ESCOLA
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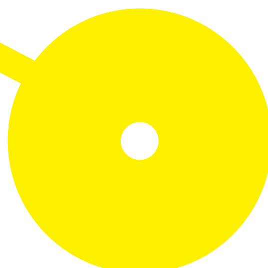
TÉCNICAS LABORATORIAIS DE BIOPATOLOGIA - PATOLOGIA MOLECULAR

# Decoding Immunomodulatory Interactions: Exploring the crosstalk between bone marrow-derived mesenchymal stem cells out the bag and peripheral blood mononuclear cells for potential in-vivo immunosuppression

Raquel Leão Monteiro

06/**2024**



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# Decoding Immunomodulatory Interactions: Exploring the crosstalk between bone marrow-derived mesenchymal stem cells (BM-MSCs) out the bag and peripheral blood mononuclear cells (PBMCs) for potential in-vivo immunosuppression


**Autor**

Raquel Leão Monteiro

**Orientador**

PhD/Sérgio Manuel Bernardo Machado Lopes/Serviço de Terapia Celular do Instituto Português de Oncologia do Porto

PhD/Ana Filipa Quintela Vieira/Escola Superior de Saúde do Instituto Politécnico do Porto



Relatório de estágio apresentado para cumprimento dos requisitos necessários à obtenção do grau de Mestre em **Técnicas Laboratoriais de Biopatologia** – Ramo de Especialização em **Patologia Molecular** pela Escola Superior de Saúde do Instituto Politécnico do Porto.

**Agradecimentos**

I sincerely appreciate EPOP, highlighting Drª Susana Roncon, the director of the IPO Cellular Therapy Department, for accepting my entrance into the team. Thank you for your outstanding work and positive influence on the team's endeavours.

Special gratitude is owed to Drº Sérgio Machado Lopes, my research supervisor, whose unwavering support, and dedicated involvement at every stage were integral to completing this thesis. His expertise, friendship and constant inspiration have been invaluable. Your strength and commitment are truly commendable, and I deeply appreciate your mentorship during tough times.

I am also thankful to the devoted whole team at the Cellular Therapy Department in IPO who received me with open arms. Your steadfast dedication to advancing medical research and delivering outstanding patient care is truly commendable. I cherish the fond memories we created during these months.

Besides, a sincere thank you to all stem cell and blood donors, with special recognition for those unrelated individuals who selflessly shared the precious gift of life without hesitation. Your generosity and commitment to making a difference are truly admirable. Thanks for your invaluable contribution and for being the lifeline for those in need.

I also want to acknowledge the support and inspiration received from other faculty members and professors, both from ESS, i3S and FMUP. Thank you for believing in me! Last, but not least, I would like to express my gratitude to my parents, friends, and boyfriend for their unwavering support, and understanding. Your love brought me to where I am today.

Yours truly,

**Resumo**

O transplante alogénico de células estaminais hematopoiéticas é crucial para doenças hematológicas, mas complicações surgem quando células imunes do dador começam a atacar tecidos do paciente. Uma barreira laboratorial no estudo desta alorreatividade é a ausência do nicho hematopoiético. Dessa forma, utilizamos células estaminais mesenquimais derivadas da medula óssea (BM-MSCs), de sacos de colheita tipicamente descartados, para avaliar o seu potencial na proliferação de células mononucleares do sangue periférico (PBMC) *in-vitro* e decifrar interações para propor mecanismos de imunomodulação *in-vivo.*

PBMCs marcadas com CFSE, foram co-cultivadas por 6 dias com PBMCs irradiadas numa proporção 2:1 para mimetizar alorreatividade, com ou sem BM-MSCs. Por citometria, averiguou-se fenótipo BM-MSCs e a proliferação/expressão de marcadores de superfície das PBMCs. Atividade metabólica foi avaliada por ensaios resazurina. Microscopia de imunofluorescência CFSE e de contraste registaram interações físicas.

BM-MSCs expandiram-se eficazmente, mantendo fenótipo adequado. Co-culturas apresentaram menos PBMCs coradas com CFSE, traduzindo-se numa redução de 18%, com atividade metabólica aumentada. Imunofenotipagem revelou diminuição de subgrupos de células T e um aumento na expressão de PD-1, sugerindo imunossupressão.

Os resultados obtidos destacam o potencial imunomodulatório das BM-MSCs recolhidas. A reutilização de sacos de colheita também oferece uma solução sustentavelmente prática, reduzindo o desperdício hospitalar e custos.



**Palavras-chave:** Doença hematológica; células mononucleares do sangue periférico; medula óssea, células estaminais mesenquimais; imunomodulação; citometria de fluxo

**Abstract**

Allogeneic hematopoietic stem cell transplant (Allo-HSCT) is crucial for hematological diseases, yet chronic complications may arise as donor immune cells start attacking patient tissues. A lab barrier to studying this alloreactivity is the absence of the hematopoietic stem cell (HSC) niche. Here, we employed bone marrow-derived mesenchymal stem cells (BM-MSCs) from discarded collection bags to assess their potential in modulating peripheral blood mononuclear cell (PBMC) proliferation *in-vitro* and decode occurring interactions to propose mechanisms of *in-vivo* immunomodulation.

Responder PBMCs, labelled with CFSE dye, were co-cultured for 6 days with irradiated stimulator cells in a 2:1 ratio to mimic alloreactivity, with or without BM-MSCs. Flow cytometry tracked BM-MSCs phenotype and PBMC proliferation/surface marker expression, while resazurin assays monitored metabolic activity over time. CFSE immunofluorescence and phase-contrast images recorded PBMC proliferation and physical interactions.

BM-MSCs expanded successfully, maintaining adequate phenotype. Co-cultures exhibited fewer CFSE-stained PBMCs, equating to an 18% reduction in responder cells, with enhanced metabolic activity. Immunophenotyping revealed downregulation of T cell subsets and increased PD-1 expression, suggesting immunosuppressive effects.

These findings underscore BM-MSCs´ immunomodulatory potential, offering a cellular therapeutic avenue. Repurposing collection bags as a source of BM-MSCs also offers a practical solution, reducing medical waste and enhancing cost efficiency.

**Keywords:** Hematological disease; peripheral blood mononuclear cells; bone marrow; mesenchymal stem cells; immunomodulation; flow cytometry

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## List of Abbreviations

**A**

Allo-HSCT- Allogeneic hematopoietic stem cell transplant

**B**

BM – Bone marrow

**C**

CBC – Complete blood count

CFSE –Carboxyfluorescein succinimidyl ester

CXCL12-Granulocyte colony-stimulating factor

**D**

DMEM-HG – Dulbecco modified Eagle's medium high glucose

**F**

FBS – Fetal bovine serum

**G**

GBD – Global burden of disease

GvHD – Graft-versus-host disease

GvT – Graft-versus-tumour effect

GM-CSF- Granulocyte-macrophage colony-stimulating factor

**H**

HLA – Human leucocyte antigens

HL – Hodgkin lymphoma

HSC – Hematopoietic stem cell

**I**

IFN – Interferon

IL – Interleukin

ISCT – International Society for Cell Therapy

IDO- Indoleamine 2,3-dioxygenase

IPO-Instituto Português Oncologia

**L**

LIF – Leukaemia inhibitory factor

**M**

MNCs – Mononuclear cells

MM – Multiple myeloma

MSC – Mesenchymal stem cell

MLR- Mixed lymphocyte reaction

**N**

NHL – Non-Hodgkin lymphoma

NO – Nitric oxide

**P**

PB – Peripheral blood

PBMC – Peripheral blood mononuclear cells

PD-1 – Programmed cell death protein 1

PGE-2 – Prostaglandin E2

**R**

ROS – Reactive oxygen species

**S**

SCF – Stem cell factor

**T**

TGF – Tumour growth factor

Th – T helper cells

TNF – Tumour necrosis factor

TPO – Thrombopoietin

**U**

UCB – Umbilical cord blood

# 1. INTRODUCTION

## 1.1) Hematooncology: Epidemiology and clinical relevance

As stated in the Global Burden of Disease (GBD) 2023, haematological malignancies continue to be a major health concern and due to the rapidly ageing population, this burden increases even more. They are very common in people over 70, and when gender is considered, there is a global male prevalence. This pattern between genders may convey distinct factors, such as genetic, hormonal balances, surrounding environment and lifestyle choices (1). Despite life expectancy has gotten better for these patients over the past few years, there is still a need to provide more insights into risk factor dynamics and understand pathogenesis to develop more precise strategies of management and treatment.

According to their neoplastic cell precursor, haematological malignancies can either be myeloid or lymphatic tumours and originate from a disruption of normal hematopoiesis believed to be driven by genetic or epigenetic lesions. This disruption overwhelms normal blood cells, creating a ripple effect of medical conditions, such as fatigue, persistent fever or infections, unusual bleeding/bruising, swollen lymph nodes, bone pain and unexpected weight loss (2). From all blood neoplasias, leukaemia, multiple myeloma (MM), non-Hodgkin lymphoma (NHL), and Hodgkin lymphoma (HL) are the most prevalent subtypes. Disease mechanisms include primary driver mutations, reprogramming and changes due to inflammation, angiogenesis, and increased hypoxia in bone marrow (BM) niche cells. Hence, the changing BM niches encourage the activation of survival pathways and induce immune evasion to uphold neoplastic cells, defend against reactive oxygen species (ROS) and resist chemotherapies (3).

Several epidemiological studies have also shown some risk factors linked to the onset of these illnesses, such as exposure to ionizing radiation, chemicals (benzene, ...), genetic disorders or viral infection (4). Nevertheless, a precise understanding of the genesis of hematological malignancies is required for their prevention and control, as it is still largely unknown.

### 1.2) Immunotherapy approaches for hematologic malignancies: HSCT

Targeted antibodies, immune checkpoint inhibitors, tumour vaccines, adoptive cell transfer therapy, CAR-T cells and stem cell transplantation are the primary components of immunotherapy available to treat hematologic malignancies (5). Currently, most are still treated by hematopoietic stem cell transplantation (HSCT) since others are still in the early stages of development and clinical trials. HSCT involves administering healthy hematopoietic stem cells (HSCs), also known as the graft, that can arise from peripheral blood (PB), umbilical cord blood (UCB) or BM to patients with impaired or diminished hematopoiesis (5). In addition, HSCT can also be applied to treat non-malignant blood disorders like thalassemia and sickle cell disease, aplastic anaemia, combined immunodeficiency, metabolic diseases, such as Hurler Syndrome (6), and even considered for some autoimmune diseases, like lupus and systemic sclerosis (7). Therefore, the overall aim of HSCT is to replace and reestablish a patient´s affected hematopoiesis to normal conditions.

As to categories available for transplantation procedures, from most to least common, there are autologous, where HSCs come from the patient itself; allogeneic, from a healthy donor (related or unrelated) compatible with the donor and finally, syngeneic, where HSCs come from a healthy identical twin. When considering allogeneic HSCT, finding a suitable donor is a necessary step. Not only they must be healthy and willing to donate but, most importantly, have a high degree of human leucocyte antigens (HLA) compatibility with the patient (8). HLA antigens are required for presenting peptides to T cells, allowing them to identify and remove abnormal elements in an individual and keeping the "self" particles from being recognized as foreigners. Besides, HLA genes exhibit high allelic frequency in the genome, which makes each person´s HLA composition very singular. Typically, a high-resolution HLA test examines 10 markers (5 genes with two loci each) and when 2 individuals share identical alleles for these, a perfect HLA match (10/10) is stipulated. Even with one or two mismatched alleles (9/10 or 8/10), the match is considered valid and an HSCT procedure may begin (9).

Each HSCT procedure encompasses distinct phases that begin with the HSCs harvest, proceeded by a preparatory phase, known as conditioning, through a brief period of chemotherapy, radiotherapy or both. Subsequently, patients undergo a period of aplasia, during which their immune system is temporarily suppressed and, finally, the transplanted

HSCs are engrafted within the recipient´s BM, leading to gradual restoration of hematopoietic functions (5, 10). The chosen approach of transplantation mostly relies on higher success rates in clinical trials for a specific illness. As an example, allogeneic transplantation is preferred in conditions in which graft-versus-tumour effect (GvT) has been shown, such as leukaemia and myelodysplastic syndromes. In this phenomenon, donor graft T cells sense and eradicate cancerous cells in the recipient's body, although the precise nature of this response remains unclear. As for lymphomas and MM, the most common course is an autologous HSCT, where GvT is mostly undetectable (11, 12). As for ease of access to transplant categories, high-dose myeloablative therapy combined with autologous or allogeneic stem cell transplantation is a successful average treatment approach that allows for additional consolidation of response. In selected cases, a less aggressive regimen with immunosuppressants may be used, although it mainly depends on the graft´s immune-competent cells to eradicate any remaining illness (13).

Following the completion of the conditioning protocol, preventive measures are initiated to address various infectious and non-infectious issues. Subsequently, the BM or PB-cell graft is administered intravenously through a central catheter. Engraftment may take 10 to 21 days and is influenced by factors like graft source, cell quantity, inflammatory milieu, and whether granulocyte colony-stimulating factor was used. If complications are absent and the recipient's clinical condition is favourable post-engraftment, then the patient is discharged and only required for preventive monitoring (8).

### 1.3) <u>Post-transplant complications: Interrogating the problem of alloreactivity</u>

Despite significant advances in the process of HSCT, major immunological problems can be frequently linked with an allogeneic HSCT. Of these problems, graft-versus-host disease (GvHD) is the most prevalent and deadly, occurring in up to 50% of patients receiving HSCT (14) with more than 10% that can die from this complication (15). It could happen within 100 days (Acute GvHD), but later onset (Persistent acute or Chronic GvHD) is also possible (16). This happens when donor-derived immunologically competent cells use intricate and distinct systems to target the patient's tissues, also known as alloreactivity (17).

While Acute GvHD is primarily dependent on donor T cells' alloreactivity response to HLA-mismatched recipient tissues, Chronic GvHD shares similarities with autoimmune conditions,

as it encompasses B cells and, therefore, an antibody-mediated response (18). Yet, reports of a comparable response to GvHD in autologous transplants have also been found (19, 20), suggesting that alloantigen recognition is insufficient to fully explain the phenomenon of alloreactivity. Hence, these immune responses could be explained due to several variables of HSCT, including conditioning regimens, tissue injury, and antigen release (21). Consequently, the immune system's ability to identify and react to alloantigens entails more complex and distinct mechanisms than first thought.

Currently, broad-spectrum immunosuppressive medications are used as first-line treatments for GvHD, providing partial clinical efficacy and remission in certain patients (20-22). However, when used over an extended period, they can greatly harm the hematopoietic milieu of the BM and, therefore, greatly impact the success of transplantation (23). Considering this, more information about this alloreactivity concern on different immune functions and surrounding BM cells is required to enable more efficient management.

Since both graft failure and GvHD stem from persistent immune system dysregulation, the current strategy for most cellular therapies to address this problem is restoring immunological homeostasis (24-26). This can be achieved through increasing regulatory activity or decreasing alloreactivity.

This immune rehabilitation attempt must, however, consider the "Janus-Face" balance of HSCT (27). This phenomenon acknowledges that while an alloreactivity´ controlled maintenance might be advantageous in overcoming oncological diseases, its uncontrolled manifestation can lead to undesirable immunological complications. As a result, cellular therapies should also raise the prospect of using immunological cross-talking to help the BM niche reach a homeostatic resolution.

In the realm of cellular therapeutics, where variations among donors are a factor, it is crucial to also possess dependable measures for assessing the potency and forecasting the effectiveness of various cellular preparations. For instance, mixed lymphocyte reaction (MLR) assays are a very common assay employed for this purpose. They allow the assessment of immunogenicity or compatibility of alloreactive lymphocytes, combining both donor and recipient lymphocytes to see how they interact. Particularly for GvHD, they have been already used to evaluate the depletion or activation of alloreactive T lymphocytes in both *in-vivo* (28, 29) and *in-vitro* models (30-33).

This crucial clinical significance emphasizes the imperative of maintaining control over alloreactivity within the laboratory setting, a task most often hindered by the absence of the natural microenvironment, referred to as the HSCs niche. To improve our comprehension of these cells' role in an alloreactive balance, it is indispensable to start identifying and classifying the cells that belong in this particular milieu.

### 1.4) Cellular microenvironment of HSCs

The cells used in HSCT, HSCs, are defined as multipotent mesoderm lineage cells capable of haematopoiesis, the ongoing production of mature blood cells such as red blood cells, platelets, megakaryocytes, and all leucocytes, including lymphocytes, monocytes, and polymorphous nuclear cells. CD34 is an important marker of human HSCs, being the CD34+ fraction known to contain stem cells able to form colonies and, therefore, able to proliferate, self-renew and differentiate into functional mature blood cells (34). However, even in an extremely pure population of CD34+ cells, the frequency of colony-forming cells is usually 20% (35). Therefore, the rehabilitation of the hematopoietic system in patients after transplant is also accompanied by other cellular populations in the BM, as they facilitate the dynamic migration of HSCs and guarantee a homeostatic production of blood cells.

Due to their close integration within the circulatory system, HSCs are mostly supported by the vascular niche present along the sinusoidal endothelium and existent peripheral blood mononuclear cells (PBMCs). Apart from these cells, HSCs surrounding niche´ also include macrophages and MSCs (36, 37). Both end up influencing HSCs migration and maintenance through the production of mediators like E-selectin, Leptin, Nestin and granulocyte-macrophage colony-stimulating factors (GM-CSF) (38, 39), plus the release of important hematopoiesis cytokines such as stem cell factor (SCF), FLT-3, thrombopoietin (TPO), interleukin 7 (IL-7), leukaemia inhibitory factor (LIF), tumour necrosis factor-β (TNF-β), and interferon-γ (IFN-γ) (40, 41). Therefore, these cellular connections, whether established directly or indirectly, underscore their pivotal role within the HSCs-specialised microenvironment.

MSCs, in particular, have already shown their engagement in direct cell-to-cell contact to enhance HSC-self renewal, along with indirect communication supplied by growth factors, hormones, and other small molecules serving as key carriers to the development and

differentiation of HSCs (38, 42, 43). To date, clinical studies in phase I/II  have shown, for example, that co-culturing human UCB-derived CD34+ HSCs *ex-vivo* with MSCs boosted their expansion without compromising their stemness and greatly enhanced patient engraftment after transplantation (44, 45).  Considering GvHD, similar trials suggest that intravenous BM-MSC infusion is a safe treatment for severe steroid-resistant or refractory aGvHD, offering notable benefits, particularly in children and when administered early in the disease (46-48).

Even so, the HSCs milieu appears to be correlated with the efficacy of hematopoiesis; therefore, discovering the interconnections between these cell populations is a significant matter of research. Here, we opted for studying BM-MSCs, not only for their widespread scientific use and prevalence in clinical trials but also due to their HLA-independent activity, allowing for universal compatibility with any donor. This knowledge may further impact the generation of successful engraftable HSCs, disease models and drug development, yielding substantial potential for new therapeutic approaches.

### 1.5) Fostering immunomodulation via MSCs

Ongoing research into BM hematopoietic regeneration and its immune niche management will impact the function and integration capacity of transplanted HSCs. Notably, MSCs play a critical role in this niche, offering the opportunity to control its proliferation and thus overcome the existing hurdle (49).

Due to their known tri-potency to differentiate into osteoblasts, chondrocytes, and adipocytes, MSCs are the ancestors of mesenchymal lineages and may derive from several sources. Within them, the BM-MSCs are the most common and studied niche for HSCT in preclinical studies, followed by those from umbilical cord and adipose tissue (49, 50).

As represented in **Figure 1**, they are responsible for immunomodulation, supporting haematopoiesis, exerting stimulatory or inhibitory effects on other cell types and ongoing turnover of tissues as inherent regenerative promoters (51). More specifically, their plasticity allows them to be drawn to the inflammatory mediators continuously released from tumorous malignancies, leading to the aggregation of several immune cells in the microenvironment [52].

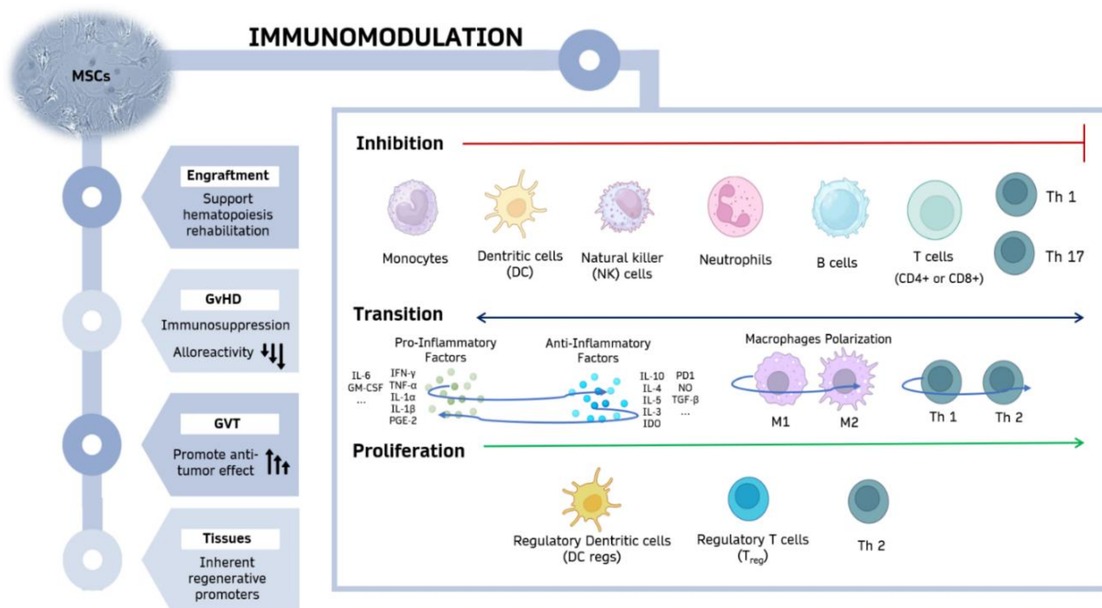


**Figure 1.** *Enhancing HSCT success via MSCs´ effects.* Abbreviations: GvHD, graft-versus-host disease; GvT, graft-versus-tumour; Th- T helper cells; IDO, indoleamine 2,3-dioxygenase; TGF-β, transforming growth factor-β; PD-1, programmed cell death protein 1; IL, interleukin; INF, interferon; PGE-2, prostaglandin E2; TNF-α, tumour necrosis factor α; NO, nitric oxide; GM-CSF, granulocyte-macrophage colony-stimulating factor. *Figure drawn on Adobe Illustrator.*

MSCs immunomodulation properties include exerting proliferative, inhibitive, and transitional effects on distinct immune cells and mediators through cell-to-cell contact or paracrine activity (**Figure 1**). In the realm of adaptative immunity, MSCs exert a profound suppressive effect on the proliferation of CD4+ or CD8+ T cell subsets, as shown in MLR assays, in a dose-sensitive way, while also inhibiting Th1/Th17 cells differentiation, through decreasing secretion of pro-inflammatory factors like IFN-γ, IL-6, IL-17 and promoting release of IL-10 and PGE2 (52). Furthermore, in inflammatory events, MSCs are known to prevent B cell proliferation, maturation and antibody secretion via the PD-1/PD-L1 pathway and partly by PGE2 (53). Particularly, this was already shown in mouse GvHD models (54, 55). All these factors end up hindering the proliferation and cytotoxicity of natural killer (NK) cells while enabling the induction of T regs CD4+/CD25+ cells´ proliferation and promoting a shift

from Th1 to Th2 cells by increasing the output of factors like IL-4, IL-10, PD-1 ligand, which is partially dependent on the IDO pathway (52).

Innate immunity is also greatly impacted by this cytokine profile shift from pro- to anti-inflammatory. For once, stimulated MSCs induce macrophage polarization due to the release of PGE2, IDO IL-23 and IL-22, favouring the formation of anti-inflammatory M2 macrophages over pro-inflammatory M1-like macrophages (56, 57). Besides, MSCs also prevent monocytes´ differentiation into dendritic cells (DCs), by stopping their cell cycle through cyclin D2 with continuous release of IL-10, IL-6 and TGF-β (58, 59). Regarding neutrophils, *in-vitro* studies have shown that MSCs may be able to control the degree of respiratory burst in activated neutrophils by reducing the generation of hydrogen peroxide and, therefore, inhibit their main response during inflammatory activation (60).

Other abilities include promotion of neovascularization, enhancement of cell viability and immunomodulation via extracellular vesicles making MSCs valuable candidates for diverse innate and adaptative immune system disorders (61, 62). Several pre-clinical attempts, both *in-vitro* and *in-vivo* have been using viable MSCs, either allogeneic or autologous, to target these immune-mediated disorders, encompassing GvHD (11, 12).

Considering it all, MSCs end up having an impact on alloreactivity, mostly due to the reduction of alloreactive donor T-cells. Recently, P. Wuttisarnwattana et al. (63) have shown this effect in *in-vivo* mouse GvHD models through CFSE dilution assays.

Nevertheless, regulating the reduction of alloreactivity via immunosuppressive effects must balance between avoiding immunological consequences while still effectively addressing the hematooncological disorder through GvT. Consequently, various studies advocate for achieving a harmonious equilibrium between these opposing forces through immune crosstalk, facilitated by MSCs (11). Moreover, the vast and varied human alloreactive cell repertoire poses a significant technological challenge in assessing the potency of MSCs to regulate alloreactivity to a beneficial level.

Overall, while MSCs exhibit promising therapeutic capabilities in orchestrating immune and alloreactive control, continued refinement, and careful consideration of multiple factors in preparing MSCs are still urgently required.

### 1.6) Key considerations in preparing BM-MSCs for cellular therapies

MSCs or MSCs-like cells can be isolated from almost any tissue of the human body, being accessibility, invasiveness of procedure and repair efficacy, factors to be considered when choosing the source (64). Considering this, our choice of using BM-MSCs from typically discarded BM collection bags offers dual benefits: it enhances environmental sustainability by curbing medical waste, while also fostering cost and scientific efficiency through resource conservation.

Not only that, but the diverse origin sources make MSCs a heterogenous population with known distinct characteristics, differentiation potential and levels of paracrine factors stimulation that also need to be adequate for the cellular therapy applied. Therefore, if these elements are further explored, along with manufacturing practices (65), this BM-MSCs source could simplify access to MSCs for clinical researchers to apply in future immunotherapeutic-specific projects.

A pro-inflammatory state in which inflammatory cytokines affect the proliferation of normal and mutant cells is a hallmark of hematologic malignancies that must be considered. Inflammation is a crucial catalyst in reshaping the neoplastic microenvironment, (3), by not only impacting haematopoiesis and alloreactivity but also by making BM-MSCs susceptible to phenotype divergence (61, 66, 67). To tackle this issue, we also proposed to evaluate occurring MSCs phenotype variation before and after each experiment performed, according to The Mesenchymal and Tissue Stem Cell Committee of the ISCT (International Society for Cell Therapy), as represented in **Table 1**. Specifically, they propose a set of guidelines to define multipotent human MSCs with regenerative potential for both scientific purposes and pre-clinical studies (68).

**Table 1.** *Standards to identify multipotent human MSCs, according to the ISCT (68)*

<table>
<tr><td rowspan="4">STANDARD PARAMETERS</td><td colspan="3">1. Adherence to plastic in standard culture conditions</td></tr>
<tr><td rowspan="2">2. Phenotype</td><td>Positive (≥ 95%)</td><td>Negative (≤ 5%)</td></tr>
<tr><td>CD90<br>CD105<br>CD73</td><td>CD45<br>CD34<br>CD14 or CD11b<br>CD19 or CD79α<br>HLA-DR</td></tr>
<tr><td colspan="3">3. In vitro multipotent differentiation: osteoblasts, adipocytes, chondroblasts (shown by staining of in vitro culture)</td></tr>
</table>

Other markers are also expressed, including Stro-1, CD9, CD10, CD13, CD29, CD44, CD49, CD51, CD54 (ICAM-1), CD117 (c-kit), CD146 (MCAM), CD166 (ALCAM), and CD61 (69), but particular combinations of them appear to be host tissue (70) or type of culture-dependent (71-73).

Prior analysis of the retained BM-MSCs´ phenotype has already pinpointed the successful establishment of prolonged cultures with adequate phenotypes. Additionally, in vitro assays demonstrated multipotent differentiation into adipocytes (74), which yield promising results for applying these cells. Yet, genetic mutations and chromosomal imbalance can also appear by prolonged in-vitro cultivation or by introducing modified MSCs (75, 76), raising the possibility that MSCs can turn carcinogenic and, therefore, calls for more regulatory attention when preparing them.

Despite these precautions, BM-MSCs are considerably easy to isolate, culture, and expand *in-vitro* (77-79), and adapt their immunomodulation effects in response to the microenvironment stimuli (80-82). Being non-hematopoietic cells, also means their function is HLA-independent (83, 84), which makes every BM-MSCs donor universal for not eliciting an immunological response and prospering with a safer infusion profile. For instance, they have already demonstrated verified safety and feasibility with beneficial effects in clinical trials mostly of phase I/II or III, whereas some have also shown placebo effects (85). The latter makes it harder to demonstrate effectiveness, although safety remains.

Selecting an appropriate basal medium, culture conditions and donor age, also allows the maintenance of inherent traits and multipotency of mesenchymal stem cells (MSCs) even during prolonged *in-vitro* culture (86). Given the rising importance of MSCs, it also becomes

crucial to produce an adequate dose of graftable MSCs (49, 87) that can consistently sustain these attributes through several passages to maximize therapeutic potential.

After that, the knowledge obtained may be applied to upscale MSC production, ensuring an ample sustainable supply for cellular therapies and, in due course, for a post-transplant therapeutic approach.

## 1.7) Project aims

Within the context presented, this study focused on evaluating the ability of BM-MSCs retained in collection bags to modulate the proliferation of PBMCs and suppress alloreactivity *in-vitro*. The goal was to demonstrate the immunomodulatory capacity of these BM-MSCs, which would be crucial for improving post-transplantation patient outcomes and mitigating chronic complications. Furthermore, another aim was to explore the interactions between BM-MSCs and PBMCs to understand the underlying mechanisms of BM-MSC-mediated immunomodulation, paving the way for innovative therapeutic strategies using this sustainable and efficient BM-MSCs source.

Specifically, the aims of this study were:

1. To evaluate BM-MSCs' immunomodulatory effects on PBMCs´ proliferation in-vitro, through two-way mixed lymphocyte reactions;
2. To characterize occurring immunophenotypic changes in PBMCs and MSCs surface by flow cytometry;
3. To elucidate mechanisms of BM-MSCs mediated immunomodulation;
4. To demonstrate the feasibility and practicality of repurposing BM-MSCs from discarded bone marrow collection bags for research studies.

# 2. MATERIALS & METHODS

## 2.1) Samples' characterisation

After obtaining ethical committee and individual consent, BM grafts were harvested from 3 healthy adult donors (HD) (median age 29 years), following specific guidelines, and intended for allogeneic HSCT at Instituto Português de Oncologia–Porto (IPO-Porto). After filtering and distribution, each collection bag/filter was rendered anonymous and transported to the laboratory for isolation of mononuclear cells (MNCs). PBMCs were obtained from 12 anonymous healthy non-mobilized donor blood samples collected in the IPO-Porto blood collection centre, 4 needed for each assay performed.

## 2.2) MNCs Isolation

### 2.2.1. Isolation and in-vitro expansion of BM-derived MSCs

After distribution and filtering were finished, collection bags/filters were brought to the lab for isolation by density gradient (74). Firstly, they were washed in 30mL RPMI 1640 (Thermo Fisher Scientific) under aseptic conditions and stirred. Then the 30mL cell suspension obtained was slowly layered on top of 20mL of Lymphoprep ™ (LOT 00219, SerumWerk) and centrifuged for 20 min at 1600 rpm. The mononuclear cells (MNCs) fraction was carefully aspirated with a pipette to a new 15 mL conical tube, topped with RPMI, and centrifuged for 10 minutes at 1100 rpm. The resulting pellet was resuspended in a fresh medium for viability and enumeration. Cells were then plated, accordingly, in T75 or 6-well culture flasks at a seeding density of 160000/cm$^2$ (88) in DMEM-low glucose (DMEM-LG) culture medium supplemented with 10% fetal bovine serum (FBS) and 1% Pen-Strep (10,000 U/mL-10,000 μg/mL, 15140122, Gibco). Every 2 days, non-adherent cells were removed by changing the culture medium and the overall morphology progression or contaminants presence was checked. MSCs were harvested when they reached ≥80% confluence, using the dissociation reagent TrypLE™ Express (REF 12604-013; LOT 1868905, ThermoFisher) standard protocol. When compared to the use of trypsin, the TrypLE enzyme´s animal-free origin eliminates the potential of pathogenic contamination hazards, plus it is designed to be RT-stable and remarkably gentle on cells. Centrifugation was performed at 1100 rpm for 10 minutes. In parallel, as a quality test procedure, 1 mL of the filtered BM was also taken to

evaluate its clonogenic proliferation, which also ascertains how many MNCs can be isolated per mL of BM aspirate collected.

### 2.2.2. Isolation of PBMCs

Using aseptic techniques, carefully transferred anticoagulant-treated blood from each blood collection tube into 50 mL sterile conical tubes. This was followed by diluting the blood with an equal volume of RPMI 1640 (1:1 dilution) and adding 5 mL of Lymphoprep ™ to a fresh 50 mL conical tube. Gently layered approximately 15 mL of diluted blood over the Lymphoprep ™ layer by positioning the pipette tip against the conical tube wall and dispensed the first 5 mL of blood dropwise to ensure a gradual introduction and prevent disruption of the separation medium and blood interface. Then centrifugation at 1600 rpm at RT for 20 minutes with the brake off to avoid disrupting the density gradient during deceleration, followed by meticulously gathering the peripheral PBMC layer using a sterile Pasteur pipette with caution to prevent excessive aspiration of Lymphoprep medium, as it could be toxic to the cells. Finally, the isolated PBMCs were transferred to a new 50 mL conical tube and resuspended with fresh medium until further use.

## 2.3) Cell viability & concentration

Each cell suspension concentration was determined through hemograms with a Sysmex XN-450™. Intrinsic cell type variability (%) was also observed. As for viability, Trypan Blue (Sigma-Aldrich, USA) and an equivalent volume of cell suspensions were combined in a hemacytometer, and under a microscope, viable and nonviable cells were counted. The ratio of viable to total cells was used to assess cellular viability.

## 2.4) Establishment of co-culture experiments between MSCs and PBMCs

For each experiment, 1 cohort of MSCs obtained was previously cultured in 6 well-plates using the culture medium described in **2.2.1.** An MSCs-filled culture flask was kept separately as fresh cell control to be used for adequate prior phenotype evaluation. The cultures were maintained in the incubator at 37ºC, 5% $CO_2$ and medium exchange was performed every 2-

3 days, up to 2 weeks. When reached 80% confluence, the co-culture experiments were performed.

### 2.4.1. Mixed lymphocyte reaction (MLR) assays

The two-way MLR assay is a functional rather than a phenotypic assay useful for evaluating T cell alloreactivity, measuring their ability to recognise and respond to foreign antigens. In this case, it was aimed to evaluate the inhibitory effect of MSCs on T-cell proliferation and other immune cells (89). On day 0, MSCs were washed, re-fed with fresh culture medium and incubated with or without freshly isolated PBMCs, either irradiated (stimulator cells, S) or not (responder cells, R). In conditions with both sets of PBMCs, they were cultured within a PBMCs responder to stimulator cells ratio of 2:1 to mimic an alloreactive condition. Stimulator cells were irradiated in a Gammacell ® 3000 Elan irradiator for 8min at 20Gy, while responder cells were labelled with the green, fluorescent dye CFSE (CellTrace™, Thermo Fisher Scientific), which stains their cytoplasm. The different conditions established allowed the evaluation of alloreactivity and immunomodulatory properties between each group of cells. Each pool of donor MSCs was either co-cultured with only responder cells (R MSCs) or with responder and stimulator cells combined (RS MSCs). Stimulator (S), responder (R), and combined PBMCs (RS) were cultured separately, in the absence of MSCs. Performed in duplicate, the positive control consisted of RS, and the negative controls consisted of R, S and MSCs cultured individually. Experimental design is shown in **Figure 2**.

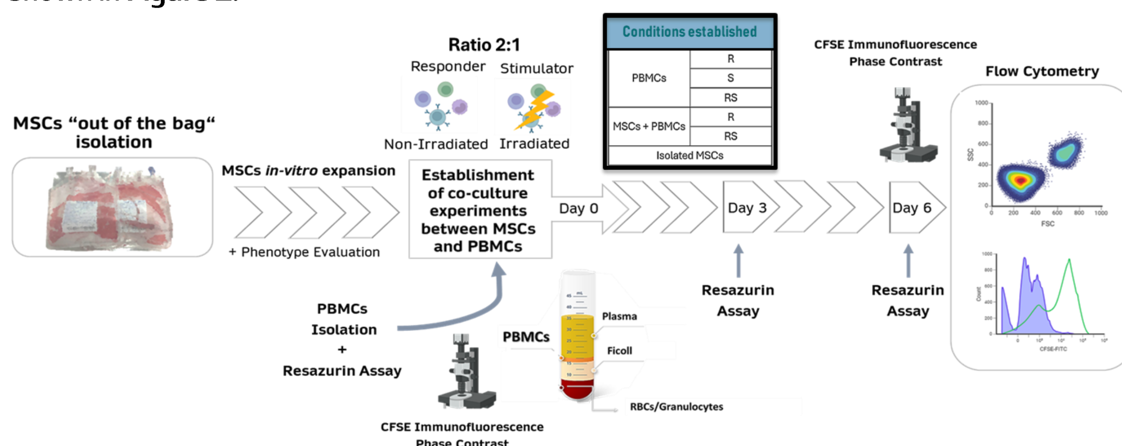


**Figure 2.** *Experimental design, including conditions established for the co-culture experiments performed.* The microscope represents both immunofluorescence and phase-contrast images obtained on day 0 and 6. Flow cytometry analysis was performed on the last day of the experiment. R, Responder cells; S, Stimulator cells.

## 2.5) Evaluation of co-culture experiments between MSCs and PBMCs

### 2.5.1. BM-MSCs phenotype evaluation

The immunophenotype of MSCs was assessed by flow cytometry before co-culture and on the last day of each co-culture experiment to evaluate any occurring phenotypic changes. Fluorochrome-conjugated monoclonal antibodies (CD45 FITC, CD34 PE, CD3 APC, HLA-DR PE, CD14 FITC, CD11-b APC, CD90 APC, CD73 PE, CD105 FITC and CD44 APC) were used to stain a 100 µl detached MSCs sample. Proceed by incubation for 15 minutes at RT in the dark and then cells were resuspended in FACSFlow to be examined with a FACSCanto II flow cytometer. MSCs were recognized by expressing less than 5% of CD14, HLA-DR surface molecule, CD45, CD34, CD3, CD11-b, CD44 and with positive expression up to 95% for CD105, CD73 and CD90, as recommended by the Mesenchymal and Tissue Stem Cell Committee of the ISCT (**Table 1**).

### 2.5.2. Cell proliferation by CFSE labelling using flow cytometry

By detecting and quantifying a fluorescent label that is equally distributed between two daughter cells after cell division, cell proliferation was followed for 6 days using the CellTrace™ CFSE Proliferation Kit. Flow cytometry analysis reveals a bright, homogeneous fluorescent signal from the initial population of cells and, since subsequent cell divisions result in larger numbers of cells, each analysis showed half the fluorescence intensity of its parent cell.

Firstly, the CellTrace™ stock solution was prepared by adding the appropriate amount of DMSO to one vial of CellTrace™ reagent and mixing thoroughly. Then, 1uL of the CellTrace™ stock solution in DMSO was added to every mL of cell suspension in PBS to create the final working solution, followed by incubating the cells for 20 minutes at 37ºC and shielding them from light. After incubation, the initial staining volume was introduced 5 times to the cells (with 1% albumin protein supplement) and it sat for 5 minutes to remove any residual free dye in the solution. Proceed by centrifuging the cells at 1100 rpm for 10 minutes and resuspending them after in a fresh pre-warmed culture medium. A second incubation was performed for a minimum of 10 minutes to ensure that the CellTrace™ reagent underwent acetate hydrolysis. Finally, flow cytometry analysis was carried out on the last day of culture on a BD FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ) using BDFACSDiva software and

further analysed with FlowJo 10.1 (FlowJo, LLC). Started by employing a specific FSC-A/SSC-A gate to only capture the population of responder cells. When feasible, 10000 events were recorded and the dilution between CD45+/CFSE+ signal with decreased signal intensity determined their proliferation.

### 2.5.3. Cell metabolic activity: Resazurin assay

A resazurin assay was performed to monitor cell metabolic activity throughout days 0, 3 and 6 of each condition. Since metabolically active cells can reduce, via mitochondrial reductase, the non-fluorescent dye resazurin to the fluorescent dye resorufin, the fluorescence measurement proportionally indicates the number of metabolic active cells present in each well. A solution of culture medium with 10% resazurin was added to each well seeded with 5000 PBMCs from all conditions. The culture plate was incubated for 3 hours at 37ºC, 5% $CO_2$, and protected from direct light with aluminium foil. Then, 100µL of each well sample was pipetted into a black 96-well plate (437111, Thermo Scientific™). Medium with 10% resazurin was used as blank. Each condition was read in triplicate and its fluorescence quantified in the FLUOstar® Omega microplate reader (BMG LABTECH) using a fluorescence excitation wavelength of 530nm and an emission of 590nm, gain 35, at room temperature (RT) and after 3 seconds of shaking. The data obtained was then processed through MARS software and exported to Excel.

### 2.5.4. Immunophenotype and cell sorting

The assessment of immunophenotypes allowed the identification, characterisation, and quantification of the distinct subpopulations of immune cells within the single-cell suspension of each condition, using multiparameter panels and specific gate strategies in flow cytometry. PBMC were tested for surface expression of markers CD45+ (all PBMCs), CD3+ (T cells), CD4+(T helper cells), CD8+(Cytotoxic T cells), CD19+ (B cells), CD56+ (NK cells), CD14+ (Monocytes) and PD1+(Programmed cell death protein 1). Particularly, PD-1, also referred to as CD279, was included because it's a surface receptor closely linked with activated T cells (90). Its role in suppressing immune responses and maintaining immune tolerance enabled us to assess the nature of immune reactions in each experimental condition. **Table 5** in **Annex I** provides a comprehensive description of the experimental setup for flow cytometry.

After initial gating of lymphocytes alongside their physical parameters of FSC/SSC, a gate for CD45+/CFSE+ population (responder cells) was determined. Subsequent steps include gating for single CD3+ cells, followed by distinguishing CD4+ T cells and CD8+ T cells. A similar discernment strategy was used for CD56+, NK cells, and CD19+, B cells. Besides, CD3+ cells were also distinguished for their expression of PD1+. As for monocytes, they were identified by including CD14+ setting gates after FSC/SSC refinement.

### 2.6) Microscopy and image analysis

Microscopy images were obtained using an inverted microscope (Olympus IX51), in both bright-field and fluorescence filters, and processed through cellSens software. As to immunofluorescence images, the fluorescence filter used was FITC to identify cells stained with CFSE.

### 2.7) Statistical analysis

All experiments were performed in triplicate and data is presented as mean ± standard deviation. Data collection, aggregation and analysis were performed with Excel 2016 (Microsoft Office), Prism 9 (GraphPad Software, Inc.) and FlowJo 10.6.2 (BD Biosciences). Statistical significance was assessed using the One Way or Two-way NOVA to compare conditions and experiments, with p-values below 0.05 considered as statistically significant.

# 3. RESULTS

## 3.1) Visual Insights: Interactions between BM-MSCs and PBMCs

### 3.1.1. Phase Contrast: Physical Interactions

To explore the physical interactions between PBMCs before and after co-culture with MSCs, phase contrast images taken on days 0 and 6 were analysed (**Figure 3**).

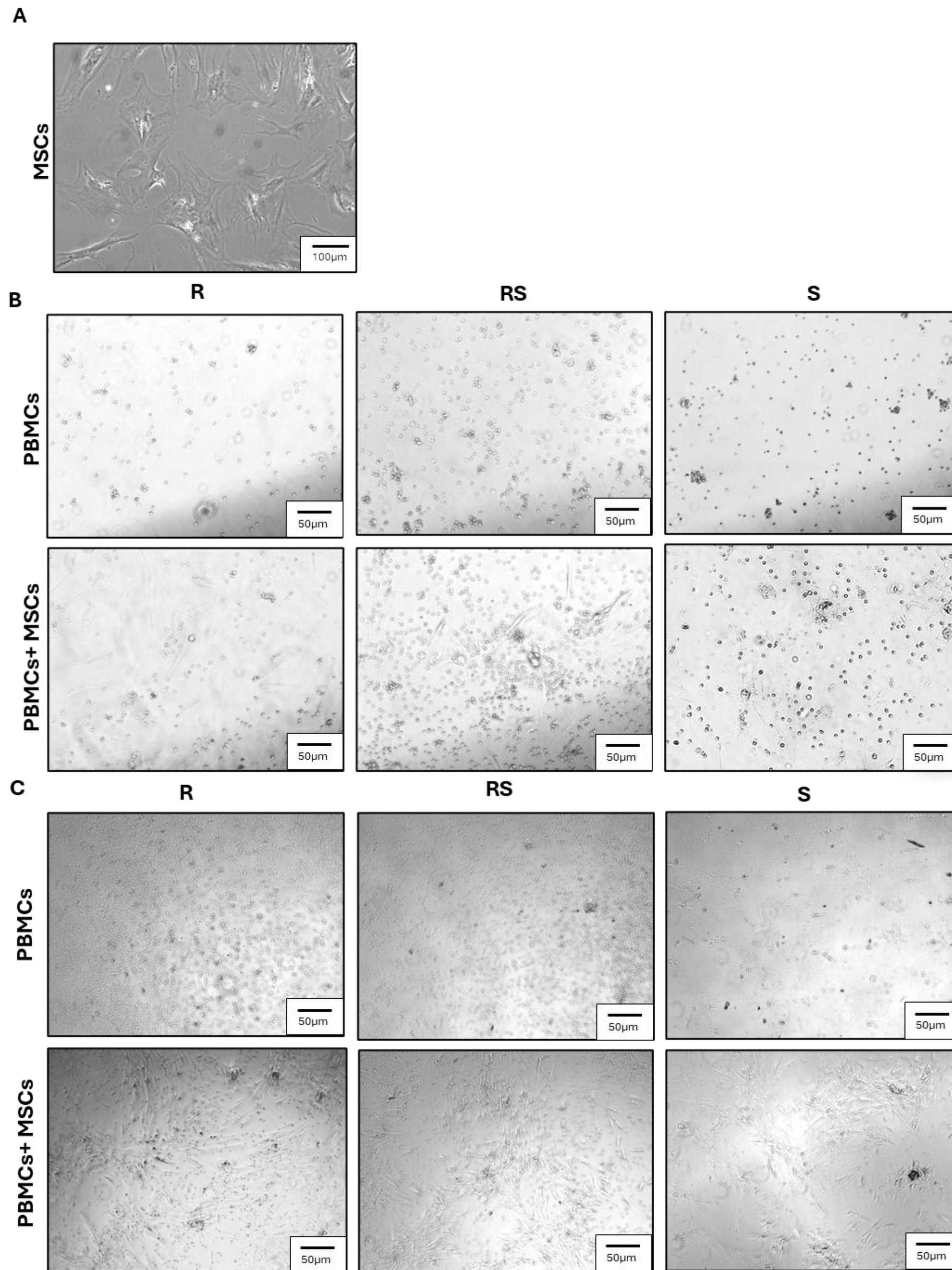


**Figure 3.** *Phase contrast images showing direct physical interactions between PBMCs and MSCs across all conditions, from day 0 and day 6.* **(A)** MSCs fresh control morphology. Scale bar = 100µm (40X). **(B)** All conditions on day 0 **(C)** All conditions on day 6. Scale bar = 50µm (20X).

The initial morphology of fresh MSCs, depicted in **Figure 3A**, revealed spindle-shaped cells adherent to plastic, resembling fibroblasts. In contrast, PBMCs display a small and round format, with a single, large nucleus that occupies most of the cell's volume. Before (**Figure 3B**) and after co-culture (**Figure 3C**), irradiated PBMCs (S) exhibited more fragments of dense nuclear chromatin compared to non-irradiated PBMCs (R), regardless of the condition. In isolated conditions, PBMCs appeared widely spaced, but upon co-culture with MSCs, they displayed a closer position with the latter cells. The total counts of PBMCs and MSCs appeared to substantially increase until the final day in most conditions, except for those involving irradiated PBMCs, where their presence was notably scarce by day 6. When comparing isolated PBMC conditions with PBMCs, either R or S, in co-culture with MSCs on day 6, a slight decrease in the number of PBMCs is suggested. In the same conditions, MSCs confluency increased, retaining a morphology and plastic adherence similar to the fresh control's.

### 3.1.2. Immunofluorescence expression of CFSE

To further investigate PBMCs proliferation, migration, and positioning, plus validate that only responder cells were labelled for the two-way MLR assay, CFSE immunofluorescence expression was inspected for all conditions (**Figure 4**).

CFSE dye forms covalent bonds with all available surface and intracellular free amines, exhibiting minimal cytotoxicity while effectively tracking proliferative activity and maintaining a consistent signal emission activity.

Negative controls, which are conditions with irradiated cells, co-cultured or not with MSCs, exhibit no CFSE signal on both days 0 and 6. However, CFSE expression is evident in the remaining conditions, with its distribution aligning exclusively with the PBMC format. Compared to day 0 (**Figure 4A**), all conditions display increased CFSE expression on day 6 (**Figure 4B**). Moreover, when contrasting the alloreactive PBMC condition with the co-cultured one on the final day, the condition involving MSCs notably shows fewer PBMCs stained with CFSE, in contrast to the observations from day 0.

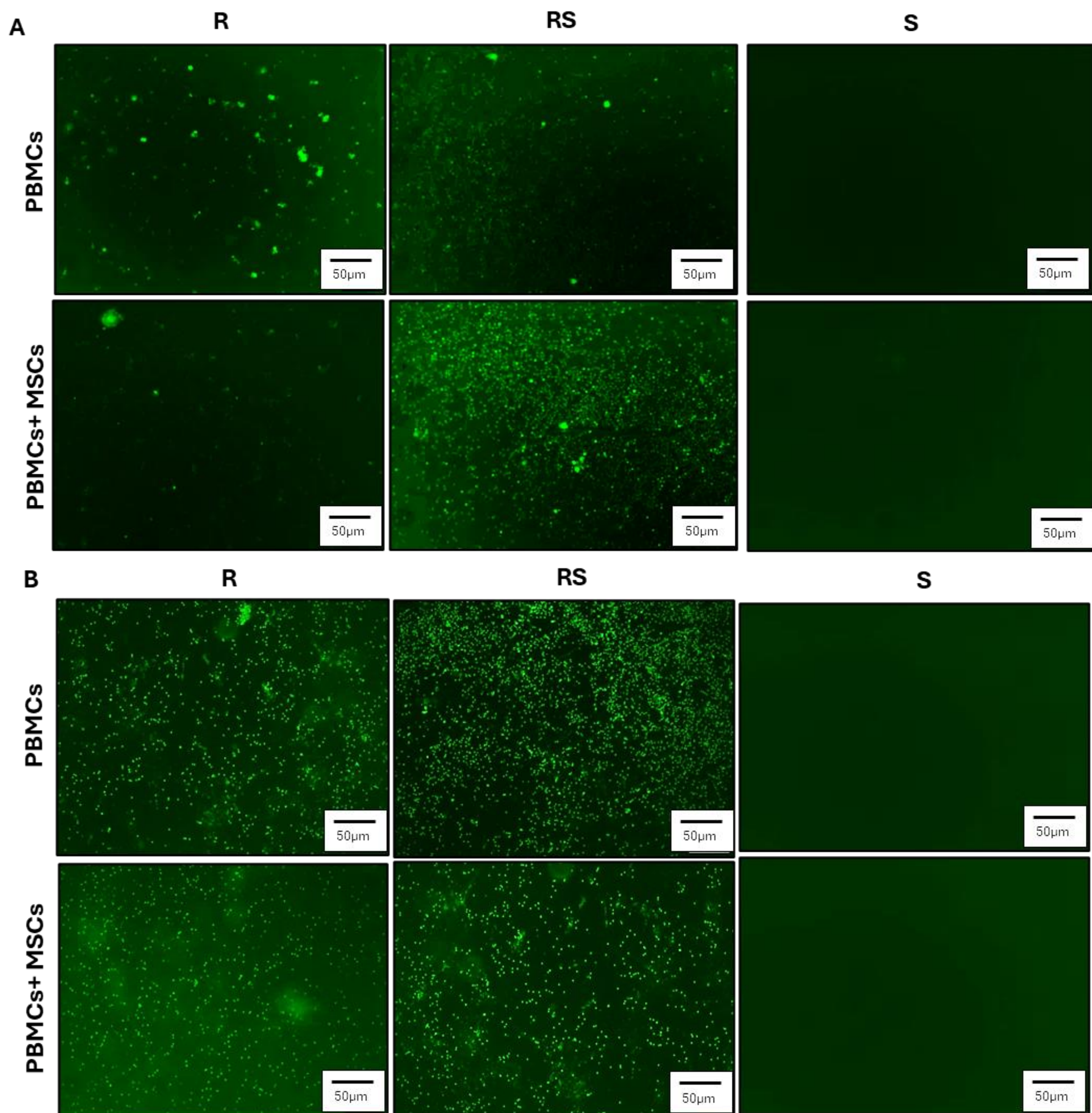


**Figure 4.** *Immunofluorescence images from the CFSE FITC (green) expression in the proliferation assay across all conditions.* **(A)** All conditions on day 0. **(B)** All conditions on day 6. Scale bar = 50μm (20X).

### 3.2) Interaction Analysis: Decoding Immunomodulation

#### 3.2.1. BM-MSCs Phenotype Evaluation

More than 80 % of the cells were considered MSCs; Contaminating cells were defined as expressing more than 5% of the hematological markers CD34, CD3, HLA-DR, and CD45. **Table 2** displays the average percentage of positive cells for each marker, while **Figure 5** shows the phenotypic profile depicted as a scatter plot.

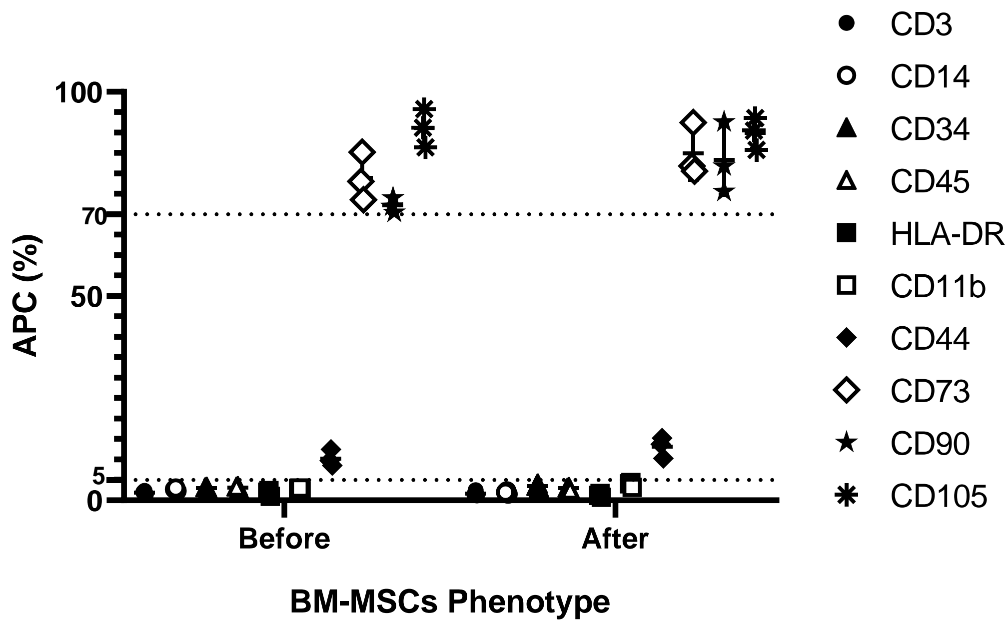


**Figure 5**. *Surface phenotypic profile of BM-MSCs assessed by flow cytometry, before experiments (passage 4) and after co-culture within the alloreactive condition RS MSCs.* The profile is depicted as a scatter plot of a panel of 9 ISCT MSC-related markers and 1 additional MSCs-related marker, CD44, featuring the mean and SD of all BM-MSCs phenotypes (n=3).

**Table 2.** *Immunophenotype of BM-MSCs obtained, for each surface antigen with standard deviation, before and after co-culture.*

| Surface antigen | BEFORE / (APC ± SD) % | AFTER / (APC ± SD) % |
|---|---|---|
| CD3 | 2.31 ± 0.28 | 1.46 ± 0.57 |
| CD14 | 2.69 ± 0.49 | 2.30 ± 0.55 |
| CD34 | 3.13 ± 0.59 | 2.41 ± 0.49 |
| CD45 | 3.46 ± 0.68 | 3.22 ± 0.95 |
| HLA-DR | 2.38 ± 0.98 | 1.69 ± 0.19 |
| CD11-b | 2.35 ± 0.57 | 4.39 ± 0.39 |
| CD44 | 9.72 ± 0.80 | 13.77± 1.95 |
| CD73 | 78.10 ± 5.23 | 72.01 ± 1.51 |
| CD90 | 94.93 ± 1.74 | 81.86 ± 7.52 |
| CD105 | 91.23 ± 3.42 | 85.77 ± 3.75 |

APC: Average Positive Cells; SD: Standard Deviation; Green, positive expression ≥ 70%; Orange, intermediate expression] 5%, 70% [; Red, negative expression ≤5%

The after-co-culture results consider MSCs phenotype upon the RS MSCs condition. While conventional metrics suggest a positivity range of up to 95%, the results indicated a distinct shift. Therefore, within this dataset, positive expression was adjusted to above 70%.

Considering this, in both instances, BM-MSCs displayed similar expression levels of CD markers, with no statistically significant differences observed. Analysis revealed minimal expression levels (<5%) of CD34, CD45, CD3, HLA-DR, CD14 and CD11-b, along substantial positive expression levels (>70%) of CD73, CD90 and CD105. CD44 was the only CD marker showing an average intermediate expression of (9.72%± 0.80) % and (13.77± 1.95) %, before and after co-culturing, respectively.

### 3.2.2. Establishment of an in-vitro co-culturing model of BM-MSCs with PBMCs

The isolated blood samples, as described, were first analysed through complete blood counts (CBC) to highlight their intrinsic variability related to red blood cells, leucocytes, and platelets composition. Afterwards, the resulting isolated PBMCs were either selected to be irradiated (stimulator cells, depicting the patient's cells' behaviour) or not (responder cells, depicting donor alloreactive cells after transplant). The average of their recovery from MNCs and viability is summarized in **Table 3**.

**Table 3.** *Average of MNC recovery and viability from blood donors (n=12), with standard deviation.*

| Sample | Recovered MNC x $10^6$ ± SD | Recovered MNC viability (%) ± SD |
|---|---|---|
| **Blood** | 1.06 ± 0.64 | 97.64 ± 0,78 |

MNC, Mononuclear cells; SD, Standard Deviation

For each CFSE assay, MSCs were obtained from one donor, and cultured for up to 2 weeks till 80% well confluence, after which different conditions for 6 days were induced: S, R, RS, R MSCs and RS MSCs. BM´s sample characterisation is shown in **Table 4**.

**Table 4**. *Characteristics of BM samples (n=3), MNC recovery and viability.*

| Sample | Recovered MNC x $10^6$ from collection bag | Recovered MNC viability (%) | CFU-GM x$10^5$ / Total |
|---|---|---|---|
| **BM1** | 1.42 | 89 | 858,4 |
| **BM2** | 1.33 | 94 | >500 |
| **BM3** | 1.51 | 91 | >500 |

MNC, Mononuclear cells; BM, Bone Marrow. F, Female; M, Male; CFU-GM, Colony forming unit's granulocyte-macrophage.

According to established product quality control criteria, cellular viability was adequate since, in all cases, the recovered MNC had a viability higher than 80%. As for the BM samples, they all fall into the expected range values of total CFU-GM, over 500 colonies per $10^5$.

Cell metabolic activity was assayed at day 0, before co-culture, and on days 3 and 6, during co-culture conditions, for all non-adherent cells (PBMCs) with resazurin assays, respectively (**Figure 6**).

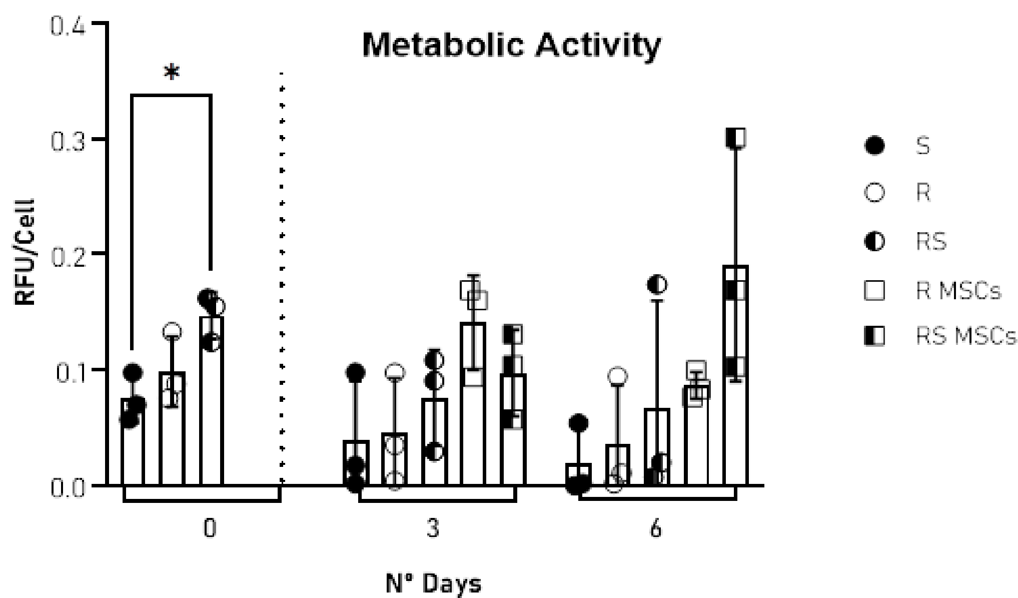


**Figure 6**. *Cell metabolic activity of PBMCs across the co-culture conditions created.* Data is shown as mean ± standard deviation, n=3 (day 0), n=5 (days 3 and 6) of relative units of resazurin fluorescence per cell measured with resazurin assays throughout days 0, 3 and 6, representing cell metabolism. Day 0 represents baseline values of the isolated PBMCs metabolism before the co-culture conditions with MSCs; *p-value < 0,05

Cell metabolic activity was assayed at day 0, before co-culture, and on days 3 and 6, during co-culture conditions, for all non-adherent cells (PBMCs) with resazurin assays, respectively. The results are presented after normalization with a 5000 cells/well concentration. While metabolic activity, shown in **Figure 6**, showed no statistically significant differences between most conditions, a slight significance emerged over time ($p$ = 0,04). Before co-culture establishment, responder cells (R) showed higher metabolic activity than stimulator cells (S). However, upon combining both cell types, RS, there was a notable and statistically significant increase in metabolic activity ($p$ = 0,03). By day 3, the cell´s metabolism decreased in these conditions, although no major differences were observed between them and the ones with MSCs (RS MSCs and R MSCs). As for PBMCs' co-culture with MSCs, the R MSCs showed higher metabolism than the alloreactive ones, RS MSCs. On the last day, the cell´s metabolism continued to decrease in most conditions except in the RS MSCs where metabolism slightly increased, ultimately emerging as the highest value of all.

### 3.2.3. PBMCs Proliferation and Immunophenotyping Insights

The immune system's response to foreign cells and antigens is critical in immunological research, with two-way MLRs being a key method for studying these interactions. **Figure 7** showcases the results obtained for the proliferation and phenotypic characterization of PBMCs, highlighting the impact of BM-MSCs on all conditions.

The proliferation of PBMCs was assessed using CFSE histograms in flow cytometry obtained from the last day of the two-way MLRs performed. The CFSE histograms demonstrate the extent of cell division, with distinct peaks corresponding to the rounds of proliferation.

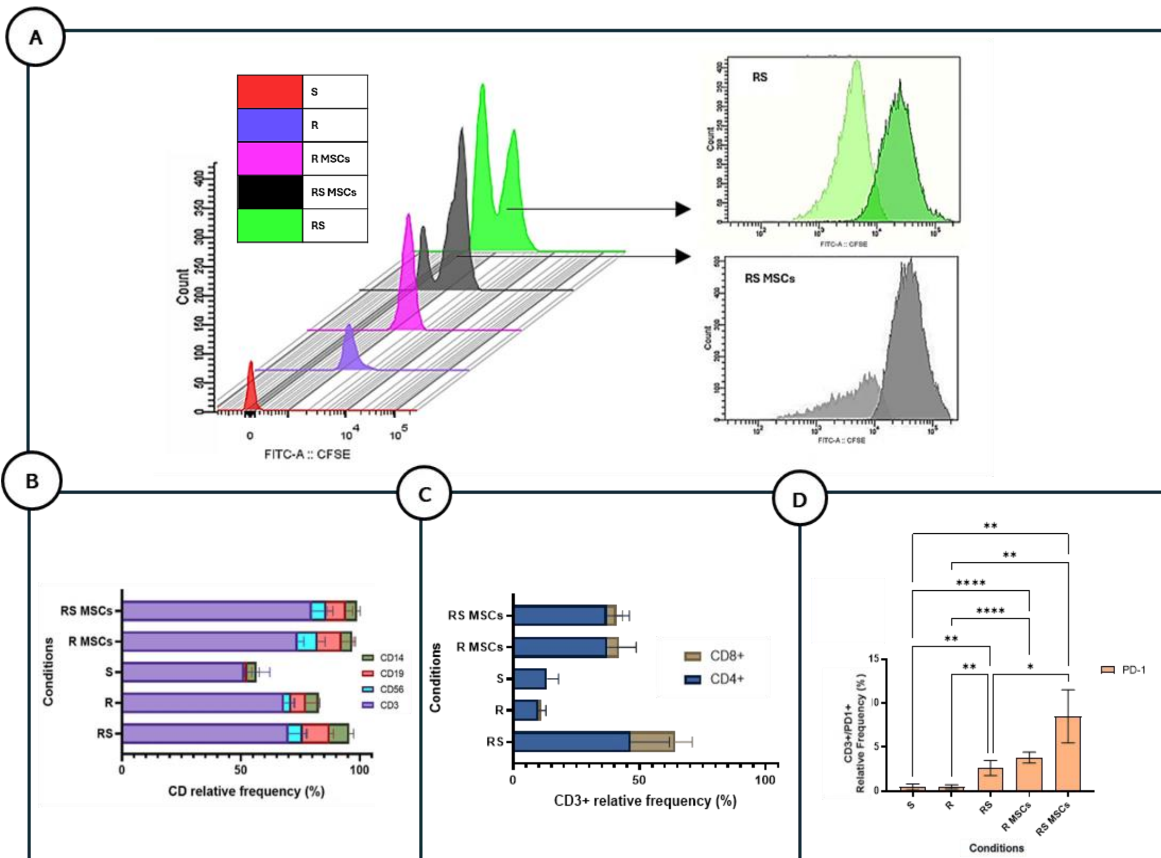


***Figure 7**. PBMCs proliferation and immunophenotyping* **(A)** Representative CFSE histograms obtained from the two-way MLR assays, showcasing PBMCs proliferation of all conditions, with further emphasis on histograms of RS and RS MSCs conditions. Intense green and grey represent nonproliferating cells from the first generation. ***Decrease in PBMC proliferation by 18% in the presence of MSCs.*** **(B)** Relative frequency of T cells (CD3+), monocytes (CD14+), NK (CD56+) and B (CD19+) cells of each condition among responder cells CFSE+/CD45+. S condition was the exception with a specific CFSE-/CD45+ gate used to analyse these populations. ***T cells monopolize immune responses.*** **(C)** Relative frequency of CD4+ and CD8+ T cell subsets. Bar charts indicate the mean relative frequency of each population(%)*± SD.* ***CD8+ were greatly affected by MSCs presence*** **(D)** Relative frequency of PD-1 surface expression on T cells*p<0.05,** p<0.01,**** p<0.0001. ***Increased PD-1 expression in RS MSCs indicates reduced immune activation by MSCs.***

Starting with the negative controls, S and R, both display a single minor peak with null or high CFSE fluorescence intensity, respectively. Similarly, the R MSCs conditions showcase a singular peak with high CFSE intensity, but with a higher count of cells. As for the positive control, RS, the second peak, the proliferative one, is higher compared to the first one. Contrarily, the RS MSCs condition shows a significantly pronounced reduction in the proliferative peak compared to the first one, although the majority of the cells remained with high fluorescence intensity. Both histograms from RS and RS MSCs were emphasized on the right side of **Figure 7A**. When comparing the proportion of proliferating responder cells, an average of 72% in the absence versus 54% in the presence of MSCs was found, indicating about 18% reduction in proliferation.

**Figure 7B** and **Figure 7C** illustrate the relative frequencies of distinct PBMC populations across conditions, obtained on the last day of culture using flow cytometry. Notably, we assessed populations using different gating strategies for conditions with responder cells CFSE+/CD45+, while adapting a CFSE-/CD45+ gate for the condition with only stimulator cells, S . **Figure 7B** shows that T cells (CD3+) constituted the predominant population in most conditions, comprising more than half of the immune cell milieu, with no major differences between them. The S condition exhibited the lowest proportion of T cells, albeit still close to 50%. Most significant variations were observed in the frequencies of monocytes (CD14+), B cells (CD19+) and NK cells (CD56+). Specifically, when comparing RS with R, NK cells, monocytes, and B cells proportions are marginally higher by approximately 2%, 3%, and 6%, respectively. As for the S condition, B cells and NK cells were barely present, while monocytes accounted for only 4% of the overall population. In both conditions involving MSCs, the CD3+ population remained relatively stable compared to RS. However, a significant difference was noted in monocytes and B cells. RS MSCs demonstrated a slight decrease in these populations by approximately 4% and 3%, respectively, when compared to RS.

Since T lymphocytes monopolised most of the immune milieu, we also directed our focus to populations within a CD45+/CD3+ gate, aiming to evaluate the distribution of T cell subsets, CD4+ and CD8+, in each condition **(Figure 7C).** Double-positive and double-negative populations for these subsets were not considered for evaluation. Across all conditions, CD4+ (helper T cells) emerged as the predominant subset. Notably, both the S and R conditions, similarly, exhibited the lowest proportion of CD4+ cells, approximately 10%, when compared to any other condition. In contrast, the CD4+ RS´ cells subset accounted for nearly half of the T cell population, while both RS MSCs and R MSCs displayed similar levels of helper T cells at around 40%. As a result, between RS and RS MSCs, a drop of about 10% in the CD4+ population is observed.

Looking into cytotoxic T cells (CD8+) levels, the R and S conditions showed minimal or nonexistent values, respectively. Conversely, RS exhibited the highest percentage of all, almost 18%. Finally, when comparing R MSCs or RS MSCs with RS, the CD8+ population was significantly inferior in the presence of MSCs. Both MSCs-related conditions showcased similar relative frequencies of only about 5%.

To gain even deeper insights into the types of elicited immune responses, we also examined PD-1 expression. **Figure 7D** displays the relative frequency of CD3+/PD-1+ cells under the different experimental setups. Here, in either isolated R or S cell conditions, the expression of PD-1 was hardly present and notably lower than in any other condition. Moreover, there are noteworthy differences between PBMCs cultured alone, those in an alloreactive state, and those co-cultured with MSCs, as shown by their pairwise comparisons. Particularly, in the alloreactive setting (RS), the PD-1 expression started to increase significantly to approximately 3%. This increment persisted slightly in R MSCs, to around 4%. However, the RS MSCs setup showed a substantial elevation in PD-1 expression, peaking at about 9%, thus displaying the highest PD-1 expression of all experiments.

## 4. DISCUSSION

Both graft failure and GvHD stem from persistent immune system dysregulation. Restoring immunological homeostasis through increased regulatory activity or decreased alloreactivity is the standard strategy in cellular therapies. Coupled with MSCs extracted from the typically discarded BM collection bags, our strategy wanted to address the intricate relationships within an alloreactive hematopoietic graft, a researched yet unsolved puzzle, plus uncover this MSCs' source immunomodulation abilities in it.

Firstly, mononuclear cells recovered from the BM collection bag proved once more (74) to be a viable option for the expansion of MSCs, maintaining the intended function and phenotype, according to the ISCT criteria (68). MSCs from bone marrow are naturally CD44-. However, prior research has demonstrated that *in vitro* cultivation also leads to the acquisition of CD44 expression in these cells, which helps to explain the above 5% CD44 expression seen both before and after co-culture with the alloreactive setup (73). Nevertheless, in both scenarios, they exhibited comparable expression levels of CD markers, showing no statistically significant differences. This consistency in marker expression indicates that these MSCs were able to maintain an appropriate phenotype over time, even when exposed to an alloreactive environment. Repurposing discarded bone marrow collection bags for BM-MSC expansion also offers a practical and accessible source for cellular therapies, enhancing environmental sustainability by curbing medical waste and fostering cost and scientific efficiency through resource conservation. If further explored with manufacturing practices, this MSC source could eventually facilitate easy and practical access to MSCs for clinical researchers to apply in future immunotherapeutic projects.

As for the two-way MLR assay, a bidirectional interaction between MSCs and PBMCs started to be visually uncovered by CFSE immunofluorescence and phase contrast images. At first, we noted consistent literature morphological features of both fresh MSCs and PBMCs throughout the observation period, with MSCs exhibiting a spindle shape with plastic adherence, resembling fibroblasts (78), while PBMCs displayed a small, round format with a single large nucleus (91). Over time, PBMCs were observed to position themselves closer to MSCs, suggesting a direct cell-to-cell interaction between these cell types, without altering cell morphology.

Furthermore, the irradiated PBMCs (S) consistently exhibited dense nuclear chromatin fragmentation, indicative of radiation-induced inhibition of cellular activity (92). This cellular behaviour is akin to the one observed in patients before undergoing allogeneic HSCT (93) and, therefore, correlates with our intent in irradiating these cells. Besides, these cells also showed no CFSE labelling, indicating the successful labelling of only responder cells for proliferation tracking. Across all conditions, the CFSE expression indicates the proliferation of PBMCs. However, when comparing RS with RS MSCs on the final day, the MSC condition showed fewer PBMCs stained with CFSE, suggesting the potential of MSCs to modulate immune cell behavior across time.

Regarding the observations on proliferation and metabolic activity of PBMCs, they were able to echo this dynamic interplay. For one, the measurement of metabolic activity allowed us to assess their functionality and responsiveness within the co-culture system, plus the introduction of an intermediate time point (day 3). Initially, no significant differences indicated a stable baseline, but slight significance emerged over time, highlighting temporal metabolic variations and emphasizing the need for longitudinal assessments. These optimizations are also being considered in current translational studies guidelines (94, 95).

Before co-culture, R cells showed higher metabolism than S cells, reflecting their anticipated role, and demonstrating once more the effectiveness of radiation in attenuating S cells' activity. Combining both in RS boosted metabolic activity synergistically, aligning with expectations for simulating an alloreactive setup (96). On day 3, the cells' metabolism dropped under these circumstances, but there were no discernible changes between them and the MSC-treated cells (RS MSCs and R MSCs). This may imply that MSC presence may not quite alter the metabolic trajectory during the early phases of interaction. Consequently, it reinforces the need to look into an adaptation period suitable to observe major changes or consider pre-priming MSCs for earlier desired effects (97). On the final day, metabolism mostly declined, except in RS MSCs condition, where it slightly increased, hinting at MSCs' potential role in sustaining metabolism among responder cells in an alloreactive setting (98).

Correlating this with the CFSE histograms obtained, distinct patterns of PBMCs proliferation can be seen across conditions. Negative controls (S and R) showed single minor peaks with null or high fluorescence intensity, respectively, both indicating the absence of proliferation. This aligns with the expected low proliferation rate in the absence of stimulatory

signals (99). Similarly, R MSCs maintained a singular peak of non-proliferative PBMCs, although with a higher count of cells compared to the negative controls. This suggests that while MSCs did not promote PBMC proliferation, they were effective in maintaining a larger population of active cells over time, as previously shown in the resazurin assay. However, similar studies have shown that reduced MSCs numbers may enhance isolated PBMC proliferation (100, 101). Considering that, over time, evaluating MSCs dose becomes crucial for optimizing future studies.

The positive control, RS, displayed a higher second peak compared to the first, which indicates an active stimulated proliferation, characteristic of an alloreactive setup (100). This implies that the ratio of 2:1 established between responder and stimulator cells was adequate to simulate an alloreactive state with sufficient stimulation to trigger PBMC proliferation. In contrast, the RS MSCs condition, designed to assess the inhibitory capacity of these BM-MSCs´ source on alloreactivity, demonstrated a significant reduction in the proliferative peak compared to the first one.

The presence of BM-MSCs was able to notably keep a PBMC-enhanced metabolism until the final day of culture while guaranteeing control over the number of alloreactive cells with an 18 % reduction in proliferating responder cells. Similarly, a 20% reduction was previously seen in the presence of these BM-MSCs, although with a one-way MLR analysis, the mitogenic stimuli may have altered the native state of responder cells (74).

The combined insights of proliferation and metabolic activity in RS MSCs highlight the known immunosuppressive effect of BM-MSCs but also create a bridge on the delicate "Janus-Face" balance of HSCT (102). This phenomenon recognizes that the controlled maintenance of alloreactivity can be advantageous in overcoming oncological diseases, but its uncontrolled manifestation can result in undesirable immunological complications. Thus, these results suggest the possibility of achieving a homeostatic resolution of both sets of cells, by immune cross-talking. Finally, the extensive and diverse human alloreactive cell repertoire presents a formidable technological challenge. However, employing quantitative methods is crucial for comprehending fundamental aspects of this notable immunosuppressive response. Therefore, the addition of immunophenotyping insights allowed a deeper understanding of the immune cell dynamics and the mechanisms driving the observed immunosuppression.

Starting with T cells, identified as CD3+, they were the predominant population across all conditions, emphasizing their central role in orchestrating immune reactions. They remained relatively stable in conditions involving MSCs compared to the alloreactive setup which suggests that MSCs may exert minimal direct effects on T cell dynamics in this context. Alternatively, MSCs might maintain a supportive microenvironment for T cell function without exerting pronounced changes in their overall proportions (103, 104). The lower T cell proportion in S can be attributed to the nature of stimulator cells, offering insights into baseline immune cell populations without an adequate immune response, rela (105). Since most populational deviations were seen with monocytes, NK cells, and B cells they emerge as key targets to discriminate distinct responses in cellular therapies. Comparing RS with R, the mild proportional increases seen, align with expectations, as alloreactivity typically potentiates immune differentiation. As for their reduced presence in S, it suggests they are highly susceptible to radiation, warranting further investigation. A significant decrease in monocytes and B cells was observed in the RS MSCs condition compared to RS, reaffirming the inhibitory known role of MSCs for these populations (103, 106, 107).

On the other hand, since T cells constituted our major population, we then delved to explore the differential phenotypes of CD4+ and CD8+ T cells, recognizing their paramount importance in immune regulation (108). CD4+/CD8+ and CD4-/CD8- populations were excluded due to no contextual relevance and low frequency within our scope. Here, S and R conditions, had the lowest CD4+ proportions, around 10%, which could be due to the condition´s absence of other interactions and regulatory signals essential for activating their immune repertoire. The predominance of CD4+ T cells in RS reinforces them as impactful mediators in immune alloreactivity (109, 110). However, upon introducing MSCs, the reduction of CD4+ T cell proportions from 50% to around 40% suggests an MSCs-mediated inhibitory role limiting the expansion or activation of CD4+, mirroring previous findings (111). Remarkably, RS MSCs and R MSCs displayed a similar level of helper T cells, suggesting a consistent effect of MSCs on CD4+ populations, no matter the circumstance. Analysis of CD8+ cytotoxic T cell dynamics revealed analogous correlations across conditions, albeit with lower proportions compared to CD4+ populations. In isolated PBMC conditions, R and S, minimal or non-existent CD8+ T cell activation underscores the need for specific stimuli to trigger robust immune responses (112). Conversely, RS shows heightened CD8+ proportions of 15% indicating their activation to target foreign cells, which is typical in alloreactive settings

(113, 114), while both R MSCs and RS MSCs exhibit reduced CD8+ levels, lower than 5%, akin to CD4+ trends. Once more, it reiterates MSCs' immunomodulatory effect, impacting both CD4+ and CD8+ T cell responses, shedding light on immune regulation (81).

Further analyzing PD1+ cells alongside CD4+ and CD8+ T cell dynamics, added depth to the study by examining the expression of an important immune checkpoint receptor. PD-1/PD-L1 axis is recognized for its involvement in regulating T cell activity and upholding peripheral tolerance (115). The significant increase, above all conditions, seen in PD-1 expression in the presence of MSCs within an alloreactive condition could potentially signify a regulatory response aimed at dampening excessive immune activation. MSCs have been recognized for their immunomodulatory properties, including the ability to suppress T-cell activation and inflammation (62). Therefore, the increase in PD-1 expression might suggest that MSCs are enhancing a regulatory environment, which could help mitigate the severity of GvHD, by maintaining peripheral tolerance and tissue integrity. However, it is crucial to interpret this increase in PD-1 expression within the broader context of GvHD and graft failure pathology and the specific mechanisms at play. Prolonged PD-1 signalling can also foster T-cell exhaustion and hinder immune responses (116), which might aggravate GvHD or compromise the GvT effect (117, 118). Therefore, further research would be necessary to fully understand the implications of MSC-mediated increase in PD-1 expression and its potential therapeutic significance in post-transplant complications.

As a final remark, while the results provided valuable insights, it is imperative to recognize the inherent limitations stemming from the analysis of 3 BM-MSCs samples. These numbers are ascribed to the low donation rate observed during the project and the singular utilization of a donation centre. To comprehensively affirm the robustness and generalizability of these findings, further investigations encompassing a broader spectrum of samples from multiple donation centres would be a future step.

## 5. CONCLUSION

This study aimed to explore the potential of BM-MSCs, retained in typically discarded collection bags, as a therapeutic strategy to modulate immune responses. Our approach focused on understanding the complex interactions within an alloreactive hematopoietic graft and uncovering the immunomodulatory abilities of MSCs from this novel source.

The results underscore that repurposing discarded bone marrow collection bags for MSC expansion emerges as a pragmatic solution with far-reaching benefits, including environmental sustainability and resource conservation in cellular therapies. The observed bi-directional interaction between these BM-MSCs and PBMCs, both visually and through MLR evidence, showed the pivotal role of MSCs in modulating immune cell behavior within allo-hematopoietic grafts, potentially shaping therapeutic outcomes. Moreover, the influence of MSCs on CD4+ and CD8+ T cell dynamics suggests their crucial role in limiting immune activation and fostering a regulatory environment. Additionally, the observed increase in PD-1 signalling in the presence of MSCs highlights their potential regulatory response to mitigate excessive immune activation, offering promising avenues for managing conditions such as GvHD.

Overall, these findings underscore the immense therapeutic potential of MSCs and pave the way for further research and clinical applications in HSCT. However, it's crucial to acknowledge the limitations inherent in the analysis of a limited number of BM-MSC samples and mediate future efforts to expand this sample pool for a more robust validation of the results seen.

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## Annex I

**Table 5**. *Experimental setup of PBMCs immunophenotyping for each condition performed.*

| Tube ID | Purpose | FITC | PE | PerCP | PerCP- Cy5.5 | APC | PE-Cy7 |
|---|---|---|---|---|---|---|---|
| C- | ➢ Negative control | CFSE | - | - | - | - | - |
| 1 | ➢ NK cells<br>➢ B cells | CFSE | CD19 | CD56 | - | CD45 | - |
| 2 | ➢ T cells<br>➢ Cytotoxic T cells<br>➢ T helper cells | CFSE | CD3 | - | CD4 | CD8 | CD45 |
| 3 | ➢ Monocytes | CFSE | - | CD14 | - | CD45 | - |
| 4 | ➢ PD-1 receptor | CFSE | CD279 | CD3 | - | - | - |