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Developing an Advanced Therapy Medicinal Product (ATMP) for the treatment of GvHD

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Abstract

Mesenchymal stem/ stromal cells (MSCs) have been proved to be capable to modulate the immune system through direct interactions target cell-MSC and secretion of soluble molecules that are induced or upregulated following cross-talk with target cells.

In this study, a full biological characterization of ImmuneSafe® (IS) features including identity, potency and safety which constitute the Critical Quality Attributes (CQAs) of the product was performed with purpose of providing tools that will assure the consistency and robustness of the manufacturing process or demonstrating product/ process comparability after a particular change in the manufacturing process. In order to achieve this goal a (bio) assay panel was developed and applied to IS in two different steps of manufacturing process. IS CQAs allowed a robust and reproducible characterization of the product, demonstrating their potential to be used throughout the production stage. Similar studies were also performed with similar cell types, such as human skin fibroblasts and MSCs differentiated in adipocytes and osteocytes, which were then benchmarked with IS. The results showed that none of these cell types demonstrated a comparable level of therapeutic potency to IS.

The patient enrolling protocol for IS clinical trial to treat GvHD will include the administration of immunosuppressive drugs (methylprednisolone or prednisolone) concomitantly with IS administration. The impact of these drugs on IS identity was evaluated through immunophenotype characterization and potency was evaluated through the activation of the different immunomodulatory pathways. The results showed that immunosuppressive drugs tested, methylprednisolone and prednisolone, did not seem to have a beneficial or detrimental interaction with IS.

MSCs are commonly stored in cryopreservation conditions before the deliver to the patient. However, recent studies have shown that banked fresh thawed MSCs have impaired immunomodulatory properties compared to MSCs in culture. IS response to an inflammatory microenvironment was compared in different times of release and fresh thawed cells revealed to have several responses compromised under pro-inflammatory environment. Additionally, IS secretome was also affected, since the production of several cytokines were decreased or even switched off, as well as the immunosuppressive activity of the product. For these reasons the implementation of a release culture step was found to be advantageous in order to maximize the therapeutic potency of IS.

Another important issue for cell-based therapies is the product delivery to the hospital. Cells should be formulated in a suitable excipient for intravenous infusion capable of maintaining the cell viability and therapeutic potential during the purposed product shelf-life. For this purpose

IS was formulated in a saline solution and identity and potency tests were performed. Hypothermosol was capable of maintaining at least 70% of initial cell number population with 80% of viability, as well as its identity and potency features within a 48h-window.

These studies enable a comprehensive IS characterization and the set-up of the assays to be used in the manufacturing process under GMP conditions. Additionally, no detrimental effects on the therapeutic potency of IS were associated with the interactions with the immunosuppressive drugs that will be used in the clinical trial, thus indicating the clinical results will not probably be affected by the background therapy applied to patients. The process of product release was also optimized to guarantee a cell product with maximized immunomodulatory properties and a 48-hour shelf-life was determined, which is a critical issue for the planning of IS logistics for the clinical trial.

Keywords:

Mesenchymal stem/ stromal cells, Critical Quality Attributes, immunosuppressive drugs, product release protocol, shelf-life.

Resumo

A capacidade das células estaminais do mesênquima/ estroma (MSCs) em modular o sistema imune através da interação direta entre células alvo - MSCs e através da secreção de moléculas solúveis tem vindo a ser demonstrada recentemente.

Neste estudo, foi feita uma completa caracterização do ImmuneSafe® (IS), que incluiu identidade, potência e segurança, que constituem os Atributos Críticos de Qualidade (CQAs) do processo produtivo, desenhados com o propósito de fornecer ferramentas que irão assegurar a consistência e robustez do processo de produção ou permitir a demonstração de alguma alteração especifica no produto/ processo durante o processo de fabrico. Para tal foi desenvolvido um painel de (bio) ensaios que foram aplicados ao IS em dois diferentes passos do processo produtivo. Os CQAs do IS permitiram uma robusta e reprodutível caracterização do produto, demonstrando o seu potencial uso aquando da fase de produção. Os mesmos ensaios foram feitos também com células semelhantes, como fibroblastos humanos e MSCs diferenciadas em adipcitos e osteócitos, com o objectivo de fazer *benchmarking* do IS. Os resultados mostraram que nenhum destes tipos de células tem um potencial terapêutico comparável com o IS.

O protocolo de recrutamento de pacientes para ensaio clínico do IS para tratar o GvHD, incluirá a administração de drogas imunossupressoras (metilprednisolona ou prednisolona) concomitantemente com a administração do IS. O impacto das drogas na identidade do IS foi avaliada através da caracterização imunofenótipica e a potência foi avaliada por meio da ativação de diferentes vias imunomoduladoras. Os resultados mostraram que as drogas imunossupressoras testadas, metilprednisolona e prednisolona, não parecem ter uma interação benéfica nem prejudicial com o IS.

Por norma as MSCs estão criopreservadas antes de serem injetadas no paciente. Contudo, estudos recentes demonstraram que MSCs descongeladas tem propriedades imunomoduladoras comprometidas em comparação com MSCs em cultura. A resposta do IS a um ambiente inflamatório foi comparado em diferentes tempos de libertação e as células descongeladas revelaram ter várias respostas comprometidas em ambiente pro-inflamatório. Além disso, secretoma das MSCs também foi afetado, uma vez que a produção de várias citocinas foi reduzida ou mesmo desligada, bem como a sua atividade imunossupressora. Por estas razões, um passo de libertação em cultura demonstrou ser vantajoso a fim de maximizar a potência terapêutica do IS.

Outra questão importante para as terapias baseadas em células é o envio do produto para o hospital. As células devem ser formuladas num excipiente apropriado para infusão intravenosa,

capaz de manter a viabilidade celular e o potencial terapêutico durante o período proposto de vida útil do produto. Para isso o IS foi formulado numa solução salina e posteriormente foram realizados testes de identidade e de potência. O Hypothermosol foi capaz de manter pelo menos 70% do número de células da população inicial com 80% de viabilidade, bem como as suas características de identidade e de potência dentro de um período de 48 horas.

Estes estudos permitiram uma caracterização exaustiva do IS e a definição dos ensaios a serem utilizados no processo de produção em condições GMP. Além disso, não se verificaram efeitos prejudiciais na potência terapêutica do IS associados à interação com drogas imunossupressoras que serão utilizadas no ensaio clínico, indicando assim, que os resultados clínicos provavelmente não serão afetados pela terapia envolvente aplicada aos pacientes. O processo de libertação do produto também foi otimizado de forma a garantir um produto celular com propriedades imunomoduladoras maximizadas e foi estabelecido um tempo de vida útil de 48 horas, que é uma questão crítica para o planeamento da logística do IS para o ensaio clínico.

Palavras-chave

Células estaminais do mesênquima/ estroma, Atributos Críticos de Qualidade, drogas imunosupressoras, libertação do produto, prazo de validade.

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List of abbreviations

AF	Amniotic Fluid
Ag	Antigen
AgRP	Agouti-related peptide
aGvHD	Acute GvHD
ALCAM	Activated leukocyte cell adhesion
ALP	Alkaline Phosphatase
AMT	Advanced medicinal therapy
ANG	Angiopoietin
ANGPTL4	Angiopoietin-like 4
APCs	Antigen presenting cells
AT	Adipose Tissue
ATMP	Advanced therapy medicinal product
bFGF	Basic fibroblast growth factor
BM	Bone Marrow
BMC	Bone marrow cells
CAT	Committee for Advanced Therapies
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
CFUs-F	Colony-forming units-fibroblastic
CGH	Comparative genomic hybridization
CQAs	Critical quality attributes
CTMPs	Cell therapy medicinal products
CTLs	Cytotoxic T lymphocytes
CXCL	Chemokine (C-X-C motif) ligand
DC	Dendritic cells

DP	Dental Pulp
DPBS	Dulbecco's Phosphate-Buffered Saline
EGF	Epidermal growth factor
EMA	European Medicines Agency
EPC	Endothelial progenitor cells
ESCs	Embryonic stem cells
Fb	Fibroblasts
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FISH	Fluorescence in situ hybridization
gDNA	Genomic Deoxyribonucleic Acid
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GMP	Good Manufacturing Practices
GTL	Geimsa/ Tripsin/ Leishman
GvHD	Graft-versus-Host-Disease
hBM	Human bone marrow
HB-EGF	Heparin-binding EGF-like growth factor
HG-EGF	Heparin-binding EGF-like growth factor
HBSS	Hank’s Balanced Salt Solution
HCT	Hematopoietic cell transplantation
HGF	Hepatocyte growth factor
HLA	Human leucocyte antigens
hMSC	Human mesenchymal stem cell
HO-1	Heme oxygenase -1
HSCs	Hematopoietic stem cells
HSCT	Hematopoietic stem cell transplant
HSP	Heat shock proteins

ICAM	Intracellular adhesion protein
IDO	Indoleamine -2 3-dioxygenase
IGF-1	Insulin-like growth factor 1
IL	Interleukin
IFN-γ	Interferon γ
IONO	Ionomycin
IPO	Instituto Português de Oncologia
IPC	In Process Control
IS	ImmuneSafe®
ISCT	International Society for Cellular Therapy
LAF	Leukocyte function-associated antigen-1
LIF	Leukemia inhibitory factor
LOH	Loss of heterozygosity
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinases
MNC	Mononuclear cell
6MPD	6-Methylprednisolone
MPA	Mycophenolic acid
mRNA	Messenger Ribonucleic Acid
MSC	Mesenchymal Stem Cell
PD	Prednisolone
MS	Muscle Tissue
NK	Natural killer cells
PECAM-1	Platelet/endothelial cell adhesion molecule
pDC	Plasmacytoid dendritic cells
PDGF	Platelet-derived growth factor

PD-L	Programmed death-ligand
PFA	Paraformaldahyde
PGE2	Prostaglandin E2
PIGF	Placental growth factor
PL	Placenta
PMA	Phorbol 12-myristate 13-acetate
SCF	Stem cell factor
SDF-1	Stromal cell-derived factor 1
SNPs	Single-nucleotide Polymorphism
TCR	T cell receptor
TGF-β	Transforming growth factor- beta
Tc	T cytotoxic cell
Th	T helper cell
Tie-1	Tyrosine kinase with immunoglobulin-like and EGF-like domains 1
TIMP	Tissue inhibitors of metalloproteinases
TNF-α	Tumor necrosis factor
TNFI	Tumor necrosis factor receptor type 1
TNFRII	Tumor necrosis factor receptor type 1
TPO	Thrombopoietin
Tregs	Regulatory T cells
TRM	Transplanted-related mortality
UCB	Umbilical Cord Blood
UCM	Umbilical Cord Matrix
uPAR	Urokinase-type plasminogen activator receptor
VCAM-1	Vascular cell adhesion
VEGF	Vascular endothelial growth factor
WB	Western-blot

1 Aim of studies

The aim of this thesis was the development of an Advanced Therapy Medicinal Product (ATMP), ImmuneSafe® (IS) for the treatment of Graft-versus-Host Disease (GvHD). IS has been developed by Cell2B and it is a cell therapy product, which is capable to modulate the responses of body's immunological system and promote tissue regeneration. This work is divided in 3 main studies: 1) Assay set-up for the definition of ImmuneSafe's Critical Quality Attributes (CQAs), 2) Immunosuppressive drug interactions with IS and 3) Release and Final Formulation of IS.

The purpose of CQAs study was to establish a (bio) assays panel that allow the determination of a full biological characterization of product features, including identity, potency and safety, which will enable a robust and reproducible characterization of IS and manufacturing process. The application of CQAs to control cell types, to different stages of the process and to different stress conditions was performed to detect similar discrepancies on the product/ process.

The study of immunosuppressive drugs interaction (methylprednisolone and prednisolone) with IS was determinant to verify its impact on the product features, because patients that will be recruited for IS clinical trial will be treated with methylprednisolone or prednisolone and it there is a possibility of these drugs affect the therapeutic potency of IS.

In the last part of this work, the aim was to optimize IS release step and delivery. The rationale was to determine if fresh thawed cells have impaired therapeutic potential when compared with fresh harvest cells from culture and determine the suitable time for release in culture. Relatively to ImmuneSafe's final formulation studies, the goal was to determine the product shelf-life and potency preservation after the release from the manufacturing site.

2 Introduction

2.1 Stem cells

Stem cell biology has attracted tremendous interest recently. It is hoped that will play a major role in the treatment of a number of incurable diseases via transplantation therapy [1].

Stem cells are unspecialized cells in the human body that are capable of becoming specialized cells, each with specialized cell function. In fact, stem cells are defined simply as cells meeting three basic criteria. Firsts, stem cells must be able to renew themselves throughout life, *i.e.*, the cells divide to produce identical daughter cells and thereby maintain the stem cell population. Second, stem cells must have the capacity to undergo differentiation to become specialized progeny cells. Stem cells that may differentiate into tissues derived from all three germ layer (ectoderm, endoderm, and mesoderm) are called "pluripotent". The best example of pluripotent stem cells are embryonic stem cells (ESCs) derived from inner cell mass of early embryos. In contrast with ESCs, most stem cells that have been well characterized are multipotent, *i.e.*, they have the capability of producing a limited range of differentiated cell lineages appropriate to their location. The third property of stem cells is that they may renew the tissue that they populate. All tissue compartments contain cells that satisfy the definition of "stem cells", and the rate at which stem cells contribute to replacement cells varies throughout the body [1]–[3].

Stem cells can be classified into four broad types based on their origin, stem cells from: i) embryos, ii) fetus, iii) umbilical cord, and iv) from the adult. Each of these can be grouped into subtypes, as identified on Figure 2.1 [1].

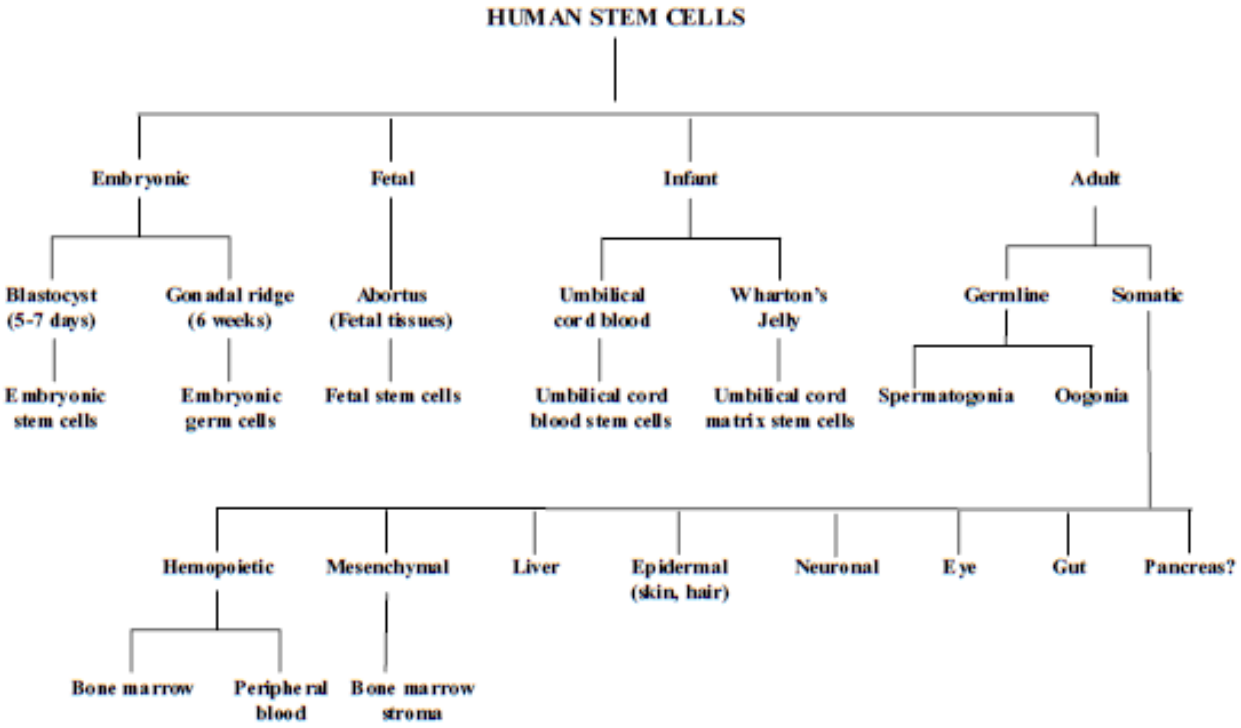


Figure 2.1 Human stem cell classification [1].

2.2 Mesenchymal Stem Cells (MSCs)

The presence of non-hematopoietic stem cells in bone marrow was first suggested by the observations of the German pathologist Cohnheim 130 years ago. His work raised the possibility that bone marrow may be the source of fibroblasts (Fb) that deposit collagen fibers as part of the normal process of wound repair. Evidence that bone marrow contains cells that can differentiate into other mesenchymal cells, as well as Fb, is now available, starting with the work of Friedenstein and colleagues. They placed whole bone marrow in plastic culture dishes and removed the nonadherent cells after 4 hours, thus discarding most of the hematopoietic cells. They reported that the adherent cells were heterogeneous in appearance, but the most tightly adherent cells were spindle-shaped and formed foci of two to four cells, which remained inactive for 2–4 days and then began to multiply rapidly. After passaging several times in culture, the adherent cells became more homogeneously fibroblastic in appearance [4].

Bone marrow is a complex tissue containing hematopoietic cell progenitors and their progeny integrated within a connective-tissue network of mesenchymal-derived cells known as stroma [5]. The mesenchymal stroma harbors an important population of cells that possess stem-like characteristics including self-renewal and differentiation capacities into several mesenchymal lineages [6], [7]. These non-hematopoietic multipotent mesenchymal stem cells (MSC), also named multipotent mesenchymal stromal cells, are found *in vivo* at low frequency and can be isolated from a variety of tissues, mostly located in perivascular niches, including Bone Marrow

(BM) as referred above, Muscle Tissue (MS), Adipose Tissue (AT) and Synovial Fluid. MSCs are also found in neonatal tissues, like Umbilical Cord Blood (UCB) and Matrix (UCM) or Placenta (PL) or Amniotic Fluid (AF) [6], [8]–[13]. Several other sources of MSCs have been identified in the last two decades: Periosteum, Pericytes, Dental pulp, Peripheral blood, Dermis, Trabecular bone, Infrapatellar pad, Muscle, Pancreas, Peridontal ligaments, Menstrual blood, Milk and Urinary tract. Although some of these sources seem promising for obtaining MSCs for clinical use, most, due to the low MSC yield and/ or invasiveness of the harvesting procedure, do not encourage further investigation [10].

Although phenotypically similar, or even identical in some cases, MSCs derived from different tissues have been shown to exhibit variable function and activity, evidenced in the level of cytokine production, gene expression, and differentiation potential. It is thought that the MSC niche, unique to each tissue origin, is at the root of these variations [6], [11].

Bone marrow derived stem cells first described by Friedenstein *et al.* are still the most frequently investigated cell type and often designated as the gold standard [6]. Most adherent cells from bone marrow aspirates do not meet the criteria of mesenchymal stem cells (MSCs), only approximately 0,01 to 0,001% of bone marrow mononuclear cells are MSCs [6], [9]. They are 10-fold less abundant than the hematopoietic stem cells (HSCs), which contributes to the organization of the microenvironment supporting the differentiation of hematopoietic cells [5].

2.2.1 MSC lineage during embryonic development

It is widely believed that MSCs derive from mesoderm. However, a recent study performed by Takashima *et al*. showed that the earliest lineage providing MSC-like cells during embryonic trunk development is indeed generated from Sox1(+) neuroepithelium intermediate rather than from mesoderm, at least in part through a neural crest intermediate stage [14], [15]. These early MSCs are then replaced, later in development, by MSCs from other origins. So MSCs derived from other developmental lineages decreases due to the increasing importance of mesodermal MSCs [16], [17]. Supporting this observation, it has been recently demonstrated that neural crest-derived cells migrate to the bone marrow through the bloodstream. These cells are still present in the adult bone marrow and can differentiate *in vitro* into neurons, glial cells and myofibroblasts. The potential link, if any, between these cells, the cells identified by Takashima *et al.* (2007) and the MSCs isolated according to Friedenstein's protocol remains to be established [17].

2.2.2 hMSCs mesengenic process and niche

Depending on the stimulus and the culture conditions employed, MSCs can form bone, cartilage, muscle, fat, and other connective tissues. These observations originally suggested that

MSCs were responsible for the normal turnover and maintenance of adult mesenchymal tissue (Figure 2.2) [12].

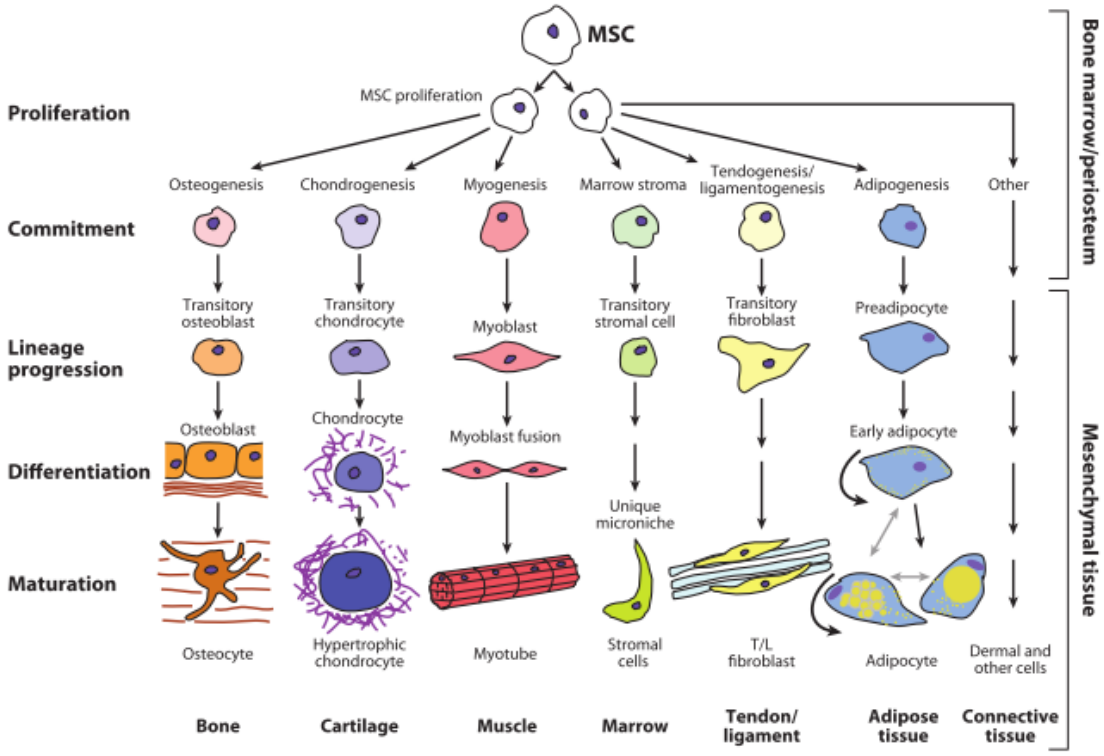


Figure 2.2 MSC mesengenic process. Human mesenchymal stem cells (hMSCs) from bone marrow may develop into bone, muscle, or adipose tissue, depending on the stimuli to which they are exposed *in vitro* [12].

The cellular origin of the MSCs remains a controversial subject. Authors defend that due to the widespread distribution of MSCs has been interpreted to indicate that the cells reside in the vascular pericyte population *in vivo* (Figure 2.3) [15]. Crisan *et al.* demonstrated that pericytes possess progenitor potential similar to MSCs (*i.e.* they can differentiate into osteocytes, chondrocytes and adipocytes), and share several surface markers associated with MSCs [9] and that it is their pleiotropic nature that allows them to sense and respond to an event in the local environment, be it injury or inflammation [12].

Whether all pericytes are MSCs or whether MSCs are the bone marrow-derived subset of pericytes is debatable since "pericytes" or "MSCs" isolated from different organs of the body can display markedly different differentiation potentials. For instance, pulp-derived pericytes/ MSCs display odontoblastic potential, while marrow-derived pericytes/ MSCs have not shown an odontoblastic phenotype. The argument is that if all pericytes were MSCs, then all pericytes should have equivalent differentiation potentials. Alternatively, it has been proposed that pericytes constitute a reservoir of tissue-specific progenitor cells, of which classically defined MSCs may only be a subset [7].

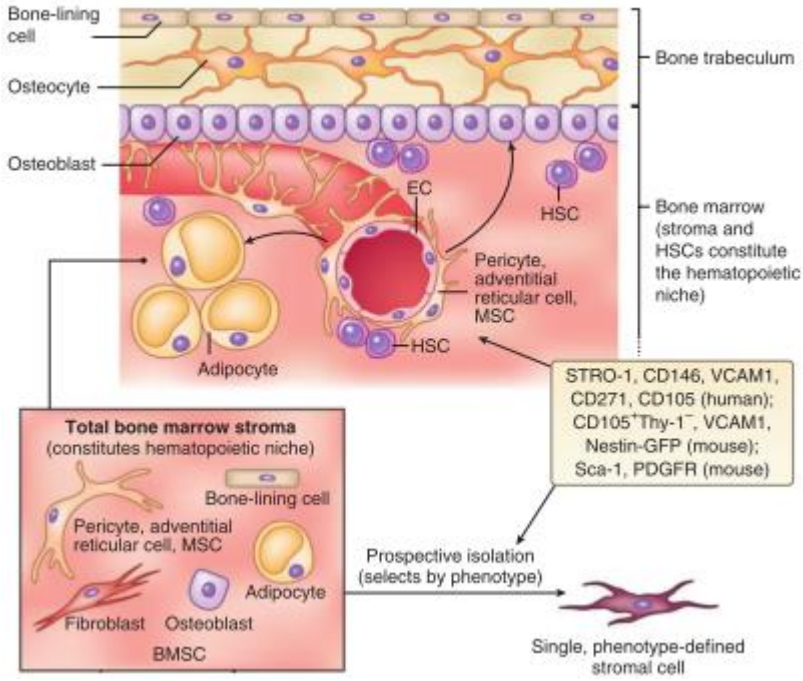


Figure 2.3 Whereabouts of MSCs. In the postnatal bone marrow, MSCs reside around sinusoids, maintain a niche for HSCs, support hematopoiesis and replenish differentiated compartment of osteoblasts and adipocytes during the tissue turnover. They also generate cartilage under specific conditions such as trauma [18].

Moreover, there is also conflicting data on pericyte contribution to MSC-derived cells in different tissues, *i.e.,* in tissues with low vascularization, such as cartilage, the pericyte contribution to MSCs will be less than in tissues with more extensive blood supplies. Feng *et al.* suggested a contribution of pericyte-derived and non-pericyte derived MSCs to cell differentiation in any given tissue depends on the extent of the vascularity and the kinetic of growth and/ or repair. This hypothesis suggests that this is an evolutionary adaptation to facilitate rapid tissue repair since stem cells can quickly accumulate at a damage site via inflammatory response [19].

2.2.3 Minimal criteria for defining multipotent MSC

The current knowledge on MSCs is still limited, in addition to the controversy around it origin, unequivocal identification systems has not yet been established. Biological and clinical interest in MSC has risen dramatically over the last two decades, this increasing interest has also generated many ambiguities and inconsistencies in the fields [20]. Currently, there is not a single marker that allows the identifications of a purified population of MSCs with a uniquely defined set of functional properties. Exhaustive phenotypic analysis has therefore been necessary to distinguish MSCs from other cells that exhibit similar fibroblastic, adherent characteristics in culture [11]. The lack of an unambiguous *in vivo* MSC marker that identifies

this cell population in different tissues is an indication that the different characteristics may be dictated by the local tissue microenvironment in which they reside [21]. Furthermore, *in vitro* and *in vivo* molecular mechanisms can be influenced by several conceptual and experimental factors, including species and tissue sources of MSCs, culture conditions, number of passages determining culture-related senescence, activation status of both MSCs and responsive immune effector cells, analytical methods and animal models used. The obvious consequence of this heterogeneity in the scientific approach to MSC physiology is that very often *in vitro* and *in vivo* data are variable, if not contradictory and reciprocally not comparable [22].

To begin to address these issues, the Mesenchymal and Tissue Stem Cell Committee of International Society for Cellular Therapy (ISCT) proposes three main criteria to define human MSC. First, MSC must be plastic-adherent when maintained in culture conditions. Second, ≥95% of the MSC population must express CD105, CD73 and CD90, as measured by flow cytometer. Additionally, these cells must lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA (human leukocyte antigen) class II, this is primarily to allow the exclusion of hematopoietic cells which may contaminate MSC cultures. Third, the cells must be able to differentiate to osteoblasts, adipocytes and chondroblasts under standard *in vitro* differentiating conditions [9], [20], [23].

Moreover, ISCT suggests that standard immune plasticity assay should be based on IFN (interferon)-γ with or without tumor necrosis factor (TNF)-α as a model *in vitro* priming agent. MSC are at default niche, displaying mostly bystander anti-apoptotic and immune homeostatic features biased towards suppression. These properties can be greatly enhanced when MSCs undergo functional polarization towards the inhibitory phenotype on exposure to various pro-inflammatory cytokines. IFN-γ remains the first key licensing agent for MSC suppressor function. There is strong consensus that cross species IFN-γ augments MSC suppressor function (through distinct effector pathways). *In vitro* MSC inflammatory "licensing" better recapitulates what probably happens *in vivo*, once MSCs are transfused into patients with dysregulated immune responses or with systemic inflammation, including sepsis. IFN-γ is sufficient for licensing and should be used to deploy a functional phenotype, however, its effect is amplified by TNF-α. The issue then arises about how MSCs are investigated after licensing [22].

As referred above, flow cytometry has been used to investigate the expression of cell-surface markers for characterizing MSC immunological properties. A traditional definition of a quiescent MSC is the expression of a major histocompatibility complex (MHC) class I, cell lacking MHC class II or co-stimulatory molecule expression [22], [24]. However, IFN-γ primed MSCs robustly upregulate markers such as MHCI and MHCII molecules, immune modulatory molecules (CD200, CD274/PD-L1/B7-H1), cytokine/ chemokine receptors (CXCR3, CXCR4, CXCR5, CCR7, CD119/ IFN-γ receptor), adhesion molecules (CD54, CD106), DNAM ligands

(CD112, CD155), NKG2D ligands (macrophage inflammatory complex A/B, UL binding protein 1,2,3) and Notch receptors (Jagged-1). Intriguingly, human MSCs do not upregulate co-stimulatory molecules (CD80, CD86) in response to IFN-γ, and immune modulators such as transforming growth factor (TGF)-β can markedly blunt MHCII upregulation in response to inflammatory stimuli [22].

Another feature that is attributed to MSCs is the capacity of MSCs to produce indoleamine 2,3-dioxygenase (IDO) and inducible nitric oxide synthase (iNOS). Activation of IDO and iNOS is a pivotal mechanism in lymphocyte inhibition with MSCs, but species-specific differences exist. For example, after inflammatory priming, human MSCs express extremely high levels of IDO and low levels of iNOS, which is opposite to that seen with mouse MSCs [25]. IDO response should be central to an in vitro regulation assay, because is generally accepted that IDO bioactivity is central to the suppressor function of human MSCs and that IFN-γ regulation leads to massive transcriptional IDO induction [22].

An array of potential complementary suppressor pathways driven by MSCs includes heme oxygenase (HO)-1, soluble HLA-G5 (sHLA-G5) and other secreted factors such as TGF-β, prostaglandin E2 (PGE2), galectin and tumor suppressor gene 6. Importantly, the MSC response to IFN-γ leads to increased expression of pro-inflammatory cytokines/ chemokines such as interleukin (IL)-6, chemokine ligand (CCL) 2, 7 and 8 [25].

2.2.4 Therapeutic applications of MSC

The evolution of MSC therapy over the years reflects a transformation in how investigators perceive these cells and their best-suited clinical applications. Initially heralded as stem cells, MSCs were first evaluated for regenerative applications, due the ability of MSCs to form tissues particularly affected by degenerative joint diseases, namely, cartilage and bone [11], [26]. MSCs have been shown to protect cells from injury and directly promote tissue repair, as they can act on several levels of endogenous repair to bring about resolution of disease [11].

Stem-cell-based therapies represent a new approach for neurodegenerative diseases. MSCs have the ability to differentiate into all mature neural cell types. In particular, in neural progenitor maintenance medium, MSCs acquire new morphological characteristics, neural markers, and electrophysiological properties, which are suggestive of neural differentiation. Several studies have revealed that the therapeutic action of MSCs is related to the release, even far from site of injection, of protective factors rather than to replacement of degenerating neurons. Such a therapeutic effect may be provided by different classes of molecules, including trophic factors, anti-inflammatory cytokines and immune-modulatory chemokines released from transplanted cells [10].

In addition to promoting tissue repair directly, MSCs have also been shown to modulate the immune system and attenuate tissue damage caused by excessive inflammation. Initial indications regarding the immunomodulation aspects of MSCs were first observed in the context of MSC transplantation studies in animals and humans. Unexpectedly, MSCs seemed to exhibit an unusual ability to evade the immune system and autologous and allogeneic MSCs could be transplanted without immune rejection [11]. Allogeneic MSC have proved to be an option with major advantages in clinical use, since the use of autologous MSC is hindered by the limited time frame for clonal expansion and the costly *in vitro* proliferation [5]. MSCs home to sites of inflammation or tissue injury and secrete considerable levels of both immunomodulatory and trophic agents [10]. Pleiotropic nature of MSCs allows them to sense and respond to an event in the local environment, be it injury or inflammation, so the local environment is key in the activation or licensing of MSCs to become immunosuppressive [12], [27]. Based on their ability to moderate T cell proliferation and function, MSCs have also been proposed as a therapeutic option in the treatment of autoimmune diseases. They have therefore been tested in a variety of animal models of diabetes, experimental autoimmune encephalomyelitis, systemic lupus erythematosus or rheumatoid arthritis [8].

MSCs and their stromal progeny secrete soluble mediators which support hematopoiesis, these function are now being characterized in the context of MSC transplantation, whereby paracrine interactions between MSCs and host cells have been shown to relate directly to the therapeutic activity of MSCs [11]. Co-injection of MSCs along with HSCs could enhance hematopoietic recovery following bone marrow transplantation and enhance HSC engraftment [8], [26]. MSCs are also intimately associated with homeostatic mechanisms that tightly regulate activity in the bone marrow microenvironment [11].

MSCs can also stimulate local angiogenesis by secretion of extracellular matrix molecules, vascular endothelial growth factor (VEGF) and MSC stabilization of new vessels by the return to their earlier pericyte phenotype, and thereby promote neovascularization of ischemic tissue [10]–[12], [21].

Many approaches have been proposed including the genetic manipulation of stem cells. The best cancer chemotherapy approach is to deliver the drug to the tumor microenvironment in order to kill tumor cells while producing the lowest collateral toxicity. Among them, MSCs represent an optimal choice to deliver anti-tumor agents due to their adaptability to culture conditions necessary for *in vitro* manipulation and their capacity for homing to pathological tissue when systemically administered [10].

All of these observations are the starting point for clinical trials that aim to treat several diseases such as myocardial infarction, multiple sclerosis, amyotrophic lateral sclerosis and leukemia

[10]. Regarding humans, up to 418 MSCs clinical trials are currently registered (http://clinicaltrials.gov, last access August 2014) and MSCs have been already been granted expand access for use in pediatric steroid-refractory acute Graft-versus-Host-Disease (GvHD) by United States Food and Drug Administration [10].

Around 418 MSCs clinical trials are currently registered, however only a few MSC-based cell therapy products have been approved in market world-wide. South Korea is leading with two MSC products registered and first authorization granted in 2011. With the approval from the Korean Food and Drug Administration (FDA) in January 2012, Cartistem has become the world's first allogeneic, off-the-shelf MSC-base product. The product contains the umbilical cord blood (UCB) - derived MSCs and it is indicated for the treatment of traumatic and degenerative osteoarthritis. In 2011 the Korean company FCB PharmiCell received Korean FDA approval for commercial sale of HeartiCellgram indicated for post-acute myocardial infarction treatment. It is autologous bone marrow-derived MSC therapy product. Similar regulatory decision has been adopted for Mesoblast product Prochymal which consists of allogeneic MSCs. Mesoblast's product Prochymal has received conditional approval in Canada and New Zealand for treatment of children with acute steroid refractory GvHD. It is also available in the United States under an Expanded Access Program for treatment of GvHD in children and adults [28], [29].

2.2.4.1 MSCs and Graft-versus-Host Disease

GvHD is a complex disease resulting from T cell recognition of a genetically disparate recipient that is unable to reject (immunosuppress) donor cells after allogeneic hematopoietic stem cell transplant (HSCT) [30].

GvHD occurs when donor T cells respond to genetically defined proteins on host cells. The most important proteins are HLA, which are highly polymorphic and are encoded by the MHC. Class I HLA (A, B, and C) proteins are expressed on almost all nucleated cells of the body at varying densities. Class II (DR, DQ, and DP) are primarily expressed on hematopoietic cells, but their expression can be induced on many other cell types following inflammation or injury. The incidence of acute GvHD is directly related to the degree of mismatch between HLA proteins and thus ideally, donors and recipients are matched at HL-A, -B, -C, and DRB1 [31].

There are two form of GvHD, acute and chronic. By definition acute GvHD (aGvHD) occurs prior to day 100, whereas chronic occurred after that time. aGvHD reflects exaggerated but normal inflammatory mechanism mediated by donor lymphocytes infused into the recipient where they function appropriately, given the foreign environment they encounter. The recipient tissues that stimulate donor lymphocytes have usually been damaged by underlying disease,

prior infection, and the transplant conditioning regimen. As result, these tissues produce molecules that promote the activation and proliferation of donor immune cells [31].

The development of aGvHD can be conceptualized in three sequential steps or phases: (I) activation of the antigen presenting cells (APCs); (II) donor T cell activation, proliferation and migration; and (III) target tissue destruction (Figure 2.4) [31].

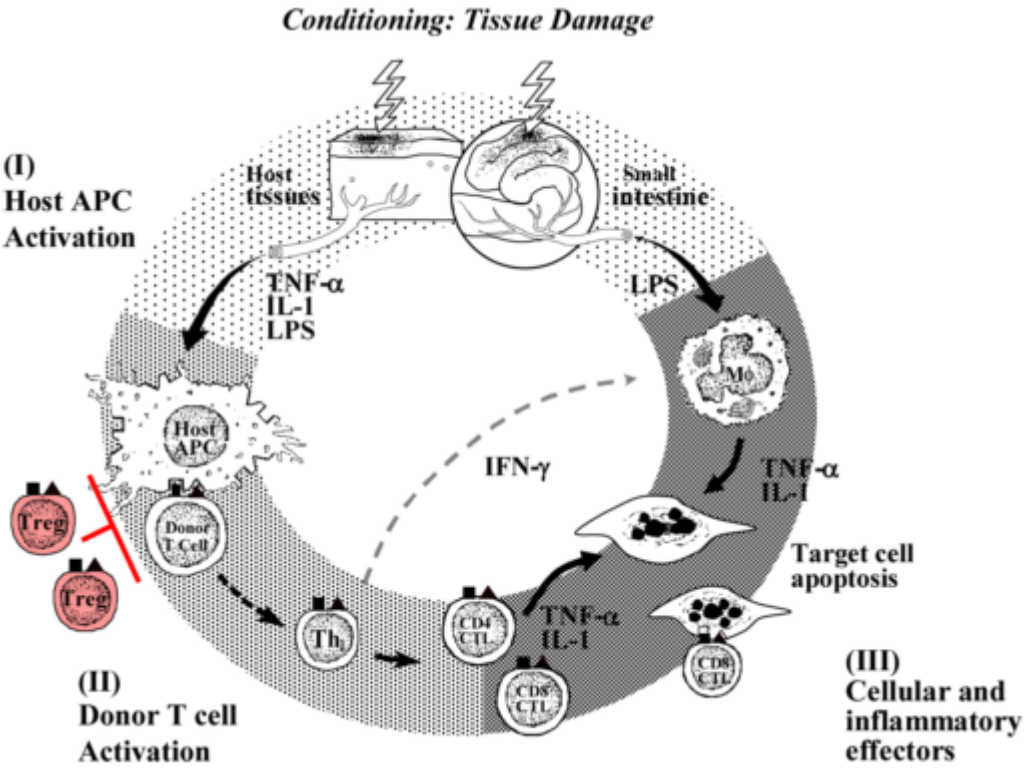


Figure 2.4 GvHD Pathophysiology [31].

Phase I: Activation of APCs: The first step involves the activation by the underlying disease and the hematopoietic cell transplantation (HCT) conditioning regime (irradiation, chemotherapy, or both). The condition regimen leads to damage to and activation of host tissue by release of several chemokines and inflammatory cytokines, TNF-α and IL-1. These cytokines can increase the expression of adhesion molecules, co-stimulatory molecules and MHC antigens on host APCs, enhancing the recognition of MHC and minor histocompatibility antigens by mature donor T cells. Damage to the gastro intestine tract from the conditioning is particularly important because it allows for systemic translocation of additional inflammatory stimuli such as microbial products including lipopolysaccharide (LPS) or other pathogen-associated molecular patterns that further enhance the activation of host APCs [31], [32].

Phase II: Donor T cell Activation, proliferation, differentiation and migration: This step is the core of the GvHD reaction, where donor T cells proliferate and differentiate in response to host APCs. Activation occurs as the result of the recognition and interaction of the T cell receptor (TCR) and co-stimulatory molecules with their cognate ligands expressed on the surface of

APC. The “danger” signals generated in Phase I augment this activation at least in part by increasing the expression of co-stimulatory molecules [30], [31]. Phase II is characterized by the proliferation of Th1 T cells and the secretion of IL-2 and IFN-γ. IL-2 and IFN-γ induce cytotoxic T lymphocytes (CTLs) and NK-cell responses, and prime additional mononuclear phagocytes to produce IL-1 and TNF-α [32].

Activated T cells migrate to GvHD target tissues (gut, liver, skin and lung) and are followed by the recruitment of other effector leucocytes [30], [31].

Phase III: Cellular and Inflammatory Effector phase: Damage to the intestinal mucosa in Phase I and by cytolytic effectors activated in Phase II allows translocation of LPS from the intestinal lumen to the circulation. Subsequently, LPS may stimulate additional cytokine production by gut-associated lymphocytes and macrophages in the gastro intestine tract and by keratinocytes, dermal fibroblasts, and macrophages within the skin. This mechanism may amplify local tissue injury and further promote an inflammatory response that, together with the CTL and NK component, leads to target tissue destruction in the bone marrow transplant host. Damage to the gastro intestine tract in Phase III increases LPS release, stimulating further cytokine production and causing additional gastro intestine tract damage. Thus the gastro intestine tract is critical to propagating the ‘‘cytokine storm’’ characteristic of acute GVHD [31], [32].

The immunomodulatory properties of MSCs have been successfully employed to treat severe steroid-resistant aGvHD, developing after allogeneic HSCT or donor lymphocyte infusion [10]. Patients treated with intravenous infusions of allogeneic MSCs no adverse events were recorded and a clinical response was noted in the majority of patients with a significant advantage in terms of survival for complete responders, as compared with partial/ non-responding patients, with significantly decreased transplanted-related mortality (TRM) [10]. Currently, there is no successful therapy for steroid-resistant aGvHD and the possible role of MSCs in this context is therefore of potential interest [5], as demonstrated on Table 1.

Table 1 Clinical experience of MSCs to prevent or to treat GvHD in allogeneic hematopoietic stem cell transplant recipients. CR- indicates complete response, PR- partial response, M/F- male/ female, NA- not available. [33], [34]

References	Indication	Patients number	Source	Results
Kebriaei *et al.* 2009	De novo acute GvHD	32 (21/10 M/F)	BM-MSC	No infusion-related toxicities or ectopic tissue formation
				94% initial response by day 28 (77% CR and 16% PR)
von Bonin *et al.* 2009	Steroid-refractory, severe acute GvHD	13 (7/6)	BM-MSC	No infusion-related side effects
				5/7 patients with initial response required additional MSC therapy
				4/9 deaths attributed to GvHD
Le Blanc *et al*. 2008	Steroid-refractory, severe acute GvHD	55 (34/21 M/F)	BM-MSC	No infusion-related side effects
				30/55 CR, 9 PC
				No relation between response and MSC HLA match
Muller *et al.* 2008	Immunologic compilation after allo-HSCT in pediatric transplant	7 (M/F not specified)	BM-MSC	No infusion-related side effects
				2 patients with severe acute GvHD did not progress to chronic GvHD
				1/3 slight improvement of chronic GvHD
Fang *et al*. 2007	Steroid-refractory, severe acute GvHD	6 (2/4 M/F)	Adipose MSC	No infusion-related side effects
				5/6 CR
				4/6 survival(18-90 months posttreatment)
Ringden *et al*. 2006	Steroid-refractory, severe acute GvHD	8 (7/1 M/F)	N/A	No infusion-related side effects
				6/8 CR
				5/8 survival(2-36 months posttreatment)
Lazarus *et al.* 2005	GvHD prevention	46 (24/22 M/F)	BM-MSC	No infusion-related toxicities or ectopic tissue formation
				No increase in incidence or severity of GvHD

2.3 Advanced Therapy Medicinal Products (ATMP)

The term "advanced therapy medical product" (ATMP) covers the following medicinal products for human use: somatic cell therapy medicinal products (CTMPs), gene therapy medicinal products, and tissue engineered [28].

For cells to be classified as medicinal products they have to fulfill at least one of the following conditions: the cells have been subjected to substantial manipulation (so that the biological characteristics, physiological functions, or structural properties relevant for the intend clinical use have been altered) and/ or these cells are not intended for use for the same function and/ or be administrated to human beings with a view to treating, preventing or diagnosing a disease through the action of its cells [28].

2.3.1 ImmuneSafe®

IS is a MSC-based therapy for application in several immune and inflammatory diseases. The rationale is to use the immunological characteristics and the immunomodulation properties of MSCs to treat patients with diseases related with the immune system, such as GvHD.

The MSCs used for the production of IS are derived from human bone marrow (hBM) of healthy donors after informed consent. After the harvest of the hBM in a hospital environment, it is processed according to Cell2B own manufacturing process. After the expansion, cells stored in a cryogenic bank are ready for the release step followed by a final formulation procedure, which makes IS ready for transportation to the hospital to be directly administered to the patient.

2.3.2 Regulatory approval of ATMPs

A number of cell therapy and tissue engineered products have been introduced into the national markets of several Member States during the last decade. Due to the novelty, complexity, and technical specificity of such products, specially tailored and harmonized rules were necessary to ensure free movement of those products within the EU. Consequently, the regulation (EC) Nº 1394/2007 on ATMPs was drafted and came into force on December 30, 2008. The regulation laid down specific rules concerning centralized authorization, supervision, and pharmacovigilance of the ATMPs (Committee for Advanced Therapies (CAT) and CAT Scientific Secretariat, 2010). The CAT has been established at European Medicines Agency (EMA) for centralized classification, certification and evaluation procedures, and other ATMP-related tasks [28].

Since 2001, when the European Directive 2001/83/CE relating to medicinal products for human use was approved, products for advanced medicinal therapy (AMT), used for gene therapy,

somatic cell therapy and for tissue engineering, have been considered as drugs. Each of these products has specific pharmacologic, metabolic and immunologic activities and the potential for treating a variety of disorders. For these reasons cellular products for AMT must meet the same stringent conditions required for drugs before they are placed on the market, in particular their activity, efficacy, safety and required dose must be defined. Furthermore they must be manipulated according to Good Manufacturing Practices (GMP) and they require testing in approved clinical trials before being commercialized [10].

2.4 Risk analysis of MSC therapy

When considering the use of *ex vivo* expanded MSCs for clinical application, some potential risks should be considered: the immunogenicity of the cells, the biosafety of medium components, the risk of ectopic tissue formation, and the potential *in vitro* transformation of the cells during expansion [10].

Due to the low frequency of mesenchymal progenitors in human tissues, the use of MSCs in vivo use requires that the cells be extensively *ex vivo* manipulated to achieve the necessary numbers that are suitable for their clinical application. MSCs are generally cultured, both under experimental and clinical grade conditions, in the presence of serum. The use of serum in the *ex vivo* expansion of MSCs might have some risks. Animal serum use can be associated with an increased risk of zoonoses transmission and trigger potential immune reactions in the recipient host, ultimately results in the rejection of the graft, especially after repeated treatments. For these reasons, animal-free additives are being considered for clinical-grade expansion of MSCs [10].

Cells propagated *in vitro* are in a proliferative state under non-physiologic conditions, this may cause accumulation of DNA damage, resulting in an increased risk for malignant transformation. Moreover, after a variable number of cell divisions, *in vitro* expanded MSCs, like every normal somatic cell, enter a senescent state and ultimately stop proliferating. Several molecular pathways have been implicated in senescence, including DNA damage and progressive shortening of telomeres. It is well known that somatic cells may activate molecular mechanisms in an attempt to circumvent senescence. Remarkably, it has been hypothesized that escape from senescence, for instance by means of telomerase activity that counteracts telomere shortening, is a crucial step in malignant transformation. However, on the available literature there are no reports of tumor formation associated with any ongoing clinical trials using MSCs. Moreover, a very recent systematic review of current clinical trials documented that MSCs therapy appears safe [10].

Consequently, MSCs expanded *in vitro* for clinical use have to be rigorously evaluated for the risk of malignant transformation. Appropriate quality control procedures to investigate this important issue should at least include: (i) release of MSCs expanded in a low number of passages, in an attempt to minimize the administration of potentially senescent cells, (ii) careful evaluation of the morphology and proliferation pattern at each culture passage, and the phenotype of the final product, (iii) demonstration of absence of genetic instability by molecular and conventional karyotyping, (iv) assessment of telomerase activity on the final product, considering that it has been documented that non-malignant human MSCs display a low/ undetectable level of this enzymatic activity, (v) DNA fingerprinting by analysis of short tandem repeats to assess the identity of the final product, (vi) whenever feasible, expansion of a sizable aliquot of the MSCs lot cryopreserved for release for further 4-5 passages, in order to demonstrate the absence of transformed MSCs which could have been present at undetectable levels in earlier passages [10][35].

3 Characterization (product and process) – metrology of critical-to-quality characteristics

A thorough characterization of product and process is fundamental to demonstrate GMP-compliance and underpins efforts to obtain product regulatory approval. Specifically, there is a need to demonstrate the critical quality attributes (CQAs): identity, safety, purity and efficacy/ potency of therapy [36], [37].

Identity of manufacture assessments can be used to determine the quality and capacity of manufacturing processes. The goal is to confirm that product contains the intended cellular and non-cellular components. Suitable characterization strategies will depend on the type of product being assessed. For advanced therapies, identifying the cell phenotype, function and mode of action will be critical for specific clinical applications. Biomarkers may be important in distinguishing different cell phenotypes, but they do not always provide a correlation to cell function. Cellular morphology can also be used to analyse cell populations using various microscopy techniques to determine if cells appear true to their phenotype [36], [37].

Safety is of prime concern to ensure therapies do not have a deleterious effect on the patient. The sterility of cell-based products also needs to be assured, confirming that the product is not contaminated with microbes or adventitious agents and, if appropriated, does not have tumorigenic potential. Cells that are cultured for extended amplification times, can lead to cellular senescence, as well as genetic and epigenetic changes. To exclude products containing cells abnormalities potentially conventional karyotyping (GTG-banding) should be combined with other techniques (Comparative genomic hybridization (CGH)/ Single nucleotide polymorphism (SNP) array or Fluorescence *in situ* hybridization (FISH)). DNA microarray technology can be used as a technique to assess relevant cellular pathways, such as senescence, as well as the recognized genetic changes that have been shown to occur with the extensive *ex vivo* expansion that is a prerequisite to obtain the cell number that are necessary for human cell-based therapy protocols. Microarray analysis measures the global expression of genes and can thereby provide an insight into genetic process expressed in stem cells [35]–[37].

Purity tests ensure that cell therapy products are free from unwanted material, including unwanted cell types, endotoxins, residual proteins, peptides or other agents used in manufacturing such as animal serum [37].

Efficacy generally refers to the ability of a product to cause a functional response in the patient, and is related to the potency of the therapy. The difference between these two terms, potency and efficacy potency, lies in the generalized capacity to be impactful (potency) and with what

intensity is this impact instituted (efficacy). Proper characterisation and understanding of cell function is the most important factor in determining whether a cell-based therapy will function effectively *in vivo*. However, as complete characterisation of some cell processes is still unknown, it is very difficult to accurately predict every consequence of a particular cell once placed within a patient. Efficacy tests should always be cell-specific, and ideally test the function of the cell that will be required in an *in viv*o situation. In some cases, *in vitro* assays can be used as surrogate measures. Such measures can often provide more sensitive and useful data than *in vivo* trials in an animal model [36], [38].

Cell viability is considered by many scientists as the primary factor for determining the cellular effect of these advanced therapies once implanted in the body. However, most viability percentages simply measure how many cells are "alive", not how many cells actively metabolizing and playing a productive role in their environment. In terms of advanced therapies, identifying the cell phenotype, function and mode of action will be critical for specific clinical applications. Biomarkers may be important in distinguish different cell phenotypes, but they do not always provide a correlation to cell function. Therefore, in terms of cell-based therapies, how the cells act in the body might be more important than their immunophenotype *in vitro*. In all these cases, though, the safety and efficacy of the product is being determined essentially by implication rather than by understanding of its mode of action [36].

3.1 ImmuneSafe® Therapeutic Potency

3.1.1 Immunomodulatory and/or immunosuppressive mechanisms of MSCs

MSCs modulate different aspects of both innate and adaptive immunity, exerting immune regulatory functions, both in *in vivo* and *in vitro*, in a wide range of immunocompetent cells, including antigen presenting cells (APC), T, B and NK lymphocytes, as depicted on Figure 3.1. In particular, it has been recently demonstrated that MSCs suppress dendritic cell (DC) activation *in vivo*, resulting in the inhibition of cytokine secretion, down-regulation of molecules involved in lymphoid organ homing with subsequent impairment of T cell priming. MSCs may also affect neutrophil and macrophage functions, by inhibiting apoptosis of resting and activated cells. The capacity of MSCs to modulate T cell responses is well documented. In particular they may inhibit T cell proliferation induced by different stimuli or direct T cells towards regulatory patterns [10].

Limiting of T cell expansion was demonstrated *in vitro* studies through MSCs inhibition of IFN-γ and TNF-α production as well as IL-10 [34], [39]. Also, IL-17 and its closest relative, IL-17-F, have recently drawn much attention in the field of immunology. IL-17 and IL-17-F are

expressed by a distinct type of T cells, T helper 17 cells and certain other lymphocytes. This cytokine play key regulatory roles in host defense and inflammatory diseases [40], [41].

Controversial results have been described on the immunomodulatory role of MSCs on B lymphocyte function, with some studies documenting MSCs inhibitory effect on B cell proliferation, differentiation and immunoglobulin secretion, and other studies demonstrating that under certain experimental conditions MSCs exert a stimulatory effect on B lymphocytes [10].

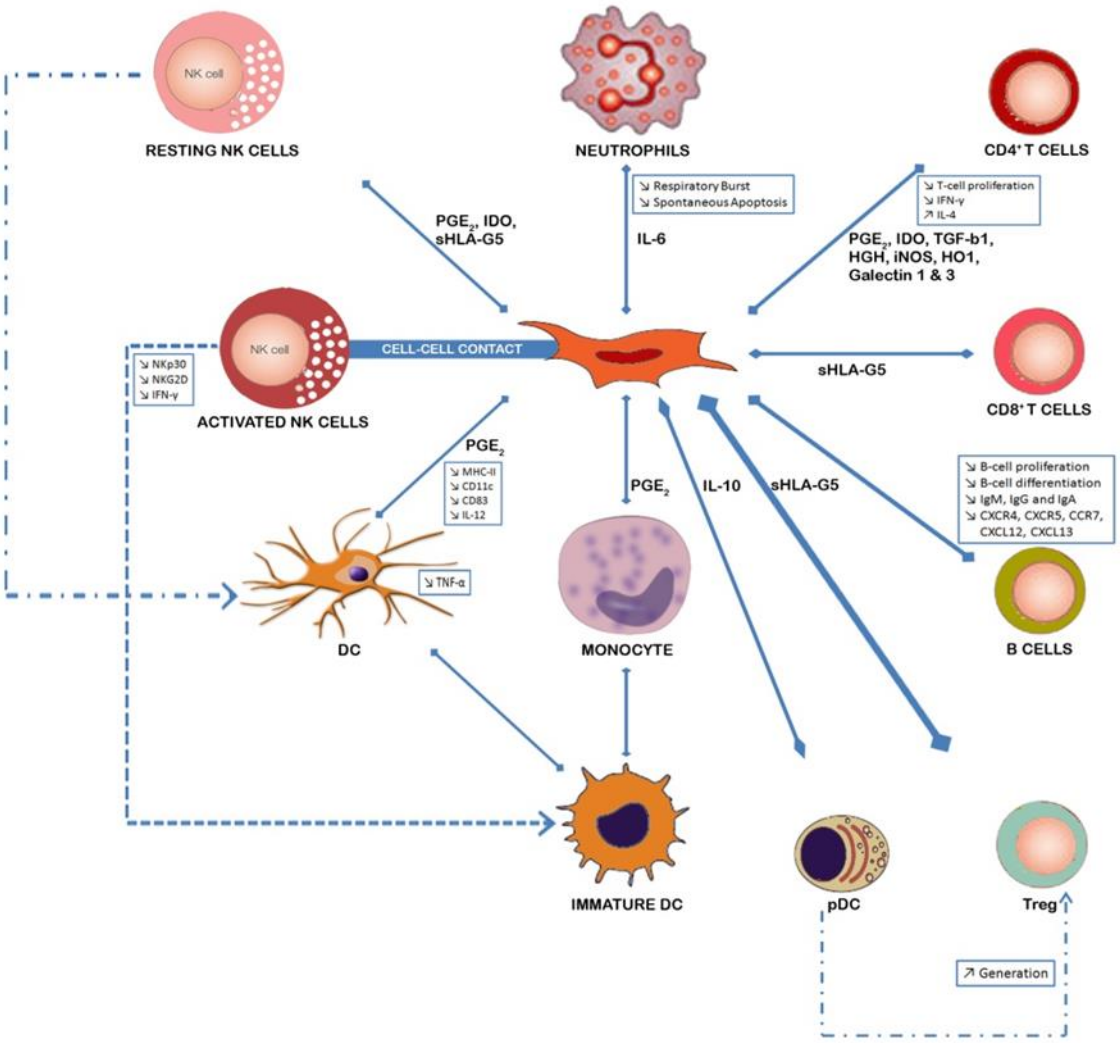


Figure 3.1 Potential mechanisms of the MSC interactions with immune cells [5].

MSCs can inhibit both the proliferation and cytotoxicity of resting natural killer (NK) cells, as well as, their cytokine production by releasing prostaglandin E2 (PGE2), IDO and sHLA-G5. Killing of MSCs by cytokine-activated NK cells involves the engagement of cell-surface receptors (thick blue line in Figure 3.1) expressed by NK cells with its ligands expressed on MSCs. MSCs inhibit the differentiation of monocytes to immature myeloid DCs, bias mature

DCs to an immature DC state, inhibit TNF-α production by DCs and increase IL-10 production by plasmacytoid DCs (pDCs). MSC-derived PGE2 is involved in all of these effects. Immature DCs are susceptible to activated NK cell-mediated lysis. MSC direct inhibition of $CD4^+$ T cell function depends on their release of several soluble molecules, including PGE2, IDO, TGF-β1, hepatocyte growth factor (HGF), inducible nitric-oxide synthase (iNOS) and HO-1. MSC inhibition of $CD8^+$ T cell cytotoxicity and the differentiation of regulatory T cells mediated directly by MSCs are related to the release of sHLA-G5 by MSCs. In addition, the upregulation of IL-10 production by pDCs results in the increased generation of regulatory T cells through an indirect mechanism. MSC-driven inhibition of B- cell function seems to depend on soluble factors and cell–cell contact. Finally, MSCs dampen the respiratory burst and delay the spontaneous apoptosis of neutrophils by constitutively releasing IL-6 [5].

The immunomodulatory properties of MSCs require cell-to-cell contact, as well as release of soluble factors, including IL-6, IL10, TGF-β, PGE2, IDO and soluble HLA-G [10], [42]. Recently it has also been demonstrated that MSCs may release microvesicles transporting functional mRNA and microRNA, a newly described mechanism of cell communication with tissue-injured cells, opening a new perspective on the MSCs action during the regenerative process [10].

The abundance of mediators identified to date suggests that MSCs exploit different immunosuppressive mechanisms under different disease conditions [41].

3.1.2 Secreted factors

As important as the effect of MSCs on immune effector function is the crosstalk effect that immune effector cells and their inducible factors may have on MSC activation and function [8], [33]. For a better framework of the role of some secreted factors by MSCs a summarized description of principal intervenient is described below.

3.1.2.1 PGE2

PGE2 is involved in the immunosuppressive activity of MSCs. PGE2 is a product of arachidonic acid metabolism that acts as a powerful immune suppressant, inhibiting T cell mitogenesis and IL-2 production, and is a cofactor for the induction of T-helper type 2 (Th_2) lymphocyte activity. Production of PGE2 by MSCs is enhanced following TNF-α or IFN-γ stimulation and its inhibition using specific inhibitors resulted in restoration of T-lymphocyte proliferation. MSC-derived PGE2 was shown to act on macrophages by stimulating the production of IL-10 and on monocytes by blocking their differentiation toward DCs [8].

3.1.2.2 Interleukins

Cytokines play a very important role in nearly all aspects of inflammation and immunity. The term interleukin has been used to describe a group of cytokines with complex immunomodulatory functions, including cell proliferation, maturation, migration and adhesion. These cytokines also play an important role in immune cell differentiation and activation. Determining the exact function of a particular cytokine is complicated by the influence of the producing cell type, the responding cell type and the phase of the immune response. ILs can also have pro- and anti-inflammatory effects, further complicating their characterization. These molecules are under constant pressure to evolve due to continual competition between the host's immune system and infecting organisms. The response of a particular cell to these cytokines depends on the ligands involved, specific receptors expressed on the cell surface and the particular signaling cascades that are activated. ILs modulate growth, differentiation and activation during an immune response [43].

3.1.2.2.1 IL-6

IL-6 is one MSC-secreted factor that has been reported to be involved in the inhibition of monocyte differentiation toward DCs, decreasing their stimulation ability on T cells. In parallel, the secretion of IL-6 by MSCs has also been reported to delay apoptosis of lymphocytes and neutrophils [8].

3.1.2.3 Chemokines

One feature that makes the use of MSCs interesting in the clinical setting is their ability to migrate to the damage tissue towards inflammatory sites after intravenous administration. Although the mechanism by which MSCs are able to migrate and home to sites of injury has not yet been elucidated, it is reasonable to assume that an increase in inflammatory chemokines concentration at the site of inflammation is the first key mediator of MSCs trafficking to the injury site. Since chemokines 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 [10].

MSC migration is broad in the absence of injury, but preferential in response to injury and inflammation, an effect mediated by chemokines receptors and gradients [33]. Upon stimulation by inflammatory cytokines, MSCs produce large amounts of chemokines, which can also attract lymphocytes through the secretion of chemokine (C-X-C motif) ligand (CXCL) 9, CXCL10 and chemokine (C-C motif) ligand (CCL) 2 [9], [27].

Furthermore, matrix metalloproteinases released by MSCs degrade the endothelial vessel basement membrane to allow extravasation into damage tissue. Chemokines receptors expression on MSCs may further be influenced by inflammatory microenvironment and even

the very soluble factors produced by MSCs themselves. Thus, paracrine and autocrine induction of chemokines and cytokines likely culminate to modulate MSC function within and migration to particular microenvironment [33].

3.1.2.4 Molecules involved in tissue repair and angiogenesis

The number of molecules known to mediate the paracrine action of cultured MSCs is very high and new molecules involved in these processes are discovered every day. Anti-apoptosis is the first expected effect when MSCs are used to treat acute lesions, the principal bioactive molecules responsible for the anti-apoptotic effect are VEGF, HGF, insulin-like growth factor (IGF) 1, stanniocalcin-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, basic fibroblast growth factor (bFGF) and IL-6 also stimulate local angiogenesis, which is particularly relevant during tissue re-organization. Mitosis of tissue-intrinsic pro-genitors 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 (ANG)-1 [10].

3.1.3 Metabolic enzymes

3.1.3.1 Indoleamine -2 3-dioxygenase (IDO)

IDO is an intracellular heme containing enzyme that take part in catabolism of tryptophan into kynurenine [44]. Upon stimulation with IFN-γ, IDO metabolizes tryptophan to kynurenine, causing depletion of local tryptophan and accumulation of toxic breakdown products [8]. Tryptophan starvation is main reason for T cell inactivation. On the other hand, IDO products such as kynurenine and oxygen radicals regulate proliferation and survival T cells [44]. IDO, however, exerts its effects mainly through the local accumulation of tryptophan metabolites rather than through tryptophan depletion [8].

3.1.3.2 Heme-oxygenase 1

Recently, a stress-responsive pathway was found to be strongly involved in MSC-mediated T cell suppression. The stress-inducible enzyme HO-1, which catalyzes the rate-limiting step of the heme degradation to biliverdin, has a suppressive effect on T cell proliferation in human and rat MSCs. Furthermore, HO-1 is a potent cytoprotective enzyme that exerts strong anti-inflammatory, antioxidative, and antiapoptotic activities through its products, especially carbon monoxide and biliverdin. This versatility, coupling direct tissue protection with immunosuppression, is of substantial relevance for transplantation immunology, where several aggressive pathologic processes have to be faced simultaneously. There are various preclinical

transplantation models evaluating HO-1, altogether suggesting beneficial effects with regards to transplantation tolerance and tissue regeneration. Regulatory T cells (Treg) induction is one of the proposed pathways how HO-1 exerts its tolerogenic effects. Tregs are pivotal for the maintenance of self-tolerance and of extraordinary interest for transplantation research because of their capability of controlling autoreactive immune cells [45].

HO-1 expression as well as immunosuppressive function is rapidly down-regulated over time in culture [42] and is shown to be produced by MSCs upon activation [8].

Any of these molecules alone does not lead to a complete abrogation of T cell proliferation, indicating their nonexclusive role. Instead, MSC-mediated immunoregulation is the result of the cumulative action displayed by several molecules [8].

To some degree, it has been implicated that full suppressive activity depends on a so-called pro-inflammatory "licensing" of MSCs composed of IFN-γ in concert with IL-1α, IL-1β, or TNF-α [42]. MSCs would not be constitutively inhibitory, but they could acquire their immunosuppressive functions after being exposed to an inflammatory environment. The culture of cells in the presence of IFN-γ and/or other inflammatory cytokines, such as TNF-α and IL-1ß, could therefore be of value in some clinical contexts where a potent immunosuppressive effect of MSCs is desirable [10].

3.2 ImmuneSafe® comparison with other cells products/ preparation and eventual manufacturing process changes

The development of an ATMP requires a full biological characterization of the product features, including identity, potency and safety, constituting the CQAs of the product. In this context, the development of specific and robust (bio) assays and the establishment of the corresponding specifications, which could be strongly correlated with ImmuneSafe's CQAs, are of paramount importance. In particular, it will provide tools to assure the consistency and robustness of the manufacturing process, either in In Process Control (IPC) settings or demonstrating product/ process comparability after a particular change in the manufacturing process.

The first part of this chapter is focused on the development of a (bio) assays panel for IS CQAs that allow a robust and reproducible product characterization, followed by the application of some (bio) assays panel to other cell products/ preparation and to evaluate the impact of eventual manufacturing process changes.

3.2.1 BM-MNCs *vs.* ImmuneSafe®

BM-MNCs fraction contains a heterologous population which consist of late-outgrowth endothelial progenitor cells (EPCs), early-outgrowth EPCs, mature endothelial cells (ECs), MSCs, hematopoietic stem cells, monocytes, $CD4^+$ T cells, $CD8^+$ T cells, B cells, NK cells, among others [46]. Because these cells were found to release a variety of different pro-angiogenic cytokines (e.g. IL-1, IL-6 and IL-8, VEGF, platelet-derived endothelial growth factor, TGF-β, basic fibroblast growth factor), the observed clinical benefit after cell-based therapy may be attributed to paracrine rather than to cellular effects [47].

hBM-MNCs have been used clinically to not only reconstitute the hematopoietic system, but to regenerate bone and to treat ischemic tissue as well [48]. Clinical trials using BM-MNCs have been performed to treat several diseases like critical/ chronic limb ischemia, liver cirrhosis, myocardial infarction, among others (e.g. NCT00761982 and NCT00282646 in http://www.clinicaltrials.gov/).

As referred above IS is obtained from BM-MNCs and the goal of this comparison, between BM-MNCs and IS, is to prove that isolated BM-MNCs do not have the same biological function and potency as isolated MSCs, especially on GvHD treatment.

3.2.2 Fibroblasts *vs.* ImmuneSafe®

Fb are considered mature mesenchymal cells that are particularly abundant in the connective of each organ and tissue. Therefore, these cells are the most frequent contaminating cell phenotype present in many cell culture systems. Fb and MSCs have an extremely similar morphological appearance, they both proliferate well and have many cell surface markers in common. MSCs lack a specific surface antigen that precisely differentiates these cells from fibroblast [49]. However, Fb analysed by Wagner *et al.* were only weak positive for CD105, while MSCs, responsible for induction differentiation, were mostly positive for CD105 [50]. Stro-1, and more recently CD146, has been claimed as specific markers for MSCs. The best way to distinguish MSCs from Fb is based on the analysis of the functional properties of these two types of cells. MSCs self-renew and retain multipotent differentiation capacity, while Fb seem more limited in both these functional properties [49].

It is widely believed that generic stromal cells such as Fb do not share the immunosuppressive effects of MSCs. Although, Fb have been reported to interact with immune system as alternative APC, either activating or down-regulating T cells and mediating indirect antiproliferative effects on lymphocytes [51]. MSC-mediated inhibition of monocyte differentiation into dendritic cells has also been documented using fibroblast. More recently, direct comparison between adult Fb

from various tissues and bone marrow MSC showed similar *in vitro* immunosuppressive potency [52].

To circumvent these issues it is imperative to increase our knowledge about Fb immunomodulatory properties and perform a side by side comparison with IS.

3.2.3 Differentiated MSC *vs*. ImmuneSafe®

MSCs have become a major focus for a potential resource in therapeutic cell-based therapies. MSCs are multipotent cells derived from stromal tissue, which have the capacity to differentiate into mesodermal and endodermal types of cells. Not only do MSCs have the capacity to differentiate into different types of cells depending on the tissue matrix, they also actively contribute to their milieu by secreting soluble products that actively participate in MSC and surrounding cell phenotype. These products can promote angiogenesis, regeneration, remodeling, immune cell activation or suppression, and cellular recruitment [38]. It has also been demonstrated that the plasticity (the ability of a cell to change its default fate) and tissue regenerative potential of MSCs may far exceed the primary use of bone marrow cells in the treatment of hematopoietic diseases [53].

Taking together the above features of MSC, it was speculate that these cells might be a tool that could be used in regenerative medicine for the treatment of degenerative diseases. Of special interest are neurological and neurodegenerative disorders, since they are the most challenging and lack effective therapies due to the limited plasticity of the central nervous system. *In vivo* experiments using different models of spinal cord injury and employing MSC alone or in combination with biomaterials revealed significant functional recovery of paralyzed limbs, reduced cavity formation in the spinal cord and better axonal regrowth through the glial scar [54].

The above properties of MSC led to the first pre-clinical and clinical trials, initially to treat myocardial infarction and later to treat stroke, amyotrophic lateral sclerosis, Parkinson's disease and other diseases of the central nervous system. Autologous MSC transplantation also has been shown to have a positive effect on patients with a severe cerebral infarct. As a result, growing interest in cell therapy approaches utilizing MSC has made these cells among the leading candidates for human application, and new trials are on the way to test these cells in patients [54].

MSCs cultured in poorly cultured conditions (cell passage performed after reach 100% of cell confluence), under extensive culture, or after stress conditions, can start to differentiate into mesenchymal lineages. For this reason, more information about differentiated MSC should be compiled and taken into account during the IS manufacturing process in case of being detected similar discrepancy.

3.3 Materials and Methods

3.3.1 ImmuneSafe® manufacturing

3.3.1.1 Collection of Bone Marrow

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

3.3.1.2 Isolation of hMSCs from Bone Marrow

BM mononuclear cells (MNC) were isolated using Sepax S-100 system (Biosafe), according to manufacturer's instructions. The Sepax cell processing system uses a rotating syringe technology that provides both separation through rotation of the syringe chamber (centrifugation) and component transfer through displacement of the syringe piston. The Sepax system allows the automated processing of blood components in a functionally-closed and sterile environment. The system uses Ficoll (GE Healthcare) to separate the low density BM-MNC, and saline solution, NaCl 0,9 % (Labesfal), to wash the cells. BM-MNCs were then plated in BD® ECM Mimetics, coated with Cell2B's recombinant Extracellular Matrices (ECM), using StemPro® MSC SFM XenoFree (Gibco) as a culture medium, or for performance comparison, BM-MNCs were plated in Corning® Polystyrene Tissue Culture Flasks, using Dulbecco's Modified Essential Medium (DMEM, Gibco) supplemented with 10% Fetal Bovine Serum MSC qualified (FBS, Gibco) at a density of 600 000 MNC/cm^2. Cell counting was performed using trypan blue (Gibco) exclusion assay.

Culture medium was changed every 3/ 4 days and cells were passaged when reached a confluence of 70-80%. The cell seeding varied according the passage number and study purpose, ranging from 1500 to 25000 cells/cm^2.

3.3.1.3 Cell cryopreservation and thawing

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

Cell thawing was performed by immersion of the cryovial at 37ºC water bath, followed by 10x dilution in culture medium. The cell suspension was then centrifuged at 1250 rpm for 7 min and cells were resuspended in the expansion culture media. Cell number and viability was determined using trypan blue exclusion assay.

3.3.2 Cell Expansion for CQAs

3.3.2.1 MSCs culture in serum-free conditions

Cells were growth in StemPro® MSC SFM XenoFree (Gibco) and due to the absence of adhesion proteins in this medium, culture vessels used are from BD® ECM Mimetics. Cells were seeded at 3000 cells/cm^2 and media was changed every 3/ 4 days. At 70-80% confluency cells were harvested from the culture flask by detaching cells using TrypLE™ solution (Gibco) for 7 min at 37ºC. Cell suspension was then centrifuged for 7 min at 1250 rpm and resuspended in the culture media. Cell number and viability was determined using trypan blue exclusion assay.

3.3.2.2 Adipocytes differentiated from MSCs

Cells were plated at 3000 cells/cm^2 (BD® ECM Mimetics) and media was changed every 3/4 days (StemPro® MSC SFM XenoFree (Gibco)). At 70-80% confluency adipogenic differentiation was induced by adding StemPro® Adipogenesis Differentiation Medium (Gibco) and incubating the cells for 15-21 days at 37ºC in the CO_2 incubator. Medium was changed every 3/ 4 days. Then the cells were harvested from the culture flask by detaching cells using TrypLE™ solution (Gibco) for 7 min at 37ºC. Cell suspension was then centrifuged for 7 min at 1250 rpm and resuspended in the culture media. Cell number and viability was determined using trypan blue exclusion assay.

3.3.2.3 Osteocytes differentiated from MSCs

Cells were plated at 3000 cells/cm^2 (BD® ECM Mimetics) and media was changed every 3/4 days (StemPro® MSC SFM XenoFree (Gibco)). At 70-80% confluency osteogenic differentiation was induced by adding StemPro® Osteogenesis Differentiation Medium (Gibco) and incubating the cells for 15-21 days at 37ºC in the CO2 incubator. Medium was changed every 3/ 4 days. Then the cells were harvested from the culture flask by detaching cells using TrypLE™ solution (Gibco) for 7 min at 37ºC. Cell suspension was then centrifuged for 7 min at 1250 rpm and resuspended in the culture media. When possible, cell number and viability was determined using trypan blue exclusion assay.

3.3.2.4 Fibroblasts

Human skin/ foreskin Fb (ATCC CRL-2522) were plated at 6000 cells/cm^2 (Corning® Polystyrene Tissue Culture Flasks) and media was changed every 3/ 4 days EMEM (ATCC) supplemented with 10% FBS (Gibco). At 80-90% confluency cells were washed with 1× Dulbecco's Phosphate-Buffered Saline (DPBS (Gibco)) and harvested from the culture flask by detaching cells using TrypLE™ solution (Gibco) for 7 min at 37ºC. Cell suspension was then

centrifuged for 7 min at 1250 rpm and resuspended in the culture media. Cell number and viability was determined using trypan blue exclusion assay.

3.3.3 Morphological analysis

Morphological analysis is a technique that enables staining cell's nuclei and cytoskeleton in order to observe morphological differences. To perform morphological analysis cells were plated in duplicate into a 24-well plate and the medium was exchanged every 3/ 4 days. When the cultures reached 80% confluency, cells were washed with DPBS 1x (Gibco) and fixed with 2% paraformaldahyde solution (PFA) (w/v) (Sigma) for 30 min at room temperature or overnight at 4ºC. Cell membranes were permeabilized with saponin/ 1x DPBS solution (50 µg/ml) (Sigma) (w/v) for 45 min at room temperature followed by incubation with rhodamin phalloidin (Molecular Probes) solution (1 µl Phalloidin/ 1ml 1× DPBS) during 60 min in the dark at room temperature. After this, cells were washed and labeled with DAPI (Molecular Probes) solution (2 drops/ml 1× DPBS) for 5 min. Cells were then washed and kept in 1× DPBS (Gibco) until observed with an optical fluorescence microscope (Axiovert 200M, Zeiss).

3.3.4 Multilineage differentiation

This assay intends to evaluate MSCs and Fb capacity to differentiate into osteogenic, adipogenic and chondrogenic lineages.

3.3.4.1 Adipogenic differentiation

Cells were plated in a 24-well plate (Corning® Polystyrene Tissue Culture Flasks: for cells grown with FBS; BD® ECM Mimetic: for cells grown without serum) with specific media culture for each cell line (EMEM (ATCC) for Fb and StemPro® MSC SFM XenoFree (Gibco) for MSCs) and media changed every 3/ 4 days until reach a confluence between 70-80%, afterwards adipogenic differentiation was induced by adding StemPro® Adipogenesis Differentiation Medium (Gibco) and incubating the cells for 15-21 days at 37ºC in the CO2 incubator. Medium was changed every 3/ 4 days.

At the end of the culture period, cells were washed with 1× DPBS (Gibco) and fixed using 2% PFA (w/v) for 30 minutes at room temperature or overnight at 4ºC. Then, cells were washed with 1× DPBS (Gibco) and stained with Oil Red-O solution (Sigma) prepared in isopropanol (Sigma) to a final concentration of 0.3% (v/v) by incubation for 1 hour at room temperature. Cells were then rinsed twice with ultrapure water and observed with an optical microscope (Primo Vert, Zeiss). The Oil Red-O staining is used to stain lipid droplets in mature adipocytes (stain red).

3.3.4.2 Osteogenic differentiation

Cells were plated in a 24-well plate (Corning® Polystyrene Tissue Culture Flasks: for cells grown with FBS; BD® ECM Mimetic: for cells grown without serum) with specific media culture for each cell line (EMEM (ATCC) for Fb and StemPro® MSC SFM XenoFree (Gibco) for MSCs) and media changed every 3/ 4 days until reach a confluence between 70-80%, afterwards osteogenic differentiation was induced by adding StemPro® Osteogenesis Differentiation Medium (Gibco) and incubating the cells for 15-21 days at 37ºC in the CO_2 incubator. Medium was changed every 3/ 4 days.

In the end of the culture period, cells were washed with 1× DPBS (Gibco) and fixed using 2% PFA (w/v) for 30 minutes at room temperature or overnight at 4ºC. First ALP (Alkaline Phosphatase) staining was performed by washing the cells with ultrapure water and incubating them for 45 minutes in the dark at room temperature with Fast Violet solution (Sigma) diluted in a concentration of 4% (v/v) in Naphthol AS-MX Phosphate Alkaline solution (Sigma). ALP staining detects the increase of activity of this enzyme producing a reddish staining in osteoblast-like cells. Afterwards von kossa staining was performed by washing the cells 3 times with DPBS (Gibco) and adding 2.5% silver nitrate solution (w/v) (Sigma) with incubation for 30 minutes in the dark at room temperature. Cells were washed 3 times with ultrapure water and observed with an optical microscope (Primo Vert, Zeiss). Von kossa staining is used to demonstrate calcium deposits that are released by osteocytes by the precipitation reaction of silver ions with phosphate in the presence of acid.

3.3.4.3 Chondrogenic differentiation

Chondrogenesis is induced using micromass culture. A cell suspension was prepared at approximately $1.6x10^7$ cells/ml and drops of 10-20 µl were seeded in 24 well ultra-low attach culture plates (Corning) under humidified conditions before addition of StemPro® Chondrogenesis Differentiation Medium (Gibco). Cells were incubated for 15-21 days at 37ºC in the CO_2 incubator. Media was carefully changed to avoid removing the aggregates every 3/ 4 days.

In the end of the culture period, cells were washed with 1× DPBS (Gibco) and fixed using 2% PFA (w/v) for 30 minutes at room temperature or overnight at 4ºC. Afterwards, cells were washed with 1× DPBS (Gibco) and stained with Alcian Blue (Sigma) solution prepared in 0.1 N HCl to a final concentration of 1% (w/v) by incubation for 30 minutes at room temperature. Cells were then rinsed three times with 0.1 N HCl and finally rinsed with ultrapure water to be observed with an optical microscope (Primo Vert, Zeiss). Alcian Blue is a dark-blue copper-containing dye, which indicates synthesis of proteoglycans by chondrocytes. Alcian blue detects

the proteoglycan aggrecan that is an indicator for cartilage formation, retaining the dark-blue stain.

3.3.5 Cells stimulation with IFN-γ and TNF-α

In order to simulate a pro-inflammatory environment and evaluate cells behavior under this type of environment, cells were stimulated with IFN-γ and TNF-α and compared with non-activated cells. The cells were stimulated between 70-80% confluence with 500 U/ml IFN-γ (R&D system) and 10 ng/ml TNF-α (Sigma) during 48h.

3.3.6 Immunophenotype characterization

Immunophenotype characterization by flow cytometry was performed to assess the maintenance of the expression of specific antigens. Cells resuspended in 1x DPBS (Gibco) were divided in FACS tubes (100 000 cells in 100µl per tube) and the corresponding antibody was added (according manufacture instructions). Appropriate isotype controls were used in every experiment. The broader panel consists: CD11b (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-A,B,C (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 labeling mixture was then incubated in the dark for 15 min at room temperature. For the washing step 2 ml of 1x DPBS (Gibco) were added to the mixture. The tubes were then centrifuged at 1000 rpm for 5 min and cell pellet resuspended and fixed in 500 µl of 2% PFA (w/v) (Sigma). The tubes were stored at 4ºC in the dark for up to 3 weeks. The samples were run in FACSCalibur equipment (BD) and analysed using Flowing Software 2.

3.3.7 Metabolic enzymes production (Western-Blot)

Metabolic enzymes in study, IDO and HO-1 are soluble proteins, which may play a role in MSC-mediated immunosuppression, and were analysed by western-blot (WB).

Cell lysates were prepared using RIPA buffer (Sigma) supplemented with Complete protease inhibitor cocktail (Roche) (1 ml/ well of a 6 well plate and 2 ml/ T25).

For western blot analysis, cell lysates were prepared and 25 µl from cleared lysate were resolved by SDS-PAGE (4%-12% Bis-Tris gels, NuPAGE, Invitrogen), transferred to PVDF membranes (iBlot system, Invitrogen) and probed with the specific primary antibodies (IDO

(anti-IDO (Indoleamine-2,3-Dioxygenase), clone 10.1 (Merck Millipore)) at a 1:5000 dilution and HO-1 (Anti- Heme Oxygenase -1 Mouse mAb (HO-1), Calbiochem) at a 1:200 dilution).

In the case of HO-1 detection 200 µl of cell lysate samples were first concentrated 4 times (10KDa Amicons, Merck Millipore) before loaded into the SDS-PAGE gel.

After electrophoresis proteins were visualized using a rabbit anti-mouse secondary antibody conjugated to HRP (Sigma) at 1:5000 and a chemioluminescence detection system (Licor, Bonsai Technologies).

Seeblue Plus2 Prestained Protein Standard (Invitrogen) and Precision Plus Protein Standard (BioRad) were used as a marker and standard, respectively.

3.3.8 Cytokine production analysis (ELISA)

Enzyme-linked immunosorbent assay (ELISA) was used to analyze and compare cytokine production from different conditions. For this study, two ELISA kits were used to detect different human cytokines, IL-6 (RayBio) and PGE2 (Arbor Assays).

Exhausted media, stimulated with IFN-γ and TNF-α and non-stimulated were collected and supernatants were stored at -20ºC until further analysis by ELISA. The ELISA was performed according to the manufacturer's instructions. Plates were read using a microplate reader, PowerWave XS (Bio-tek).

3.3.9 Secretome

Secretome analysis was performed by tebu-bio, using the profiling service on Quantibody Human Angiogenesis Array 1000.

Exhausted media, stimulated and non-stimulated were collected and supernatants were stored at -20ºC until further analysis.

3.3.10 Transcriptome

Differential gene expression, between MSC non-stimulated and stimulated with IFN-γ+TNF-α, was carried out based on their trancriptomes.

mRNA was extracted from 1-1.5x10^6 cells, pellets stored at -80ºC, using RNeasy Mini Kit (Quiagen) with β-ME for cellular lysis, QIAshredder for cellular homogenization, digested with DNase and eluted in 50 µl.

Transcriptome analysis was performed by Geneinseq, Next Gen Sequencing Unit at Biocant.

The transcriptomes were sequenced as single-end reads in the Ion Proton (Life Technologies) high throughput sequencing platform. Data was processed using the Trinity package and the differential gene analysis determined with R/bioconductor packages.

3.3.11Immunosuppression potential

Immunosuppression effect of MSC in lymphocyte sub-populations (T helper, T cytotoxic and NK) were performed in partnership with Blood and Transplantation Centre of Coimbra. Immunosuppression potential of MSCs was based in the suppression of two cytokines production, TNF-α and IL-17, on lymphocytes.

3.3.11.1 Mononuclear cells isolation from peripheral blood

Blood samples were obtained from healthy donors after informed consent.

Blood samples, collected in heparin, were diluted in saline solution (1:2) and layered over Lymphoprep (STEMCELL Technologies) in a ratio of 2:1 (diluted blood: Lymphoprep) and centrifuged at 1200 g, 20 minutes, at 18ºC. Mononuclear layer was aspirated and washed with Hank's Balanced Salt Solution 1x (HBSS) (Gibco), 430 g, 15 min at 18ºC. Pellet was resuspended in 1ml of RPMI 1640 (Gibco). Cell number and viability was determined using trypan blue exclusion assay.

3.3.11.2 Co-culture of MSC, BM-MNC, Fb or Differentiated cells with MNC from peripheral blood

MNCs isolated from peripheral blood and allogeneic human MSCs were co-cultured in a 24 well plate (TPP). The ratio between MSC and MNC was 1:2 (250 000 MSC to 500 000 MNC) in RPMI 1640 (Gibco) plus 10% FBS for 24h, at 37ºC with 5% of CO_2 and 90% humidity. After incubation the cells were activated or not, in presence of 50 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma), 1 µg/ml of ionomycin (IONO) (Boehringer Mannheim) and 10 µg/ml brefeldin A from *Penicillium brefeldiamun* (Sigma), during 4 hours, in the same environment.

Different stimulation approaches were performed for MNC:

MNC + PMA + Ionomicina and MNC + PMA + Ionomicina + MSC.

3.3.11.3 Functional characterization of T cell subpopulations

For functional characterization study of T cell subpopulations through pro-inflammatory cytokine production, sample was incubated with the following monoclonal antibodies CD3 Pacific Blue (clone UCHT1, BD Pharmigen), CD8 Allophycocyanin Elite 7 (clone SK1, BD), CD4 Phycoerythrin Cyanine 7 (clone SFCI12T4D11, Beckman Coulter), CD45Ra Allophycocyanin (clone HI100, BD) and incubated in the dark for 10 min. After incubation 100 µl of Fix reagent of Intraprep™ kit (Beckman Coulter) was added and incubated for 10 min.

Then the cells were washed with 2 ml of 1x DPBS (Gibco) and centrifuged at 430 g for 5 min. Cell pellets were resuspended in 100 µl of Permeabilization reagent of Intraprep^{TM} kit and were added the following intracytoplasmic monoclonal antibodies: TNF-α Fluorescein isothiocyanate and IL-17 Phycoerythrin and incubated for 10 min. After incubation the cells were washed with 2 ml of 1x DPBS (Gibco) and centrifuged at 430 g for 5 min. In the end, the cells were resuspended in 250 µl of 1x DPBS (Gibco) and acquired in a flow cytometer (FACS Canto TM II (BD)).

3.3.11.4 Acquisition and analysis by flow cytometry

Cells were acquired in a flow cytometer FACS Canto TM II (BD), using the software FACSDiva 6.1.2 (BD).

Data were afterwards analysed using software Infinicyt 1.5 (Cytognos).

Selection of different T cell population were based: T cells are $CD3^+$, T cytotoxic are $CD3^+$ and $CD8^+$, T helper are $CD3^+$ and $CD4^+$ and NK are $CD3^-$ and $CD45Ra^+$.

3.3.12Karyotype

Chromosome analysis were performed at *"Instituto Nacional de Saúde Doutor Ricardo Jorge"* through GTL (Geimsa/ Tripsin/ Leishman) banding, followed by microscopy analysis.

3.3.13Single-nucleotide Polymorphism (SNPs)

Gene amplification/ deletions and loss of heterozygosity (LOH) were evaluated on two IS batches (each batch included BM-MNC, MSC in P2 and P4 in ECM28 and P4 DMEM) at Genomic Unit at Biocant, after genomic DNA (gDNA) purification using DNeasy® Blood & Tissue kit (Quiagen) according manufacturing instructions for cultured cells (cell pellets with 1-1.5x10^6 cells, stored at -80ºC).

After purification gDNAs were precipitated using Ethanol (Scharlau)/ Isopropanol (Sigma) precipitation. Glycogen (Fermentas) (20 mg/ml) was added. The mixtures were incubated at -80ºC overnight. After incubation, the mixture was centrifuged at 13000 g for 10 min, the pellet was washed with 70% cold ethanol. The pellets were allowed to dry and resuspended in a suitable volume (±50 µl) of steriled miliQ water.

gDNA was quantified spectrophotometrically using Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies) and fluorometry by Qubit 2.0 Fluorometer (Invitrogen by Life Technologies) using Quant-iT Pico Green dsDNA Assay kit (Invitrogen/Molecular Probes by Life Technologies).

gDNA labelling with Cy3 was performed using SureTag Complete DNA Labeling kit (Agilent), according manufacture instructions.

The array used was Sure Print G3 Human Cancer CGH+SNP Microarray Kit 180 K (Agilent). This array has more resolution in regions with higher chromosomal instability.

3.3.14 Data analysis

Data are presented as mean ± standard error, and were analysed with GraphPad Prism 6 software, t test- one per row. *P* value <0,05 was considered to be statistically significant.

3.4 Results and Discussion

For a full biological characterization of IS features, a set of (bio) assays panel were defined to be performed along the manufacturing process, in passages 2 and 4. Full biological characterization of IS was set in P2 and P4 because in manufacturing process will correspond to cryopreservation steps. The two cryopreservation steps were establish in IS manufacturing mainly for economic reasons and thus allow the exclusion of batches that do not correspond to defined product parameters in early passages. (Bio) assays panel include morphology analysis, multilineage differentiation potential, immunophenotype characterization, IDO and HO-1 detection, cytokine quantification, secretome, transcriptome, immunosuppression potential, karyotype and SNPs.

3.4.1 (Bio) assay panel for ImmuneSafe® CQAs

3.4.1.1 ImmuneSafe® morphology

MSCs proliferate *in vitro* as plastic-adherent cells, have fibroblast-like morphology and grow in monolayer cultures [16], [20]. IS batches were stained with DAPI and rhodamin phaloidin, for visualization of nucleus and actin filaments, respectively. Images of two IS batches (BM21 and BM26 in P2 and P4) were presented on Figure 3.2.

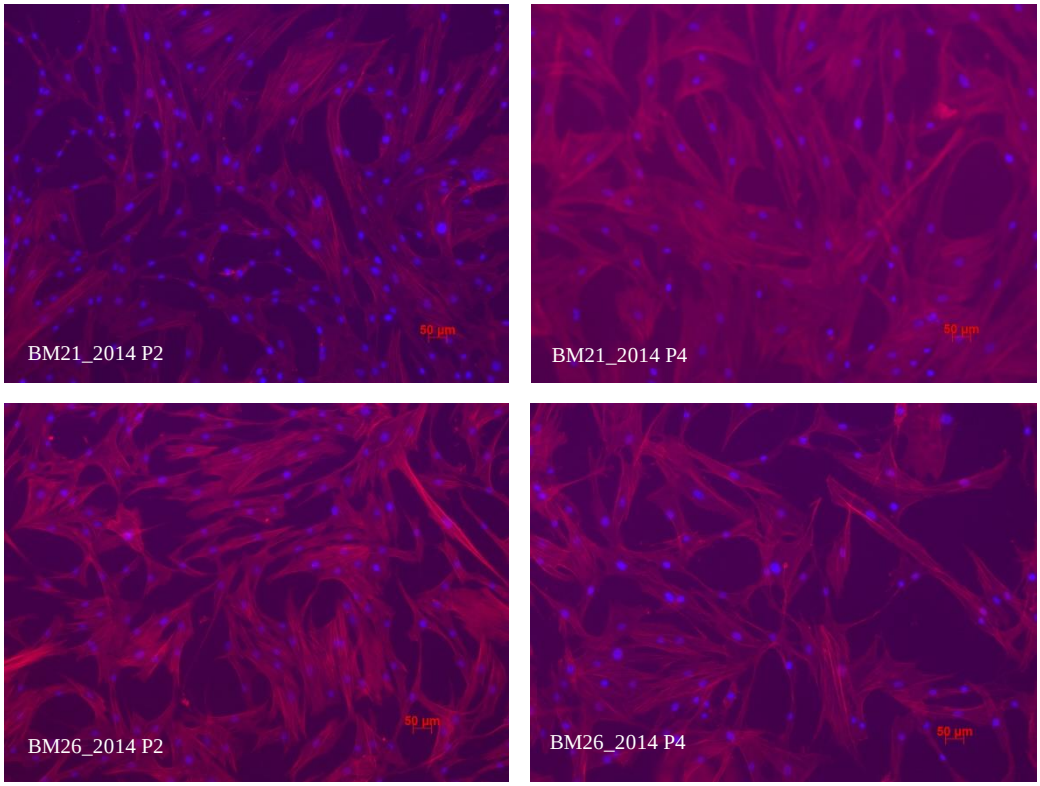


Figure 3.2 ImmuneSafe® morphology in P2 and P4 of BM21 and BM26_2014, stained with DAPI and rhodamin phalloidin.

All IS batches grew in monolayer culture and displayed spindle-shaped morphology, without other cells types visualized in culture. This morphology analyses indicate that Cell2B manufacturing process, without serum, seems to be an efficient method for MSCs isolation from BM. Relevant morphological differences were not verified between P2 and P4, suggesting that Cell2B manufacturing process does not alter the MSCs morphology.

3.4.1.2 ImmuneSafe® multilineage differentiation potential

Another identity feature of MSCs is the ability to differentiate in osteocytes, chondrocytes and adipocytes *in vitro* [20]. All analysed IS batches demonstrated the capacity to differentiate in the three main mesodermal lineage (adipocytes, osteoblasts and chondrocytes) and this feature was maintained throughout the expansion (P2 and P4), as summarized on Figure 3.3.

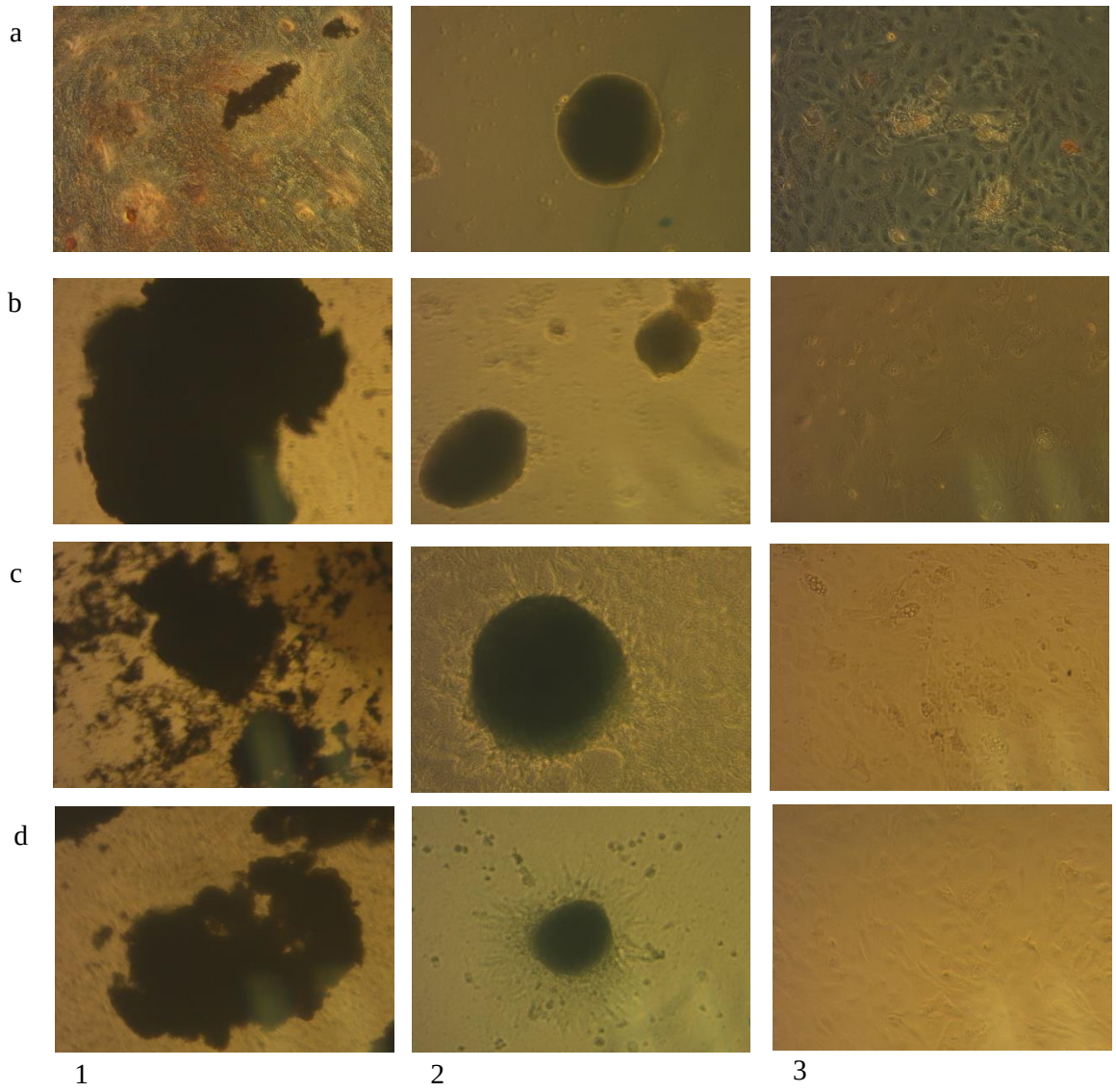


Figure 3.3 ImmuneSafe® differentiation of BM21 and BM26_2014, in P2 and P4. (a) BM21 in P2 (b) BM21 P4 (c) BM26 P2 and (d) BM26 P4, (1) Osteogenesis, (2) Chondrogenesis and (3) Adipogenesis differentiation.

3.4.1.3 ImmuneSafe® immunophenotype

The last identity test performed to IS was the immunophenotype characterization using identity surface markers. Furthermore, others surface markers were also tested, such as membrane proteins and others markers envolved on immune system regulation.

IS unstimulated immunophenotype was compared with IS immunophenotype under pro-inflamatory environment (IFN-γ + TNF-α) to identify the surface markers who responded to this pro-inflamatory stimulus and may be part of IS mechanism of action *in vivo* for immunological diseases.

Phenotypically, MSCs express a number of markers, none of each are specific to MSCs. It is generally agreed that adult human MSCs do not express the hematopoietic markers CD45, CD34, CD14, or CD11. They also do not express the co-stimulatory molecules CD80, CD86, or CD40 or the adhesion molecules CD31 (platelet/endothelial cell adhesion molecule (PECAM)-1), CD18 (leukocyte function-associated antigen-1 (LFA-1)), or CD56 (neuronal cell adhesion molecule-1), but they can express CD105 (SH2), CD73 (SH3/4), CD44, CD90 (Thy-1), and Stro-1 as well as the adhesion molecules CD106 (vascular cell adhesion molecule (VCAM)-1), CD166 (activated leukocyte cell adhesion molecule (ALCAM)), CD54 (intercellular adhesion molecule (ICAM)-1), and CD29 [55].

The analysis of IS immunophenotype (presented in Figure 3.4 and Figure 3.5) demonstrate that IS identity corresponded typical MSC phenotype. IS did not express hematopoietic markers (such as CD11b, CD19, CD14, CD34, CD45), HLA-class II (MSC only express HLA-DR surface molecules in presence of IFN-γ but not in an unstimulated state [20]), CD31 and co-stimulatory molecule like CD80, nonetheless, IS expressed CD73, CD90 and CD105.

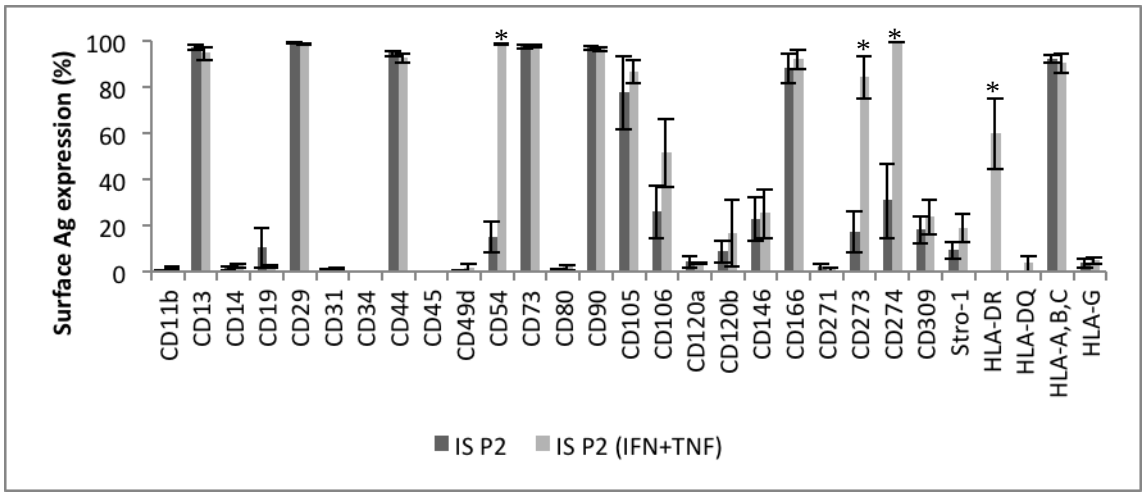


Figure 3.4 Percentage of ImmuneSafe® surface antigens expression in P2, without and with IFN-γ + TNF-α stimulation. (N=5) **P*<0.05

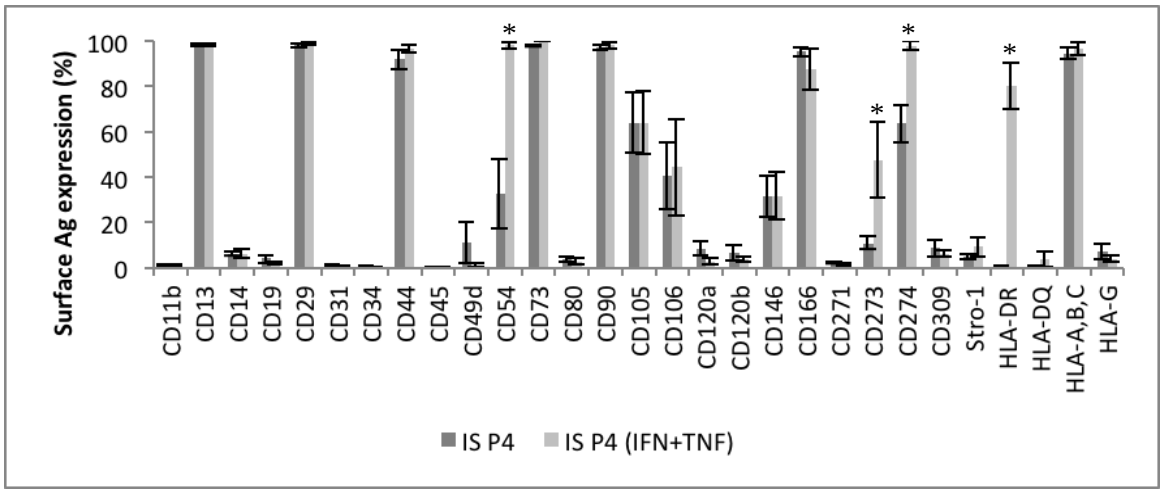


Figure 3.5 Percentage of ImmuneSafe® surface antigens expression in P4, without and with IFN-γ + TNF-α stimulation. (N=5) **P*<0.05

Cell-cell adhesion mediated by CD54 (ICAM-1) and CD106 (VCAM-1) is critical for T cell activation and leukocyte recruitment to the inflammation site and, therefore, plays an important role in evoking effective immune responses. ICAM-1 and VCAM-1 are upregulated by inflammatory cytokines, and such upregulation render MSCs more adhesive to T cells [56]. On IS ICAM-1 was upregulated under pro-inflammatory environment, (*P*<0.05) while the expression of VCAM-1 was maintained after stimulation with IFN-γ + TNF-α.

Under the pro-inflammatory environment CD273 (Programmed death (PD-L) 2) and CD274 (PD-L1) responded by increasing their expression (p<0.05), a phenomenon also described on the literature where IFN-γ upregulates the expression of markers of immune modulatory molecules (CD200, CD274/PD-L1/B7-H1) [22]. Some *in vitro* studies suggest that PD-L1 and PD-L2 can inhibit T cell proliferation and cytokine production, whereas others indicate that PD-L1 enhance T cell activation. The reasons for the contradictory results of those functional studies are not clear but may reflect different preparations of PD-L immunoglobulin fusion protein acting as agonists or antagonists [57].

CD44 and CD166 (ALCAM) were constitutively expressed. CD44 is a multifunctional receptor involved in cell-cell and cell-ECM interactions, cell traffic, lymph node homing, presentation of chemokines and growth factors to traveling cells, and transmission of growth signals. CD44 also participates in the uptake and intracellular degradation of hialuronic acid, as well as in transmission of signals mediating hematopoiesis and apoptosis [58] and CD166 plays an important role in mediating adhesion interactions between thymic epithelial cells and $CD6^{+}$ cells during intrathymic T cell development [59].

CD120a and CD120b, also known as TNFRI and TNFRII (tumor necrosis factor receptor type 1 and 2) respectively, were expressed at very low basal levels for both conditions. The main

function of CD120a is leading apotptosis, inflammation, tumor necrosis and cell differentiation and CD120b when complexed with TNFRI, recruits anti-apoptotic molecules [59].

Although Stro-1 has been claimed as a specific marker for MSCs [49] it was not significantly expressed by IS. This could be due the fact that Stro-1 has been identified *in vivo* [22] and it expression is down regulated in culture [23].

Relevant differences were not observed between IS in P2 and P4. IS showed identical immunophenotype and response to IFN-γ and TNF-α stimulus in both passages. These results indicate one more time that Cell2B manufacturing process enable hMSC isolation from hBM, with capacity to react to a pro-inflammatory environment and retain this capacity along expansion.

3.4.1.4 IDO and HO-1 production by ImmuneSafe®

IS soluble proteins from cell lysates of IS in P2 and P4, non-stimulated and stimulated with IFN-γ and TNF-α were separated in a SDS-Page gel, followed by WB detection in order to verify IS capacity to produce IDO and HO-1 under a pro-inflammatory environment.

IDO is widely produced by human cells and tissues during inflammatory responses by IFN-γ and other inflammatory cytokines [44]. Additionally, IDO activation is considered a pivotal mechanism in lymphocyte inhibition with MSCs [22] through the catalysing of tryptophan conversion to kynurenine [6]. HO-1 plays an important role on immune regulation [9] and was also shown to be produced by MSCs upon stimulation [8].

Based on the results showed on Table 2, IDO was only expressed under pro-inflammatory environment. On the other hand, HO-1 expression was not up regulated by pro-inflammatory environment as referred in the literature. Moreover, HO-1 expression was not verified in all analysed batches. A qualitatively comparison of IS in P2 and P4, IS in the earlier passage apparently demonstrate a higher expression level of HO-1. HO-1 expression and immunosuppressive function is referred on the literature to be down regulated over time in culture [42].

Table 2 Rating of IDO and HO-1 production by IS in P2 and P4, without stimulation and stimulated with IFN-γ and TNF-α. (P2, N=3; P4, N=6)

			Results	
Sample	**Passage**	**Condition**	**IDO**	**HO-1**
ImmuneSafe	P2	-TNF-IFN	-	+
			-	+
			-	-
		+TNF+IFN	++	+
			++	+
			+	-
	P4	-IFN-TNF	-	+
			-	-
			-	-
			-	-
			-	-
			-	-
		+TNF+IFN	+++	-
			+	-
			+	-
			+	-
			+	-
			+	-

IDO production under a pro-inflammatory environment by IS is indicative that IS was capable to immunomodulate the immune system.

3.4.1.5 ImmuneSafe® Secretome

3.4.1.6 ImmuneSafe® cytokine production (ELISA)

In order to determine if IS has the ability to respond to a pro-inflammatory environment also by producing anti-inflammatory cytokines PGE2 and IL-6, the exhausted culture media from non-stimulated and stimulated with IFN-γ and TNF-α cultures were analysed by ELISA, data shown on Figure 3.6.

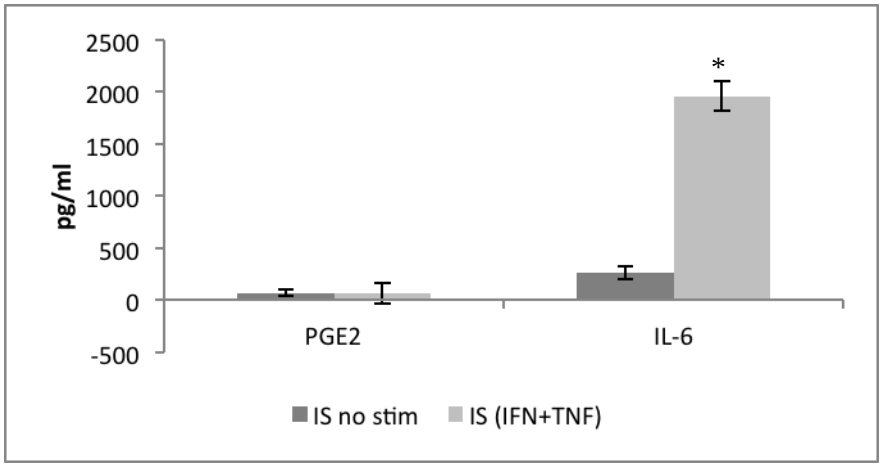


Figure 3.6 Comparison of PGE2 and IL-6 production by ImmuneSafe®, in culture without stimulation and stimulated with IFN-γ and TNF-α. (PGE2, N=2; IL-6, N=4) **P*<0.05

Based on Figure 3.6, PGE2 production by MSCs was not enhanced under a pro-inflammatory environment (unstimulated 65.5 pg/ml and stimulated 59.6 pg/ml). This contradicts what is reported on the literature that PGE2 production by MSCs is enhanced following TNF-α or IFN-γ stimulation [8]. This result may indicate that PGE2 is not a stable protein and could be degraded during the storage of exhausted medium at -20ºC or IS does not respond to pro-inflammatory environment increasing the secretion of PGE2.

On the other hand, IL-6 production was highly promoted under pro-inflammatory environment (*P*=0,0003) (unstimulated 264.0 pg/ml and stimulated 1962.1 pg/ml). Djouad *et al.* showed high levels secretion of IL-6 and VEGF by murine MSC, which are directly correlated to the inhibition of T cell proliferation [60]. IL-6 secreted by MSCs is considered the major soluble factor, if not the only one, responsible for the anti-apoptotic effect of MSCs [61].

3.4.1.7 ImmuneSafe® secretome (tebu-bio)

Exhausted medium of five IS batches non-stimulated and stimulated with IFN-γ and TNF-α were also analysed for other cytokines. Table 3 summarizes the cytokines that were up regulated, down regulated and unaffected under a pro-inflammatory environment. These results suggest IS can active several metabolic pathways and switch off another's in response to a pro-inflammatory environment.

Table 3 Impact of pro-inflammatory environment on ImmuneSafe® cytokine production. Up regulated: at least 2 fold increase; Down regulated: at least 2 fold decrease;

Family	No effect	Up regulated	Down regulated
Chemokines	CCL2	CCL1, CCL5, CCL7,CCL8, CCL13, CXCL1, CXCL5, CXCL10, CXCL11, CXCL16	-
Metalloproteinases	TIMP-1, TIMP-2	MMP-1, MMP9	-
Interleukins	IL-1b, IL-2, IL-4, IL-10, IL12p70	IL-1a, IL-6, IL-8 (CXCL8), IL-17	IL-12p40
Pro-inflammatory	TNF-β	-	-
Hematopoiesis related	-	TPO, G-CSF, GM-CSF	-
Angiogenese/ Tissue repair	bFGF, EGF, VEGF, Tie-1, PDGF-BB, PIGF	Angiogenin, ANG-2, HB-EGF, HGF, Angiostatin, VEGF-R3, VEGF-D, TGFα, IGF-1, LIF	ANG-1, FGF-4, VEGF-P2, Tie-2, PECAM-1
Inflammatory response	TGFβ1	Follistatin, TGFβ3	Activin A
Cell metabolism	-	ANGPTL4, AgRP, Leptin	-
Cell migration	-	uPAR	-

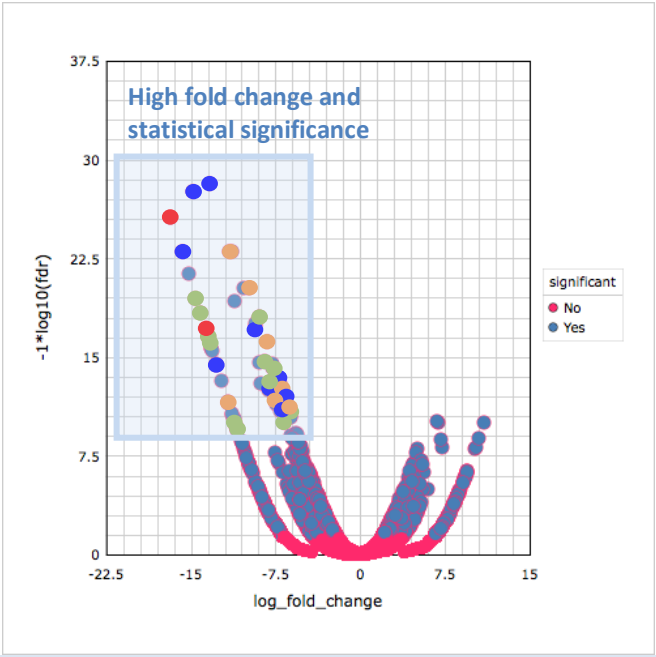
Chemokines are involved in a diverse range of biological processes, including leukocyte trafficking, hematopoiesis, angiogenesis, and organogenesis [4]. Metalloproteinases released by MSCs complement chemokine function through degradation of the endothelial vessel basement membrane to allow extravasation into damage tissue [37]. Accordingly to the results presented on Table 3, IS can have the capacity to home to site of injury through migration cross endothelium and support hematopoiesis, angiogenesis, and organogenesis.

Through the secretion of ILs, IS can have the capacity to modulate growth, differentiation and activation during an immune response [43]. IL-17 mediate biological function via surface receptors on target cells, intervening on regulatory role on host defense and inflammatory diseases [40]. IL-8 is a chemokine (CXCL8) secreted by several cell types and is produced upon stimulation with inflammatory stimuli. It functions as a chemoattractant, and is also a potent angiogenic factor and exerts a variety of functions on leukocytes and particularly on neutrophils [62]. IL-1a is pleiotropic cytokine involved in various immune responses, inflammatory processes, and hematopoiesis [63].

In the presence of damaged tissue, MSCs have been shown to be able to support and induce tissue repair by giving rise to a regenerative microenvironment. This capacity is highlighted by the IS capacity to secreted several proteins that are anti-apoptotic and that can simulate local angiogenesis. Among them are angiogenin that is capable to stimulate angiogenesis [64], ANG-2 a key regulator of angiogenesis that exerts context-dependent effects on ECs [65], HGF a potent mitogen [66], VEGF a highly specific mitogen for vascular endothelial cells [67], LIF that affects cell growth by inhibiting differentiation [68].

3.4.1.8 ImmuneSafe® Transcriptome

A differential gene expression analysis were carried out for 2 samples (non-stimulated and stimulated with IFN-γ + TNF-α) of total RNA from one IS batch. The transcripts with higher fold change (with high statistical significance) under pro-inflammatory environment are present on the upper left side of the volcano graph on Figure 3.7.



Chemokines	CCL5, CCL7, CCL8, CXCL1, CXCL2, CXCL3, CXCL5, CXCL8, CXCL9, CXCL11
Interleukins	IL-1a, IL-1b, IL-4I1, IL-6, IL-15RA, IL-18BP, IL-32
Antigens	CD38, CD40, CD54 (ICAM-1), CD74, HLA-DQA1, HLA-DQB1, HLA-DRA, HLA-DRA1, HLA-DRB1, HLA-DRB5, TNFS13B, TNFAIP3, TNFSF10
Others	IDO, CSF2 (GM-CSF)

Figure 3.7 Differential mRNA expression between ImmuneSafe® non stimulated and stimulated with IFN-γ + TNF-α.

The majority of transcripts that were highly expressed by IS, under pro-inflammatory environment are in concordance with previous characterization studies of IS (immunophenotype, secretome and metabolic enzymes). mRNA of CCL5, CCL7, CCL8, CXCL1 and CXCL5 was highly expressed after stimulation matching with secretome results observed before. The same trend was also observed for IL-1a and IL-6. On the other hand, protein expression of IL-1b was not improved after stimulation (was maintained), contrasting with mRNA expression that was enhanced after stimulation.

mRNA expression of CD54 (ICAM-1), HLA-DQ and HLA-DR haplotypes were enhanced after stimulation. From the three, HLA-DQ when analysed by cytometry was not expressed, neither under pro-inflammatory environment. Although CD120a (TNFRI) and CD120b (TNFRII) were not detected on IS surface by immunophenotype analysis, the mRNA of genes belonging to the tumour necrosis factor superfamily were highly expressed (e.g. TNFS13B, TNFAIP3, TNFSF10).

Importantly, IDO mRNA was one of the transcripts with higher fold change after IFN-γ + TNF-α stimulation, corroborating with previous obtained results and confirming IDO production as one of the immunomodulatory pathways activated by IS under pro-inflammatory environment.

3.4.1.9 ImmuneSafe® immunosuppression potential

IS immunosuppression potential was based on the ability of MSCs to suppress the activity of T cells and NK cells (pro-inflammatory cytokines production), the main cellular players in GvHD development.

In a co-culture system with activated PBMCs, IS was able to suppress the production of pro-inflammatory cytokines, IL-17 on T cells (T cytotoxic and T helper) and TNF-α on T cells and NK as shown on Figure 3.8.

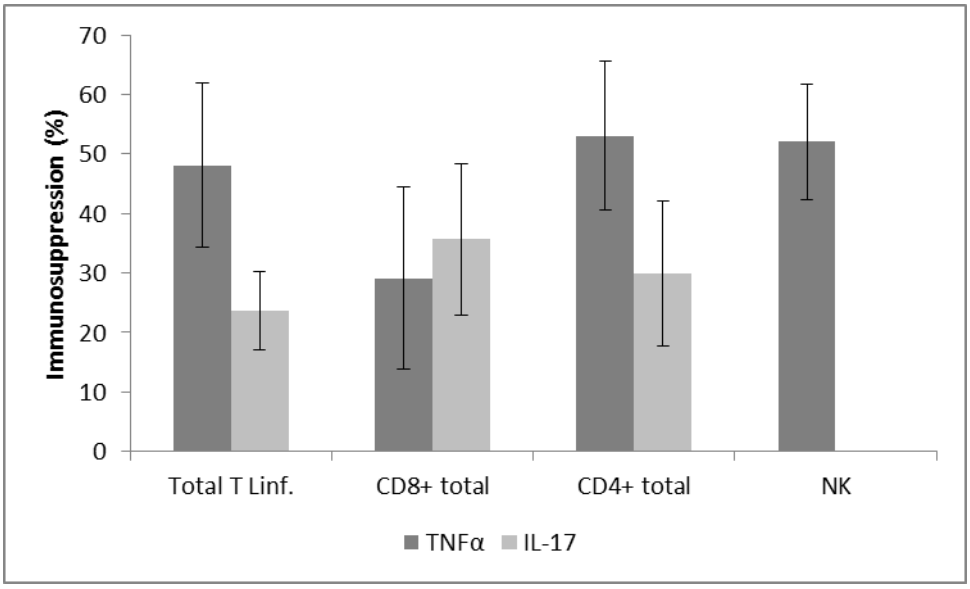


Figure 3.8 Immunosuppression of T lymphocytes, Tc, Th and NK cells by ImmuneSafe®, based on the suppression of TNF-α (N=4) and IL-17 (N=3) production.

IS suppressed 48.1% of TNF-α production on total T lymphocytes, 29.1% on T cytotoxic, 53.1% on T helper and 52.1% on NK cells. Regarding IL-17 suppression, IS suppressed 23.6% the production of IL-17 on total T lymphocytes, 35.7% on T cytotoxic and 30.0% on T helper cells.

These results are in conformity with literature where MSCs have been consistently reported that have suppressive effect on CD4+ T helper cells and CD8+ cytotoxic T lymphocytes [41] and have the capacity to inhibit NK cells proliferation and cytokine production, but only under pro-inflammatory environment [33].

3.4.1.10 ImmuneSafe® Karyotype

Karyotype analysis of five IS batches presented a normal karyotype, 46 XX or 46 XY depending on the donor gender.

3.4.1.11 SNPs of ImmuneSafe®

Two batches of MSCs were analysed to detect any structural genomic alterations. For this purpose DNA from IS in P2 and P4 and MSC isolated and expanded using a FBS-supplemented system in P4 were compared with initial sample DNA from BM-MNCs.

The results did not showed any structural genomic alterations when compared with initial sample. So, in both cellular propagation processes, standard method (FBS-supplemented system) and Cell2B IP preserved the DNA integrity.

3.4.2 BM-MNCs *vs.* ImmuneSafe®

IS CQAs were compared with BM-MNCs CQAs to demonstrate that BM-MNCs do not have the same biological/ therapeutic function and Cell2B manufacturing process have an high contribution in the final product achievement.

3.4.2.1 BM-MNCs morphology

As shown on Figure 3.9, BM-MNCs (a) are mainly composed by hematopoietic cells and present morphological features significantly different from IS. The large majority are non-adherent smaller cells with a round shape. On the other hand, IS (b) are long and thin with large nucleus.

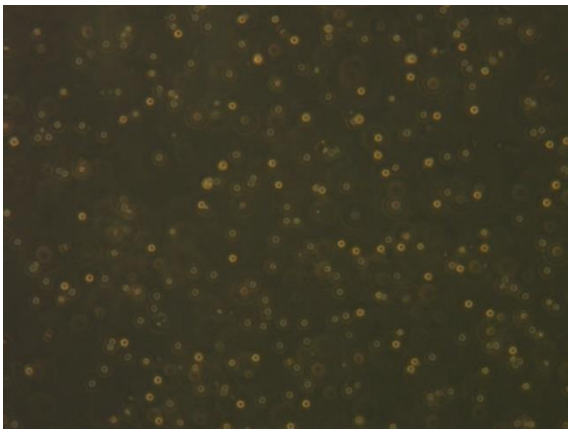
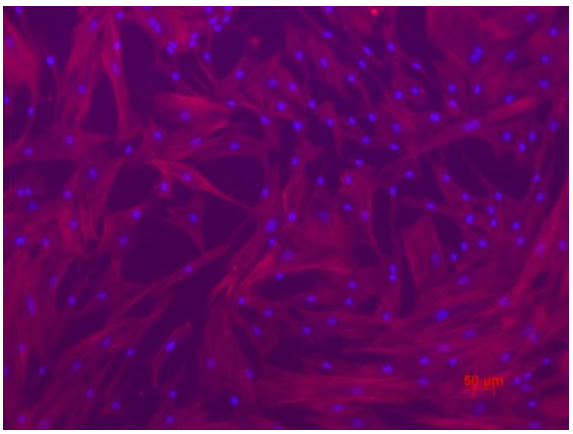
a  b 

Figure 3.9 Morphological differences between BM-MNC and ImmuneSafe®. (a) BM-MNCs under optical microscope. (b) ImmuneSafe® stained with DAPI and rhodamin phalloidin under optical fluorescent microscope.

3.4.2.2 Immunophenotype comparison between BM-MNC and ImmuneSafe®

The immunophenotype of BM-MNC is considerably different from IS immunophenotype, as shown on Figure 3.10, demonstrating that the culture process is determinant to achieve a unique MSC population from the BM sample.

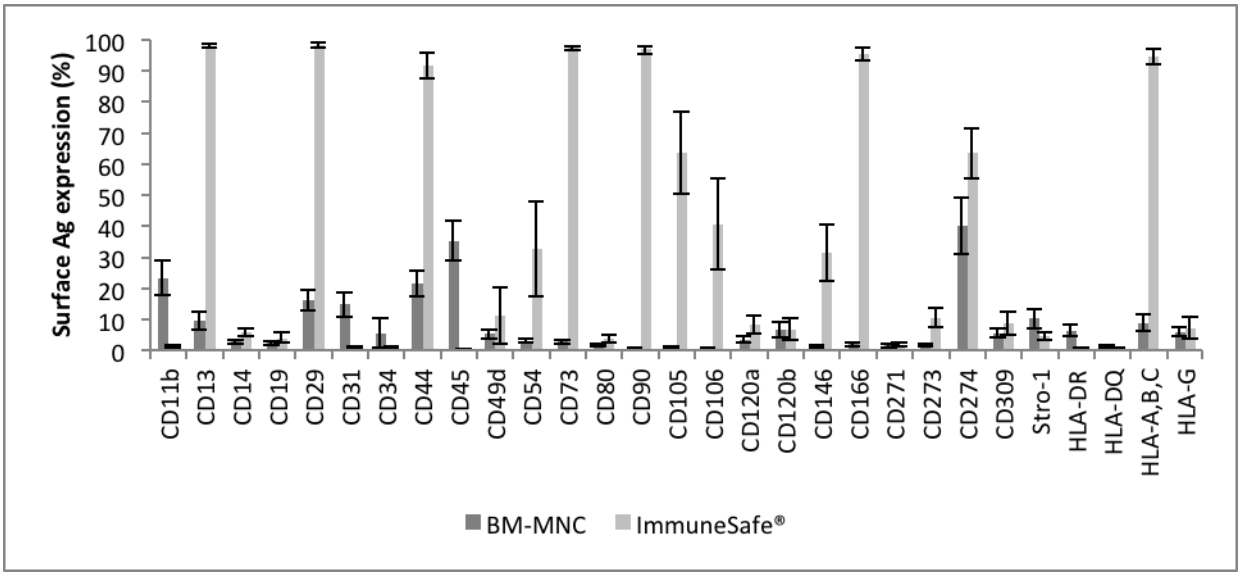


Figure 3.10 Comparison of percentage surface antigens expression between BM-MNCs (N=12) and ImmuneSafe®, under unstimulated stated (N=5).

From the 29 markers analysed 13 of them were statistically significant, with a $P<0.05$ (CD13, CD29, CD44, CD45, CD54, CD73, CD90, CD105, CD106, CD146, CD273, CD166 and HLA-A,B,C).

BM-MNCs expressed some hematopoietic markers (CD11b and CD45), CD11b that is critical for the transendothelial migration of monocytes an neutrophils and it was also involved in granulocyte adhesion, phagocytosis, and neutrophil activation, CD29 that is broadly expresses on a majority of hematopoietic and non-hematopoietic cells, epithelial cells and mast cells and it main function is cell-cell and cell-matrix interaction, CD31 (PECAM-1) that is expressed on monocytes, platelets, granulocytes, endothelial cells and lymphocyte subset and it main function is cell adhesion and signal transduction, CD44 that is expressed on all leukocytes, endothelial cells, hepatocytes, and MSCs and it main function is leukocyte attachment and rolling on endothelial cells, stromal cells and ECM, CD274 (PD-L1) is involved in the co-stimulatory signal, essential for T lymphocyte proliferation and production of IL-10 and IFN-γ [59].

Cell2B propagation method enables the achievement of MSC population immunophenotypically different from the source, BM-MNCs, that may indicate an improvement on the therapeutic function of the product and an increase of clinical applications besides regenerative medicine.

3.4.2.3 IDO and HO-1 production by BM-MNCs

IDO and HO-1 WB results of BM-MNCs lysates were summarized on Table 4. The only protein that was detected was HO-1 at 6h in culture, in both conditions. These results show that BM-MNCs do not share the same capacity to express IDO under pro-inflammatory environment as observed by IS.

Table 4 Rating of IDO and HO-1 production by BM-MNC, without stimulation and stimulated with IFN-γ and TNF-α. (-) not expressed (+) expressed (N=3)

Sample ID		Result	
Time	**Condition**	**IDO**	**HO-1**
T0	-	-	-
		-	-
		-	-
T6	-TNF-IFN	-	+
		-	+
		-	-
	+TNF+IFN	-	+
		-	+
		-	+
T48	-TNF-IFN	-	-
		-	-
		-	-
	+TNF+IFN	-	-
		-	-
		-	-

HO-1 expression by BM-MNCs was not reported on the literature, only is referred a combination of cell therapy using allogeneic bone marrow cells (BMC) with gene therapy leading overexpression of HO-1that might be particularly beneficial in the setting of reperfused myocardial infarction (MI). Because BM cells survival after intracoronary delivery to the myocardium is reduced due to inflammatory and proteolytic environment in the setting of MI, it is important to modify the cells in order to increase their resistance against apoptosis. Such property can be achieved by transduction of the BMC with HO-1 gene. HO-1 is a key enzyme in metabolism of heme and its products (biliverdin, carbon monoxide, iron) have proven anti-oxidant, anti-inflammatory, angiogenic and anti-apoptotic properties. Its expression is upregulated in the peri-infarct area after MI and HO-1 knockout aggravated ischaemia reperfusion injury in experimental MI. To sum, the expression of HO-1 increases the survival of BM cells after myocardial delivery [69].

As IDO was not detected on performed BM-MNC lysates and IDO production by BM-MNCs is not referred in the literature, could indicate that BM-MNCs do not have the capacity to inhibit lymphocyte proliferation as IS, essential for the treatment of GvHD.

3.4.3 Fibroblasts *vs.* ImmuneSafe®

IS CQAs were compared with human Fb CQAs to demonstrate that Fb do not have the same biological/ therapeutic function. This issue was raised because adult Fb from various tissues were compared with and bone marrow MSC and showed similar *in vitro* immunosuppressive potency [52]. Fb have been shown to interact with immune system, either activating or down-regulating T cells and mediating indirect antiproliferative effects on lymphocytes [51] or inhibiting monocyte differentiation into dendritic cells.

3.4.3.1 Fibroblast morphology

Fb and MSCs have similar morphological appearance [49]. Human Fb display a spindle-shaped morphology (presented on Figure 3.11), similar to the IS morphology.

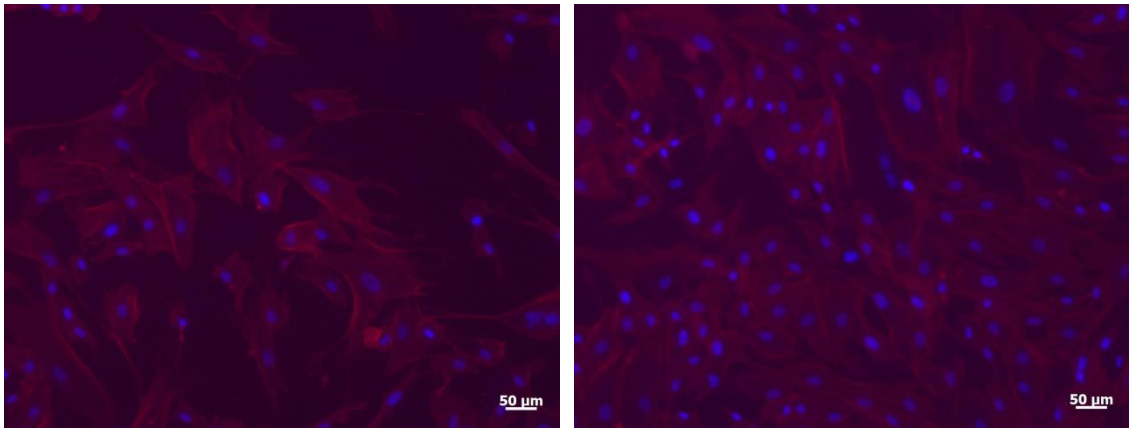


Figure 3.11 Morphology of human skin fibroblasts.

3.4.3.2 Fibroblasts multilineage differentiation potential

One way to distinguish Fb from MSCs is based on shelf-renew and differentiation capacity, wherein Fb seem to be more limited in both functional properties [49]. Human dermal Fb demonstrated the ability to differentiate into osteogenic and chondrogenic lineages but displayed poor capacity for adipogenesis (Figure 3.12).

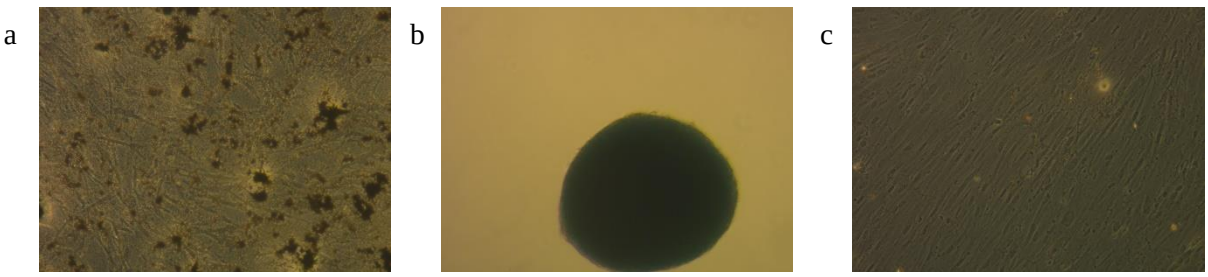


Figure 3.12 Human fibroblast differentiation (a) Osteogenesis (b) Chondrogenesis (c) Adipogeneis**.**

3.4.3.3 Fibroblasts vs. ImmuneSafe® immunophenotype

Fibroblast immunophenotype characterization was also performed with and without stimulation with IFN-γ and TNF-α, presented on Figure 3.13, for further comparison with IS immunophenotype, shown on Figure 3.14 and Figure 3.15, in order to identify some differences on surface expression between them that can be useful for their identification and potency under pro-inflammatory environment.

Upon activation with pro-inflammatory cytokines, human Fb up-regulated the expression of several membrane proteins, as shown on Figure 3.13, including some with immunomodulatory activity, such as CD54 (ICAM-1) and CD273 (PD-L2).

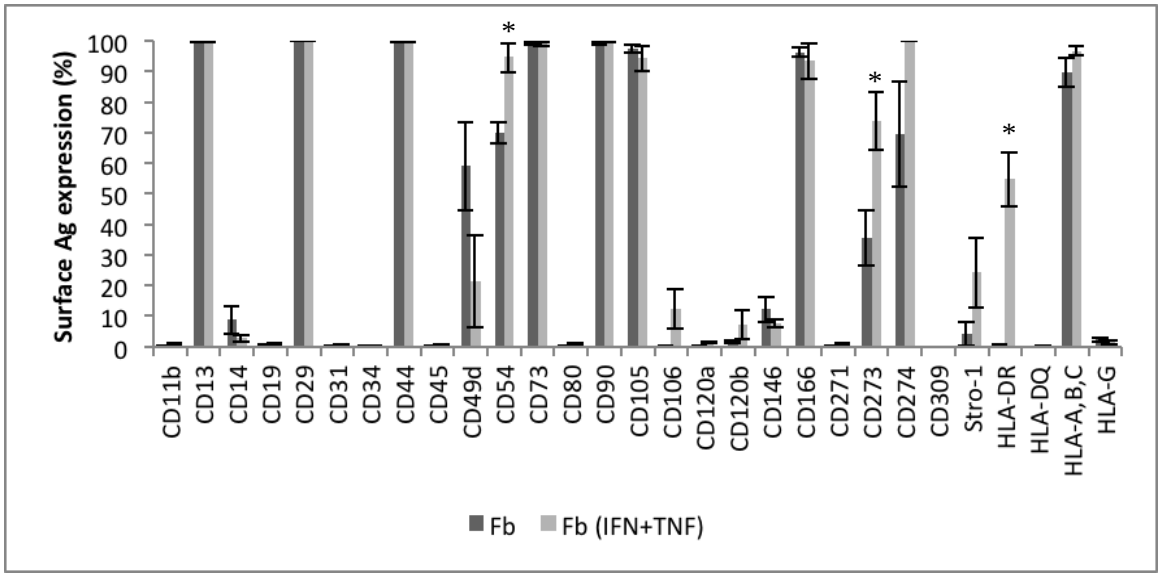


Figure 3.13 Percentage of fibroblasts surface antigen expression, with and without IFN-γ + TNF-α stimulation. (N=3) *P<0.05

As shown on Figure 3.14, human Fb immunophenotype resembles MSCs typical antigen presentation (positive for CD13, CD73, CD90 and CD105), and negative for most hematopoietic markers. Although, some differences (statistically significant) between fibroblast and IS were observed, namely on CD49d, CD106 (VCAM-1) and CD273 (PD-L2) expression. CD49d and CD273 (PD-L2) were highly expressed by human Fb when compared with IS, on the other hand CD106 was highly expressed by IS than human fibroblast.

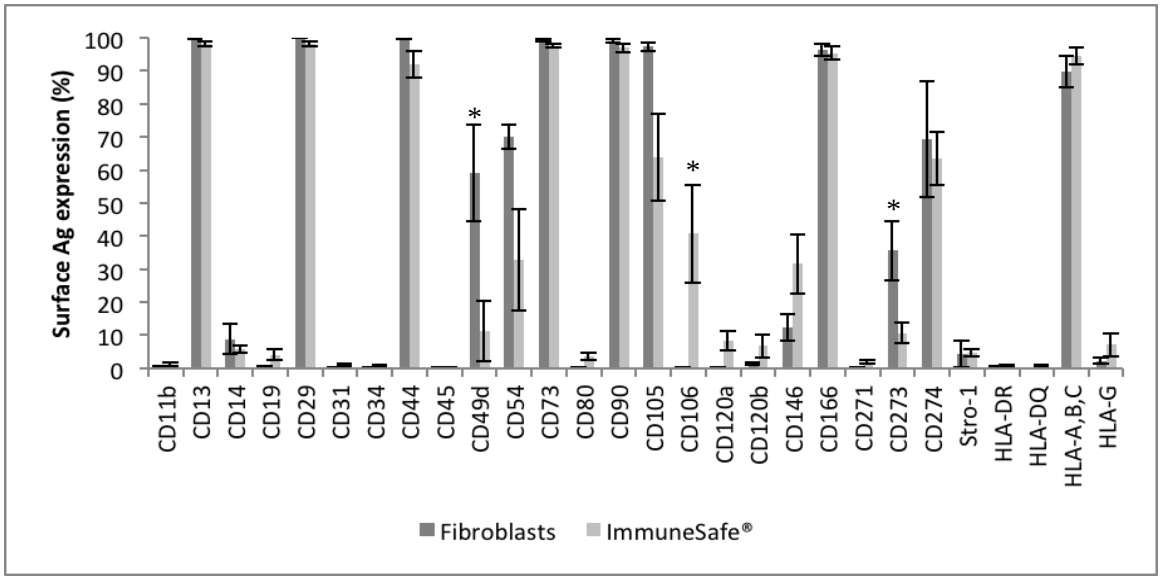


Figure 3.14 Comparison of percentage antigen surface expression between fibroblasts, under unstimulated environment. (N=3) and ImmuneSafe® (N=5). *P<0.05

Despite the differences observed on Figure 3.15 on expression of some antigens between Fb and IS under pro-inflammatory environment, they are not statistical significant.

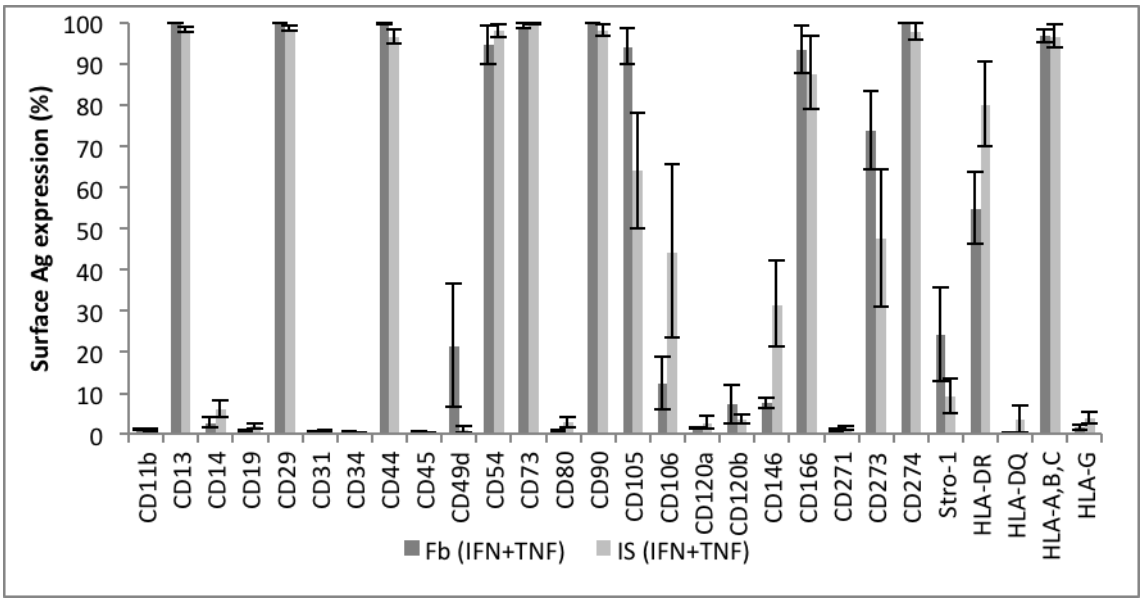


Figure 3.15 Comparison of percentage antigen surface expression between fibroblasts (N=3) and ImmuneSafe® (N=5), after stimulation with IFN-γ + TNF-α.

Distinguish Fb from MSCs and extrapolate it potential *in vivo,* based on immunophenotype were not straightforward. Since Fb and MSCs have many cell surface markers in common [49] and some literature are not in conformity with obtained results. Wherein Wagner *et al.* referred that Fb were only weak positive for CD105, while MSCs, responsible for induction differentiation, were mostly positive for CD105 [50]. This does not match with our results, where CD105 was higher expressed by human Fb than IS. Although Fb used were not the same,

Fb used in this work were ATCC CRL-2522 and Wagner *et al.* used ATCC CRL-1635 [70] both derived from human newborn foreskin fibroblast. Another difference is the culture media used for Fb expansion, in this work was used EMEM and Wagner *et al.* used DMEM [70].

Recently, CD146 has been claimed to be a specific marker for MSCs [49] and through the comparison of CD146 expression between IS and Fb, CD146 showed higher expression on IS than on Fb.

3.4.3.4 Fibroblasts cytokine production

PGE2 and IL-6 production by Fb was evaluated under non-stimulated state, stimulated with IFN-γ, stimulated with TNF-α and with both cytokines (Figure 3.16).

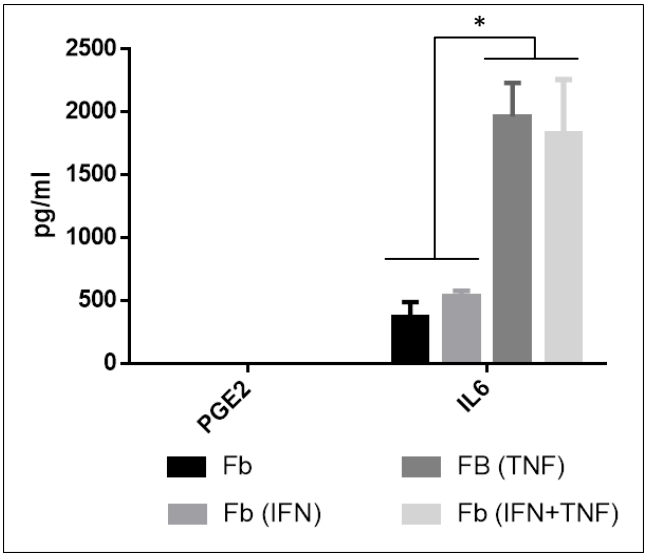


Figure 3.16 Evaluation of PGE2 and IL-6 production by fibroblasts under different stimulus. (N=2). *P<0.05

Fb did not seem to produce PGE2 in any of the tested conditions: without stimulation, stimulated with IFN-γ, stimulated with TNF-α and both, as present on Figure 3.16. However, IL-6 production by human Fb was increased upon activation, particularly in presence of TNF-α. IL-6 is documented in the literature to be produced by fibroblast and thereby mediate the inhibition of monocyte differentiation into dendritic cells [52].

PGE2 and IL-6 production by human Fb revealed a similar response to pro-inflammatory environment when compared with IS. IS did not seems to produce large amount of PGE2, in both analysed conditions and showed similar response to the stimulation with IFN-γ + TNF-α, producing IL-6 in the same range of values (IS: 1962.1 pg/ml and Fb:15345 pg/ml). Despite the lower amount of PGE2 produced by IS, human fibroblast did not produce PGE2 at all. As PGE2 is important on immunomodulation of immune system, this could be an indication that Fb do not have this capacity.

3.4.3.5 IDO and HO-1 production by fibroblasts

IDO bioactivity is generally accepted to be central for the suppression function of human MSC and IFN-γ regulation leads a massive transcriptional IDO induction [22] for that reason this criterion was evaluated on human fibroblast. HO-1 was also studied because on the literature HO-1 is indicated to be involved in MSC-mediated T cell suppression and to be a potent cytoprotective enzyme that exerts strong anti-inflammatory, antioxidative, and antiapoptotic activities [45].

Fibroblast did not show the capacity to express IDO, although HO-1 was sporadically detected after TNF-α or IFN-γ + TNF-α stimulation (Table 5).

Table 5 Rating of IDO and HO-1 production by fibroblasts, without stimulation and stimulated with IFN-γ and TNF-α. (-) not expressed (+) expressed (N=2)

Sample ID		**Results**	
Name	**Condition**	**IDO**	**HO-1**
Fibroblasts	-IFN-TNF	-	-
		-	-
	+IFN	-	-
		-	-
	+TNF	-	-
		-	+
	+IFN+TNF	-	-
		-	+

Fb have been shown to react to stress factors producing HO-1. Panchencko *et al.* demonstrated that skin Fb able to respond to hypoxia by HO-1 mRNA induction [71]. HO-1 expression in primary human lung Fb were also detected after cigarette smoke extract exposure, because cigarette smoke contains many components capable of eliciting oxidative stress, which may induce HO-1, a cytoprotective enzyme [72]. Vile *et al.* had observed an adaptive response to UVA-dependent oxidative stress in human skin cells. Where their results clearly implicate HO-1 as the initial inducible mediator in this adaptive process and implicate ferritin as an important oxidant stress-inducible antioxidant in these cells [73].

3.4.3.6 Fibroblasts immunosuppression potential

Immunosuppressive potential on T cells by human Fb was evaluated in order to determine if Fb have the same immunosuppressive potency as IS. Despite the variability of results presented on Figure 3.17, Fb showed the ability to supress the production of TNF-α by T cells and NK. Contrarily, there is an activation of IL-17 production on T cells, particularly by cytotoxic $CD8^+$ cells.

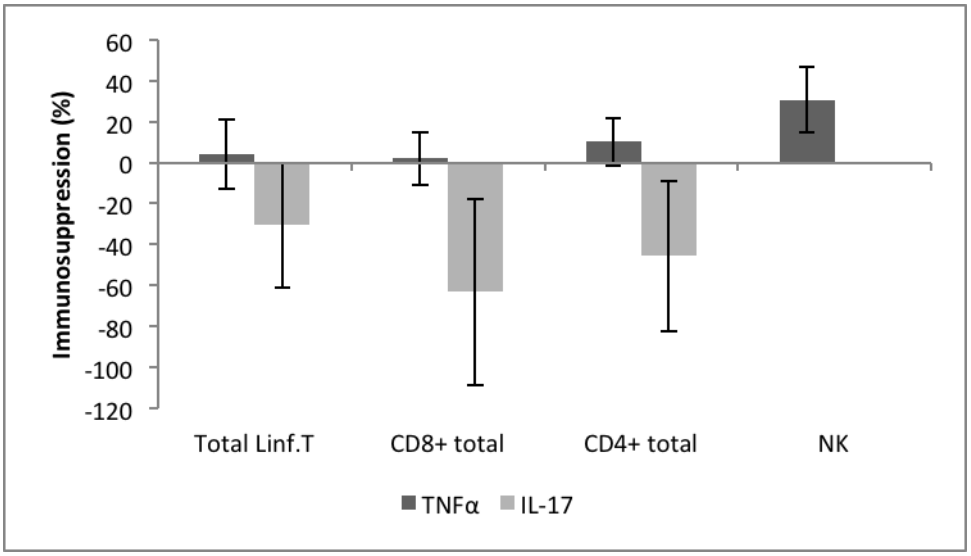


Figure 3.17 Immunosuppression of T lymphocytes, Tc, Th and NK cells by fibroblast, based on the suppression of TNF-α (N=5) and IL-17 production (N=4).

Comparing IS with human Fb, IS has higher and more consistent immunosuppressive potential and IS can suppress the production of both studied cytokines, TNF-α and IL-17 in all analysed T lymphocytes. These results are in agreement with theory that Fb do not share the same immunosuppressive effect of MSCs [51] and in disagreement with opposite literature where indicates that adult Fb from various tissues and bone marrow MSC showed similar *in vitro* immunosuppressive potency [52].

3.4.4 Differentiated MSC *vs.* ImmuneSafe®

IS CQAs were compared with differentiated MSCs CQAs (adipo and osteo) in order to detect poorly cultured conditions, extensive culture, or other stress conditions during the IS manufacturing process, because MSCs under cited deviation can start to differentiate into mesenchymal lineages.

3.4.4.1 Adipocytes MSC vs. ImmuneSafe®

3.4.4.1.1 Adipocytes MSC Immunophenotype

Adipocytes differentiated from MSCs did not share the same immunophenotype with IS, several differences on the expression of surface antigens were presented on Figure 3.18 and Figure 3.19. IS presented a higher percentage of expression when compared with adipocytes MSC for several markers, such as CD13, CD29, CD44, CD73, CD90, CD105, CD106 (VCAM-1), CD166 (ALCAM) and HLA-A,B,C under non stimulated environment and the referred plus CD54 (ICAM-1), CD273 (PD-L2), CD274 (PD-L1) and HLA-DR in pro-inflammatory environment.

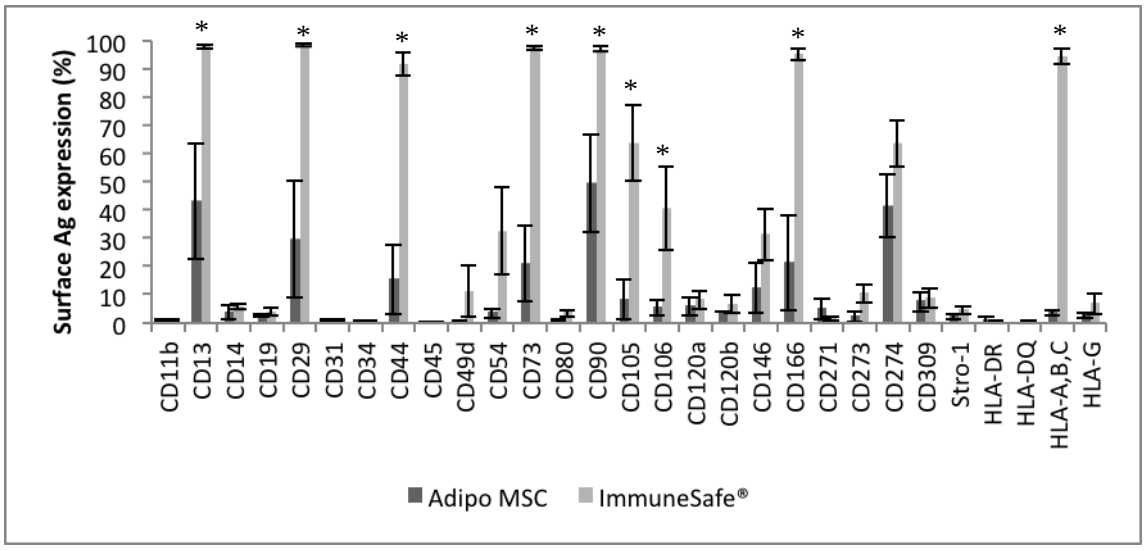


Figure 3.18 Comparison of percentage surface antigen expression between adipocytes MSC (N=3) and ImmuneSafe® (N=5) under unstimulated environment.* *P*<0.05

These results indicate that immunophenotype of IS changed after differentiation into adipocytes as well as it function, namely on adhesion (e.g. CD54 and CD106) and immune modulation (e.g. CD274). CD106 expression is strongly down regulated in MSCs after differentiation to adipo-, osteo-, and chondrocytes, suggesting that it may indeed be a marker of the most potent/ undifferentiated cells within expanded MSC cultures [23].

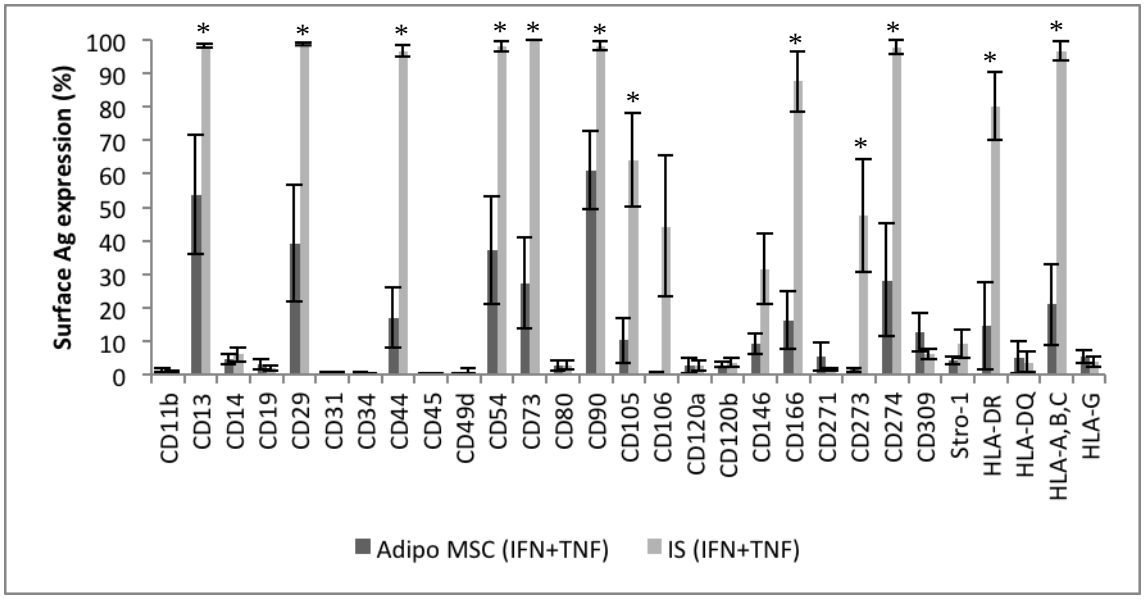


Figure 3.19 Comparison of percentage surface antigen expression between adipocytes MSC (N=3) and ImmuneSafe® (N=5), after IFN-γ +TNF-α stimulation. **P*<0.05

The decrease on HLA-A,B,C (MHC-I) expression, under unstimulated state and under pro-inflammatory environment and HLA-DR (MHC-II) expression after IFN-γ and TNF-α by adipocytes are referred in the literature, where is cited that adipogenesis reduced the expression of both HLA class I and II, in particular, no class II was detectable before or after IFN-γ treatment [74].

3.4.4.1.2 Adipocytes MSC cytokine production

As shown on Figure 3.20, pro-inflammatory cytokines did not influence the PGE2 production by adipocytes MSC. Although, MSCs differentiated into adipogenic lineage maintained the capacity to produce IL-6 when activated with pro-inflammatory cytokines. The same behaviour was observed on IS. To sum, the capacity to react to a pro-inflammatory environment producing IL-6 was not altered with the differentiation of MSCs into adipocytes.

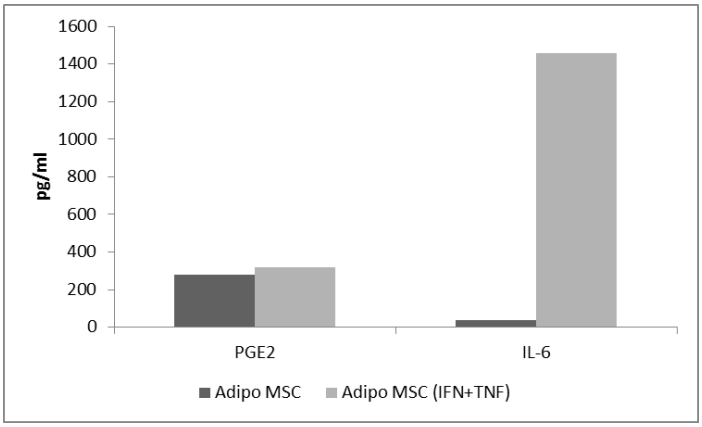


Figure 3.20 Comparison of PGE2 and IL-6 production by adipocytes MSC, in culture without stimulation and stimulated with IFN-γ + TNF-α. (N=1)

3.4.4.1.3 IDO and HO-1 production by adipocytes MSC

MSC differentiated into adipogenic lineage maintained the capacity to produce IDO under pro-inflammatory environment, as observed on Table 6. HO-1 was consistently expressed under pro-inflammatory environment but occasionally expressed under an environment without stimulus. The capacity to react to a pro-inflammatory environment producing IDO and sometimes HO-1 remains unaltered after MSC differentiation into adipocytes.

Table 6 Rating of IDO and HO-1 production by adipocytes MSC, without stimulation and stimulated with IFN-γ and TNF-α. (-) not expressed (+) expressed (N=2)

		Results	
	Condition	**IDO**	**HO-1**
Adipo MSC	-IFN-TNF	-	-
		-	+
	+IFN+TNF	+++	+
		++	+

3.4.4.1.4 Adipocytes MSC immunosuppression potential

Despite the variability of results presented on Figure 3.21, MSCs differentiated into adipogenic lineage did not show the ability to supress the production of TNF-α and IL-17 on T lymphocytes under a pro-inflammatory environment. The only exception was on $CD8^+$ cells, T cytotoxic cells, which revealed suppression of IL-17 production. Immunosuppression capacity was not preserved after differentiation into adipogenic lineage.

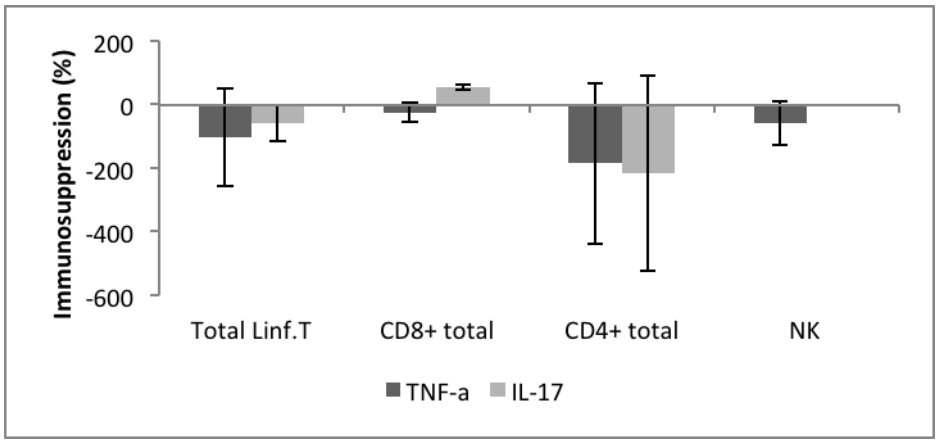


Figure 3.21 Immunosuppression of T lymphocytes, Tc, Th and NK cells by adipocytes MSC, based on the suppression of TNF-α and IL-17 production (N=2).

3.4.4.2 Osteocytes MSC vs. ImmuneSafe®

Application of CQAs to differentiated MSCs into Osteocytes and further comparison with IS, as the same purpose as the referred for Adipo CQAs. Evaluation of eventual discrepancies on manufacturing process, namely poorly cultured conditions, extensive culture and stress conditions.

3.4.4.2.1 Osteocytes MSC Immunophenotype

Osteocytes differentiated from MSCs did not share the same immunophenotype with IS, several differences on the expression of surface antigen were presented on Figure 3.22 and Figure 3.23. IS presented an higher percentage of Ag expression when compared with Osteocytes MSCs, like CD13, CD29, CD44, CD73, CD90, CD105, CD106 (VCAM-1), CD166 (ALCAM), CD274 (PD-L1) and HLA-A,B,C under non stimulated environment and the referred plus CD54 (ICAM-1), CD273 (PD-L2), and HLA-DR under pro-inflammatory environment.

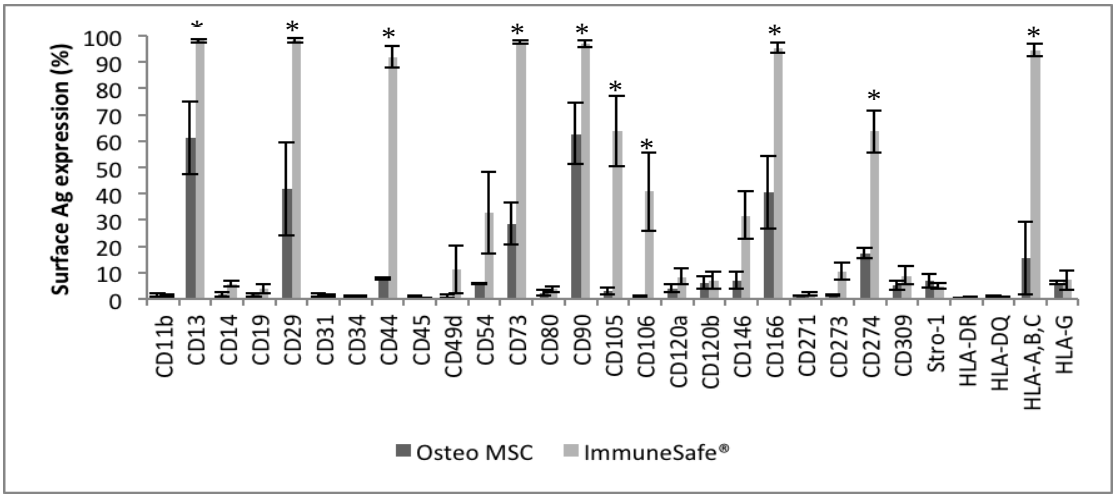


Figure 3.22 Comparison of percentage antigen surface expression between osteocytes MSC (N=3) and ImmuneSafe® (N=5) under unstimulated environment. ***P*<0.05

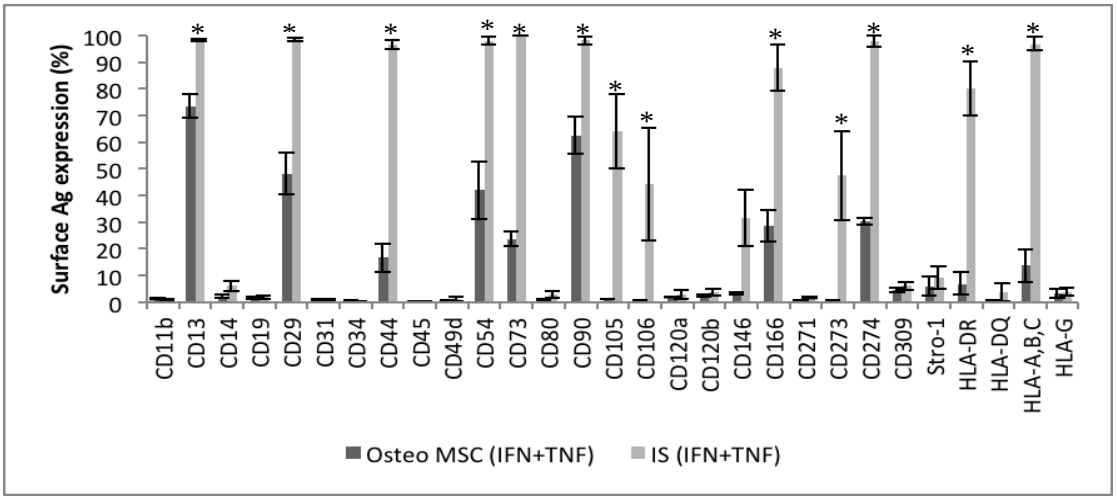


Figure 3.23 Comparison of percentage surface antigen expression between osteocytes MSC (N=3) and ImmuneSafe® (N=5), after IFNγ + TNFα stimulation. **P*<0.05

These results indicate that immunophenotype of MSC changed after differentiation into osteocytes as well as it function, namely on adhesion (e.g. CD54 and CD106) and immune modulation (e.g. CD274). CD106 expression is strongly down regulated in MSCs after differentiation to adipo-, osteo-, and chondrocytes, suggesting that it may indeed be a marker of the most potent/ undifferentiated cells within expanded MSC cultures [23].

3.4.4.2.2 Osteocytes MSC cytokine production

As shown on Figure 3.24, MSCs differentiated into osteogenic lineage maintained the capacity to produce IL-6 when activated with pro-inflammatory cytokines. Pro-inflammatory cytokines did not influence the PGE2 production by osteocytes MSCs. The same behaviour was observed with IS.

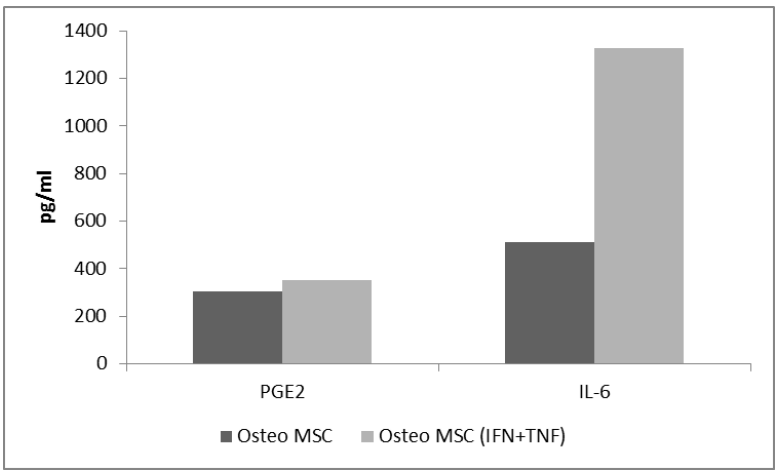


Figure 3.24 Comparison of PGE2 and IL-6 production by Osteocytes MSC, in culture without stimulation and stimulated with IFN-γ + TNF-α. (N=1)

3.4.4.2.3 IDO and HO-1 production by MSC Osteocytes

MSC differentiated into osteogenic lineage maintained the capacity to produce IDO under pro-inflammatory environment, as observed on Table 7. HO-1 was only detected under an unstimulated environment. The capacity to react to a pro-inflammatory environment producing IDO and sometimes HO-1 remains unaltered after MSC differentiation into osteocytes.

Table 7 Rating of IDO and HO-1 production by osteocytes MSC, without stimulation and stimulated with IFN-γ and TNF-α. (-) not expressed (+) expressed (N=2)

		Results	
	Condition	**IDO**	**HO-1**
Osteo MSC	-IFN-TNF	-	+
		-	+
	+IFN+TNF	+++	-
		++	-

3.4.4.2.4 Osteocytes MSC Immunosuppression

Despite the variability of results presented on Figure 3.25, MSCs differentiated into osteogenic lineage did not show the ability to supress the production of TNF-α and IL-17 by T lymphocytes in a pro-inflammatory environment. The only exception was $CD8^+$ cells, T cytotoxic cells that revealed suppression of IL-17 production. Immunosuppression capacity was not preserved after differentiation into osteogenic lineage.

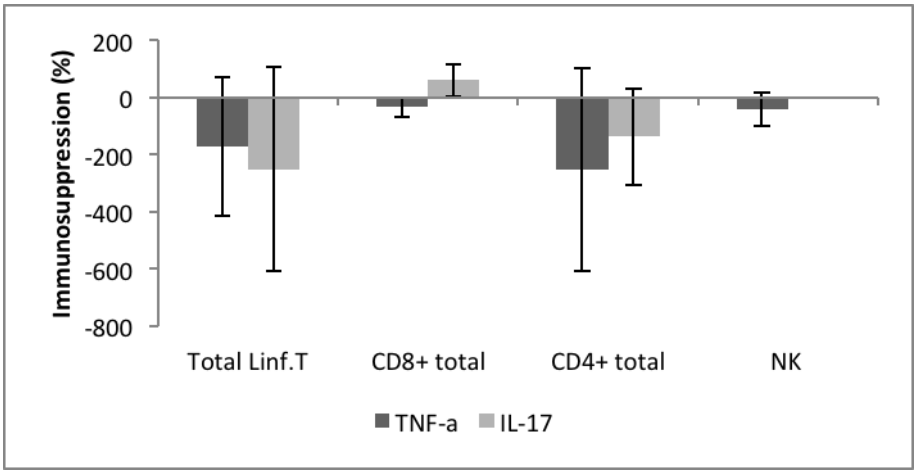


Figure 3.25 Immunosuppression of T lymphocytes, Tc cells, Th cells and NK cells by osteocytes MSC, based on the suppression of TNF-α and IL-17 production (N=2).

4 Immunosuppressive drugs interaction with ImmuneSafe®

About 35%-50% of HSCT recipients will develop aGvHD. Those who do not respond to primary therapy, which usually consist of glucocorticoids (steroids) are associated with considerable high morbidity and mortality. Different types and dosages of steroids are used in the initial management of aGvHD, but methylprednisolone (6MPD) given at a dosage of 2 mg/kg/day or prednisolone 2.5 mg/kg/day is more frequently administered. About 50% of patients with aGvHD can be treated with first line treatment, but if it is resistant to corticosteroids prognosis become dismal. New drugs, new anti-bodies (Abs) or increases immunosuppression, and immunomodulatory procedures such as extracorporeal photopheresis (ECP) may induce remission of GvHD, but problems involving infections or side effects still exist [75], [76].

The interactions between MSCs and the immunosuppressive drugs have also been studied in recent years. Different studies shown that commonly used immunosuppressive drugs such as tacrolimus, rapamycin or mycophenolic acid (MPA) did not induce short-term toxicity on MSCs [77], [78]. In fact, although these drugs induced a reduction on MSC proliferative capacity and differentiative potential, the immunomodulatory properties of MSCs were not affected [78]. Different authors reported that MSCs could potentiate the immunomodulatory activity of some drugs, namely MPA [77] or cyclosporin A [79]. In the proposed clinical trial for IS, the therapy administered to the enrolled patients will consist on methylprednisolone or prednisolone together with IS. Until now, no adverse effects have been reported in the literature regarding the complementary use of both MSCs and methylprednisolone [76]. In fact, this standard treatment is also used in different clinical trials involving the treatment of acute GvHD with MSCs (*e.g.* NCT00136903 and NCT01589549 in www.clinicaltrials.gov) with no adverse effects reported.

4.1 Materials and methods

Nowadays, MSC therapy seems to be one of the most promising last-line treatment for refractory aGvHD, therefore is highly important to evaluate the impact of immunosuppressive drugs on IS therapeutic potential. Methylprednisolone and prednisolone were the two immunosuppressive drugs in focus in this study at 2 mg/Kg/day and 2.5 mg/Kg/day dosage *in vivo*, so an *in vitro* approach was taken into account to verify if immunosuppressive drugs influence on IS therapy.

4.1.1 Cell expansion

MSCs were growth in StemPro® MSC SFM XenoFree (Gibco) and due to the absence of adhesion proteins in this medium, culture vessels used are from BD® ECM Mimetics. Cells were plated at 3000 cells/cm^2 and media was changed every 3/ 4 days.

4.1.2 Interaction between immunosuppressive drugs and ImmuneSafe®

At 70-80% confluency four different conditions were set: (1) cells without stimulation and drug, (2) cells with immunosuppressive drug (0,4 mg/l Methylprednisolone_Solumedrol (Pfizer) or 0,5 mg/l Prednisolone_Deltacortene (Bruno Farmaceutici), (3) cells stimulated with IFN-γ (500 U/ml) + TNF-α (10 ng/ml) and (4) cells stimulated with IFN-γ + TNF-α plus immunosuppressive drug, see 3.3.5 for stimulation dosage.

After 2 days in culture, exhausted medium was collected for further cytokine analysis by ELISA. One well of each condition was lysed using RIPA buffer (Sigma) supplemented with Complete protease inhibitor cocktail (Roche) for IDO and HO-1 WB detection, see 3.3.7. The remaining cells were harvested for membrane proteins analysis by cytometry: CD44 (clone BJ18), CD54 (clone HCD54), CD106 (clone STA), CD120a (clone 55R-286), CD120b (clone TR75-89), CD166 (clone 3A6), CD273 (clone MIH18), CD274 (clone 29E.2A3), (all purchased from BioLegend), see 3.3.6.

4.2 Results and Discussion

4.2.1 Interaction of immunosuppressive drugs with IS membrane proteins

The influence of methylprednisolone and prednisolone on MSCs membrane proteins was tested *in vitro,* using concentrations of 0,4 mg/l and 0,5 mg/l, respectively. According Figure 4.1 and Figure 4.2, immunosuppressive drugs tested, methylprednisolone and prednisolone, did not seem to have a beneficial or detrimental interaction with IS. CD54 (ICAM-1) and CD106 (VCAM-1) seem to be the most sensitive membrane proteins to immunosuppressive drugs. However, only CD106 expression seems to be influenced with methylprednisolone addition, with statistical significance (P<0.05). However, under a pro-inflammatory environment, which will be the environment that IS will find *in vivo,* such difference on CD106 expression was not verified.

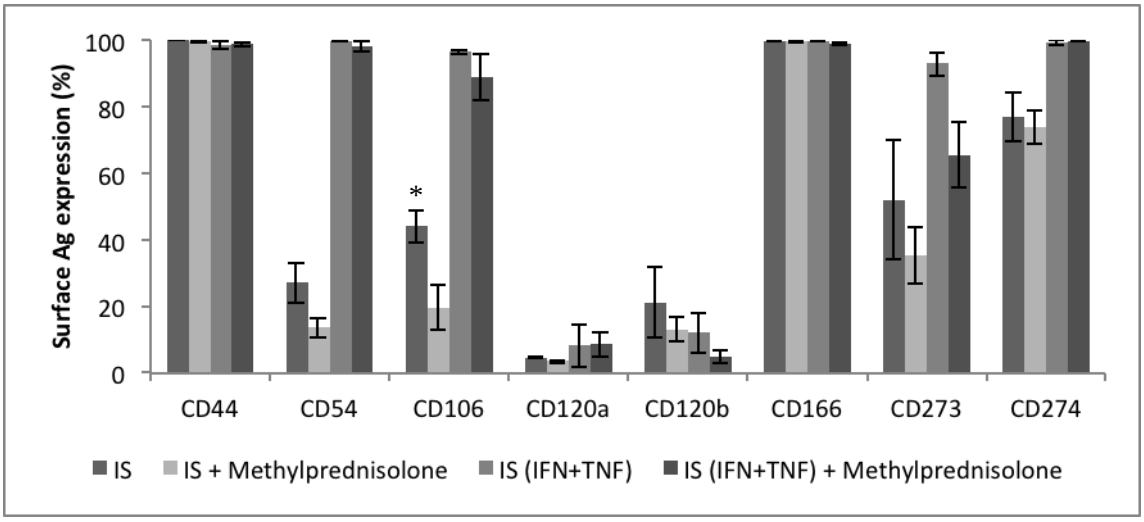


Figure 4.1 Methylprednisolone interaction with ImmuneSafe® membrane proteins. (N=3) *P<0.05

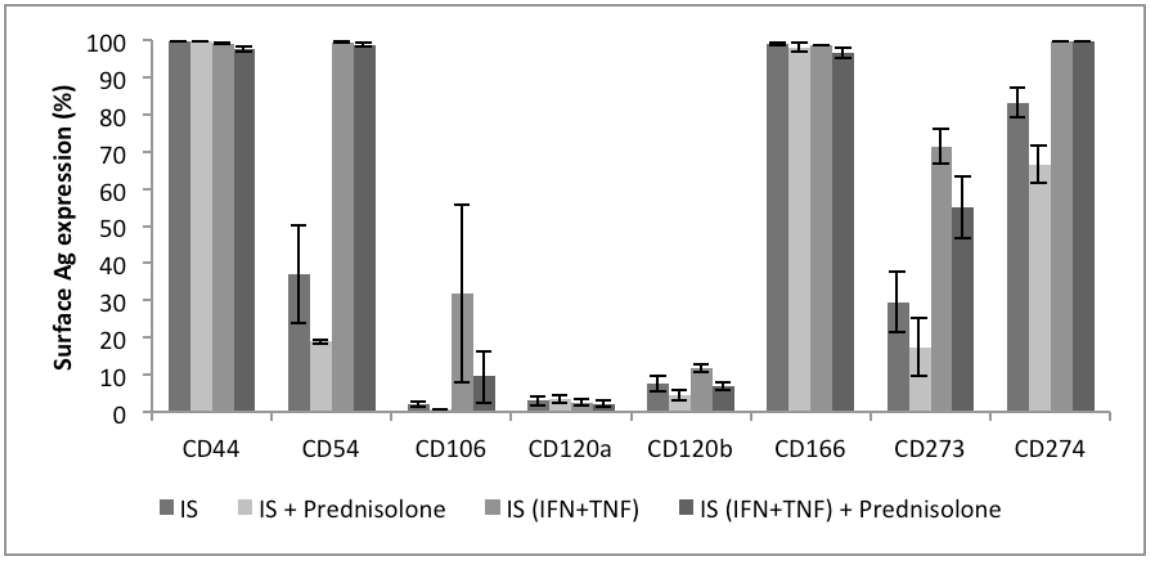


Figure 4.2 Prednisolone interaction with ImmuneSafe® membrane proteins. (N=3)

4.2.2 Influence of immunosuppressive drugs on cytokine production (IL-6)

Immunosuppressive drugs only seem to affect IL-6 production of IS under an environment without inflammatory cytokines, as observed on Figure 4.3 and Figure 4.4, but without statistical significance and IL-6 production under pro-inflammatory environment was maintained even in presence of immunosuppressive drugs. Nonetheless, the IS response to an inflammatory environment is the most important, since IS *in vivo* will find an inflammatory environment.

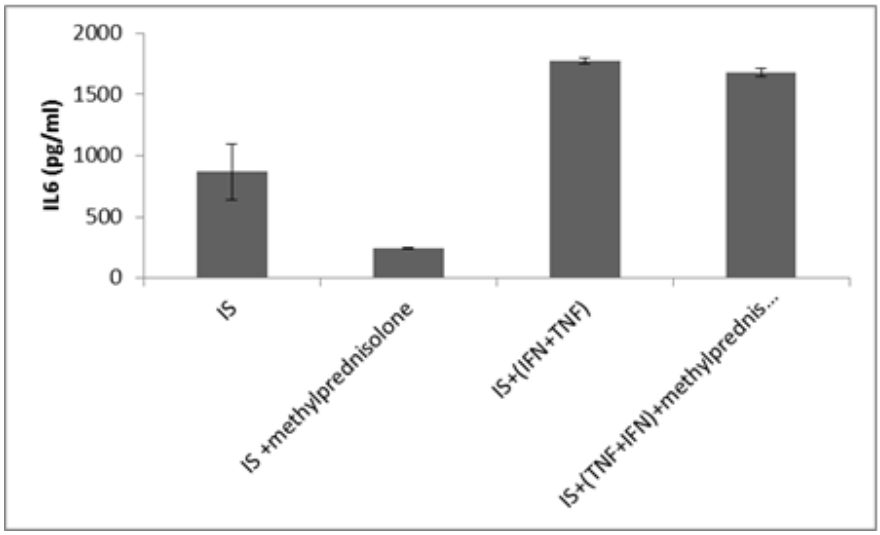


Figure 4.3 Influence of methylprednisolone on IL-6 production by ImmuneSafe®. (N=3)

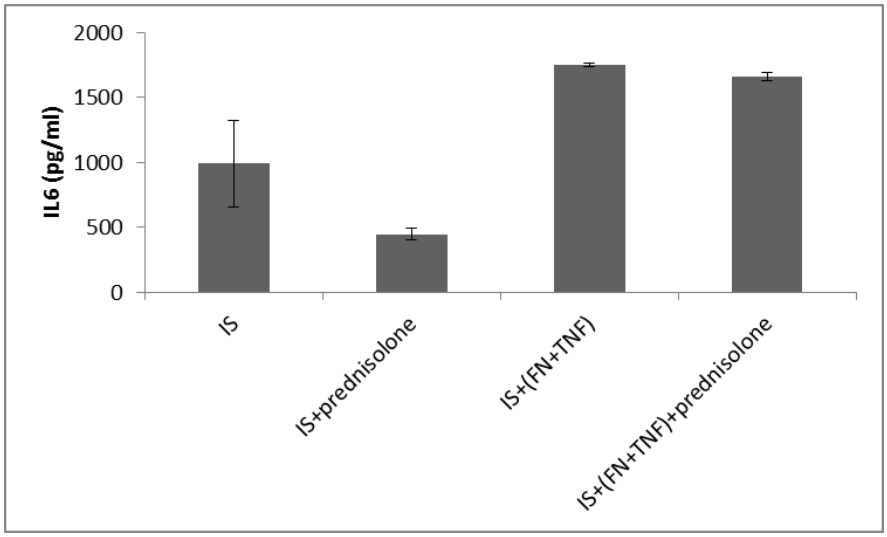


Figure 4.4 Influence of prednisolone on IL-6 production by ImmuneSafe®. (N=3)

4.2.3 Influence of immunosuppressive drugs on IDO and HO-1 production

Based on Figure 4.5 and Figure 4.6, methylprednisolone and prednisolone did not seem to influence the IDO production on three different IS batches analysed. IS and IS plus immunosuppressive drug did not express IDO unless stimulated with IFN-γ + TNF-α and IS under pro-inflammatory environment expressed IDO in the same level between the same batch.

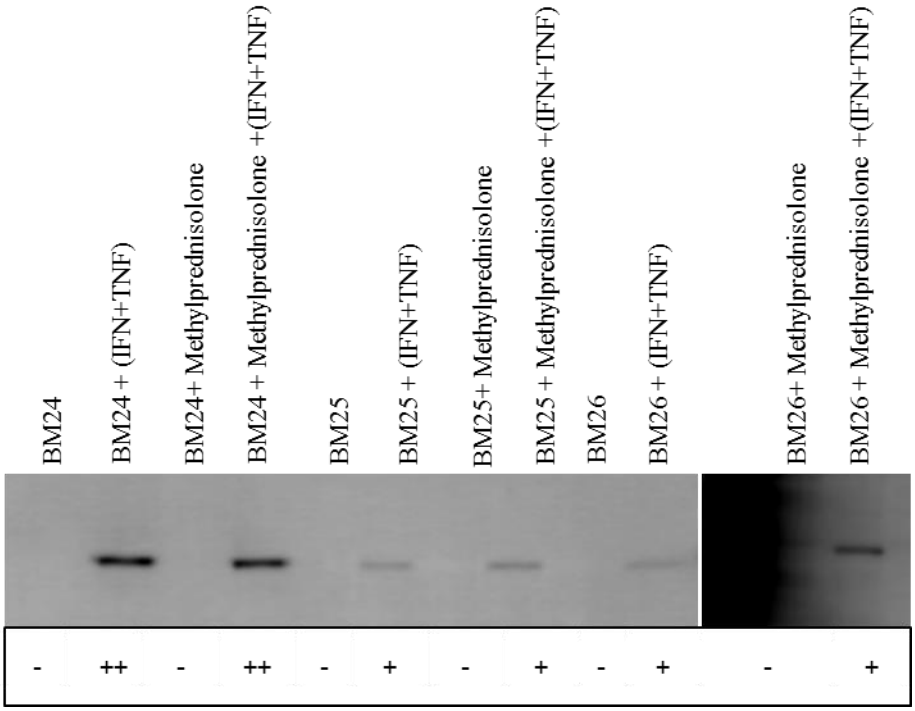


Figure 4.5 Influence of methylprednisolone on IDO production by ImmuneSafe®, without stimulation and stimulated with IFN-γ and TNF-α, detected by WB. (-) not expressed (+) expressed (N=3)

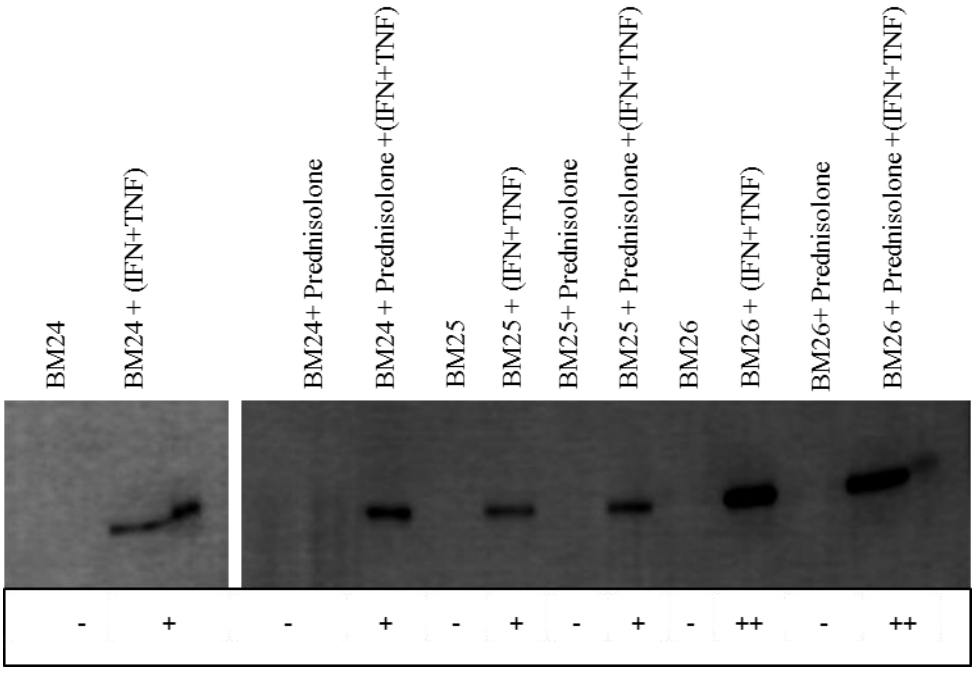


Figure 4.6 Influence of prednisolone on IDO production by ImmuneSafe®, without stimulation and stimulated with IFN-γ and TNF-α, detected by WB. (-) not expressed (+) expressed (N=3)

HO-1 was punctually expressed along the experience and it was not possible to observe a tendency, as shown on Table 8. For example, HO-1 was expressed on BM24 (IS batch 24) on methylprednisolone experiment (unstimulated environment, pro-inflammatory environment) but

not on prednisolone, where the controls were the same, the only difference was that the experiments were performed in different days. The same was verified on BM26.

Table 8 Influence of methylprednisolone and prednisolone on HO-1 production by ImmuneSafe®, without stimulation and stimulated with IFN-γ and TNF-α. (-) not expressed (+) expressed (N=3)

Condition/ IS batch	BM24	BM25	BM26
-IFN-TNF	+	-	-
+TNF+IFN	+	-	-
+Methylprednisolone	+	-	-
(+TNF+IFN)+Methylprednisolone	-	-	-
-IFN-TNF	-	-	+
+TNF+IFN	-	-	+
+Prednisolone	-	-	-
(+TNF+IFN)+Prednisolone	-	-	-

5 ImmuneSafe® release and shelf-life evaluation in Hypothermosol

The supply process of a cell product must fit with the processes available at the ultimate destination in the hospital - critically this depends on whether the product is shipped at room temperature, shipped at a cold chain temperature (4ºC) or cryopreserved – the latter requiring temporary storage in freezers and subsequent cell resuscitation before reaching the patient. Cell therapies are relatively fragile living materials and require careful management of transport and the supply chain – achieving a consistent and long as possible shelf-life is critical to achieve this goal, as is the design of transport packaging and preservation systems [36].

Freeze-thawed cells have shown impaired therapeutic properties compared to culture-derived fresh MSCs, which are used in many experimental and pre-clinical studies to demonstrate efficacy [80]. Freshly thawed MSCs are unable to suppress T cell proliferation *in vitro*, which correlates with impaired up regulation of IDO in response to IFN-γ. MSC biochemical response to IFN-γ and/ or the protein synthesis machinery is compromised. Moreover, dead MSC found in the freshly thawed MSC sample promote T cell hyperproliferation. Cryopreservation negatively affects the immunosuppressive properties of MSC in a reversible manner, and is associated with heat-shock stress response initiated during the thawing process. A culture recovery period of at least 24h is able to restore the immunosuppressive properties of MSC, including transcriptional IDO responsiveness to IFN-γ and down-regulation of heat shock proteins (Hsp) expression [81].

Patients treated with freshly harvested cells in low passage had a 100% response rate, twice the response rate of 50% observed in a comparable group of patients treated with freeze-thawed cells in higher passage [80].

5.1 Materials and methods

During the manufacturing process of IS, cells are cryopreserved and one final culture step is required for the cells to recover full biological function before the administration into the patient. In order to guarantee the highest product quality, the time between cell thawing and infusion into patient should be the minimum, and therefore avoid compromising the IS efficacy.

Cells were cultured in StemPro® MSC SFM XenoFree (Gibco) in BD® ECM Mimetics vessels. The different characterization tests were performed in the following steps: pre-cryopreservation, release cells at 0h (cells after thawing), 24h, 48h and 72h (post-thaw in culture) and post-formulation at variable times (cells formulated in HypoThermosol (BioLife Solution) at 2M

cells/ml at 4ºC). In each time point cell number and viability (trypan blue exclusion assay), immunophenotype (identity and membrane proteins) of non-stimulated cells and stimulated with IFN-γ and TNF-α (see 3.3.6 and 3.3.5), secretome of exhausted media (see 3.3.9 and 3.3.5), IDO and HO-1 by Western-blot of non-stimulated and stimulated cells with IFN-γ and TNF-α (see 3.3.7 and 3.3.5), and immunosuppression (see 3.3.11) were evaluated.

In addition to the previously mentioned tests on shelf-life experiment, excipient media was collected at each time point for secretome analysis (see 3.3.9) and pH was measured (Metter-toledo AG, FiveEasy^{TM}FE20).

5.2 Results and Discussion

The goal of this experiment is to optimize/ simulate the last part of IS manufacturing (release and shipping/ delivery) tuning suitable times for each step and increase the knowledge about IS product.

5.2.1 Cell number and viability on ImmuneSafe® release and shelf-life

As shown on Figure 5.1, after cell thawing the recovery time in culture was advantageous for the cell recovery, in terms of cell number and viability. The cells took at least 48h to approach the initial cell number seeded ($2.5x10^5$ cells/cm^2), this show that some of considered live cells were compromised, because did not have the capacity to adhere to the culture flask and consequently were not recovered.

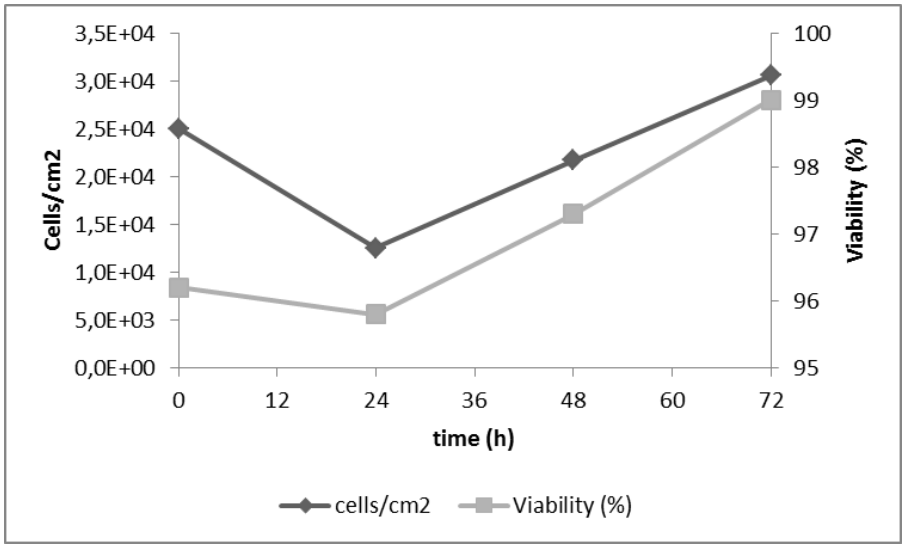


Figure 5.1 Tracking of cell number and viability of cell recovery after release at 3 different times (24h, 48 and 72h). (N=1)

MSCs formulated in Hypothermosol demonstrated to have a long longevity, as shown on Figure 5.2, after 7 days 55.5% of initial MSC population was still counted and with a viability of 65%.

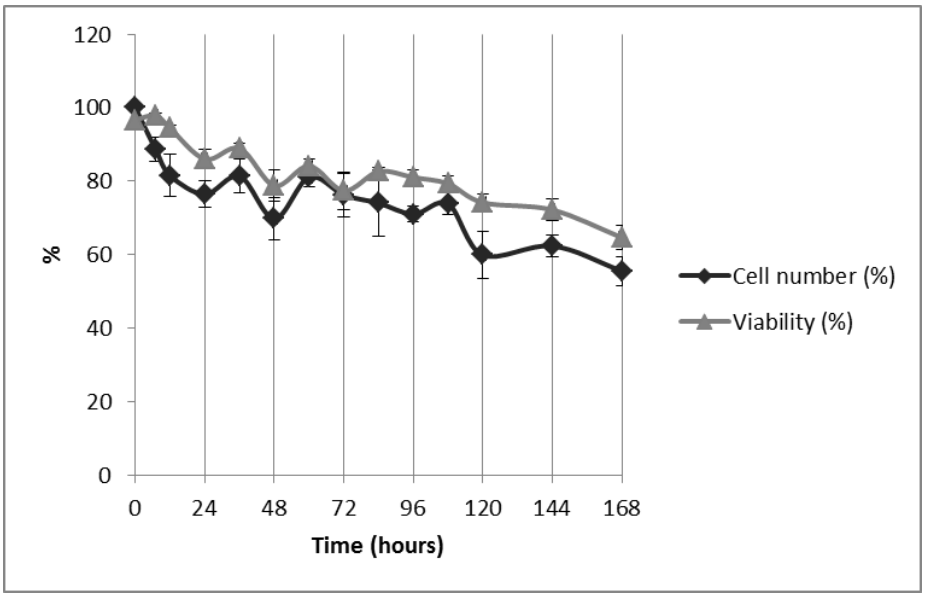


Figure 5.2 Tracking of cell number and viability of MSCs stored in Hypothermosol for 7 days at 4ºC. (N=10)

Based on the results present on Figure 5.2 the window for IS delivery was established within 48h, so based on these results, cell number and viability within this time will not be a problem, because at least 70% of initial population will be present and 80% viable.

5.2.2 pH variation during ImmuneSafe® release and shelf-life

pH levels is a good indicator of cell growth. As observed on Figure 5.3, pH of released cells decreased over the time, indicating that lactic acid was given off as a by-product of cell metabolism reflecting the increase in total biomass and cellular metabolism. On the other hand, IS formulated Hypothermosol was maintained within the pH around 7.7 which is a stable pH that does not influence negatively the IS.

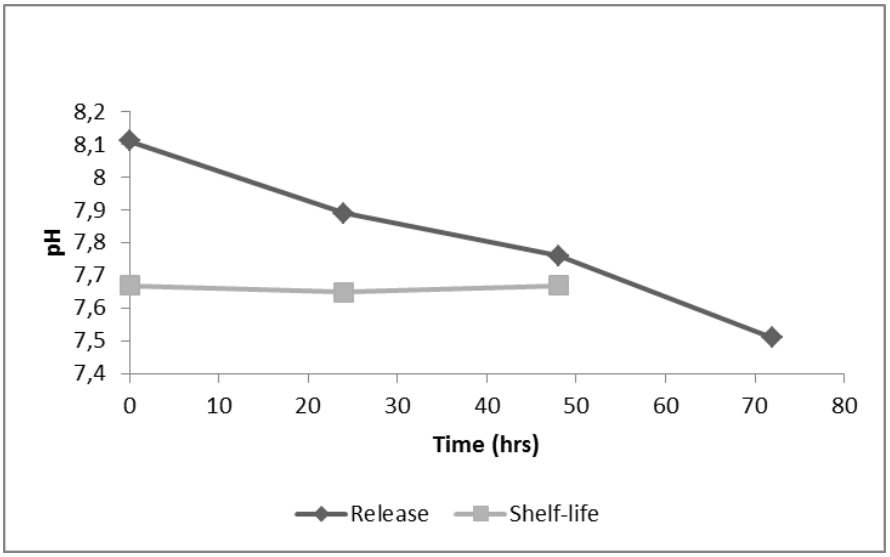


Figure 5.3 pH variation during release and shelf-life experiment. (N=1)

5.2.3 Immunophenotype on ImmuneSafe® release and shelf-life

5.2.3.1 Identity

IS identity of all analysed points of release correspond to the expected, positive for CD73, CD90 and CD105 and negative for hematopoietic markers (CD14, CD19, CD34 and HLA-DR), as shown on Figure 5.4.

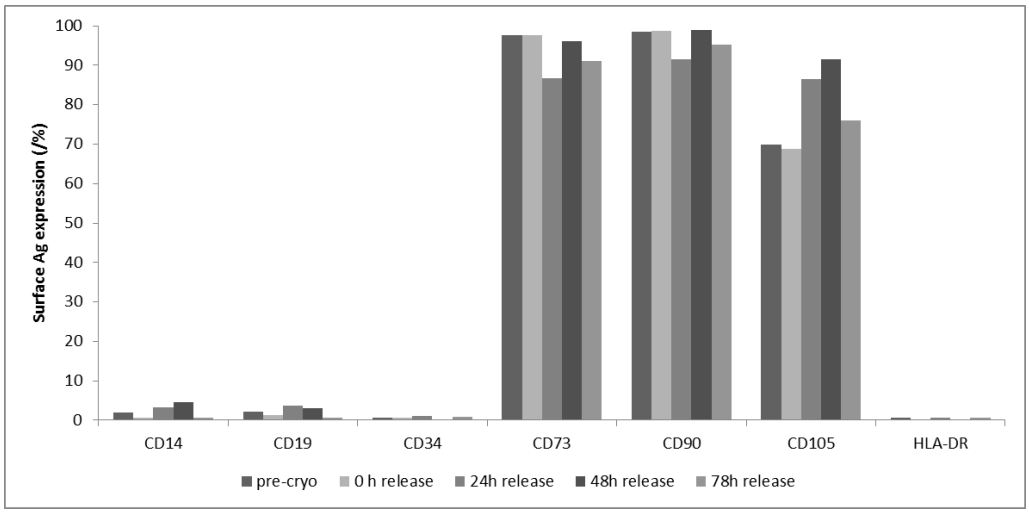


Figure 5.4 IS Identity panel at different times of release, under unstimulated environment. (N=1)

According Figure 5.5, the expression of HLA-DR was increased under pro-inflammatory environment, in all time points analysed, the only exception was cells after thawing (0h release), suggesting that MSCs after thawing do not have all biological function operational. HLA-DR is involved on peptide presentation to $CD4^+$ T cells [59].

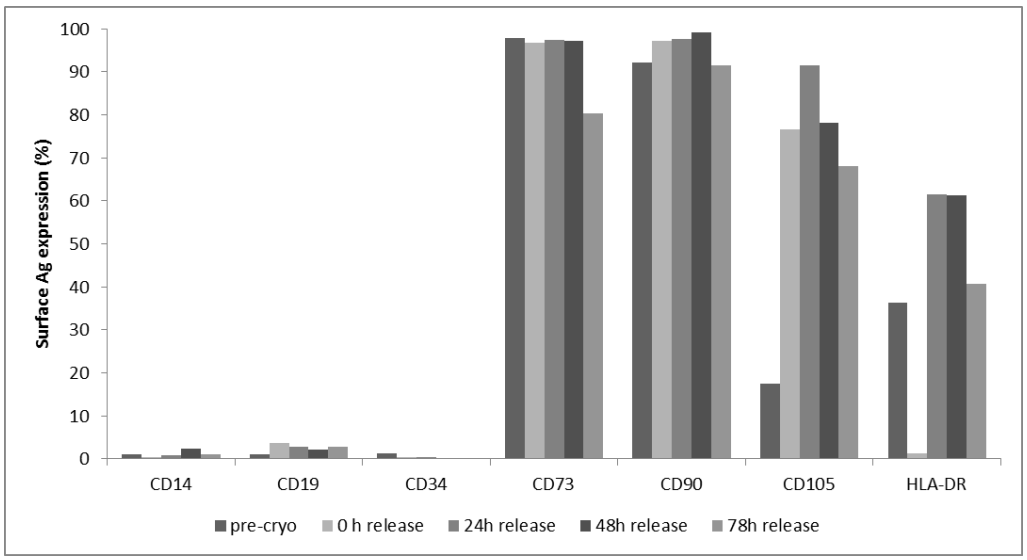


Figure 5.5 IS Identity panel at different times of release, under IFN-γ + TNF-α stimulation. (N=1)

IS identity of all analysed points of shelf-life corresponded to the expected, positive for CD73, CD90 and CD105 and negative for hematopoietic markers (CD14, CD19, CD34 and HLA-DR), as shown on Figure 5.6. As expected, HLA-DR was up regulated in all different times of shelf-life under pro-inflammatory environment (Figure 5.7). IS formulation in Hypothermosol at 4ºC did not influence their identity.

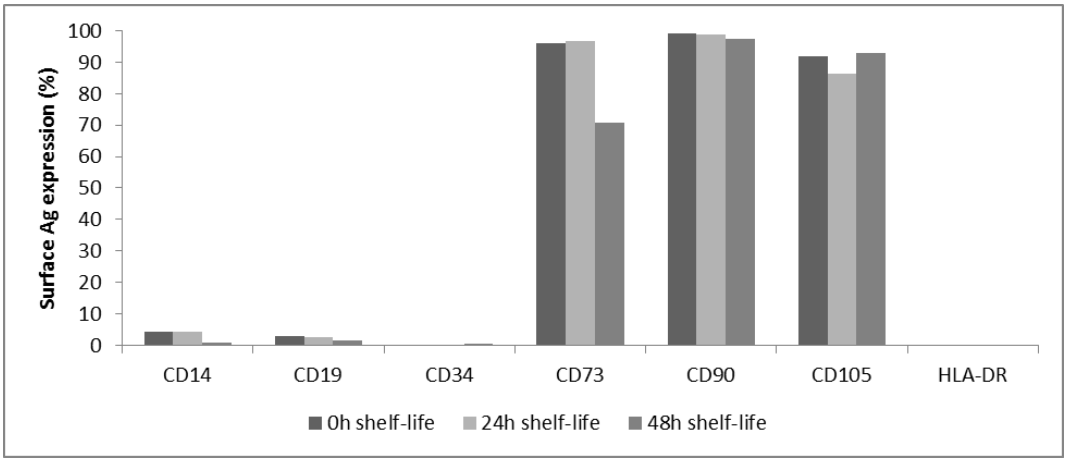


Figure 5.6 Identity panel of MSCs at different times of shelf-life, under unstimulated environment. (N=1)

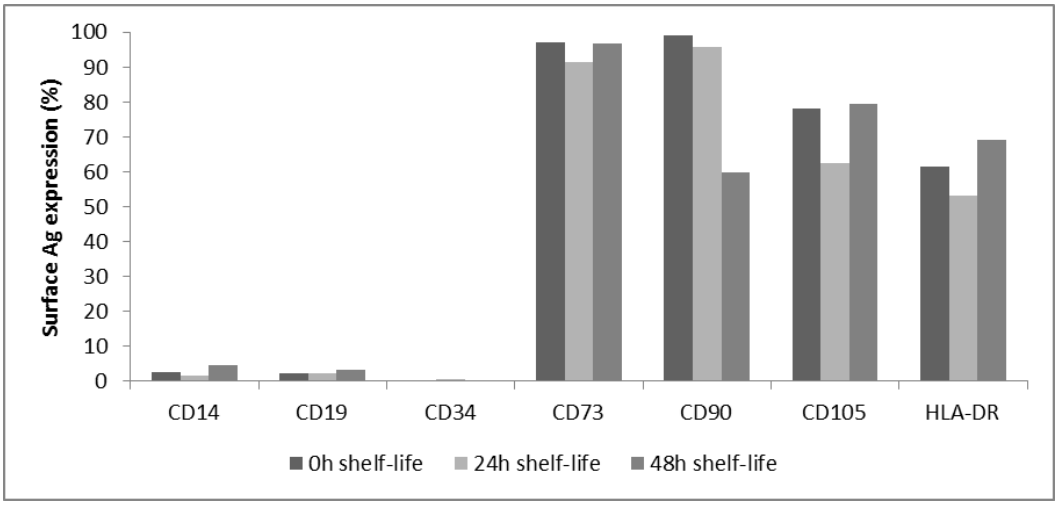


Figure 5.7 Identity panel of MSCs at different times of shelf-life, after IFN-γ + TNF-α stimulation. (N=1)

5.2.4 Membrane proteins on ImmuneSafe® release and shelf-life

The analysis of membrane proteins showed that it is necessary a release step for the cells to recover from cryopreservation, the major difference was on CD274 expression (Figure 5.8 and Figure 5.9). CD274 responded to the pro-inflammatory environment increase it expression (as observed on IS 3.4.1.3), the only exception was cells after thawing (0h release), indicating one

more time that the cells need a step in culture for a complete recovery of biological function. The remaining membrane proteins analysed showed similar behaviour when compare to IS (3.4.1.3).

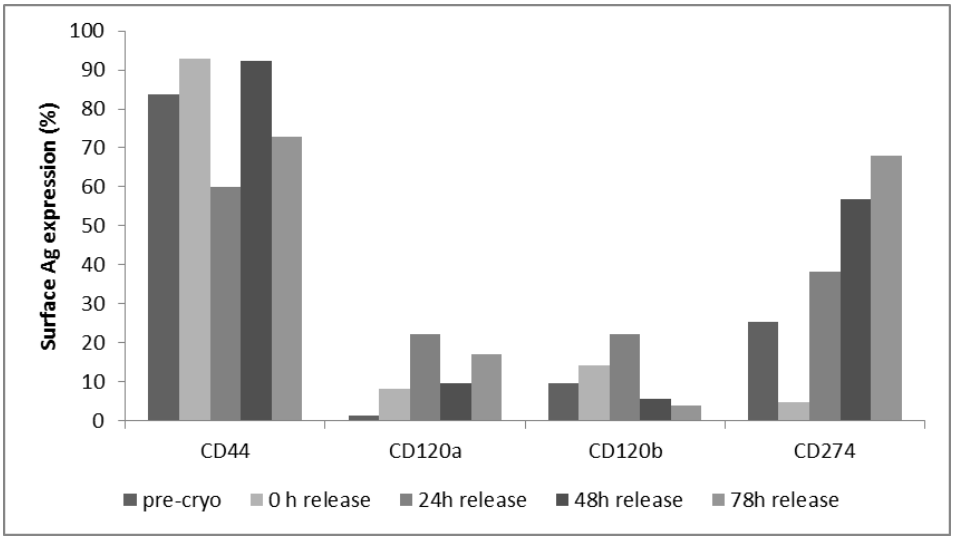


Figure 5.8 IS membrane proteins expression at different times of release, under unstimulated environment. (N=1)

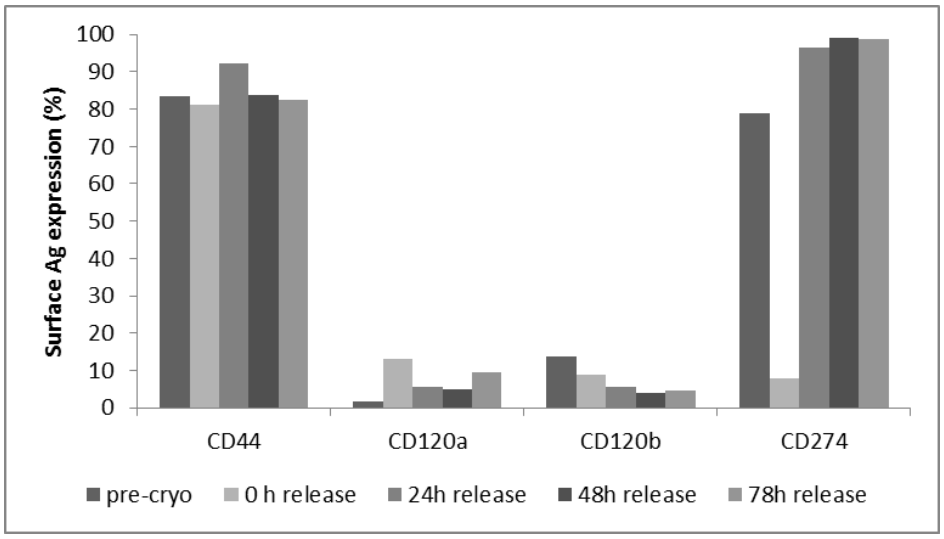


Figure 5.9 IS membrane proteins expression at different times of release, after IFN-γ + TNF-α stimulation. (N=1)

Comparing Figure 5.10 with Figure 5.11, the major difference was on CD274 expression. CD274 responded to the pro-inflammatory environment increase it expression (as observed on IS 3.4.1.3). The remaining membrane proteins analysed showed similar behaviour when compare to IS (3.4.1.3).

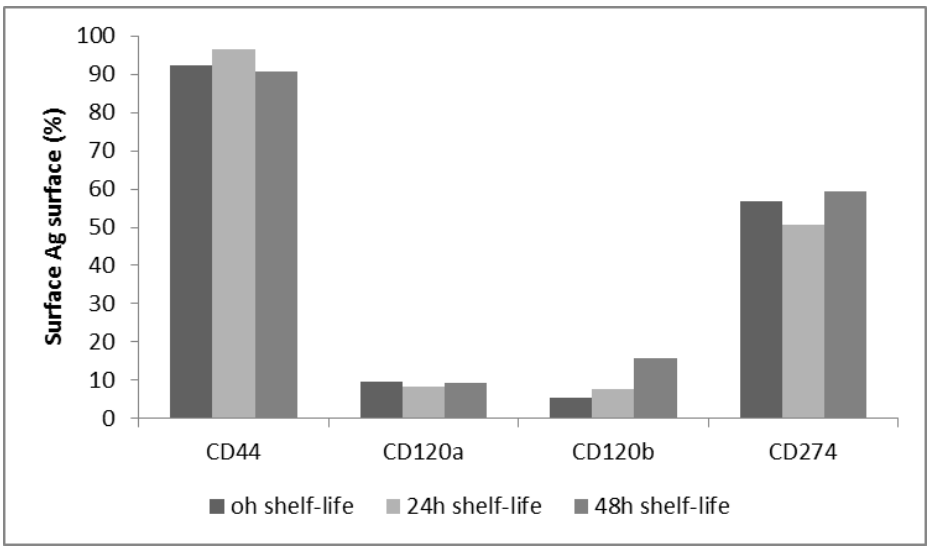


Figure 5.10 IS membrane proteins expression at different times of shelf-life, under unstimulated environment. (N=1)

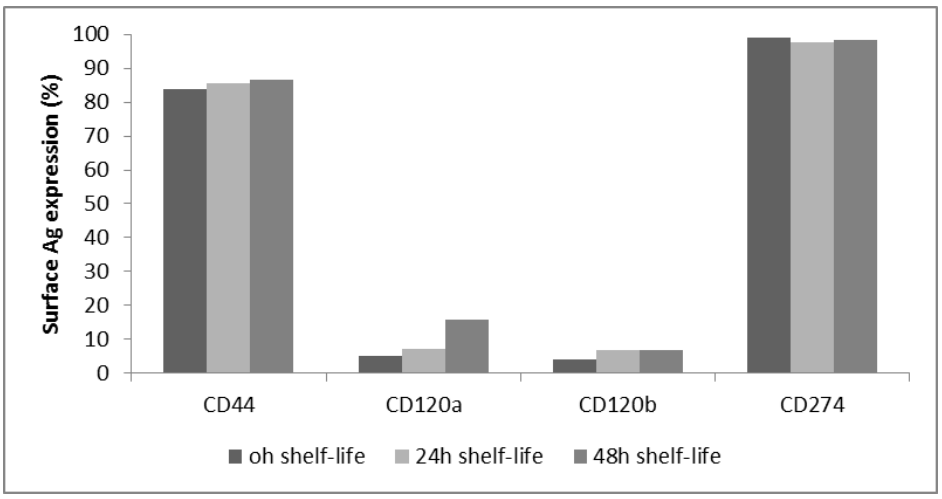


Figure 5.11 IS membrane proteins expression at different times of shelf-life, after IFN-γ + TNF-α stimulation. (N=1)

IS formulation in Hypothermosol at 4ºC did not influence the membrane proteins analysed.

5.2.5 Secretome on ImmuneSafe® release and shelf-life

5.2.5.1 Impact of cryopreservation on secretome

Secretome of exhausted media (non-stimulated and stimulated with IFN-γ and TNF-α) from cells before and after been cryopreserved were compared in order to verify the impact of cryopreservation on cytokine production and thereby the capacity to respond to a pro-inflammatory environment (Table 9).

Table 9 Impact of cryopreservation on cytokine production and capacity to respond to an inflammatory environment. (N=1)

Family	ID	no stim	IFN+TNF
Interleukins	**IL-1a**		Off
	IL-17		Off
	IL-6	4	
Chemokines	**CCL1**		272
	CCL8		3
	CCL7	12	
	CCL13		11
	CXCL16	Off	13
Metalloproteinases	**MMP-9**	23	Off
Hematopoiesis related	**TPO**		Off
	G-CSF		4
	GM-CSF		5
Angiogenesis/ Tissue repair	**Angiogenin**	38	19
	ANG-2		0ff
	ANG-1	9	5
	Angiostatin	3	
	IGF-1		Off
	FGF-4	Off	Off
	VEGF	2	5
	VEGFR3		2
	LIF		21
	PIGF		Off
	HGF		Off
	HB-EGF		Off
	Follistatin	12	46
	TGFβ3	2	Off
Cell metabolism	**ANGPTL4**	Off	Off
	gRP		Off
	Leptin		Off
Cellmigration	**uPAR**	3	18

Reduction on cytokine production after cryopreservation (x)
Unaffected

As shown on Table 9, cryopreservation had a huge impact on cytokine production, especially under a pro-inflammatory environment. From 58 analysed cytokines, 9 of them decreased at least 2 fold its production and 18 were switched off. These results support the pre conceptualized idea that the cells should be delivery fresh, after a release step in culture. If IS were administrated after thawing, IS would have diverse biological process compromised. The abolishing of some ILs production after thawing is an indication that IS would not have the capacity to modulate growth, differentiation and activation during an immune response [43].

Suppression on production of several chemokines after thawing will be reflected on chemoattractant role of IS on biological processes, including leukocyte trafficking, hematopoiesis, angiogenesis, and organogenesis [4] that will be diminished. Furthermore, IS after thawing may not have the capacity to home to site of injury through migration cross endothelium and support hematopoiesis, angiogenesis, and organogenesis.

5.2.5.2 Shelf-life secretome

Secretome of supernatants collected at different times of shelf-life (IS+Hypothermosol at 4ºC) were compared with secretome of same cells but stimulated with IFN-γ + TNF-α, in order to verify if the cells, from different time points during shelf-life, have the capacity to respond to a pro-inflammatory environment and thus evaluate the influence of delivery time in the product.

MSCs formulated in Hypothermosol secreted some interleukins during shelf-life, namely IL-1b, IL-2 and IL12P40, as shown on Figure 5.12 (a). IL-1b and IL-12p40 seems to have a tendency to increase over the time, although IL-2 is the opposite.

MSCs from shelf-life responded to the pro-inflammatory environment increasing the production of others interleukins, like IL-6 and IL-8 (Figure 5.12 (b)). IL-1b and IL-12p40 production, under pro-inflammatory environment, seems to be influenced with elapsed time in Hypothermosol.

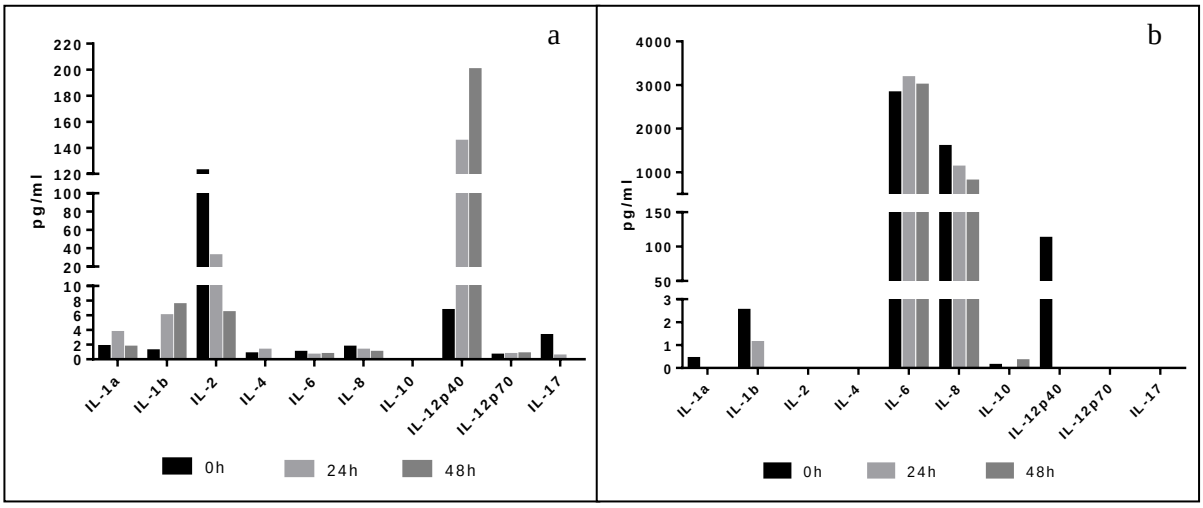


Figure 5.12 Interleukins production during shelf-life experiment. Interleukins production by ImmuneSafe® formulated in Hypothermosol at three different times of shelf life (left graph_a). Interleukins production by MSCs from shelf-life experiment at three different times stimulated with IFN-γ + TNF-α (right graph_b). (N=1)

IL-6 production under pro-inflammatory environment by IS after formulation in Hypothermosol within 48h indicate that IS could be able to inhibit T cell proliferation [60] and mediate anti-apoptotic effect through IL-6 secretion [61] and IL-8 production indicate that IS will have chemoattractant function and is also a potent angiogenic factor [62] under similar inflammatory environment.

On Figure 5.13(a) several chemokines were detected on supernatant at different time points of shelf-life, but is not clear a tendency over time. Almost every chemokines analysed responded to the pro-inflammatory environment increasing it production, as presented on Figure 5.13 (b).

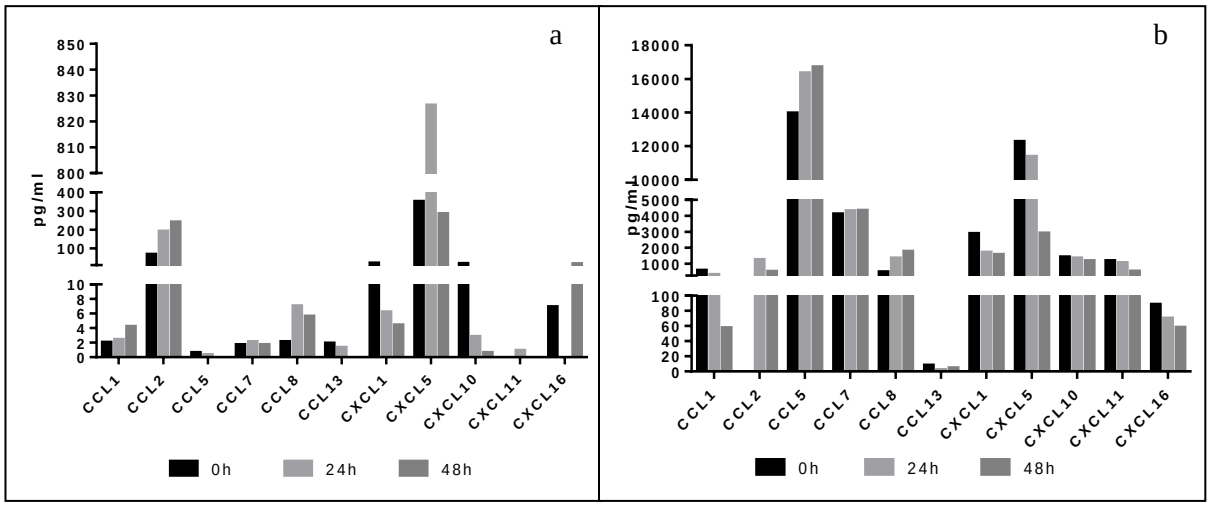


Figure 5.13 Chemokines production during shelf-life experiment. Chemokines production by ImmuneSafe® formulated in Hypothermosol at three different times of shelf life (left graph_a). Chemokines production by MSCs from shelf-life experiment at three different times stimulated with IFN-γ + TNF-α (right graph_b). (N=1)

The up regulation of chemokines production under pro-inflammatory environment referred on Figure 5.13 (b) indicate that IS could enhancing it chemoattractant role and several biological processes, including leukocyte trafficking, hematopoiesis, angiogenesis, and organogenesis [4] in response to a similar pro-inflammatory environment.

From analysis of data present in Figure 5.14, IS formulated in Hypothermosol produced some cytokines that are responsible for angiogenesis and tissue repair, namely bFGF, HGF, ANG-1, VEGF, Tie-1 and TGFα. After simulation with IFN-γ + TNF-α some continued to be produced, like ANG-1 and VEGF, and others were activated, like Angiogenin, IGF-1, PDGF-BB and PECAM-1. In some cases the results were variable over time, indicating that more replicate should be made. IS have the capacity to secrete several proteins that can simulate local angiogenesis and that are anti-apoptotic, indicate that IS under similar environment will be able to support and induce tissue repair that is highly affected by GvHD.

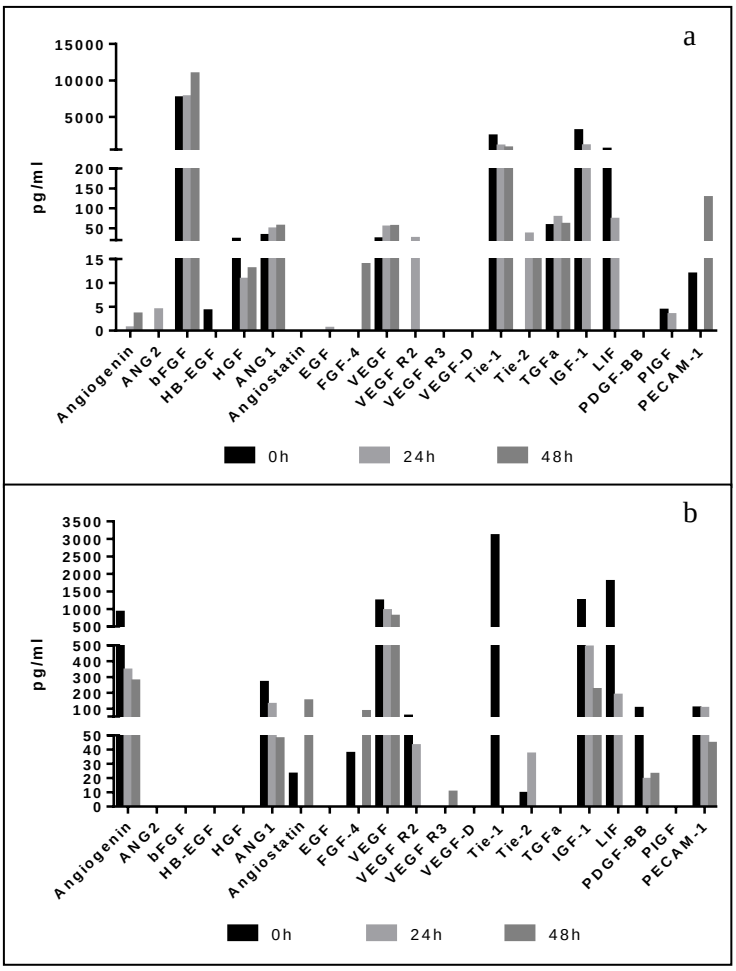


Figure 5.14 Angiogenesis/ tissue repair cytokines production during shelf-life experiment. Angiogenesis/ tissue repair cytokines production by ImmuneSafe® formulated in Hypothermosol at three different times of shelf-life (graph_a). Angiogenesis/ tissue repair cytokines production by MSCs from shelf-life experiment at three different times stimulated with IFN-γ + TNF-α (graph_b). (N=1)

Some cytokines responsible for inflammatory response, Activin A and Follistatin, were also detected on shelf-life supernatant, as shown on Figure 5.15 (a). Under pro-inflammatory environment Activin A was highly expressed in all time points analysed (Figure 5.15 (b)).

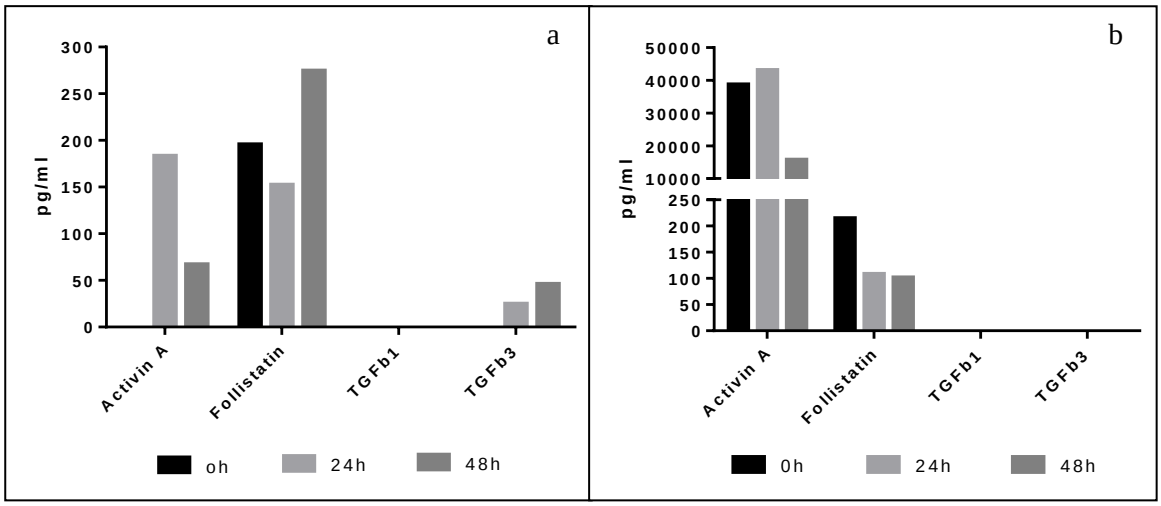


Figure 5.15 Inflammatory response during shelf-life experiment. Inflammatory response analysis of ImmuneSafe® formulated in Hypothermosol at three different times of shelf-life (left graph_a). Inflammatory response analysis of MSCs from shelf-life experiment at three different times stimulated with IFN-γ + TNF-α (right graph_b). (N=1)

5.2.6 IDO and HO-1 production on ImmuneSafe® release and shelf-life

IDO was up-regulated under pro-inflammatory environment in almost release and shelf-life times under pro-inflammatory environment, as shown on Table 10. The only exception was the cells on shelf-life at 24h, this result was not concordant with remaining results because the cells from previous time point (0h) and the next (48h) expressed IDO, probably IFN-γ and TNF-α were not added to the culture.

It is worth mentioning IDO expression after cell thawing, could be an artefact due the two days in culture under stimulation with IFN-γ and TNF-α.

Table 10 Rating of IDO and HO-1 production during ImmuneSafe® release and shelf-life, without stimulation and stimulated with IFN-γ and TNF-α. (-) not expressed (+) expressed (N=1)

		Result	
Sample ID	**Condition**	**IDO**	**HO-1**
Pre-cryo	-TNF-IFN	-	-
	+TNF+IFN	++	-
Release, 0h	-TNF-IFN	-	-
	+TNF+IFN	+	-
Release, 24h	-TNF-IFN	-	-
	+TNF+IFN	++	-
Release, 72h	-TNF-IFN	-	-
	+TNF+IFN	+	-
shelf-life, 0h	-TNF-IFN	-	-
	+TNF+IFN	+	-
shelf-life, 24h	-TNF-IFN	-	-
	+TNF+IFN	-	-
shelf-life, 48h	-TNF-IFN	-	-
	+TNF+IFN	+	-
shelf-life, 5 days	-TNF-IFN	-	+
	+TNF+IFN	++	+

5.2.7 Immunosuppression on ImmuneSafe® release and shelf-life

According Figure 5.16, IS in culture before cryopreservation, showed high suppression capacity of TNF-α production in all immune cells analysed (57.8% on total T cells, 54.5% on T cytotoxic, 75.0% on T helper and 85.4% on NK cells). After thawing, IS did not show the capacity to suppress TNF-α production in any T lymphocytes analysed and this capacity was gradually recovered during the time in culture reaching it maximum potential at 48h in culture (85.7% on total T cells, 73.1% on T cytotoxic, 90.5% on T helper and 79.6 on NK cells).

Accordingly, after cryopreservation IS should have a release step in culture for at least 48h, to recover it immunosuppression potential that is compromised by cryopreservation.

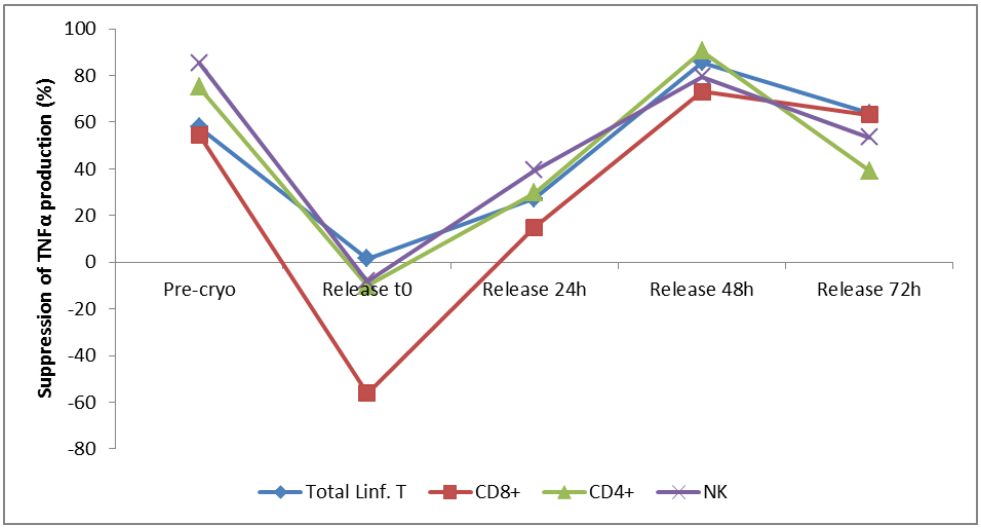


Figure 5.16 Immunosuppression of T lymphocytes, Tc, Th and NK cells by ImmuneSafe® on different times of release, based on the suppression of TNF-α production (N=1).

After IS formulation in Hypothermosol, IS decreased the capacity to suppress TNF-α in all analysed T lymphocytes when compared with IS from 48h of release, this decrement could be due the stress of being in another solution and at -4ºC (Figure 5.17). Although, the time in Hypothermosol seems to be advantageous for IS to recover it immunosuppression capacity. To confirm this indication of IS adaption to a new solution, the immunosuppression capacity of IS from different times of shelf-life in Hypothermosol should be evaluated at least two more times.

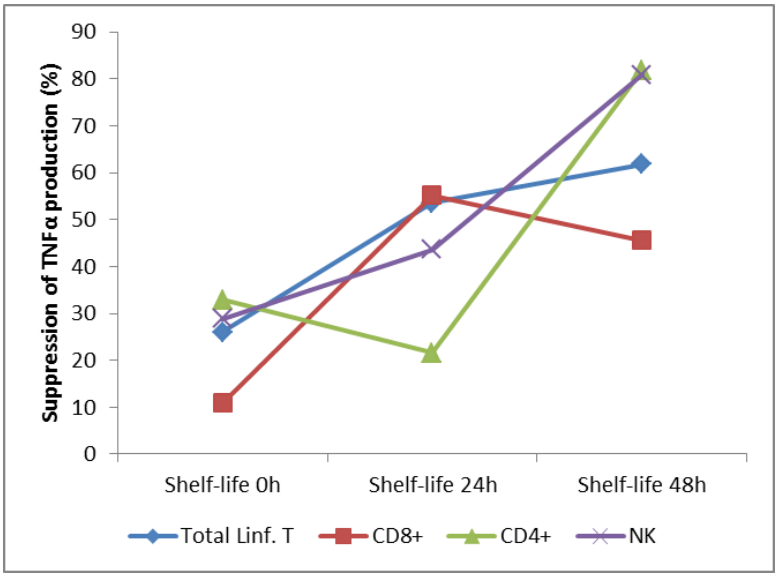


Figure 5.17 Immunosuppression of T lymphocytes, Tc, Th and NK cells by ImmuneSafe® on different times of shelf-life in Hypothermosol, based on the suppression of TNF-α production (N=1).

6 Conclusion

This work contributed to the development of an ATMP, IS and it was divided in three parts: assay set-up for the definition of ImmuneSafe's CQAs, Immunosuppressive drug interaction with IS and Release and Final Formulation of IS.

In what concerns to assay set-up for the definition of ImmuneSafe's CQAs, which include safety, identity and potency in two points of manufacturing process, Cell2B propagation method enabled the achievement of a MSC population, which presented a spindle shape morphology with capacity to differentiate in the three main mesodermal lineage (adipocytes, osteoblasts and chondrocytes), without cross contamination with hematopoietic cells. Furthermore, IS is capable to react to pro-inflammatory environment by up-regulating the expression of several surface antigens involved on immune response modulation, such as CD54 (ICAM-1), CD273 and CD274, and the production of metabolic enzymes (IDO), chemokines, metalloproteinases, interleukins and several other cytokines. The activation of these immunomodulatory pathways allows IS to migrate to sites of injury and induce an anti-inflammatory microenvironment, which was demonstrated by its capacity to suppress the production of IL-17 by T lymphocytes (total, $CD8^+$ and $CD4^+$) and TNF-α by T lymphocytes and NK cells. It is also important to highlight that Cell2B propagation method is a safe expansion method, because it was able to preserve a normal karyotype and any gene amplification/ deletions and loss of heterozygosity were identified.

Although BM-MNCs are also currently being tested as a cell therapy product, to regenerate bone and to treat ischemic tissue as well and to treat several diseases, like critical/ chronic limb ischemia, liver cirrhosis, myocardial infarction [48], [82]. IS showed different biological characteristics and physiological functions, which attested the importance of the designed manufacturing process to obtain a cell product with therapeutic potential to treat immunological diseases.

The benchmarking of IS with similar cell population was also performed. Human Fb and IS presented identical morphology, similar immunophenotype and PGE2 and IL-6 production under pro-inflammatory environment. However, human Fb showed limited adipogenic differentiation capacity, did not produce IDO under pro-inflammatory environment, and were not able to suppress TNF-α production of T lymphocytes (total, $CD8^+$ and $CD4^+$) and NK cells in an consistent way and lead to an increase of IL-17 production by T lymphocytes (total, $CD8^+$ and $CD4^+$). Based on the obtained results human skin Fb do not share all potency parameters as revealed by IS, which makes them not suitable as therapeutics for immunological-related diseases.

Differentiation as indicatory of deviation during the MSCs expansion, namely poorly cultivation, extensive culture, varied stress conditions, can be easily detected by immunophenotype analysis and immunosuppressive capacity. MSCs differentiated in adipocytes and osteocytes presented a decrease of specific surface Ag expression and did not show the ability to suppress the production of TNF-α and IL-17 on analysed lymphocytes under a pro-inflammatory environment, excepting on $CD8^+$ cells, T cytotoxic cells, which revealed suppression of IL-17 production. However, PGE2, IL-6 and IDO production was maintained even after MSCs differentiation in adipocytes and osteocytes.

On immunosuppression drugs interaction with IS, immunosuppressive drugs tested, methylprednisolone and prednisolone, did not seem to have a beneficial or detrimental interaction with IS. Identity and potency of the cell product were not significantly affected. It was verified a decrease on surface antigen expression of CD106 (VCAM-1) and on IL-6 production, both under unstimulated state, which was not so relevant because IS will find *in vivo* an inflammatory environment and the ability to react to a pro-inflammatory environment was maintained even in presence of immunosuppressive drugs. These results thus indicate that the therapeutic performance of IS is not expected to be affect during the clinical trial due to interactions with these immunosuppressive drugs.

The last part of developed work was focused on finding the most suitable timing for product release and evaluate IS shelf-life. The results suggest that a culture release step at least for 48h was advantageous, after cell thawing, for the cells to recover all biological function, namely the capacity to respond to a pro-inflammatory environment expressing HLA-DR and CD274, secretion of several cytokines (interleukins, chemokines, metalloproteinases, etc.) and the capacity to immunosuppress the production of TNF-α of T lymphocytes (total, $CD8^+$ and $CD4^+$) and NK cells. IS shelf-life study revealed that Hypothermosol is a suitable excipient for product deliver at 4ºC, since it enables the maintenance of cellular viability and cell number as well as the capacity to respond to pro-inflammatory environment secreting several cytokines, IDO and immunosuppress the production of TNF-α by T lymphocytes and NK cells. If we assume that the deliver time to the hospital will be within 48h, then at least 70% of initial population will be injected into the patient with 80% of viability and IS potency *in vitro* was assured.

In conclusion, IS have several advantages when compared with MSCs that have been used in previous clinical trials. IS is free of animal components, will be delivered as freshly harvested cells in low passage and will be produced under GMP in a robust large scale and not under punctual autologous expansion dependent on the needs. Furthermore, IS under pro-inflammatory environment acts through its primary mechanism of action that it is based on the production of a variety of soluble factors, which interact and modulate the responses of body's

immunological system and promote tissue regeneration, beyond the activation of many receptors that are involved on cell-cell or cell-ECM interaction.

7 Future work

Despite the definition of IS CQA's, the characterization of the cell product should continue to be performed at different levels. For instance, the impact of different culture conditions must be evaluated in order to improve the manufacturing process. For instance, understand how overconfluent passages and extensive culture (beyond the population doublings level of IS) will affect the product is crucial to increase IS quality parameters. Also, studies with advanced techniques such as proteomics or lipidomics will bring new and complementary information regarding the response of IS to pro-inflammatory states, which will contribute to increase the knowledge on the mechanism of action and eventually define the clinical development of IS for other inflammatory and immunological disease.

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