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

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Mesenchymal stem/stromal cells (MSCs) have proved to be able to modulate the immune system by the secretion of cytokines and other soluble factors which makes them strong candidates to be used in Advanced Therapy Medicinal Products (ATMP) for the treatment of GvHD and other immune diseases; ImmuneSafe® is one of the products based on MSCs with the intention of preventing and treating GvHD. In this work, the benchmarking of ImmuneSafe® was performed using MSCs that were previously administered to patients (MSC IPO) and with positive results in treating and preventing GvHD. The tests performed suggested that, besides having similar characteristics and activity, ImmuneSafe® has a higher potency in what relates to the immunosuppressive activity. The mechanism of action (MoA) of ImmuneSafe® was also studied and the results indicated that IDO is one of the central players in its immunomodulatory activity. Therefore, IDO appeared to be the most interesting protein to be used as a candidate for the potency assay, crucial for the clinical application of ImmuneSafe®. The activation profile of ImmuneSafe® was further analyzed using several pro-inflammatory cytokines. The overall results suggested that ImmuneSafe® reacts to those pro-inflammatory environments by releasing different soluble factors and altering the level of expression of some membrane proteins. Overall, the first steps were given in order to study ImmuneSafe®'s MoA and in understanding its responses to several pro-inflammatory environments. Further investigation is still crucial in order to achieve trustful results and develop an effective potency assay.

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

Introduction

"*The stem cells in adult mammalian tissues (and the property of stemness) are difficult to define conceptually, largely impossible to identify morphologically and are associated with functions and attributes that commonly confuse rather than clarify their identity and role*" [1]. In 1990 things were at this point; 24 years later it is still a challenge to fully define stem cells.

Mesenchymal Stem/Stromal Cells (MSCs)

MSCs were first isolated from bone marrow, but it is now known that they reside in almost every type of connective and neonatal tissues. They are characterized as an heterogeneous population of cells that respect the three minimal criteria defined by the ISCT [2]: *i)* adherence to plastic (very useful for the isolation of the cells); *ii)* specific surface antigen (Ag) expression (≥95% of the MSCs must express CD105, CD73 and CD90; ≤2% of the MSCs must lack expression of CD45, CD34, CD14 or CD11b, CD79a orCD19 and HLA-II); *iii)* multipotent differentiation potential into osteoblasts, adipocytes and chondroblasts under standard *in vitro* differentiating conditions.

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

MSCs as treatment for Graft-versus-Host Disease (GvHD)

GvHD is the most frequent complication after allogeneic hematopoietic stem cell transplantation (HSCT). It is a consequence of interactions between antigen-presenting cells (APCs) of the recipient and immunocompetent donor cells and is associated with considerable morbidity and mortality,

particularly in patients who do not respond to primary therapy (steroids). It is known that these immunologically competent cells are T-cells and that GvHD can develop in various clinical settings when tissues containing T-cells are transferred from one person to another [3]. The major target organs of acute GvHD are skin, liver, and intestinal tract, although other organs can be affected.

There are two types of GvHD: acute (aGvHD) and chronic (cGvHD). The development of aGvHD can be (described in three steps see Figure 1): *i)* effects of conditioning*, ii)* T cell activation and *iii)* cellular and inflammatory effector phase.

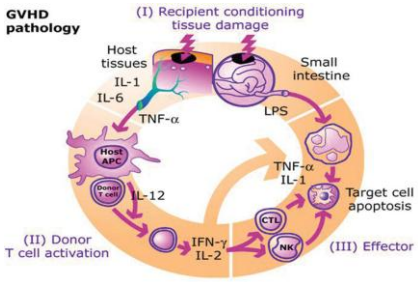

Figure 1 - GvHD pathology [4]

Additional complexity has been recently added with the description of regulatory cell populations that can contribute for the treatment of GvHD: Regulatory T-cells (Tregs), regulatory APCs and mesenchymal stem cells (MSCs) [3]–[5].

The clinical efficacy of MSCs in the treatment of GvHD was first described by Le Blanc [6]*;* since then, other clinical studies have shown the efficacy of MSCs in the treatment of this disease but the mechanism of action still needs further investigation [7].

MSCs Mechanism of Action (MoA)

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

It has already been demonstrated that MSCs are able to suppress T cell proliferation and induce split T cell anergy [8]. It has also been sown that MSCs can inhibit the differentiation of monocytes into dendritic cells (DCs) and inhibit B cell activation, proliferation and differentiation to immunoglobulin (Ig)-producing cells [9]. They are also believed to be involved in the production of regulatory T cells (Tregs) [10].

Interestingly, MSCs seem to have pro-inflammatory and immunosuppressive phenotypes. Following this concept, MSCs are not spontaneously immunosuppressive but require "licensing" or activation to exert their immunosuppressive effects. This activation is dependent on "signals", molecules that are released *in vivo* and can trigger MSCs response [12].

MSCs have shown to exert their immunomodulatory effects through the release of a variety of soluble factors, mainly cytokines [9]. A summary of the main soluble factors involved are described below.

Metabolic Enzymes

Heme oxygenase-1 (HO-1) is also known as heat shock protein. It is one of three isoenzymes from the heme oxygenases family that catalyze the oxidative degradation of heme to biliverdin, free divalent iron and CO. The only inducible form, HO-1, has been described as an anti-inflammatory and immunosuppressive molecule [13]. In human MSCs, HO-1 inhibition with SnPP completely abolished the suppressive effect of MCs [13].

Indoleamine 2,3-dioxygenase (IDO) is an enzyme that catabolises tryptophan into kynurenine metabolites that regulate T-cell proliferation [9], [12]. It is inducible by stimulation of MSCs with IFN-γ or TLR3 and TLR4. IDO degrades the essential amino acid tryptophan and, in concert with other enzymes, gives rise to metabolites of tryptophan that inhibit lymphocyte proliferation [14].

Secreted Factors

Prostanglandin E2 (PGE2) is a rapidly released and short acting small lipid mediator that is known to have a role in immunomodulation [12]. Some studies showed that PGE2 is produced when MSCs are in co-culture with monocytes and it is possible to induce its production by stimulating the MSCs with IFN-γ and TNF-α, as well as by TLR3 ligands (but

not TLR4) [9]. It is now known that PGE2 suppress the T-cell activation and proliferation both *in vitro* and *in vivo* and has an important role in MSC reprogramming of macrophages and dendritic cells.

Interleukins (IL) are a complex group of cytokines with complex immunomodulatory functions, including cell proliferation, migration, maturation, adhesion and also in immune cell differentiation and activation. ILs can have pro- and anti-inflammatory functions which makes their characterization quite difficult and distinguishes them from chemokines in general [15].

Chemokines are low-molecular weight molecules members of the cytokine family of regulatory proteins. They are considered secondary pro-inflammatory mediators that are induced by primary pro-inflammatory molecules and are known by their ability to recruit well-defined subsets of leukocytes. Through the recruitment of leukocytes, chemokines lead to the activation of host defence mechanisms. There are two major chemokine subfamilies: Chemokine (C-X-C motif) Ligand (CXC) and Chemokine (C-C motif) Ligand (CC) [16].

Metalloproteinases released by MSCs complement the chemokine function by degrading the endothelial vessel basement membrane to allow extravasation into damage tissue and the ratio between matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMP) appears to be important in MSC function [17].

Membrane Proteins

CD54 and CD106, also known as Inflammatory Cytokine-Induced Intracellular Adhesion Molecule-1 (ICAM-1) and Vascular Cell Adhesion Molecule-1 (VCAM-1), are membrane surface molecules that may be critical in the immunomodulatory effect of the MSCs [18]. They are known to mediate the cell adhesion that is a possible critical factor in the MSCs immunossuppressive action.

CD166, also known as ALCAM, is a positive selection marker for MSCs and it is also known to be expressed on activated T cells and monocytes and also to play a role in mediating adhesion interactions between thymic epithelial cells and $CD6^+$ cells during intrathymic T cell development [19].

CD44 is a cell surface glycoprotein that is involved in cell-to-cell interaction, migration and adhesion. It is an adhesion molecule involved in leukocyte attachment to and rolling on endothelial cells, homing to peripheral lymphoid organs and to the sites of inflammation, and leukocyte aggregation [20].

Programmed death 1 (PD-1) is a transmembrane protein that is believed to be involved in the suppression of the immune system. Direct contact of MSCs and target cells can lead to the inhibition of cell proliferation via engagement of PD-1 to its ligands PD-L1 (CD274) and PD-L2 (CD273), leading to the target cells to modulate the expression of different cytokine receptors and transduction molecules for cytokine signaling and allowing, for example, the inhibition of T-cell receptor-mediated lymphocyte proliferation [21].

ImmuneSafe®

ImmuneSafe® is an Advanced Therapy Medicinal Product (ATMP) developed by Cell2B and based on MSCs with the purpose to treat several immune and inflammatory diseases such as GvHD. Upon stimulation by a pro-inflammatory environment, ImmuneSafe® modulates the responses of body's immunological system and promotes tissue regeneration by the production of soluble factors such as cytokines.

Materials and Methods

Bone Marrow Samples

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

Bone Marrow Mononuclear Cells Isolation Procedure

Bone Marrow Mononuclear Cells (BM-MNC) isolation was performed using Sepax (BioSafe) system. At the end of the process, the kit was removed from the Sepax and the

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

Immunophenotype Characterization

In order to prepare the cells for immunophenotypic analysis, the cell suspension was centrifuged for 7 minutes at 1250 rpm and resuspended in DPBS. 100 µl of the homogeneous cell suspension was added to each tube and the corresponding fluorochrome-conjugated antibodies were administered to the tubes (5 or 10 µl, according to the concentration of the antibody solution). Appropriate isotype controls were used in every experiment. The mixture was incubated for 15 minutes in the dark, at room temperature. To wash the excess of antibody following staining, 2 mL of DPBS were added to each tube and the tubes were then centrifuged for 7 minutes at 1200 rpm. The cell pellet was resuspended in 400-500 µl of 2% paraformaldehyde (PFA) in order to fix the cell-antibody mixtures and the tubes were stored at 4°C in the dark. The samples were run in FACSCalibur equipment and analysed using Flowing Software 2 (Perttu Terho (Turku Centre for Biotechnology, University of Turku, Finland)).

Pro-inflammatory stimulation

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

For the activation profile experiments, cells were also stimulated with: Lipopolysaccharides (LPS, Sigma-Aldrich®)-1µM, interleukin-2 (IL-2, R&D Systems™)-25µM, Interleukin-12 (IL-12), IFN-γ+TNF-α+ IL-2, TNF-α+IL-1, TNF-α+IL-1+IL-6. The last three cytokines (IL-12, interleukin-6 (IL-6) and interleukin-1 (IL-1)) were obtained from the R&D Systems™ and the concentration used in the assays were the same, 10 ng/mL.

Cytokine Production Analysis

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

Immunosuppressive Studies

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

Western Blot Analysis

The detection of Indoleamine 2,3-dioxygenase (IDO), was performed by Western Blotting at the Analytical Services Unit of IBET laboratories (Oeiras, Portugal). Cell lysates were prepared using RIPA buffer (Sigma) supplemented with complete protease inhibitor cocktail (Roche). Anti-IDO clone 10.1 (Merck Millipore) at a 1:5000 dilution was used to detect protein expression on whole cell protein extracts isolated from MSCs by immunoblot analysis.

Immunomodulatory Pathways Blocking Studies

The enzymatic activity of IDO was blocked using two stereoisomers with potentially different biological properties, 1-methyl-D-tryptophan (1-MT-D-T, Sigma.Aldrich®) and 1-methyl-L-tryptophan (1-MT-L-T, Sigma.Aldrich®), each one at the concentration of 2mM. After incubation at 37ºC, 5% CO_2, the exhausted media was stored at -20ºC to be later on analyzed and cell lysates were prepared for western blot analysis. Cells were also sent to CHC for immunossupressive studies.

For the membrane proteins study, six antibodies were used in order to block these proteins: anti-CD44, anti-CD54, anti-CD106, anti-CD166, anti-PD-L1 and anti-PD-L2. The antibodies were all obtained from R&D Systems™. The usual immunophenotype characterization protocol was performed (including controls). Immunossupressive studies were also performed. The concentrations for each of the antibodies were the following: anti-CD44-20 µM; anti-CD54-25 µM; anti-CD106-25 µM; anti-CD166-25 µM; anti-PD-L1-50 µM and anti-PD-L2-30 µM.

Results and Discussion

ImmuneSafe® benchmarking with MSCs with proven clinically efficacy

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

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

ImmuneSafe® and MSC IPO demonstrated similar results with ImmuneSafe® surpassing MSC IPO in the potency tests. From this analysis, it can be indirectly assumed that ImmuneSafe® have clinical potential for the treatment of several immune diseases and, in particular, GvHD.

Immunomodulatory pathways blockage studies

Although the mechanism of action of MSCs for immunological and inflammatory diseases has been under intense study, it is not yet fully understood [22]–[25]. A specific strategy was used to block the different immunomodulatory pathways believed to be involved in their immunomodulatory properties and then evaluate its impact on the product's immunosuppressive capacity.

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

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

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

Impact of several stimuli in the immunomodulatory profile of ImmuneSafe®

With the goal of understanding the impact of the different disease stages on ImmuneSafe®'s immunomodulatory activity (namely the activation of the different immunomodulatory pathways), cells were stimulated with different combinations of the cytokines that characterize the GvHD development stages: TNF-α, IFN-γ, LPS, IL-2, IL-12, IFN-γ+TNF-α+IL-2, TNF-α+IL-1 and TNF-α+IL-1+IL-6.

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

The results obtained suggested that ImmuneSafe® responds differently to the pro-inflammatory environments to which it was subjected. These responses will affect its therapeutic action through the release of several cytokines and other soluble factors. Overall, these results suggested that IFN-γ was the pro-inflammatory cytokine that triggered a higher number of responses by ImmuneSafe® when added alone to the cell cultures. The most pro-inflammatory environment, however, appeared to be the one caused by the simultaneous addition of IFN-γ and TNF-α which was already expected [26]. Understanding the environments that trigger ImmuneSafe®'s immunomodulatory mechanism is extremely important for its clinical application and together with further experiments, these analysis could contribute for that knowledge.

Conclusions and Future Work

There is still a lot of work left to do in this area. The main problem still resides in understanding how this cells act *in vivo*; the *in vitro* tests are crucial to give the first steps but are not enough. A potency test would be one of the most important achievements for the clinical application of ImmuneSafe® and any other cell product. Understanding the mechanism of action of the cells and which are the factors that trigger its responses would be the first step.

Besides, tests at the DNA or RNA level would lead to more conclusive and trustful results and conclusions, *i.e*., RT-PCR, sRNA, microarrays and others.

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

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