In the same line, more recent studies showed that MSC induced human memory T cells, whose identification was based on CD45RO expression, to express IL-17, which did not occur in the naive compartment [254,280].

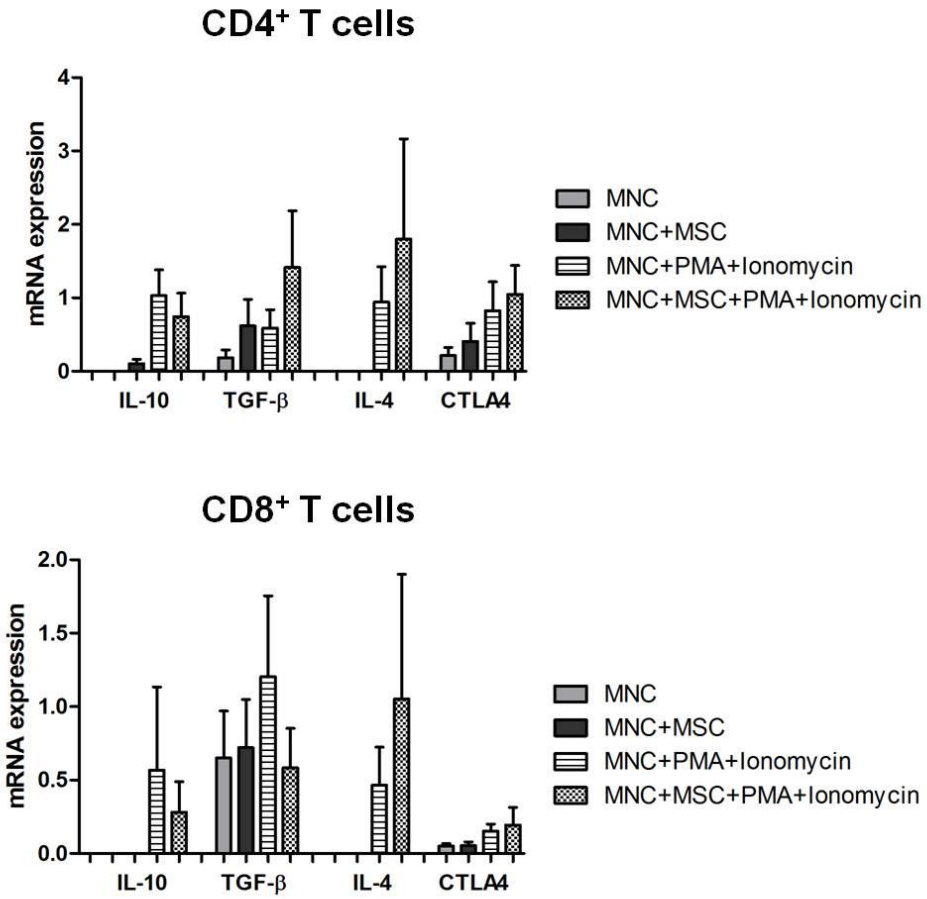

Figure 16. mRNA expression of IL-10, TGF-β, IL-2, IL-4 and CTLA-4 in $CD4^+$ and $CD8^+$ T cells. Semi-quantitative analysis of IL-10, TGF-β, IL-2, IL-4 and CTLA-4 mRNA expression in FACS-purified $CD4^+$ and $CD8^+$ T cells from the following culture conditions: non-stimulatred MNC (MNC); non-stimulated MNC co-cultured with MSC (MNC+MSC); MNC stimulated with PMA plus ionomycin (MNC+PMA+ionomycin); MNC stimulated with PMA plus ionomycin in co-culture with MSC (MNC+MSC+PMA+ionomycin). Statistically significant differences were considered when $p<0.05$ for Wilcoxon paired-sample test, comparing MNC+PMA+ionomycin vs. MNC+MSC+PMA+ionomycin. IL, interleukin; TGF-β, transforming growth factor β; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; MNC, mononuclear cells; PMA, phorbol myristate acetate; MSC, mesenchymal stromal cells.

At this point, our results and previously published data support the idea that MSC differently regulate the distinct T cell functional compartments, which can impact the outcome of MSC cell therapy.

Concerning other pro-inflammatory cytokines, MSC induced a decreased percentage of $CD4^+$ and $CD8^+$ T cells expressing IL-17 and IL-9, and of CD4+ T cells producing IL-6. Conversely, at mRNA level, we observed a tendency to an increase of the anti-inflammatory IL-4 and TGF-β in $CD4^+$ T cells, as described by others [180,181,231,236]. It has been attributed to MSC the ability to inhibit Th17 differentiation, from mouse and human T cells, and decrease the

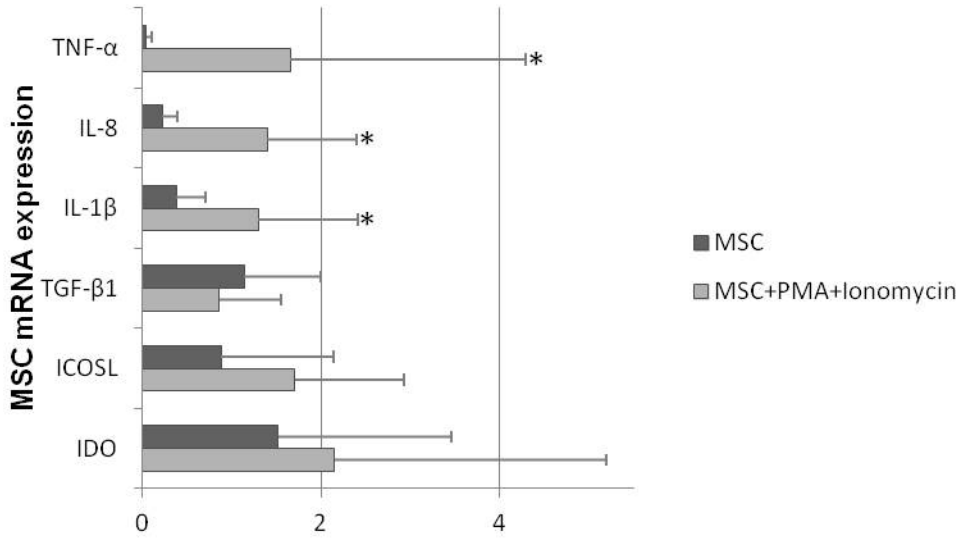

Figure 17. mRNA expression of TNF-α, IL-8, IL-1 β, TGF-β, ICOSL and IDO in MSC. Semi-quantitative analysis of TNF-α, IL-8, IL-1 β, TGF-β, ICOSL, IDO mRNA expression in non-stimulated MSC (MSC) and in MSC stimulated with PMA plus ionomycin (MSC+PMA+ionomycin). Statistically significant differences were considered when $p<0.05$ for Wilcoxon paired-sample test, comparing MSC vs. MSC+PMA+ionomycin. TNF-α, tumor necrosis factor-α; IL, interleukin; TGF-β, transforming growth factor β; ICOSL, inducible costimulatory ligand; IDO, indoleamine-2,3-dioxygenase; MSC, mesenchymal stromal cells, PMA, phorbol myristate acetate.

expression of IL-17 in differentiated Th17, an effect assigned to prostaglandin E2 [9,263,271,272,275,277]; however, the effect of MSC on T cells expressing IL-17 is controversial and some studied show that Th17 function can be enhanced by MSC [251,252,254,263,280]. Nevertheless, the different experimental approaches used may explain the conflicting data.

Beyond the direct impairment of Th1 and Th17 immune responses, by reducing the expression of their typical effector cytokines (TNF-α, IFNγ, IL-2 and IL-17), MSC can also induce the expression of the anti-inflammatory cytokine IL-10 in both Th1 and Th17 cells [9,264].

IL-6 is a cytokine with recognized pro- and anti-inflammatory properties. Roughly, anti-inflammatory biological effects can be ascribed to IL-6 classic signaling via membrane IL-6 receptor (IL-6R), while pro-inflammatory function is associated to trans-signaling mediated by soluble IL-6R (sIL-6R) [491]. The essential role of this cytokine in inflammation was confirmed by several animal models of autoimmune inflammatory diseases, where the blockage of IL-6 trans-signaling prevented the inflammatory process. As an inflammatory state contribute to proteolytic cleavage of IL-6R, resulting in the release of sIL-6R, trans-signaling is likely to be enhanced under this condition; besides, IL-6 is necessary for Th17 differentiation and maintenance [488]. MSC-mediated reduction of IL-6 expression in $CD4^+$ T cells is in agreement

with the decreased expression of the other pro-inflammatory cytokines evaluated in the present study, namely the percentage of T cells producing IL-17.

The biological effects of IL-9 still poorly described. This cytokine is produced by Th9, Th2, Treg and Th17 cell subsets and plays an important role in the immune response against helminthes and in allergic inflammation [638-640]. Despite promoting Th2 cytokine production, enhancing the regulatory function of Treg and suppressing the expression of the pro-inflammatory cytokines TNF-α, IL-12 and IFNγ under certain experimental conditions; IL-9 is also able to expand Th17 cell population and to promote inflammation, as the adoptive transfer of Th9-polarized cells induces experimental autoimmune encephalomyelitis and experimental autoimmune uveitis [638,641]. Recent works showed that *in vivo* systemic administration of MSC is beneficial in allergic airways inflammation mouse models, by increasing IL-10 and decreasing IL-4, IL-5 and IL-13 cytokine secretion [249,259-261]. To the best of our knowledge, IL-9 had never been evaluated in *in vivo* MSC-mediated suppression of allergic inflammation in animal models; also, this is the first study reporting the inhibition of IL-9-producing $CD4^+$ and $CD8^+$ T cells by MSC, opening new possibilities in the investigation of MSC in allergic inflammation.

6. CONCLUSIONS

MSC constitute a possible therapeutic approach for immune-mediated disorders, however these pathologies are very heterogeneous in what concerns to the effector T cell subset involved and to the distribution of T cells among their functional compartments. Thus, a detailed knowledge of the effect of MSC over each individual functional compartment of $CD4^+$ and $CD8^+$ T cells became essential to predict and understand their outcome as cell therapy. Here, we investigated whether human BM-MSC altered the expression of pro-inflammatory cytokines among the $CD4^+$ and $CD8^+$ T cell functional compartments naturally occurring in the peripheral blood.

Overall, our results showed that MSC inhibits TNF-α, IFNγ and IL-2 protein expression in all $CD4^+$ and $CD8^+$ T cell functional compartments, except for IFNγ expression in naive $CD4^+$ T cells, where MSC had no suppressive effect. Interestingly, MSC-derived inhibition was stronger for $CD4^+$ than $CD8^+$ T cells, concerning IL-2 and TNF-α, whereas it was more pronounced in $CD8^+$ T cells for IFNγ.

It is noteworthy that MSC showed distinct inhibitory patterns for the different functional compartments of T cells. MSC-derived inhibition of TNF-α expression was more marked among naive, CM and EM T cells, for both $CD4^+$ and $CD8^+$ T cell subsets; CM $CD4^+$ T cells and effector and EM $CD8^+$ T cells were the functional compartments displaying a higher degree of suppression of IFNγ expression; and the inhibition of IL-2 production was more pronounced among effector, EM and CM $CD4^+$ T cells and CM $CD8^+$ T cells.

Finally, we verified that, under our experimental conditions, MSC co-culture reduced the percentage of both $CD4^+$ and $CD8^+$ T cells expressing IL-17, IL-9 and of $CD4^+$ T cells producing IL-6. The influence of MSC on IL-9 can open new possibilities of research regarding the clinical use of MSC in allergic inflammation.

In the present study, we showed that MSC differentially regulate the functional compartments of T cells. Also, the pattern of inhibition of the functional compartments of $CD4^+$ T cells differed from that observed in $CD8^+$ T cells. Our data revealed that the distinctive distribution of T cells among activation/differentiation compartments of immune system disorders will condition the therapeutic effect of MSC.

CHAPTER 5 |

OVERALL DISCUSSION AND CONCLUSIONS

OVERALL DISCUSSION

1. DIFFERENCES AMONG THE IMMUNOSUPPRESSIVE ABILITIES OF HUMAN MSC DERIVED FROM BONE MARROW, ADIPOSE TISSUE, AND UMBILICAL CORD MATRIX – OBJECTIVE 1

Because the differences of the immune suppressive capabilities of human MSC arising from different tissues were a theme of debate, we investigated how MSC derived from BM, AT, and UCM influenced T, B, and NK cell activation and proliferation; mRNA expression of molecules with an important role in the immune function (IL-2 in T cells, and TNF-α, granzyme B, and perforin in NK cells); and mRNA expression of the master transcription factors for T cell differentiation into Th1, Th2, and Treg cells (T-bet, GATA3, and FoxP3, respectively), within each T cell activation compartment. This study is described in chapter 2.

1.1. Human MSC derived from BM, AT, and UCM differentially inhibit lymphocyte activation and proliferation

To explore whether MSC isolated from BM, AT, and UCM have distinct inhibitory abilities over mitogen-induced lymphocytes' activation and proliferation, we co-cultured PHA-stimulated PBMC with either, BM, AT, or UCM-derived MSC. We found striking differences among MSC arisen from the three sources: AT-MSC displayed a stronger inhibitory effect over B cell, $CD4^+$ T cell, and $CD8^+$ T cell proliferation and activation, and over $CD56^{dim}$ and $CD56^{bright}$ NK cell activation; whereas UCM-MSC had a milder inhibitory action. Of note, UCM-MSC influenced neither B cell proliferation and activation, nor $CD56^{bright}$ NK cell activation, under our experimental conditions. Remarkably, in the presence of AT-MSC the proportion of $CD4^+$ and $CD8^+$ T cells in non-activated stage ($CD69^-CD25^-HLA\text{-}DR^-$) was higher than that observed for BM and UCM-MSC, which may be perceived as an ability to block T cell activation in an early phase of the activation process.

In contrast to our results, Prasanna and colleagues reported that UCM-MSC displayed a stronger inhibitor effect than BM-MSC over T cell proliferation induced upon PHA or MLR stimulation of PBMC [39]; likewise, Bárcia et al. described that UCM-MSC inhibited T cell proliferation in CD3/CD28 plus IL-2-stimulated PBMC to a greater extent than BM-MSC [37]; in turn, Najar et al. didn't find significant differences among the suppressive effect of UCM-MSC

and BM-MSC over T cell proliferation induced by MLR or PHA plus IL-2 stimulation of purified T cells [26].

Considering cell activation, our data pointed out that UCM-MSC had the lowest ability to suppress activation of $CD4^+$ T cells, $CD8^+$ T cells (measured by CD69, CD25, and HLA-DR expression), and $CD56^{dim}$ NK cells (measured by CD69 and CD25 expression), upon PHA stimulation of PBMC, in comparison to BM-MSC and AT-MST; besides, UCM-MSC were unable to inhibit activation of B cells and $CD56^{bright}$ NK cells. In the same line, Prasanna and colleagues showed that, in PHA-stimulated PBMC, BM-MSC were able to inhibit IFNγ secretion and CD28 expression by T cells to a greater extent than UCM-MSC; remarkably, UCM-MSC induced higher levels of CTLA-4 on T cell surface than BM-MSC [39]. On the other hand, Najar et al. showed that, upon stimulation of purified T cells with PHA plus IL-2, UCM-MSC inhibited T cell activation (measured by CD38 expression) to a greater extent than their BM counterparts [26].

Notwithstanding, the studies comparing AT-MSC and BM-MSC are more consistent, indicating that AT-MSC display a higher immunosuppressive capacity. Indeed, AT-MSC exhibited a stronger inhibitory effect over the proliferation of $CD4^+$ and $CD8^+$ T cells, either for PBMC stimulated with PHA (in our study), for PHA plus IL-2-stimulated purified T cells, or MLR [26]. In the same line, AT-MSC possess a stronger suppressive effect over PHA-induced $CD4^+$ and $CD8^+$ T cell activation, compared to BM-MSC, evaluated by our group based on the expression of CD69, CD25, and HLA-DR, and by others [26] by CD38 expression.

Similarly, in our study, AT-MSC were more effective in suppressing PHA-induced activation (measured by CD69 and CD25 expression) and proliferation of B cells than BM-MSC. In the same line, a superior inhibitory activity of human AT-MSC, compared to BM-MSC, over Ig production by B cells had been demonstrated by Bochev et al [318]; whereas other studies corroborate the inhibitory ability of human MSC over B cell proliferation [305,306,312-315], as detailed in chapter 1, section 3.3.2.1, showing this inhibitory effect is achieved by an arrest in G0/G1 phase of the cell cycle induced by BM-MSC [305,315]. Human MSC were also shown to impair B cell function as Ig-producing cells [55,305,306,312,314,318,319] and differentiation into plasma cells [306,309,314,315], which can be a consequence of the defective B cell activation observed in the presence of MSC. These subjects are thoroughly described in chapter 1, sections 3.3.2.2 and 3.3.2.3.

Similarly, to what was observed for T and B cells, AT-MSC were more effective in suppressing PHA-induced activation of $CD56^{dim}$ NK cells, and $CD56^{bright}$ NK cells (measured by CD69 and CD25 expression) than BM-MSC, under our experimental conditions. Inhibition of

CD69 upregulation was also described by others for IL-2-activated NK cells [102], though other studies obtained contradictory results [33,326]. Corroborating with MSC-derived suppression of NK activation, there are numerous studies describing the suppressive effect of human MSC over NK cell function, described in detail in section 3.4.2.2 of the 1st chapter.

Our results demonstrated that human MSC from different sources possess important differences concerning their immunosuppressive ability, which may influence the final outcome obtained in *in vitro* assays or in clinical applications.

1.2. The influence of human MSC derived from BM, AT, and UCM over IL-2 mRNA expression within each one of the distinct activation compartments of T cells

According to our results, all the three types of MSC studied were able to efficiently inhibit IL-2 mRNA expression in earlier activated T cells (which corresponds to the activation compartment with higher IL-2 mRNA expression after PHA stimulation). Remarkably, BM-MSC are more efficient in inhibiting IL-2 mRNA expression in T cells, as compared to AT or UCM-MSC. Accordingly, a previous study had shown that human BM-MSC were more efficient in inhibiting IL-2 secretion (IL-2 protein level measured in the culture supernatant) than UCM-MSC, when co-cultured with PHA-stimulated PBMC [39]. The impairment of IL-2 production may contribute to the inhibitory activity displayed by MSC over T cell proliferation. However, studying the overall IL-2 mRNA levels within purified T cell subsets, or the protein levels in the culture supernatant, give an incomplete information. If in the one hand mRNA translation to protein is a complex process which can be further regulated and, therefore, may not reflect the cytokine protein levels, on the other hand, knowing the total mRNA or total protein levels isn't informative about what is happening at single-cell level. In fact, these approaches give no information about whether MSC exert an equal inhibitory effect over all T cells, or if their inhibitory function is specially focused on specific T cells subsets, which equally will lead to the reduction of total IL-2 mRNA or protein levels. The study described on chapter 4 sought to overcome that shortcoming.

1.3. The influence of human MSC derived from BM, AT, and UCM on TNF-α, granzyme B, and perforin mRNA expression within the distinct activation compartments of NK cells

Attending to NK cells, our study showed that MSC derived from BM, AT, and UCM suppress TNF-α and perforin expression at mRNA level, which is in agreement with other studies describing a reduced protein levels of TNF-α [327], IFNγ [75,76,102,236,326,327,329], granzyme A [33], and granzyme B [102], though was also described that BM and AT-MSC have no inhibitory effect over granzyme B expression by IL-2-activated NK cells [33]. Of note, we found no effect of MSC over perforin mRNA expression in NK cells, which was verified as well by others at protein level [33]. Moreover, human MSC possess the ability to suppress other effector molecules which participate in NK cell immune function and, consequently, impair NK cell cytolytic activity, as detailed in section 3.4.2 of the 1st chapter.

1.4. Regulation of T-bet, FoxP3, and GATA3 mRNA expression within the distinct activation compartments of T cells, by human MSC derived from BM, AT, and UCM

Concerning the master transcription regulators of T cell differentiation, we verified that MSC did not alter mRNA of GATA3, but increased the mRNA expression of T-bet and FoxP3 in activated T cell compartments. Of note, FoxP3 mRNA upregulation was more pronounced in the presence of AT-MSC, compared to BM and UCM-MSC.

Despite a great number of studies described MSC promote Treg cell generation and expansion, both *in vitro* and *in vivo* (detailed in chapter 1, section 3.1.2.5), only recently the potential of human MSC derived from different tissues to induce Treg cells was tested, being demonstrated that BM-MSC and UCM-MSC possess equal ability to generate Treg cells when in co-culture with anti-CD3/CD28 plus IL-2-stimulated T cells [37].

Concerning the increased T-bet mRNA expression we observed in the purified T cell activation compartments, it is apparently in opposition to several studies describing that human and mouse MSC inhibit Th1 cell differentiation and function [129,231,236,251,262,263], as detailed in chapter 1, section 3.1.2.3. Nevertheless, all these studies inferred that MSC inhibited Th1 cell differentiation and function based on the decreased levels of IFNγ in the culture supernatant or on the decreased percentage of IFNγ-

producing $CD4^+$ T cells, not by investigating T-bet expression on T cells. Recently, our group showed that human BM-MSC decreased the amount of IFNγ produced *per* cell (measured as MFI) [244], which is consistent with the hypothesis that a decreased IFNγ level in the culture supernatant may be a consequence of a reduced ability of Th1 cells to produce this cytokine, rather than a direct deleterious effect of MSC over T-bet expression by T cells.

Recently, Pianta and colleagues [255] reported a reduction on the percentage of $CD4^+$T-bet^+ T cells, in MLR, in the presence of conditioned medium derived from human amniotic-MSC. However, we had demonstrated that when only the activated compartments of T cells are analyzed, human MSC derived from the BM, AT, and UCM induce an increase in T-bet mRNA expression in PHA-stimulated T cells. As the proportion of activated T cells is highly reduced under the influence of MSC, it is expectable that, even if the augment of T-bet mRNA was reflected at protein level in activated T cells, the percentage of $CD4^+$T-bet^+ T cells and of IFNγ-producing cells among total $CD4^+$ T cells would be reduced.

Altogether, it seems that human MSC are able to hamper Th1 immune response by different mechanisms, depending on the microenvironmental conditions: MSC can either inhibit Th1 cell differentiation, perhaps by reducing the number of Th1 cells generated upon stimulation, as consequence of the impairment of T cell proliferation and activation, but without interfering with the sense of T cell proliferation (further studies assessing T-bet protein expression within the T cell activation compartments will be need to elucidate this point); or can directly inhibit the effector functions of fully differentiated Th1 cells [251].

2. Immunosuppressive effect of human BM-MSC on peripheral blood monocyte subsets and mDC – Objective 2

There are an increasing number of studies describing the suppressive effect of human MSC over T cells, being demonstrated that T cell suppression is achieved not only by a direct inhibitory effect of MSC over T cells (described in section 3.1 of the 1st chapter), but also indirectly, by MSC modulation of antigen-presenting cell function, biasing these cells toward a tolerogenic phenotype (detailed in chapter 1, section 3.5 and 3.6). Nevertheless, regarding this field of research, we found limited data concerning the effect of human MSC over freshly isolated peripheral blood DC, as the majority of the published data refers to *in vitro* generated

monocyte-derived DC; and concerning monocytes, the influence of human MSC on the recently described peripheral blood monocyte subsets (classical, intermediated, and non-classical monocytes) had never been assessed. The study described in the chapter 3 intended to clarify the effect of human BM-MSC over the naturally occurring peripheral blood mDC and to explore whether BM-MSC differentially affected the function of the distinct subsets of peripheral blood monocyte subsets.

2.1. Human BM-MSC differentially inhibit TNF-α and CCL4 (MIP-1β) protein expression in the distinct monocytes subsets and mDC

Our results showed that MSC efficiently inhibited TNF-α and CCL4 (MIP-1β) production by monocytes and mDC, both by decreasing the percentage of producing cells and by reducing the amount of cytokine produced *per* cell (measured as MFI). mDC were more susceptible to MSC-driven suppression than monocytes and, among the distinct monocyte subsets, the lowest degree of inhibition was observed in classical and non-classical monocytes for CCL4 and TNF-α, respectively.

According to our results, upon LPS plus IFNγ stimulation a percentage above 80% of TNF-α producing cells is observed among all monocytes subsets; nevertheless, when analyzing the amount of cytokine produced *per* cell (measured as MFI), non-classical monocytes appear as the main producers of TNF-α; interestingly, this cell subset is the most resistant to MSC-derived suppression. Accordingly, previous studies showed the deleterious effect of BM-MSC on monocyte pro-inflammatory function by reducing TNF-α and IL-12 expression, and their allostimulatory abilities [236,248,355], while increasing the expression of IL-10 [52,285,375]; altogether hampering the development of Th1 immune response.

Consistently with our observations, human MSC derived from the BM, AT, and amniotic tissue also inhibit LPS-induced TNF-α expression, as well as IFNγ and IL-12 production, by monocyte-derived immature mDC [31,351]; likewise, naturally occurring peripheral blood slanDC [333] and mDC [236] also experienced a decreased TNF-α expression when co-cultured with human BM-MSC. Interestingly, BM-MSC co-culture with peripheral blood DC also resulted in the impairment of IL-12 production and induction of IL-10, ILT3, and ILT4 expression [236,333]. Moreover, MSC-treated slanDC fail to trigger a Th1 immune response [333]. Overall, the published data support that MSC impair the pro-inflammatory functions of DC while promote the development of a tolerogenic phenotype.

CCL4 is the CCR5 ligand, and has an important role in the chemoattraction of T cells and monocytes [642], therefore, by suppressing the expression of this chemokine, BM-MSC hinder monocyte and mDC interaction with T cells. The strong inhibition observed for mDC assumes great biological significance given their important role as antigen-presenting cells. In the same line, CCL4 expression is also strongly inhibited in intermediate and non-classical monocytes. The intermediate monocytes have been described to have the highest ability to present antigens to T cells, among all peripheral blood monocyte subsets [369], whereas non-classical monocytes were shown to express the highest levels of HLA-DR and CD86, which is predictive of antigen presentation abilities, and include the slanDC, with recognized antigen-presenting cell activity [332,335,368]. These data suggest that MSC impair T cell chemoattraction toward the cell subsets that have the capability to stimulate them most efficiently.

2.2. Effect of human BM-MSC on CCR7 and CD83 protein expression in monocytes and mDC

Under our experimental conditions, neither CCR7 nor CD83 were inhibited by MSC in monocytes and mDC. The observed augmented HLA-DR expression in $CCR7^+$ *versus* $CCR7^-$ cells further confirm the activated state of the cells bearing CCR7 at the cell surface.

In opposition to our study, wherein no biologically significant alterations on HLA-DR expression were observed for LPS plus IFNγ-activated monocytes subsets and mDC in the presence of BM-MSC, previous studies demonstrated that human BM-MSC, AT-MSC, UCM-MSC, and UCB-MSC hamper HLA-DR upregulation in monocytes [248,268,355]. Concerning the influence of MSC on CD83 upregulation after DC stimulation, there are contradictory data in the literature, being reported that MSC hinder CD83 upregulation [25,31], but also that they do not affect CD83 upregulation [53], or further upregulate CD83 and CCR7 expression upon monocyte-derived immature DC stimulation with LPS or a pro-inflammatory cytokine cocktail [353,354]. Thus, it seems that MSC regulation of the expression of activation markers by DC is a complex process probably influenced by several factors.

2.3. Human BM-MSC differentially regulate cytokine and chemokine mRNA expression in mDC, classical and non-classical monocytes

MSC decreased mRNA expression of IL-1β and IL-6 in classical monocytes, but not in non-classical monocytes; in addition, MSC also reduced mRNA transcripts of CCL3, CCL5, CXCL9, and CXCL10 in classical and non-classical monocytes, and IL-1β and CXCL10 in mDC.

The decreased mRNA expression of IL-1β and IL-6 in classical monocytes, and IL-1β in mDC, is consistent with the described suppression of other pro-inflammatory cytokines mediated by human BM-MSC, such as TNF-α, IL-6, and IL-12, in monocytes and DC [236,333,355]. The decreased mRNA expression of CCL3, CCL5, CXCL9, and CXCL10, if reflected at protein levels, may impair T cells (attracted by CCL3 and CCL5, which binds to CCR1 and CCR5 receptors) and, more specifically, Th1 cells (expressing CXCR3, the receptor for CXCL9 and CXCL10) chemotaxis toward monocytes and mDC [642], which is in the same line than MSC-driven CCL4 suppression observed in this study at protein level. Of note, CCL5 also binds to CCR3 receptor, expressed by eosinophils, basophils, mast cells, and Th2 cells [642], suggesting that MSC can also difficult the development of a Th2 immune response.

3. Immunosuppressive effect of human BM-MSC on naturally occurring peripheral blood T cell functional compartments, and on peripheral blood T cells producing IL-17, IL-9 and IL-6 – Objective 3

Though the inhibitory effect of human MSC over IL-2, IFNγ, and TNF-α had previously been described in the literature, an experimental approach that analyzed MSC suppressive effect within each individual T cell functional compartment (naive, central memory (CM), effector memory (EM), and effector) was missing. The description of MSC-driven IL-2, IFNγ, and TNF-α suppression in overall $CD4^+$ and $CD8^+$ T cells gives no information about the effect of MSC at a single cell level, and don't allow to distinguish whether the suppressive action embraces all T cells or is more focused on specific T cell subsets. T cells included in distinct functional/differentiation compartments have differential activities. This subject assumes great relevance when considering the clinical use of MSC in disorders of immune etiology, wherein T cells' distribution among the activation, differentiation, or functional compartments

is commonly disturbed. In this setting, to get insight of how MSC influence the function of each specific T cell functional compartment may help to predict the clinical benefit of MSC therapy in the distinct diseases, or even in distinct patients. The influence of human BM-MSC on the distinct functional compartments of $CD4^+$ and $CD8^+$ T cells was the subject of the study described in chapter 4.

3.1. Immunosuppressive effect of human BM-MSC on IL-2, IFNγ, and TNF-α expression by naturally occurring peripheral blood naive, central memory, effector memory, and effector T cells

Our results showed that co-culture with BM-MSC decreased the percentage of PMA plus ionomycin-stimulated T cells producing IL-2, IFNγ, and TNF-α, as well as IFNγ and TNF-α MFI, among $CD4^+$ and $CD8^+$ T cells from all the functional compartments studied, except for IL-2 and IFNγ in naive $CD4^+$ T cells, and IL-2 in naive $CD8^+$ T cells, because only a residual percentage of cells expressed the mentioned cytokines.

The suppressive effect of human BM-MSC over T cell cytokine production was previously described in the supernatant protein levels of IL-2 [39,237,243] and IFNγ [236,237]; in agreement, MSC-induced reduction of IL-2, IFNγ, and TNF-α T cell expression was also found in mouse [129,231,251,263], and is detailed in chapter 1, section 3.1.2.

According to our results, the degree of inhibition for TNF-α and IL-2 was higher for $CD4^+$ T cells than for $CD8^+$ T cells, and the opposite was verified for IFNγ. Analyzing the suppressive effect among the distinct functional compartments of $CD4^+$ T cells, we observed that effector and EM $CD4^+$ T cell subsets were more resistant to MSC-induced inhibition of TNF-α production; whereas the degree of inhibition for IL-2 and IFNγ was equivalent among all the functional compartments, except for naive $CD4^+$ T cells, wherein only a residual percentage of cells expressed the abovementioned cytokines. Therefore, the $CD4^+$ T cell subsets that produce higher levels of TNF-α (both by having a higher percentage of TNF-α-producing cells and higher MFI) were the less inhibited.

Concerning $CD8^+$ T cells' functional compartments, we observed that effector and EM $CD8^+$ T cell subsets presented the highest degree of inhibition on IFNγ production, and the lowest degree of inhibition on TNF-α and IL-2 production (once again, only a residual percentage of naive $CD8^+$ T cells produce the aforementioned cytokines). Accordingly, mouse

BM-MSC also display a more marked inhibition on IFNγ-producing memory $CD8^+$ T cells, compared to naive $CD8^+$ T cells [291]. And, similar to what was observed for $CD4^+$ T cells, the $CD8^+$ T cell subsets that produce higher levels of TNF-α (both by having a higher percentage of TNF-α-producing cells and higher TNF-α MFI) were the less inhibited, in our study.

The inhibition of IL-2 production by T cells is likely to contribute to MSC-driven suppression of T cell proliferation. IL-2 produced by $CD4^+$ T cells is essential to induce both $CD4^+$ T cell (acting autocrinally) and $CD8^+$ T cell proliferation, and IL-2 produced by $CD4^+$ T cells was inhibited by MSC to a greater extent than that produced by $CD8^+$ T cells. Besides the promotion of T cell clonal expansion, IL-2 also contributes to T cell differentiation into effector cells and activates NK cells [230]. The importance of the suppression of IL-2 secretion in MSC-derived impairment of T cell proliferation is notorious in the studies in mouse and baboons demonstrating that the addition of exogenous IL-2 to immune cells and MSC co-culture restore T cell proliferation [129,242].

The higher inhibitory degree exerted by MSC over the $CD8^+$ T cell subsets that produce the highest levels of IFNγ (EM and effector compartments) shows a clear inhibitory action of MSC over the Th1 immune response. In fact, IFNγ contributes to Th1 cell differentiation and exert other important roles in the context of a Th1 immune response, such as the activation and differentiation of CTL; activation of NK cells, DC, and macrophages; increase of macrophage's phagocytic and anti-microbial activity; upregulation of MHC-I, MHC-II, and IL-12 expression, and increase of the antigen-presentation skills of antigen-presenting cells; moreover, IFNγ also induces Ig class switching in B cells to IgG [230].

Finally, the milder action of MSC over the T cell subsets producing the highest amounts of TNF-α (EM and effector compartments) is consistent with the lower MSC suppressive action over the cytotoxic activity of fully differentiated effector $CD8^+$ T cells reported in MLR [62]. In fact, TNF-α is an important effector molecule with cytotoxic functions, capable of induce apoptosis after binding to the receptor TNFR1 in the target cells. Its pro-inflammatory activity includes the upregulation of chemokine and adhesion molecules expression, contributing to the formation of the immune cell infiltrate in sites of inflammation; and the increase of the phagocytic and anti-microbial activity by macrophages [230,643]. Accordingly, our results and the previously published data suggest that the immunosuppressive action of MSC over immune effector mechanisms is more moderate over cells with a higher degree of differentiation.

3.2. Immunosuppressive effect of human BM-MSC on naturally occurring peripheral blood T cells expressing IL-17, IL-9, and IL-6

We found that MSC decreased the percentage of $CD4^+$ and $CD8^+$ T cells producing IL-9, IL-17, and co-expressing IL-17 and TNF-α; as well as the percentage of $CD4^+IL\text{-}6^+$ T cells.

MSC-mediated impairment of Th17 cell differentiation [9,251,263,275-279] and the suppression of IL-17 secretion by fully differentiated Th17 [9,263,275,278,281] had already been demonstrated for human and mouse, though there are studies reporting the enhancement of Th17 differentiation [252,254,280] or function [251,262] under the influence of MSC, as detailed in chapter 1, section 3.1.2.4. Here, we reported that MSC exerted an inhibitory effect over the naturally occurring Th17 and Tc17 cells from healthy individuals peripheral blood, by decreasing the percentage cells producing IL-17, alone or in combination with TNF-α, and the amount of TNF-α produced *per* cell (measured as MFI) which, overall, is consistent with the MSC role in the regulation of the inflammatory response.

Despite an increasing knowledge on the anti and pro-inflammatory functions of IL-6, their role in Th17 differentiation and B cell Ig production, the function of Th6 still elusive [233,488,491,644]. We verified a decreased percentage of Th6 cells induced by MSC, which is consistent with their suppressive effect over IL-6 produced by mDC [333], human mast cells [399], mouse macrophages [381], and the decreased IL-6 serum levels detected among MSC-treated CIA mice [182].

The observation that MSC decreased percentage of $CD4^+$ and $CD8^+$ T cells expressing IL-9, is in agreement with a recent work showing that T cells cultured with conditioned medium derived from human amniotic-MSC displayed a reduced IL-9 protein levels in the culture supernatant [255]. IL-9 is involved in the response against helminthes and in allergic inflammation; promotes the expansion and activity of mast cells (including the upregulation of IL-5 and IL-13 secretion by mast cells); and both anti-inflammatory and pro-inflammatory effects had been ascribed to this cytokine [638,641]. These new findings may broaden the research field concerning the MSC influence on allergic inflammation.

CONCLUDING REMARKS

Overall, we found that human MSC derived from different tissues (BM, AT, and UCM) displayed a distinct suppressive ability over T, B, and NK cell activation and/or proliferation. Those differences were more relevant for B cell and $CD56^{bright}$ NK cell activation, in which UCM-MSC did not present an inhibitory effect, in opposition to MSC derived from the other tissues. Among the three types of MSC tested, AT-MSC presented the highest suppressive activity. We also found that MSC hindered PHA-induced T cell activation, but didn't affected their sense of polarization, inferred by the mRNA expression of T-bet which was not inhibited under the influence of MSC.

We described an inhibitory effect induced by BM-MSC on the protein and mRNA expression of pro-inflammatory cytokines and chemokines by peripheral blood mDC and peripheral blood monocyte subpopulations (classical, intermediate, and non-classical monocytes); remarkably, the influence of BM-MSC was distinct for the different monocyte subsets. The inhibition of cytokine and chemokine expression was not accompanied by the inhibition of activation/maturation markers expression. Thus, despite displaying an activated/mature phenotype, mDC and monocytes presented a hampered pro-inflammatory function which may hinder the development of inflammatory immune responses.

Likewise, human BM-MSC were also shown to inhibit pro-inflammatory cytokine production by all $CD4^+$ and $CD8^+$ T cell functional compartments; notwithstanding, the distinct T cell functional compartments displayed a different pattern of cytokine inhibition. This may impact the therapeutic outcome of MSC-based therapies for diseases with a different distribution of T cells among the functional compartments. Furthermore, the description of the inhibitory effect of BM-MSC on Th9 and Tc9 cells may bring new insights in the beneficial therapeutic effect of MSC in allergic inflammation animal models.

Thus, though the immunosuppressive activity of human MSC is unequivocal, it is important to have in mind that their tissue of origin influences their suppressive effect, and these differences have different manifestations in the distinct main lymphocytes subpopulations ($CD4^+$ and $CD8^+$ T cells, B cells, and $CD56^{dim}$ and $CD56^{brigth}$ NK cells). When focusing our attention on particular antigen-presenting cell subsets (mDC, and classical, intermediate, and non-classical monocytes) and specific $CD4^+$ and $CD8^+$ T cell functional compartments (naive, central memory, effector memory, and effector compartments), it

becomes obvious that they are not evenly affected by the suppressive action of human BM-MSC.

All these factors are likely to affect the clinical outcome of MSC-based therapies and, therefore, according to our results, the choice of the MSC source, the representativeness of each monocyte subpopulation, and the profile of T cell distribution among the functional compartments should be taken into account in *in vivo* assays, to further understand how they actually impact the clinical outcome of MSC-based therapies and, perhaps, to explain some incongruities observed among different diseases or different subjects with the same diagnosis.

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