Depicting the mechanism of action of an ATMP for the development of a potency assay

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Abstract

Mesenchymal stem/stromal cells (MSCs) have been subject of interest in the last years and the *in vitro* studies have demonstrated the wide potential of these cells. They have proved to be able to modulate the immune system by the secretion of cytokines and other soluble factors which makes them strong candidates to be used in Advanced Therapy Medicinal Products (ATMP) for the treatment of GvHD and other immune diseases; ImmuneSafe® is one of the products based on MSCs with the intention of preventing and treating GvHD.

The benchmarking of ImmuneSafe® was performed using MSCs that were previously administered to patients (MSC IPO) and with positive results in treating and preventing GvHD. The tests performed allowed the characterization of MSC IPO and the comparison of the results with the ones obtained for ImmuneSafe® suggested that, besides having similar characteristics and activity, ImmuneSafe® have a higher potency in what relates to the immunosuppressive activity.

The mechanism of action (MoA) of ImmuneSafe® was also studied by blocking several pathways believed to be involved in the immunomodulatory properties of the cells. The results indicated that IDO is one of the central players in the immunomodulatory activity of MSCs. Therefore, IDO appeared to be the most interesting protein to be used as a candidate for the potency assay, crucial for the clinical application of ImmuneSafe®.

The activation profile of ImmuneSafe® was analyzed using several pro-inflammatory cytokines. The overall results suggested that ImmuneSafe® reacts to those pro-inflammatory environments by releasing different soluble factors and altering the level of expression of some membrane proteins. The combination of IFN-γ and TNF-α lead to the pro-inflammatory environment with higher production of soluble factors: chemokines, metalloproteinases, inflammatory molecules and proteins involved in cell metabolism and cell migration.

Overall, the first steps were given in order to study ImmuneSafe®'s MoA and in understanding its reactions to several pro-inflammatory environments. Further investigation is still crucial in order to achieve trustful results and develop an effective potency assay.

Keywords

Mesenchymal stem/stromal cells, benchmarking, mechanism of action (MoA), Activation profile, cytokines, potency assay.

Resumo

As células estaminais do mesênquima (MSCs) têm sido alvo de interesse nos últimos anos e os estudos *in vitro* já demonstraram o seu vasto potencial. As MSCs já provaram a sua capacidade de modular o sistema imunitário através da secreção de citocinas e outros factores solúveis, o que as torna fortes candidatas a serem usadas em "Advanced Therapy Medicinal Products" (ATMP) para o tratamento do GvHD e outras doenças imunitárias; o ImmuneSafe® é um dos produtos derivados de MSCs e tem como objectivo prevenir e tratar o GvHD.

O "benchmarking" do ImmuneSafe® foi efectuado usando MSCs que foram previamente administradas a pacientes (MSC IPO) e que obtiveram resultados positivos no tratamento e prevenção do GvHD. Os testes efectuados permitiram a caracterização das MSC IPO e a comparação dos resultados com os obtidos para o ImmuneSafe® sugere que, apesar de ambos apresentarem características e actividades semelhantes, o ImmuneSafe® tem uma potência superior no que diz respeito aos estudos de imunossupressão.

O mecanismo de acção (MoA) do ImmuneSafe® foi também estudado através do bloqueio de vias metabólicas que se acredita estarem envolvidas nas propriedades imunomodulatórias das células. Os resultados indicaram que o IDO é um dos "jogadores" mais importantes na actividade imunomodulatória das MSCs. Assim, o IDO sobressaiu como a proteína com maior potencial para ser candidata ao desenvolvimento do ensaio de potência, o que é de extrema importância para a aplicação clínica do ImmuneSafe®.

O perfil de activação do ImmuneSafe® foi analisado usando várias citocinas pró-inflamatórias. Os resultados gerais sugeriram que o ImmuneSafe® reage aos diferentes ambientes pró-inflamatórios ao libertar vários factores solúveis e alterando o nível de expressão de proteínas membranares. A combinação do IFN-γ com o TNF-α resultou no ambiente pró-inflamatório em que ocorreu maior produção de factores solúveis: quimiocinas, metaloproteases, moléculas inflamatórias e proteínas envolvidas no metabolismo e migração celulares.

No geral, foram dados os primeiros passos no estudo do mecanismo de acção do ImmuneSafe® e no entendimento das suas reacções aos diversos ambientes pró-inflamatórios.

É de extrema importância proceder a mais estudos de forma a atingir resultados mais fiáveis e desenvolver um ensaio de potência eficaz.

Palavras-chave

Células estaminais do mesênquima, benchmarking, mecanismo de acção, perfil de activação, citocinas, ensaio de potência.

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List of Abbreviations

1-MT	1-Methyl-Tryptophan
AF	Amniotic Fluid
Ag	Antigen
aGvHD	acute Graft versus Host Disease
ALCAM	Activated Leukocyte Cell Adhesion Molecule
ANG	Angiopoietin
ANGPTL4	Angiopoietin-like 4
APC	Antigen-Presenting Cells
ASC	Adult Stem Cells
AT	Adipose Tissue
ATMP	Advanced Therapy Medicinal Product
bFGF	Basic Fibroblast Growth Factor
BM	Bone Marrow
BM-MSC	Bone Marrow Mesenchymal Stem Cells
CCL	Chemokine (C-C motif) Ligand
CD	Cluster of Differentiation
cGvHD	chronic Graft versus Host Disease
CHC	Centro de Histocompatibilidade de Coimbra
CHS	Centro de Histocompatibilidade do Sul
CTL	Cytotoxic T Lymphocyte
CXCL	Chemokine (C-X-C motif) Ligand
DC	Dendritic Cells
DPBS	Dulbecco's Phosphate-Buffered Saline
DT	Dental Pulp
ECM	Extracellular Cell Matrix
EGF	Epidermal growth factor
ELISA	Enzyme-Linked Immunosorbent Assay
ESC	Embryonic Stem Cells
FSC	Fetal Stem cells
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GvHD	Graft versus Host Disease
HA	Hyaluronic Acid
HB-EGF	Heparin-binding EGF-like growth factor
HBSS	Hank's Balanced Salt Solution
HCT	Hematopoietic Cell Transplant

HGF	Hepatocyte growth factor
HLA	Human Leukocyte Antigen
HO-1	Heme Oxygenase 1
HSC	Hematopoietic Stem Cells
HSCT	Hematopoietic Stem Cell Transplantation
IBB-IST	Instituto de Biotecnologia e Bioengenharia-Instituto Superior Técnico
iDC	immature Dendritic Cells
IDO	Indoleamine -2 3-dioxygenase
IFN-γ	Interferon γ
IL	Interleukin
ICAM-1	Induced Intracellular Adhesion Molecule-1
Ig	Immunoglobulin
IGF	Insulin-like Growth Factor
iNOS	Inducible Nitric Oxide Synthase
IPOLFG	Instituto Português de Oncologia de Lisboa Francisco Gentil
IPO-Porto	Instituto Português de Oncologia do Porto
iPSC	induced Pluripotent Stem Cells
IS	ImmuneSafe®
ISC	Infant Stem Cells
ISCT	International Society for Cellular Therapy
LCA	Leukocyte Common Antigen
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MK886	3-[1-(p-Chlorobenzyl)-5-(Isopropyl)-3-t-Butylthioindol-2-yl]-2,2-Dimethylpropanoic
MLR	Mixed Lymphocyte Reaction
MMP	Matrix Metalloproteinase
MNC	Mononuclear Cell
MoA	Mechanism of Action
mRNA	Messenger Ribonucleic Acid
MSC	Mesenchymal Stem/Stromal Cells
MT	Muscle Tissue
NIH	National Institutes of Health
NO	Nitric Oxide
NK	Natural Killer
NSB	Non-Specific Binding
PBMC	Peripheral Blood Mononuclear Cell
PECAM-1	Platelet/endothelial cell adhesion molecule
PD-1	Programmed Death 1

PD-L	Programmed cell Death Ligand
PDGF	Platelet-derived growth factor
PFA	Paraformaldehyde
PGE2	Prostaglandin E2
PL	Placenta
PMA	Phorbol 12-Myristate 13-Acetate
RNA	Ribonucleic Acid
RPM	Rotations Per Minute
RT-PCR	Real Time Polymerase Chain Reaction
SCF	Stem cell factor
SEM	Standard Error of the Mean
SnPP	Tin Protoporphyrin IX Dichloride
sRNA	small RNA
TBI	Total Body Irradiation
TGF-β	Transforming Growth Factor β
Th	T helper cells
Tie	Tyrosine kinase with immunoglobulin-like and EGF-like domains
TIMP	Tissue inhibitors of metalloproteinases
TLR	Toll-Like Receptor
TNF-α	Tumor Necrosis Factor α
TNFR	Tumor Necrosis Factor Receptor
TPO	Thrombopoietin
Treg	Regulatory T cells
TSG-6	TNF-α Stimulated Gene 6 protein
UCB	Umbilical Cord Blood
UCM	Umbilical Cord Matrix
uPAR	Urokinase-type plasminogen activator receptor
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular endothelial growth factor

1 Aim of studies

The aim of this thesis was to study, using a systematic approach, the Mechanism of Action (MoA) of ImmuneSafe® and compare it with other similar cellular products.

This project was divided in three sections. First of all, the ImmuneSafe® was compared with a similar cellular product previously used in clinical studies and with positive results in treating and preventing graft versus host disease (GvHD).

The second task was to determine ImmuneSafe®'s MoA by depicting the role of its different immunomodulatory pathways by singularly inactivating each pathway.

The last task was to study the activation profile of ImmuneSafe® by stimulating the cells with different pro-inflammatory cytokines, characteristic of GvHD, and evaluating the modifications in the activation of the different immunomodulatory pathways.

Overall, this project is expected to improve the knowledge on ImmuneSafe®'s mechanism of action and a side-by-side comparison with other MSC products previously used in clinical studies. In addition, it is expected to give the first steps in finding the best potency assay for this product.

2 Introduction

"*The stem cells in adult mammalian tissues (and the property of stemness) are difficult to define conceptually, largely impossible to identify morphologically and are associated with functions and attributes that commonly confuse rather than clarify their identity and role*" (Potten & Loeffler 1990). In 1990 things were at this point; 24 years later it is still a challenge to fully define stem cells.

2.1 Stem Cells

Stem cells are cells that have the ability to replicate themselves for unlimited or prolonged periods of time and also to differentiate into various cell types. It is believed that a stem cell keeps its "stemness" until it receives a signal to develop into a specialized cell (Bongso & Lee 2005). This way, it is possible to distinguish stem cells from other cell types by two characteristics: *i)* they are unspecialized cells capable of renewing themselves through cell division; and *ii)* under the right stimulus, they can be induced to become tissue- or organ-specific cells (Institutes of Health 2009). Stem cells can be classified according to their differentiation potential or according to their sources. Regarding its differentiation potential, they can be designed totipotent, pluripotent and multipotent. Totipotent cells have the ability to differentiate into every extraembryonic, somatic or germ cell known in the mammalian development; they can give rise to a new organism. Plutipotent cells, unlike totipotent cells, cannot give rise to a new organism but can differentiate into any cell of the body, including the germ layers (endoderm, ectoderm and mesoderm) and some extraembryonic tissues. Multipotent cells can differentiate into only a limited range of cell types.

There is a recent type of stem cells called induced Pluripotent Stem Cells (iPSCs). In 2006 Yamanaka and Takahashi were able to reprogram differentiated cells into a state of pluripotency (Takahashi & Yamanaka 2006). iPSCs can theoretically be obtained from any dividing cell of the body and were an important discovery since it has the potential to overcome all the ethical issues related to the use of Embryonic Stem Cells (ESCs). However, the use of these cells in patients is limited since the tumourogenicity is much higher when compared to the use of ESCs. Since further investigation is needed in order to overpass these problems, iPSCs are now being faced as cellular disease models and drug screening models but in the future they have the potential to have a much wider application; the capacity to reprogram any cell into a pluripotency sate and inducing its differentiation into any cell that the patient need will greatly diminish the rejections by the immune system (Ben-David & Benvenistry 2011; Takahashi & Yamanaka 2006).

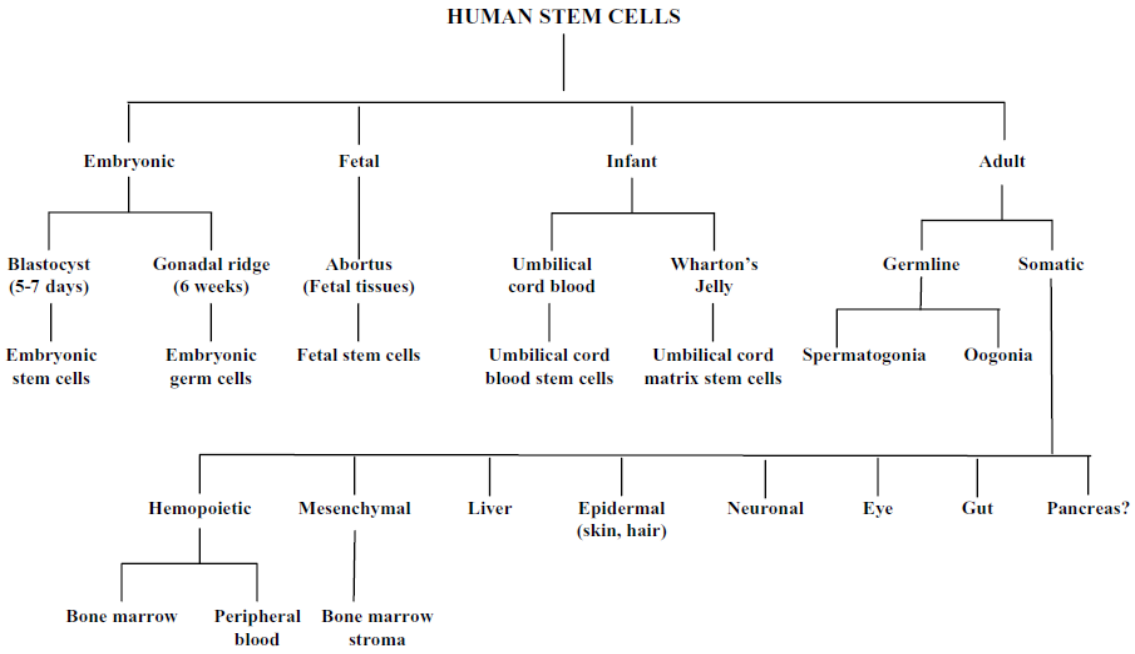

Figure 1 - Human Stem Cell classification. "Stem Cells - From Bench to Bedside" (Bongso & Lee 2005)

According to their sources, stem cells can be classified in Embryonic Stem Cells (ESCs), Fetal Stem Cells (FSCs), Infant Stem Cells (ISCs) and Adult Stem Cells (ASCs) (see Figure 1). For instance, Mesenchymal Stem Cells (MSCs) are an example of ASCs and can be found in a wide variety of tissues and so are Hematopoietic Stem Cells (HSCs) that are mainly found in the peripheral blood and also in the bone marrow (Bongso & Lee 2005).

2.2 Mesenchymal Stem/ Stromal Cells (MSC)

In 1970, Friedenstein *et al.* discovered the first evidence of fibroblast-like cells present in the bone marrow of rodents that were hematopoietic supportive cells and with osteogenic potential (Friedenstein et al. 1970). Six years later, Friedenstein *et al.* made an advance in the study of MSCs and described a method for "stromal cells" isolation from whole bone marrow aspirates based on differential adhesion properties (Friedenstein et al. 1976). However, it was only in the late 1980s that Owen and Friedenstein reported heterogeneity of the bone marrow stromal cells for the first time (Owen & Friedenstein 1988; Owen 1988). Later, these cells were called mesenchymal stem cells and researchers started to study their therapeutic potential. This notion of mesenchymal stem cell was popularized by Arnold Caplan; he proposed that MSCs gave rise to bone, cartilage, tendon, ligament, marrow stroma, adipocytes, dermis, muscle and connective tissue (Caplan 1991).

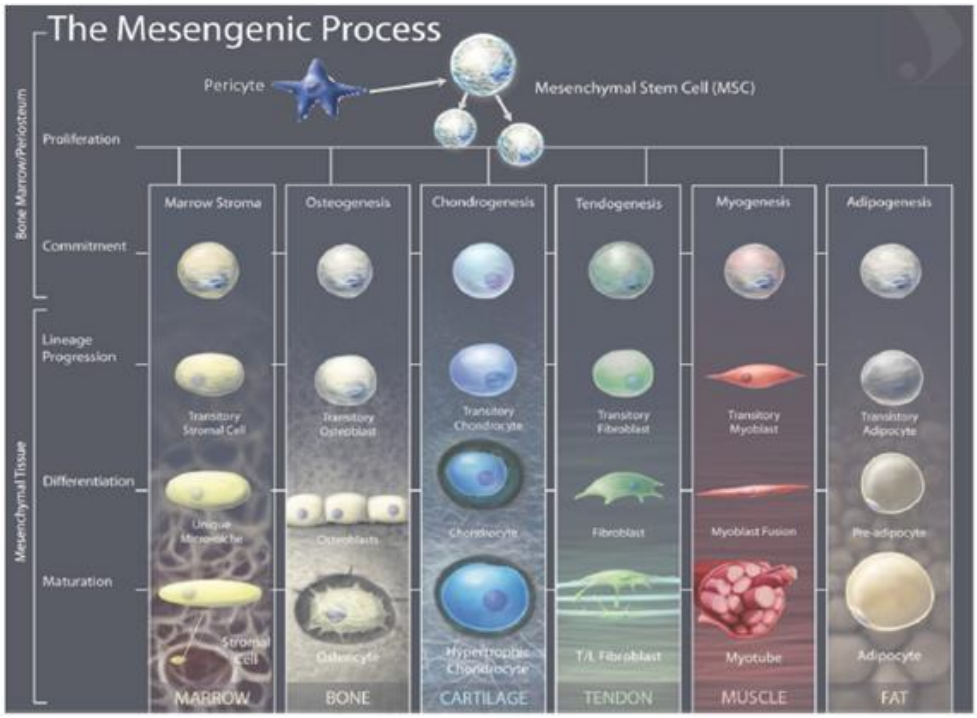

Figure 2 - The mesengenic process (Caplan & Correa 2011)

The "stemness" of these cells has been subject of controversy since the population of cells isolated *in vitro* from the bone marrow and other sources is not uniform (Horwitz et al. 2012). In order to solve that problem, the International Society for Cellular Therapy (ISCT) proposed the term "mesenchymal stromal cells" and the international acronym MSC is valid for both designations. The term "mesenchymal stem cells" is now reserved for the subset of mesenchymal cells that demonstrate stem cell activity by rigorous criteria (Horwitz et al. 2005).

The fact is that very little is known about the phenotypic characteristics of MSCs *in vivo*, their anatomical localization, their contribution to organogenesis and postnatal tissue. Although there are already some successful experiments, it is still missing an accurate assay that would test their ability to self-renew and differentiate *in vivo* (Kronenberg 2009); knowing the stimulus they need *in vivo* to differentiate into a specific type of cell, for example, would be of extremely utility for the regenerative medicine.

MSCs were first isolated from bone marrow, but it is now known that they reside in almost every type of connective and neonatal tissues. MSCs can be found in the bone marrow (BM), adipose tissue (AT), dental pulp (DP), muscle tissue (MT), umbilical cord blood (UCB) and matrix (UCM), placenta (PL-MSCs) or amniotic fluid (AF-MSCs). They are characterized as an heterogeneous population of cells that respect the three minimal criteria defined by the ISCT (Dominici et al. 2006): *i)* adherence to plastic (very useful for the isolation of the cells); *ii)* specific surface antigen (Ag) expression (≥95% of the MSCs must express CD105, CD73 and CD90; ≤2% of the MSCs must lack expression of CD45, CD34, CD14 or CD11b, CD79a orCD19 and HLA-II); *iii)* multipotent differentiation potential into osteoblasts, adipocytes and chondroblasts under standard *in vitro* differentiating conditions.

Perivascular cells were identified in multiple human organs including skeletal muscle, pancreas, adipose tissue, and placenta. These cells exhibited osteogenic, chondrogenic, and adipogenic potentials and expressed MSC markers, suggesting blood vessel harbor a reserve of progenitor cells that may be integral to the origin of the elusive MSCs and other related adult stem cells (Crisan et al. 2008). These characteristics suggest that MSCs may be pericytes but the contrary is not true (Caplan & Correa 2011). However, more recent studies comparing messenger RNAs from MSCs and pericytes have shown crucial differences between these two types of cells.

The *in vitro* studies with MSCs have generated important information confirming that these cells have application in a wide variety of areas; they act as support for hematopoiesis and promote graft survival and engraftment of HSCs, have important immunomodulatory properties and can actively participate in tissue regeneration (Caplan & Ph 1980).

In a clinical setting, several trials are using MSCs for various conditions including orthopedic injuries, graft versus host disease (GvHD) following bone marrow transplantation, cardiovascular diseases, autoimmune diseases and liver diseases (Caplan & Ph 1980).

2.3 Graft-versus-Host Disease (GvHD)

GvHD is the most frequent complication after allogeneic hematopoietic stem cell transplantation (HSCT). It is a consequence of interactions between antigen-presenting cells (APCs) of the recipient and immunocompetent donor cells and is associated with considerable morbidity and mortality, particularly in patients who do not respond to primary therapy (steroids). It is known that these immunologically competent cells are T-cells and that GvHD can develop in various clinical settings when tissues containing T-cells are transferred from one person to another (Ferrara et al. 2009). The major target organs of acute GvHD are skin, liver, and intestinal tract, although other organs can be affected.

When the first allografts were performed, it was shown that patients given marrow from donors other than monozygotic twins were likely to develop the so called "secondary disease" (Devergie 2008). When the bases for the HSCT were established, it became obvious that GvHD would be a formidable problem even when the donor is a "perfectly" matched sibling, meaning they have identical antigens of the major histocompatibility complex (MHC), termed HLA (human leukocyte antigen) in humans (Deeg 2007).

In 1966, Billingham defined the three necessary conditions for a patient given allo-HSCT to develop GvHD: *i)* administration of immunocompetent cells; *ii)* histo-incompatibility between donor and recipient; and *iii)* inability of the recipient to destroy or inactivate the transfused or transplanted cells (Billingham 1996).

There are two types of GvHD: acute (aGvHD) and chronic (cGvHD). The traditional definition was based on the time of onset after transplantation (less or more than 100 days after HSCT) but is no longer valid. The National Institutes of Health (NIH) Consensus Conference proposed two

subcategories for both aGvHD and cGvHD based on signs and symptoms rather than time of onset. For aGvHD (absence of features consistent with cGvHD) there is *i)* classic aGvHD (before day 100) and *ii)* persistent, recurrent, or late aGvHD (after day 100, often upon withdrawal of immunosuppression). For cGvHD there is *i)* classic cGvHD (no signs of aGvHD), and *ii)* an overlap syndrome in which features of both acute and chronic GvHD are present (Billingham 1996). The incidence of the severity of aGvHD is determined by the extent of involvement of the three principal target organs (skin, liver and gastrointestinal tract). The disease can be classified as grade I (mild), II (moderate), III (severe) and IV (very severe) (Ferrara et al. 2009).

The risk of aGvHD increases with the use of unrelated donors, multiparous female donor, older age of the recipient, graft type and certain conditioning regimens. Although the central role of HLA matching in HSCT is well established, it has become apparent that other genetic systems affect the development of GvHD. The incidence of aGvHD is directly related to the degree of mismatch between HLA proteins and ranges from 35-45% in recipients of full matched sibling donor grafts to 60-80% in recipients of one-antigen HLA-mismatched unrelated donor grafts (Ferrara et al. 2009).

The development of aGvHD can be described in three steps (see Figure 3):

i. **Effects of conditioning**. The first step consists in preparing the body for the transplant and includes total body irradiation (TBI) and/or chemotherapy. This results in tissue damage and the release of proinflammatory cytokines, such as tumor necrosis factor α (TNF-α) and interleukin 1 (IL-1), into the circulation. As a consequence, expression of MHC antigens and adhesion molecules is increased, enhancing the activation of host dendritic cells (DCs), which are antigen-presenting cells (APCs), necessary for the initiation of primary and secondary immune responses. TBI also causes damage of the gastrointestinal tract, allowing immunostimulatory microbial products such as lipopolysaccharides (LPS) to enter into systemic circulation, activating the innate immune system and promoting the inflammatory cytokine cascade.
ii. **T-cell activation**. The second phase consists on the interaction of donor T-cells with host APCs. DCs are the most potent APCs and are activated by inflammatory cytokines, microbial products and necrotic cells. These "danger signals" mature DCs and induce T-cell activation whereas immature DCs induce T-cell tolerance. After activation, T-cells express Interferon γ (IFN- γ), interleukin 2 (IL-2) and TNF-α, among others, leading to T-cell expansion.
iii. **Cellular and inflammatory effector phase**. The last phase is a complex cascade of effectors mediated by cellular effectors such as cytotoxic effector cells (CTLs) and natural killer (NK) cells, and inflammatory cytokines such as TNF-α, IL-1 and macrophage-derived nitric oxide (NO). Interactions of innate (LGL/NK cells) and adaptive (alloreactive T-cells) immune responses lead to organ damage. There is recent evidence that B cells can contribute for the development of (chronic) GvHD but host B cells may attenuate the effects by the secretion of IL-10 (Devergie 2008; Ferrara et al. 2009; Reddy & Ferrara 2009).

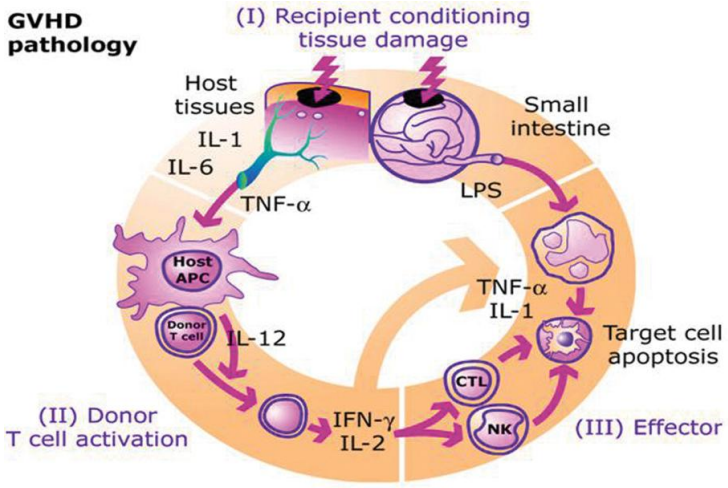

Figure 3 - GvHD pathology. (Reddy & Ferrara 2009)

A study by Ju *et al* focused on the cytokine gene expression by peripheral blood mononuclear cells (PBMCs) in 30 patients who were subject of allo-HSCT and showed that IFN-γ, IL-2, IL-12 and IL-18 were significantly more frequent in patients with aGvHD (Ju et al. 2005).

The participation of IL-17A has also been described to contribute to aGvHD. IL-17A has been reported to protect organisms against extracellular bacteria and fungi due to its capacity to recruit neutrophils to the areas of infection. In 2000 it was proposed that IL-17 couldn't be classified as Th1 or Th2, but as a new lineage of IL-17-producing cells named Th17 (Infante-Duarte et al. 2000).

Additional complexity has been recently added with the description of regulatory cell populations that can contribute for the treatment of GvHD: Regulatory T-cells (Tregs), regulatory APCs and mesenchymal stem cells (MSCs) (Devergie 2008; Reddy & Ferrara 2003; Ferrara et al. 2009).

2.4 MSCs as treatment for GvHD

The immunomodulatory feature of MSCs makes them very strong candidates for the treatment of several immune diseases, including GvHD. The most common treatment consists in steroids, however in cases of severe steroid-resistant aGvHD, the outcome is poor and the overall survival is low. In these cases MSC-based therapy may be used as secondary line of treatment.

MSCs are an attractive therapeutic approach during or after HSCT. They can minimize the toxicity of the conditioning phase while inducing hematopoietic engraftment and decreasing the incidence and severity of GvHD. In some studies MSCs were co-transplanted with HSCs while in others were added after the transplant; it remains unclear which is the best option (Lin & Hogan 2011). Both are valid therapeutic approaches, but the first is called prophylaxis (or preventive medicine) and intends to prevent the disease, as opposed to disease treatment which is the option where MSCs are infused after the disease onset.

The clinical efficacy of MSCs in the treatment of GvHD was first described by Le Blanc (Le Blanc et al. 2008). The paper describes a 9-year-old boy with steroid-resistant grade IV aGvHD that showed a complete response after receiving haploidentical third-party MSCs. Since then, other clinical studies have shown the efficacy of MSCs in the treatment of this disease but the mechanism of action still needs further investigation (von Bonin et al. 2009).

2.5 MSCs Mechanism of Action

MSCs mechanism of action is a vast area of investigation. In this work, the focus is the mechanism of action concerning their utilization in the treatment of aGvHD.

The mixed lymphocyte reaction (MLR) has become an integral tool in the field of immunological research. It provides a convenient *in vitro* method to study the process of normal T-cell activation and proliferation in primary culture (O'Flaherty et al. 2000). Since the discovery that human MSCs can suppress allogeneic MLRs, their immunosuppressive properties have been subject of intensive investigation. The general conclusions are that *ex vivo* expanded human MSCs can modulate the function of T cells, NK cells, DCs, B cells and macrophages (Stagg & Galipeau 2013).

It has been shown that, when incorporated as a third party in a MLR, MSCs can suppress T cell proliferation and induce split T cell anergy. The addition of exogenous IL-2 can overcome this anergy (Glennie et al. 2005). They have also shown to promote the expansion of $Fox3^{+}$Tregs through cytokine modulation, especially by inducing DCs to produce IL-10. The production of prostaglandin E2 (PGE2) have also shown to be involved in the suppressive activity against Th17 and even in the conversion of Th17 into a regulatory state. The inhibition of NK cells is also related with PGE2 and indoleamine 2,3 - dioxygenase (IDO). Interestingly, MSCs exposed to activated NK cells tend to be killed whereas MSCs activated with INF-γ are a potent modulator of MSC-NK cell cross-talk (Spaggiari et al. 2008). When co-cultured with blood monocytes, MSCs have shown to suppress their differentiation into DCs. The co-culture of MSCs and mature DCs lead to a significant decrease in the expression of the costimulatory molecules CD80 and CD86 and inhibit as much as 50% the production of TNF-α in response to LPS (Stagg & Galipeau 2013). It was also established that MSCs can inhibit B cell activation, proliferation and differentiation to immunoglobulin (Ig)-producing cells. Another study suggests that this inhibition is related to the proteolyc processing of MSCs-derived CCL2 into N-terminal quadrapeptide cleaved CCL2 by matrix metalloproteinases (MMPs). Concerning macrophages, a study by Németh *et al* reported that the administration of MSCs could reprogram macrophages *in vivo*. The MSCs activated with LPS or TNF-α release PGE2 which enhance the production of IL-10 by the macrophages (Németh et al. 2009). The general impact of MSCs on immune system cells is depicted in Figure 4.

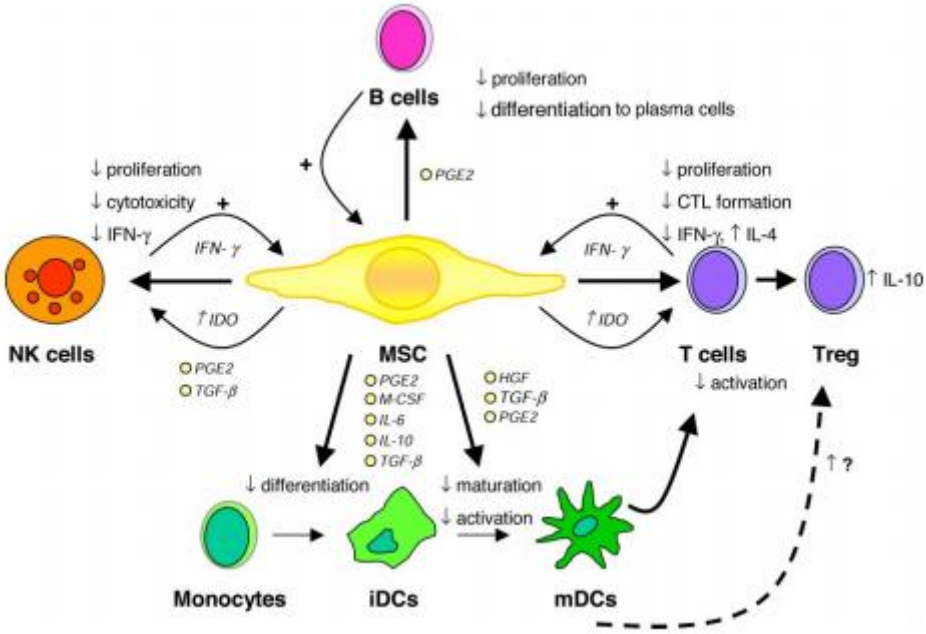

Figure 4 - Immunomodulatory properties of mesenchymal stromal cells. (Nauta & Fibbe 2007)

This gives an idea of the complex mechanism by which MSCs exert their action. A summary of the principal soluble factors involved in this complexity is at Table 1.

Table 1 - Soluble factors involved in the immunomodulatory properties of MSCs. Adapted from (Stagg & Galipeau 2013)

Secreted Factors
Galectins
Prostaglandin E2 (PGE2)
Insulin-like growth factor (IGF)-binding proteins
Transforming growth factor β (TGF- β)
Human leucocyte antigen-G5 (HLA-G5)
N-terminal qudrapeptide cleaved CCL2
TNF-α stimulated gene 6 protein (TSG-6)
Metabolic enzymes
Indoleamine 2,3-dioxygenase (IDO)
Heme oxygenase 1 (HO-1)
Inducible nitric oxide synthase (iNOS)
Cell surface proteins
Programmed cell death ligands 1 and 2 (PD-L1 and PD-L2)
Cytokine-induced intracellular adhesion molecule-1 (ICAM-1 or CD54) and Vascular cell adhesion molecule-1 (VCAM-1 or CD106)
Activated leukocyte cell adhesion molecule (ALCAM or CD166)
CD44

A curious fact about MSCs is that they seem to have pro-inflammatory and immunosuppressive phenotypes. Considerable data support an anti-inflammatory effect of MSCs in immune cells but there are also conflicting reports suggesting that MSCs enhance immune cells survival, for example, in the clearance of pathogens or necrotic cells. A recent idea suggesting that MSCs sense the environment can be the explanation for these questions. Following this concept, MSCs are not spontaneously immunosuppressive but require "licensing" or activation to exert their immunosuppressive effects. This activation is dependent on "signals", molecules that are released *in vivo* and can trigger MSCs response. Some of these molecules are pro-inflammatory cytokines and have already been tested *in vitro*, like IFN-γ, TNF-α or IL-1β (Th1 cytokines). Their effect is to trigger the production of other molecules by MSCs, like IDO, PGE2, TGF-β, TSG-6, and anti-inflammatory Th2 cytokines (Chen & Nuñez 2010; English 2013). Toll-like receptors (TLRs) signaling have also been implicated in the licensing of MSCs. Activation of TLRs expressed by MSCs in an infectious environment is believed to act as a fail-safe mechanism, impeding their immunosuppressive activity and inducing their pathogen clearance activity. A recent study reported that human MSCs primed with inflammatory cytokines are licensed to acquire anti-microbial functions. There are some discrepancies in the results; for example, IDO, known to be essential for the immunosuppressive effects of MSCs, have also shown to be involved in the anti-microbial functions. Depending on the degree of tryptophan depletion, IDO appears to drive MSCs towards anti-microbial rather than immunosuppressive functions (Phinney & Sensebé 2013).

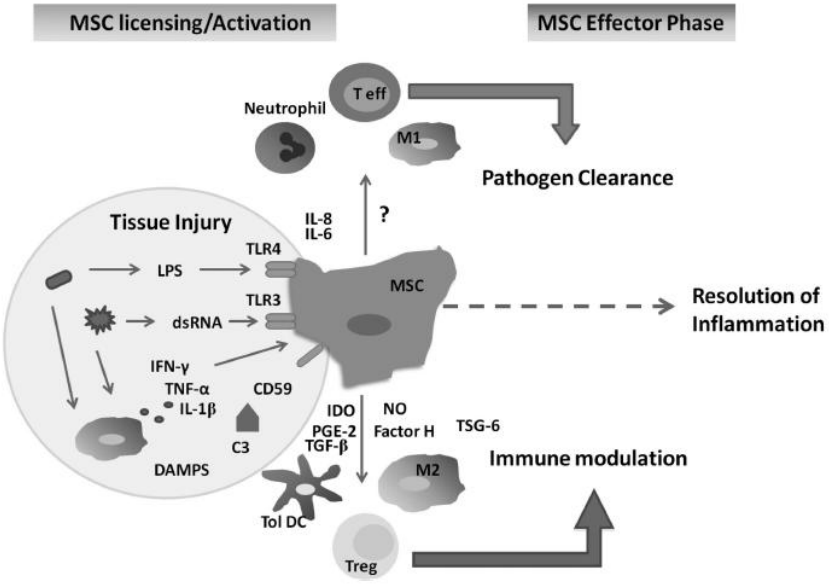

Figure 5 - MSC licensing/activation. (English 2013)

In order to study the immunosuppressive characteristics of MSCs *in vitro*, it is necessary to create an inflammatory environment. It can be achieved with pro-inflammatory cytokines such as IFN-γ, TNF-α or IL-1. In a study by François *et al* (François et al. 2012), they state that better results are achieved using a combination of these cytokines: IFN-γ and TNF-α.

Among all the pathways described, we can distinguish some metabolic enzymes (HO-1, IDO, NO), secreted factors (PGE2, TGF-β, HLA-G5, IL-6, chemokines, etc) and also cell surface proteins (CD44, CD54, CD106, CD166, PDL-1 and PDL-2).

2.5.1 Metabolic Enzymes

2.5.1.1 Nitric oxide (NO)

There are several studies indicating that NO is critical for the immunosuppressive activity of MSCs since NO production via iNOS has an inhibitory effect against macrophages and T-cells. However the importance of NO appears to be species dependent; in contrast to mouse MSCs, human MSCs does not rely on NO for their immunosuppressive effect but rather on other metabolic enzymes such as IDO (Stagg & Galipeau 2013).

2.5.1.2 Heme oxygenase-1 (HO-1)

Heme oxygenase-1 (HO-1) is also known as heat shock protein. It is one of three isoenzymes from the heme oxygenases family that catalyze the oxidative degradation of heme to biliverdin, free divalent iron and CO. The only inducible form, HO-1, has been described as an anti-inflammatory and immunosuppressive molecule (Chabannes et al. 2007).

The initial studies have suggested that HO-1 expression by regulatory T cells (Tregs) regulates Treg-mediated immunossupression. However, data from studies of HO-1 knockout mice have shown that, in fact, HO-1 indirectly affects Treg function. HO-1 might be involved in the regulation of dendritic cells (DCs), inhibition in the production of reactive oxygen species and inflammatory cytokines such as IL-12, IL-6 and type I IFNs, while promoting IL-10 production (Stagg & Galipeau 2013; Chabannes et al. 2007) but its major effect is related to the activation and induction of Tregs, which are also under evaluation for the treatment of GvHD occurring in bone marrow transplanted patients.

There is a study where is demonstrated that human MSCs promote the induction of Tregs very similar to those naturally occurring thymic forms (nTreg-like), $IL10^{+}$Tr1, and TGF-β^{+}Th3 cells (Mougiakakos et al. 2011). The levels of HO-1 were significantly higher when normally cultured cells were stimulated with IFN-γ. However, in licenced MSCs, HO-1 is no longer required for the immunosuppressive effect, which is instead afforded by PGE2 release. In a study by Chabannes *et a*l, they cocultured MSCs and T cells from adult rats and used an HO-1 inhibitor, tin protoporphyrin IX (SnPP). In the absence of any treatment, MSCs from adult rats inhibited T cell proliferation by 90% (1:1) and this suppression was not altered when SnPP was added. However, in human MSCs, HO-1 inhibition with SnPP completely abolished the suppressive effect of MCs (Chabannes et al. 2007). Mougiakakos *et al* (Mougiakakos et al. 2011) proposed that human MSCs may perform multi-step immunosuppression that involves different factors at different stages of the process.

2.5.1.3 Indoleamine 2,3-dioxygenase (IDO)

IDO is an enzyme that catabolises tryptophan into kynurenine metabolites that regulate T-cell proliferation (English 2013; Stagg & Galipeau 2013). It is inducible by stimulation of MSCs with IFN-γ or TLR3 and TLR4. IDO degrades the essential amino acid tryptophan and, in concert with other enzymes, gives rise to metabolites of tryptophan that inhibit lymphocyte proliferation (Gieseke et al. 2007). The mechanism by which IDO exerts its immunosuppressive activity is probably related to the induction of monocytes differentiation into an IL-10-secreting M2 immunosuppressive macrophages ($CD14^+/CD206^+$) (François et al. 2012). It has also been demonstrated that IDO is involved in the differentiation of $CD4^+$T cells into $Foxp3^+$Tregs (Fallarino et al. 2006). In some studies, the blockage of IDO with a pharmacologic inhibitor, 1-methyl tryptophan (1-MT), has totally abrogated its suppressive effect (Chen et al. 2008; Schmidt et al. 2012; Liu et al. 2010).

There are some studies with contrary results stating that IFN-γ, instead of helping in the inhibition, helps in the stimulation of PBMC's proliferation (see Figure 6). Obviously, IFN-γ plays an ambiguous role in various *in vitro* settings and this may be different *in vivo* (Gieseke et al. 2007) .

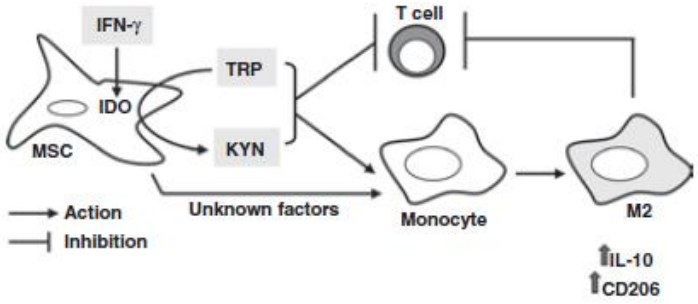

Figure 6 - Ambiguous role of INF-gamma. (François et al. 2012)

2.5.2 Secreted Factors

2.5.2.1 Prostaglandin E2 (PGE2)

Prostaglandin E2 (PGE2) is a rapidly released and short acting small lipid mediator that is known to have a role in immunomodulation. The synthetic pathway of PGE2 involves the Cyclooxygenase (COX) enzyme (COX-1 and COX-2) production of prostaglandin H2 from arachinoid acid, followed by conversion of prostaglandin H2 into prostaglandins via prostaglandin synthases (English 2013).

Some studies showed that PGE2 is produced when MSCs are in co-culture with monocytes and it is possible to induce its production by stimulating the MSCs with IFN-γ and TNF-α, as well as by TLR3 ligands (but not TLR4) (Stagg & Galipeau 2013). A study concerning an experimental arthritis model has also identified IL-6 as required for the production of PGE2, however it can be exclusive of that disease (Bouffi et al. 2010). It is now known that PGE2 suppress the T-cell activation and proliferation both *in vitro* and *in vivo* and has an important role in MSC reprogramming of macrophages and dendritic cells. A study by Spaggiari *et al* demonstrated that the MSCs effect concerning DCs is

restricted to the early stages, *i.e.*, to the cytokine-induced progression from monocytes to iDCs. Addition of MSCs in a very low proportion (1:10) prevented this differentiation as revealed by the persistence of CD14 (monocyte marker) and by the lack of expression of CD1a (iDC marker). MSCs produce PGE2 constitutively but this production increases when MSCs are cocultured with monocytes. Besides, the addition of the PGE2 inhibitor NS-398 to the cocultures almost completely restored DC maturation and function (Spaggiari et al. 2009).

2.5.2.2 Interleukins (IL)

Interleukins (IL) are a complex group of cytokines with complex immunomodulatory functions, including cell proliferation, migration, maturation, adhesion and also in immune cell differentiation and activation. ILs can have pro- and anti-inflammatory functions which makes their characterization quite difficult and distinguishes them from chemokines in general. The response of a particular cell to ILs depends on the ligands involved, specific receptors expressed on the cells and the particular signaling cascade activated (Brocker et al. 2010).

Interleukin-6 (IL-6) is one of the most important and referred interleukins when considering the immunomodulatory functions of MSCs. It is a cytokine that is highly expressed in the bone marrow stroma and is known for its role in bone homeostasis. In some papers, it is referred as a component responsible for the "stemness" of MSCs (Pricola et al. 2009). Additionally, it is involved in inflammation and infection responses and in regenerative, metabolic and neural processes (Scheller et al. 2011).

There is also evidence that IL-6 is essential for the production of PGE2 and is also believe to be important for the Treg cells generation (Bouffi et al. 2010). IL-6 used to be recognized as a pro-inflammatory cytokine, however more recently it was discovered that it has also regenerative and anti-inflammatory properties. It is important to distinguish between *trans-signaling* and *classic-signaling*. The activation of cells that only express gp130 via the IL-6/sIL-6R complex is called *trans-signaling* whereas activation of cells via the mbIL-6R in complex with IL-6 is called *classic-signaling*. It was shown that the pro-inflammatory activity of IL-6 is dependent on *trans-signaling*. On the other hand, the regenerative/anti-inflammatory activity depends on *classic-signaling*. IL-6 is also known to interfere in DC maturation, but is not as crucial as PGE2 (Spaggiari et al. 2008).

2.5.2.3 Human Leukocyte Antigen-G5 (HLA-G5)

HLA-G is a non-classic human leukocyte antigen (HLA) class I molecule. It shares with MSCs some inhibitory properties on immune cells since it can alter NK and T-cell functions, as well as DC's maturation. There are seven isoforms of this molecule including membrane bound HLA-G1, -G2, -G3 and -G4 and soluble HLA-G5, -G6, and -G7. In a study by Selmani *et al*, they identified IL-10 as the key factor for the production of HLA-G5 (Selmani et al. 2008). In fact, IL-10 and HLA-G5 seem to be regulated according to an amplification feedback loop and both are essential for the immunosuppressive action. The conclusions of this study are that HLA-G5 secreted by MSCs contributes to the suppression of the NK lytic activity and IFN-γ secretion, the direct inhibition of allogeneic T-cell responses, the increase of IL-10 concentration in the alloreaction environment and

the expansion of $CD4^{+}CD25^{high}FOXP3^{+}$Tregs. They also state that it is required a cell-to-cell contact between MSCs and T-cells.

2.5.2.4 Chemokines

Chemokines are low-molecular weight molecules members of the cytokine family of regulatory proteins. They are considered secondary pro-inflammatory mediators that are induced by primary pro-inflammatory molecules such as IFN-γ, TNF-α or IL-1 and are known by their ability to recruit well-defined subsets of leukocytes. Through the recruitment of leukocytes, chemokines lead to the activation of host defence mechanisms. There are two major chemokine subfamilies: Chemokine (C-X-C motif) Ligand (CXC) and Chemokine (C-C motif) Ligand (CC) (Groves & Jiang 1995).

One of the interesting features of MSCs is their ability to migrate to the damage tissue after intravenous administration. Although this mechanism is not yet well understood, some studies report that is due to an increase in the concentration of chemokines in the sites of injury. Since chemokine receptors and essential molecules for the transmigration of leukocytes from blood to tissue are strongly expressed by MSCs, this could explain the MSCs mechanism of transport, homing, adhesion and transmigration across endothelium (Girolamo et al. 2013). A study by Ren *et al* based on mouse MSCs reported that the full immunosuppressive activity of MSCs is only achieved when IFN-γ is added in combination with TNF-α, IL-1α or IL-1β; besides, the licensing of MSCs with IFN-γ+TNF-α induces the release of high levels of several chemokines such as CXCL9, CXCL10 and CXCL11, which are ligands for the CXCR3 receptor (Ren et al. 2008). This will allow the recruitment of lymphocytes in proximity with MSCs, and thus NO (important in the immunocuppressive mechanism in mice).

Metalloproteinases released by MSCs complement the chemokine function by degrading the endothelial vessel basement membrane to allow extravasation into damage tissue and the ratio between matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMP) appears to be important in MSC function (Kasper et al. 2007).

2.5.2.5 Molecules involved in tissue repair and angiogenesis

When related to the angiogenesis and tissue repair functions, there are several molecules that should be considered. The desired anti-apoptotic effect is mostly due to vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin-like growth factor 1 (IGF-1), TGF-β and granulocyte macrophage colony-stimulating factor (GM-CSF). The same molecules, in addition to phosphatidylinositol-glycan biosynthesis class F protein (PIGF), MCP-1 (or CCL2), basic fibroblast growth factor (bFGF) and IL-6 also stimulate local angiogenesis, which is particular important during tissue re-organization. Mitosis of tissue-intrinsic progenitors or resident stem cells has been demonstrated to be activated by the secretion of stem cell factor (SCF), leukemia inhibitor factor (LIF), macrophage colony-stimulating factor (M-CSF), stromal cell-derived factor 1 (SDF-1) and angiopoietin-1 (ANG-1) (Girolamo et al. 2013).

2.5.3 Membrane proteins

The membrane proteins here referred are CD44, CD54, CD106, Cd166, PD-L1 and PD-L2. Special reference is made due to their important role in the immunomodulatory activity of MSCs.

2.5.3.1 CD54 and CD106

CD54 and CD106, also known as Inflammatory Cytokine-Induced Intracellular Adhesion Molecule-1 (ICAM-1) and Vascular Cell Adhesion Molecule-1 (VCAM-1), are membrane surface molecules that may be critical in the immunomodulatory effect of the MSCs (Ren et al. 2010). They are known to mediate the cell adhesion that is a possible critical factor in the MSCs immunossuppressive action. These two molecules are inducible by the concomitant presence of IFN-γ and TNF-α (or IL-1) and are required for lymphocyte-MSC adhesion, playing an important role in MSC-mediated immunossuppression.

CD54 is weakly expressed in leukocytes, epithelial an resting endothelial cells, as well as other cell types. When cells are not under a pro-inflammatory environment, the level of expression is negligible and increases when the pro-inflammatory cytokines are added. It is also believed to be a costimulatory receptor that depends upon cell identity; during MHC class I restricted antigen presentation, CD54 acts as a signaling molecule when expressed on APCs; however on CD8+T cells it behaves as a costimulatory receptor (Sheikh & Jones 2008).

CD106 (VCAM-1) is activated by endothelial cells and certain leukocytes such as macrophages. There is also a study where they describe CD106 as a subpopulation of MSCs with "*unique immunoregulatory activity*" (Yang et al. 2013).

2.5.3.2 CD166

CD166, also known as ALCAM, is a transmembrane glycoprotein member of the immunoglobulin superfamily of proteins. It is a positive selection marker for MSCs and it is also known to be expressed on activated T cells and monocytes and also to play a role in mediating adhesion interactions between thymic epithelial cells and $CD6^{+}$ cells during intrathymic T cell development (Biolegend 2014).

2.5.3.3 CD44

CD44 is a cell surface glycoprotein that is involved in cell-to-cell interaction, migration and adhesion. It is an adhesion molecule involved in leukocyte attachment to and rolling on endothelial cells, homing to peripheral lymphoid organs and to the sites of inflammation, and leukocyte aggregation. An increase in levels of CD44 is a hallmark of T-cell activation. The CD44-hyaluronic acid (HA) interaction is used for activated T-cell extravasation into sites of inflammation. Migration of lymphocytes to inflammatory tissues is regulated by cytokines, growth factors, ant their receptors (Zhu et al. 2006).

2.5.3.4 PD-L1 and PD-L2 (CD274 and CD273)

Programmed death 1 (PD-1) is a transmembrane protein that is believed to be involved in the suppression of the immune system. Direct contact of MSCs and target cells can lead to the inhibition

of cell proliferation via engagement of PD-1 to its ligands PD-L1 and PD-L2, leading to the target cells to modulate the expression of different cytokine receptors and transduction molecules for cytokine signaling and allowing, for example, the inhibition of T-cell receptor-mediated lymphocyte proliferation. PD-L1 is widely expressed on APCs and hematopoietic cells, whereas PD-L2 is only expressed on macrophages and DCs (Augello et al. 2005).

PD-L1 (or CD274) is a member of the B7 family of immune proteins that provide signals for both stimulating and inhibiting T cell activation. Its interaction with PD1 inhibits T-cell proliferation and cytokine production (Biolegend 2014; R&D Systems 2014; Augello et al. 2005).

PD-L2 (or CD273) is also a member of the B7 family of proteins that provide signals for regulating T-cell activation and tolerance; it inhibits T cell responses via PD-1 binding, stimulates T cells via alternative receptor binding and promotes tumor immunity (Biolegend 2014; R&D Systems 2014; Augello et al. 2005).

2.6 ImmuneSafe®

ImmuneSafe® (IS) is an Advanced Therapy Medicinal Product (ATMP) developed by Cell2B and based on MSCs with the purpose to treat several immune and inflammatory diseases such as GvHD. Upon stimulation by a pro-inflammatory environment, ImmuneSafe® modulates the responses of body's immunological system and promotes tissue regeneration by the production of soluble factors such as cytokines. MSCs for the production of ImmuneSafe® are obtained from healthy donors with their previous consent. Cells are expanded in a completely animal-origin free process and cryopreserved. After a final preparation they are ready to be sent to the hospitals and administered to the patients.

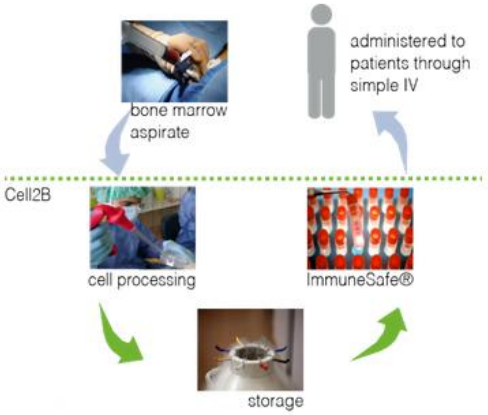

Figure 7 - ImmuneSafe® production. (Cell2B 2014)

3 Materials and Methods

3.1 Bone Marrow Samples

Human bone marrow samples were obtained either from Instituto Português de Oncologia de Lisboa Francisco Gentil (IPOLFG), Instituto Português de Oncologia do Porto (IPO-Porto) or from a commercial source (Lonza®, USA). All the samples were collected by authorized personnel and after informed consent from healthy donors.

3.2 Bone Marrow Mononuclear Cells Isolation Procedure

Bone Marrow Mononuclear Cells (BM-MNC) isolation was performed using Sepax (BioSafe) system.

The Sepax cell processing system uses a rotating syringe technology that provides both separation through rotation of the syringe chamber (centrifugation) and component transfer through displacement of the syringe piston.

The system allows the automated processing of blood component in a functionally-closed and sterile environment and consists in a Sepax Main Unit and axial displacement drive to the chamber on the single-use separation kit, as well as drive to the directional valves, and also a single-use kit that will keep the sample in a sterile environment during the complete operation. It uses Ficoll (GE Healthcare®) to separate the low density BM-MNC, and a saline solution (NaCl, Labesfal) to wash the cells.

At the end of the process, the kit was removed from the Sepax and the sample was collected from the output bag and washed with DPBS 1x (Gibco®). The cell suspension was centrifuged for 7 minutes at 1500 rpm. The supernatant was discarded, cells were resuspended in StemPro® MSC SFM XenoFree (Gibco®) and cell number and viability was determined using the trypan blue staining method. The cells were then cryopreserved or cultured in 6-well plates previously coated with Cell2B proprietary ECM, at 37ºC, 5% CO_2 in a humidified atmosphere. After 72 hours the non-adherent cell fraction was removed and medium was renewed every 4 days.

3.3 Cell culture

Upon reaching a 70-80% confluence, the culture medium was carefully removed and discarded and cells were washed with DPBS 1x. Cells were then incubated with TrypLE^{TM} (Gibco®) for 7 minutes at 37ºC. Afterwards the cell suspension was diluted 3x in culture medium, centrifuged for 7 minutes at 1250 rpm and resuspended in culture medium. Cell number and viability were determined using the Trypan Blue Exclusion Method. Cells were then seeded within a density range of 3000 to 6000 cells/cm^2.

3.4 Cryopreservation and Cell Thawing of MSCs

After harvesting cells and centrifugation, cells were resuspended in cryopreservation medium, Synth-a-Freeze™ (Gibco®), transferred to cryovials and stored in liquid nitrogen (-196°C).

Cells were thawed by quickly warming the cryovial in a water bath at 37ºC and diluting the cell suspension 10x in culture medium. The cell suspension was centrifuged at 1250 rpm for 7 minutes. The cells were then resuspended in culture medium and cell number and viability were determined.

3.5 Immunophenotype Characterization

In order to prepare the cells for immunophenotypic analysis, the cell suspension was centrifuged for 7 minutes at 1250 rpm and resuspended in DPBS. 100 µl of the homogeneous cell suspension was added to each tube and the corresponding fluorochrome-conjugated antibodies were administered to the tubes (5 or 10 µl, according to the concentration of the antibody solution). Appropriate isotype controls were used in every experiment. The panel of antibodies consists on: CD11 (clone ICRF44), CD13 (clone WM15), CD14 (clone M5E2), CD19 (clone 6D5), CD29 (clone TS2/16), CD31 (clone WM59), CD34 (clone 581), CD44 (clone BJ18), CD45 (clone HI30), CD49d (clone 9F10), CD54 (clone HCD54), CD73 (clone AD2), CD80 (clone 2D10), CD90 (clone 5E10), CD105 (clone 43A3), CD106 (clone STA), CD120a (clone 55R-286), CD120b (clone TR75-89), CD146 (clone SHM-57), CD166 (clone 3A6), CD271 (clone ME20.4), CD273 (clone MIH18), CD274 (clone 29E.2A3), CD309 (clone HKDR-1), HLA-ABC (clone W6/32), HLA-DQ (clone HLADQ1), HLA-DR (clone LN3), HLA-G (clone 87G) and Stro-1 (STRO-1) (all purchased from BioLegend). The mixture was incubated for 15 minutes in the dark, at room temperature. To wash the excess of antibody following staining, 2 mL of DPBS were added to each tube and the tubes were then centrifuged for 7 minutes at 1200 rpm. The cell pellet was resuspended in 400-500 µl of 2% paraformaldehyde (PFA) in order to fix the cell-antibody mixtures and the tubes were stored at 4°C in the dark. The samples were run in FACSCalibur equipment and analysed using Flowing Software 2 (Perttu Terho (Turku Centre for Biotechnology, University of Turku, Finland)).

3.6 Pro-inflammatory stimulation

Some of the cultures were grown with no stimulation while others were stimulated when they reached 60%-70% confluency with interferon gamma (IFN-γ, 500 U/mL) and tumor necrosis factor alpha (TNF-α, 10 ng/mL), both pro-inflammatory cytokines. When stimulated, cells were incubated for 48h at 37ºC, 5% CO_2. After incubation, media was removed, aliquoted and stored at -20ºC until further analysis. Cell counting was performed and cells were immediately analyzed or stored at -80ºC until further analysis.

For the activation profile experiments, cells were also stimulated with: Lipopolysaccharides (LPS, Sigma-Aldrich®)-1µM, interleukin-2 (IL-2, R&D Systems™)- 25µM, Interleukin-12 (IL-12), IFN-γ+TNF-α+ IL-2, TNF-α+IL-1, TNF-α+IL-1+IL-6. The last three cytokines (IL-12, interleukin-6 (IL-6) and

interleukin-1 (IL-1)) were obtained from the R&D Systems™ and the concentration used in the assays were the same, 10 ng/mL.

3.7 Cytokine Production Analysis

The production of cytokines under the different culture conditions was evaluated by analyzing the exhausted medium (stored at -20ºC). The analysis was performed by either Enzyme-linked immunosorbent assays (ELISA for PGE-2 and IL-6) and using the Quantibody Human Angiogenesis Array 1000 (external service provided by Tebu-bio).

3.7.1 Prostaglandin E2 (PGE2) analysis

The DetectX® PGE2 High Sensitivity Immunoassay Kit was used to quantitatively measure PGE2 present in the exhausted medium from the different culture conditions. All reagents from the kit were prepared according to manufacturer's indication.

100 µl of each sample or standard was added to each well of the 96-well plate. Then, 125 µl of the Assay Buffer was added to the non-specific binding (NSB) wells, while 100 µl of the same buffer were added to the wells to act as maximum binding wells. 25 µl of the DetectX Prostanglandin E2 conjugated were added to each well, as well as 25 µl of the DetectX Prostaglandin E2 High Sensitivity Antibody (exception made for the NBS wells). Then, the plate is covered and shaken for 15 minutes at room temperature, after what it is incubated for 16 hours at 4°C. After incubation, the solution was discarded and the wells were washed 4 times with 300 µl of the wash buffer and blotted against clean paper to remove the remaining wash buffer. After this, 100 µl of TMB Substrate Reagent was added to each well and the plate was incubated for 30 minutes at room temperature. In the end of the incubation time, 50 µl of Stop Solution is added to each well and the absorbance is read at 450 nm (PowerWave™ XS, Bio-tek).

The concentration of PGE2 was calculated using the absorbance read at 450 nm and the standard curve.

3.7.2 Interleukin-6 (IL-6) analysis

The RayBio® Human IL-6 ELISA kit is an *in vitro* enzyme-linked immunobsorbent assay. The DetectX® PGE2 High Sensitivity Immunoassay Kit was used to quantitatively measure IL-6 present in the exhausted medium from the different culture conditions. All reagents from the kit were prepared according to manufacturer's indication.

100 µl of each sample or standard were added to the corresponding well and the plate was then covered and incubated for 2.5 hours at room temperature with gentle shaking. After incubation, the solution was discarded and the well were washed 4 times with wash solution and finally the plate was inverted and blotted against clean paper in order to remove any remaining wash buffer. 100 µl of the previously prepared biotinylated antibody was added to each well and incubated for 1 hour at room temperature with gentle shaking. The solution was discarded and the wells were washed 4 more

times. After the second washing step, 100 µl of the Streptavidin Solution previously prepared was added to each well and incubated for 45 minutes at room temperature with gentle shaking. The solution was discarded and the wells were washed 4 more times. After this, 100 µl of TMB One-Step Substrate Reagent was added to each well an incubated for 30 minutes at room temperature in the dark with gentle shaking. After the incubation, 50 µl of Stop Solution was added to each well and the absorbance was read at 450 nm immediately (PowerWave^{TM} XS, Bio-tek).

The concentration of IL-6 was calculated using the absorbance read at 450 nm and the standard curve.

3.8 Immunossupressive studies

The immunossupressive studies were carried out at Centro de Histocompatibilidade de Coimbra (CHC), Instituto Português do Sangue e Transplantação. The objective was to quantify the level of suppression of TNF-α and IL-17 production by T-lymphocytes and NK cells induced by MSCs in a co-culture system.

3.8.1 PBMC isolation

Peripheral blood mononuclear cells (PBMC) were isolated from the peripheral blood samples obtained from healthy donors after informed consent. The blood samples in heparin were diluted in physiological serum (1:2) and layered over Lymphoprep (StemCell Tecnhologies, Vancouver, Canada) in a ratio of 2:1 (diluted blood:Lymphoprep). The mixture was centrifuged at 1200g for 20 minutes and at 18°C. The mononuclear layer, between the Lymphoprep and the plasma layers, was aspirated into a Falcon tube (Beckon Dickinson Bioscience, BD) and washed with Hank's Balanced Salt Solution 1x (HBSS). The mixture was centrifuged for 15 minutes at 430g and 18°C and the pellet was resuspended in 1 mL of RPMI 1640 (Gibco). Cell number and viability were determined by the Trypan Blue Exclusion Method.

3.8.2 Co-culture system

The co-culture of PBMC and allogeneic human MSCs was performed in a 24-well plate (TPP, Sweeden) in a ratio of 1:2 (250 000 MSCs and 500 000 MNCs) and in a RPMI 1640 (Gibco) with 10% FBS for 24h, at 37°C with 5% CO_2 and 90% humidity. After incubation cells were activated, or not, with 50 ng/mL of Phorbol 12-myristate 13-acetate (PMA) (Sigma, Saint Louis, MO, USA), 1µg/mL of Ionomycin (Boehringer Mannheim, Germany) and 10 µg/mL of Breferdin A (Golgi plug-Sigma, Saint Louis, MO, USA) during 4 hours in the same environment.

Two different conditions were tested: MNC+PMA+Ionomycin and MNC+PMA+Ionomycin+MSC.

For the functional characterization study of T cell subpopulations through pro-inflammatory cytokine production, the following monoclonal antibodies were added to a 250 µl sample: CD3 Pacific Blue (clone UCHT1. BD Pharmigen, San Diego, CA, EUA), CD8 Allophycocyanin Elite 7 (clone SK1, BD, San José, CA, EUA), CD4 Phycoerythrin Cyanine 7 (clone SFCI12T4D11, Beckman Coulter, Brea,

EUA), CD27 Phycoerythrin Cyanine 5 (clone 1ª4CD27, BC, Brea, EUA), CD45Ra Allophycocyanin (clone HI100, BD, San José, EUA). After the addition of the antibodies, the tubes were incubated in the dark for 10 minutes and 100 µl of the Fix reagent from the Intraprep™ kit (Beckman Coulter, Brea, EUA). The mixture was incubated for 10 more minutes and after that time the cells were washed with 2 mL of DPBS (Gibco) and centrifuged for 5 min at 430g. The pellet was resuspended in 100 µl of the permeabilization agent from the Intraprep™ kit and incubated in the dark for 10 minutes. The intracytoplasmic monoclonal antibodies TNF-α Fluorescein isothiocyanate and IL-17 Phycoerythrin were added and the mixture was incubated for 10 minutes. Cells were washed again with DPBS 1x (Gibco) and centrifuged for 5 minutes at 430g. In the end, cells were resuspended in 250 µl of DPBS (Gibco) and acquired in a flow cytometer FACS Canto™ II (BD) using the software FACSDiva 6.1.2 (BD). Data were afterwards analyzed with the software Infinicyt 1.5 (Cytognos, Salamanca, Spain).

3.9 Western Blot analysis

The detection of Indoleamine 2,3-dioxygenase (IDO) was performed by Western Blotting at the Analytical Services Unit of IBET laboratories (Oeiras, Portugal).

Cell lysates were prepared using RIPA buffer (Sigma) supplemented with complete protease inhibitor cocktail (Roche). The mixture was kept on ice for five minutes and the lysate obtained was stored at 4°C for further analysis. Anti-IDO clone 10.1 (Merck Millipore) at a 1:5000 dilution was used to detect protein expression on whole cell protein extracts isolated from MSCs by immunoblot analysis.

3.10 Immunomodulatory pathways blocking studies

For the blocking studies, different strategies were used for the two main immunomodulatory pathways. For all the assays, positive and negative controls were used.

3.10.1 Metabolic Enzymes

The purpose was to block the enzymatic activity of one metabolic enzyme: indoleamine-2,3-dioxygenase (IDO). IDO was blocked using two stereoisomers with potentially different biological properties, 1-methyl-D-tryptophan (1-MT-D-T, Sigma.Aldrich®) and 1-methyl-L-tryptophan (1-MT-L-T, Sigma.Aldrich®), each one at the concentration of 2mM.

After incubation at 37ºC, 5% CO_2, the exhausted media was stored at -20ºC to be later on analyzed and cell lysates were prepared for western blot analysis. Cells were also sent to CHC for immunossupressive studies.

3.10.2 Membrane Proteins

For the membrane proteins study, six antibodies were used in order to block these proteins: anti-CD44, anti-CD54, anti-CD106, anti-CD166, anti-PD-L1 and anti-PD-L2. The antibodies were all obtained from R&D Systems™. Upon reaching a 60-70% confluency, cells were stimulated with the pro-inflammatory cytokines as described above. Afterward the exhausted media was removed and

stored for further analysis and cells were detached from the culture vessel and transferred into falcon tubes were they were incubated with the blocking antibodies in culture media, at room temperature and in the dark for 60 minutes. At the end of the incubation time, cells were centrifuged at 1250 rpm for 7 minutes and the usual immunophenotype characterization protocol was performed (including controls). Immunossupressive studies were also performed in three different combinations: 1) a "cocktail" with the blocking antibodies for adhesion molecules CD106, CD54, CD66 and CD44; 2) another one with only the blocking antibodies for the PD-L1 and PD-L2 and 3) a cocktail with blocking antibodies for all membrane proteins. The concentrations for each of the antibodies were the following: anti-CD44-20 µM; anti-CD54-25 µM; anti-CD106-25 µM; anti-CD166-25 µM; anti-PD-L1-50 µM and anti-PD-L2-30 µM.

4 Results and Discussion

In this chapter, the results from the studies performed during this work are presented. Section 4.1 focuses on the comparison between ImmuneSafe® and an MSC-derived product previously used in clinical studies. Section 4.2 is related to the mechanism of action of ImmuneSafe® in which several immunomodulatory pathways were blocked and the immunossuppressive potential was tested. Finally, in section 4.3, the activation profile of ImmuneSafe® is analyzed under several pro-inflammatory environments which are believed to be involved in the development of GvHD.

4.1 ImmuneSafe® benchmarking with MSCs with proven clinically efficacy

The goal of the first part of this project was to benchmark the ImmuneSafe® against other cell products that were previously used in clinical trials/studies. The usual procedure with new therapeutic approaches is to do the benchmark with similar products that are already being commercialized; however only one cell-based product is currently approved for the treatment of pediatric GvHD in Canada and New Zealand (Prochymal®) and prescription is required.

In 2007 a consortium was established between Instituto de Biotecnologia e Bioengenharia-Instituto Superior Técnico (IBB-IST), IPO Francisco Gentil Lisboa and Centro de Histocompatibilidade do Sul (CHS) focused on developing an *ex vivo* isolation and expansion procedure of human MSCs under Good Manufacturing Practices (GMP) conditions for cellular therapy.

Seven patients received expanded allogeneic MSC for the treatment and prevention of GvHD. Three patients were treated for aGvHD, one for cGvHD and the last three received a co-infusion of MSC with an hematopoietic cell transplant (HCT).

Two of the patients treated for aGvHD showed a complete response and the third patient died shortly after cell infusion due to MSC non-related complications. The success rate is of 66%, which is similar to the results described in other clinical studies (Le Blanc et al. 2008; Ringdén et al. 2006). The patient treated for cGvHD only had a partial response which is also in agreement with the data found in the literature (Perez Simon et al. 2011).

In the cases of co-infusion with HCT, patients received MSC infusions at day 0 and four days later. The results indicate that co-infusion of MSC enhance hematopoietic engraftment and may be effective in preventing the onset of GvHD post-HCT; only one of the three patients developed grade I skin GvHD.

Due to the positive clinical outcome of these studies, MSC IPO were used to benchmark ImmuneSafe® in this study.

MSC isolated from three donors used in the clinical study (referred as MSC IPO in this chapter) were used to compare with ImmuneSafe® through a series of characterization studies: immunosuppressive activity, immunophenotype characterization and production of cytokines and metabolic enzymes.

For confidentiality reasons, the results in this section are not presented.

4.1.1 Conclusion

The analysis of the immunophenotype, immunosuppressive activity, secretome and metabolic enzymes of the MSC IPO and the posterior comparison of the results with the ones obtained for ImmuneSafe® allowed some conclusions to be taken. Since the results appeared to be similar, it is safe to say that MSC IPO are now fully characterized MSCs with the characteristics described in the literature. Besides the immunophenotype, all the properties were similar but ImmuneSafe® appeared to have a potency superior to MSC IPO. This conclusion comes essentially from the results of the immunosuppressive studies and also from the western blot analysis. The immunosuppressive capacity of ImmuneSafe® surpassed the results obtained for MSC IPO and the qualitative analysis of the production of IDO seemed to detect increased levels of protein on ImmuneSafe®'s extracts than with MSC IPO. This conclusion is only qualitative and a quantitative analysis of IDO expression would be required.

Since there is no information available concerning the other cell products in the market for the benchmark, MSC IPO were used for that purpose. ImmuneSafe® and MSC IPO demonstrated similar results with ImmuneSafe® surpassing MSC IPO in the potency tests. From this analysis, it can be indirectly assumed that ImmuneSafe® have clinical potential for the treatment of several immune diseases and, in particular, GvHD.

4.2 Immunomodulatory pathways blockage studies

Although the mechanism of action of MSCs for immunological and inflammatory diseases has been under intense study, it is not yet fully understood (Nauta & Fibbe 2007; Horwitz & Prather 2009; Kim & Cho 2013; Yi & Song 2012). With the aim of gaining a more in-depth knowledge of ImmuneSafe®'s MoA, this study consisted in blocking several pathways believed to be involved in their immunomodulatory properties and then evaluate its impact on the product's immunosuppressive capacity. A specific strategy was used to block the different immunomodulatory pathways.

For confidentiality reasons, the results in this section are not presented.

4.2.1 Conclusion

The results obtained for the immunomodulatory pathways blockage study are only a very preliminary analysis of the mechanism of action of ImmuneSafe®. In order to obtain definite conclusions, it would be necessary to run several additional experiments.

The more obvious are related to the blocking agents concentrations; the results show that, generally, the blockage was not achieved, at least not totally. The tests would have to be repeated with several

blocking agents concentrations until the blockage was total and confirmed by the appropriate technique. After the confirmation, it would be necessary to repeat the immunosuppressive studies and interpret the results. Additionally, after a successfully achieved blockage, RT-PCR or other tests at the mRNA level would be of extremely importance for a more precise investigation; sRNA, for example, could be used to block the production of the proteins at the gene level.

IDO appears to be one of the most important "players" in the immunomodulatory activity of MSCs. Even though the blockage was not achieved, at least not totally, the addition of one of the blocking isoforms abolished partially the percentage of immunosuppression by the cells. CD106 and CD166 may also have an important role although more tests would be necessary to confirm this supposition. Once again, a quantitative analysis for the production of all the proteins would be of utmost importance.

Since a potency test would be of utmost importance at a production level, the result here obtained together with the results described in the literature and the further investigation required, nonetheless, IDO appears to be a strong candidate.

4.3 Impact of several stimuli in the immunomodulatory profile of ImmuneSafe®

The onset and development of GvHD has been well studied and it is characterized by different stages (see Figure 3 in section 2.3, (Reddy & Ferrara 2003)). With the goal of understanding the impact of the different disease stages on ImmuneSafe's immunomodulatory activity (namely the activation of the different immunomodulatory pathways), cells were stimulated with different combinations of the cytokines that characterize the GvHD development stages: TNF-α, IFN-γ, LPS, IL-2, IL-12, IFN-γ+TNF-α+IL-2, TNF-α+IL-1 and TNF-α+IL-1+IL-6.

For confidentiality reasons, the results in this section are not presented.

4.3.1 Conclusion

In this chapter the results for the impact of several inflammatory environments in ImmuneSafe® were evaluated. The purpose was to mimic the *in vivo* environment to which the cells are subjected through the development of GvHD and identify the responses of the cells to those environments.

The results here presented suggested that ImmuneSafe® responds differently to the pro-inflammatory environments to which it was subjected. These responses will affect its therapeutic action through the release of several cytokines and other soluble factors.

Overall, these results suggested that IFN-γ is the pro-inflammatory cytokine that triggers a higher number of responses by ImmuneSafe® when added alone to the cell cultures. The most pro-inflammatory environment, however, appeared to be the one caused by the simultaneous addition of IFN-γ and TNF-α which was already expected (François et al. 2012).

Understanding the environments that trigger ImmuneSafe®'s immunomodulatory mechanism is extremely important for its clinical application and together with further experiments, these analysis could contribute for that knowledge.

5 Conclusions and Future Work

The aim of this thesis was to study, using a systematic approach, the Mechanism of Action of ImmuneSafe® and compare it with other similar cellular products. The work performed in this thesis was divided in three parts: *i)* benchmarking of ImmuneSafe® with a cellular product previously used in clinical studies and with positive results in avoiding and treating GvHD, *ii)* studying the MoA of ImmuneSafe® by depicting the role of its different immunomodulatory pathways (metabolic enzyme, and membrane surface proteins) and *iii)* analyze the activation profile of MSCs by stimulating the cells with several pro-inflammatory cytokines and studying the alterations in their membrane surface proteins expression, metabolic enzymes production and secretome.

The cells used for ImmuneSafe® benchmarking were from the clinical study performed in IPO Lisboa. The usual comparison with other cell products already in the market was not possible since there was no available information of the same. These cells (MSC IPO) have already been administered to patients and the overall results in avoiding and treating GvHD were extremely positive. Since these MSC IPO have never been fully characterized, it was important to perform the same studies as ImmuneSafe® was subjected in order to characterize them and to study their therapeutic potency. The purpose was also to compare the results obtained and extrapolate the efficacy of ImmuneSafe® in a clinical setting. The results obtained with MSC IPO were similar to those obtained for ImmuneSafe®; however ImmuneSafe® seems to be more potent, especially in what concerns the immunosuppressive potential and the IDO production. The conclusion from this analysis is that ImmuneSafe® have a great clinical potential for the prevention and treatment of GvHD and other immune diseases, although further *in vitro* and clinical tests are still required.

The second part of this thesis was based on the study of ImmuneSafe® MoA and consisted in blocking several pathways believed to be involved in the immunomodulatory properties of the cells and evaluate their immunosuppressive capacity. For that purpose, six membrane proteins believed to have a role on ImmuneSafe® immunomodulatory ability were blocked using the respective monoclonal antibodies and their level of expression was analyzed together with the MFI. When the adhesion proteins were blocked, the immunosuppressive activity decreased, especially when the production of TNF-α was analyzed; since only CD106 and CD166 were blocked, this decrease in the immunosuppressive capacity of the cells may be related to one or both of the proteins. The FACS analysis showed that the full blockage of the adhesion molecules was not achieved (only CD273 was partially blocked) and the immunossuppressive capacity did not decreased proving that the blockage level of CD273 was not enough to diminish that capacity.

This study of the immunomodulatory pathways would have to be repeated using a higher number of samples and also with several blocking agents concentrations until the blockage was totally confirmed by western blot, ELISA or other more specific method. Other blocking methods could also be used such as sRNA that would block the production of proteins at the gene level. Absolute conclusions cannot be taken from this study without further tests, but from all the conditions tested, IDO appears to

be one of the most important players in the immunomodulatory activity of MSCs (Gieseke et al. 2007; Schmidt et al. 2012; Qian et al. 2009; Horwitz & Prather 2009). There are still a series of problems regarding the use of MSCs for clinical trials. The fact is that it is still missing a potency test that would allow the comparison of cells obtained from different donors and different sources; MSCs are an heterogeneous population and they may present major differences depending on the way they are isolated and growth. Developing a potency test would be extremely important for the production and clinical application of ImmuneSafe® and the results here obtained, together with the results described in the literature, indicate that IDO can be a strong candidate for the development of such test.

The last part of this thesis was related to the activation profile of MSCs. The effect of the stimulation of the cells with IFN-γ and TNF-α have already been studied; since the focus of ImmuneSafe® is the treatment and prevention of GvHD, it become necessary to study the *in vitro* behaviour of the cells when stimulated with cytokines believed to be the ultimate causatives of the disease. To begin, four conditions were analysed (TNF-α, IFN-γ, LPS and IL-2) for their immunophenotype, metabolic enzymes production and secretome (only TNF-α, IFN-γ and IL-2). Later, four new conditions were tested only for their immunophenotype (IL-12, IFN-γ+TNF-α+IL-2, TNF-α+IL-1 and TNF-α+IL-1+IL-6).

All the pro-inflammatory environments triggered the up regulation of chemokines, one of the most important mediators in the immunomodulatory mechanism of ImmuneSafe®. When both IFN-γ and TNF-α were added to the cell culture, there was also up regulation of IL-17, two matrix metalloproteinases, all the hematopoiesis related proteins, cytokines involved in the inflammatory response and cell metabolism and the only protein tested involved in cell migration which was never expressed in the other conditions. This highlights the important of ImmuneSafe® in a pro-inflammatory environment and gives an idea of the main "actors" in this response.

There is still a lot of work left to do in this area. The main problem still resides in understanding how this cells act *in vivo; t*he *in vitro* tests are crucial to give the first steps but are not enough. A potency test would be one of the most important achievements for the clinical application of ImmuneSafe® and any other cell product. Understanding the mechanism of action of the cells and which are the factors that trigger its responses would be the first step. This thesis presents some methods to study the MoA and the activation profile of ImmuneSafe®, however more specific tests would be necessary together with a higher number of samples tested (MSCs from several donors). As previously referred, tests at the DNA or RNA level would lead to more conclusive and trustful results and conclusions, *i.e.*, RT-PCR, sRNA, microarrays and others.

Throughout this work, ImmuneSafe® had its clinical potential evaluated, as well as its MoA and its activation profile. Altogether the results highlighted the potential of this cell-based product in the treatment of immunological diseases and, in particular, GvHD. Clinical tests are now essential in order to confirm ImmuneSafe®'s potential and also to determine the best administration regime.

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