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Isolation, expansion and functional characterization of hPL-expanded hBM-MSC for the treatment of systemic and severe acute Graft-versus-Host Disease

S.S.D. **MED/15**

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ABSTRACT

Mesenchymal Stromal Cells (MSC), firstly described by Friedenstein as bone marrow-derived non-hematopoietic adherent cells with the ability to form bone tissue *in vitro* (Friedenstein et al. 1966; 1968; 1974), are multipotent fibroblast-like stem cells with three main features, as stated by the International Society of Cellular Therapy (ISCT) (Horwitz et al. 2005, Dominici et al. 2006): i) the ability to adhere to plastic, ii) the surface expression of CD73, CD90 and CD105 molecules together with the lack of CD14, CD31, CD34, CD45 and HLA-DR; and iii) the ability to differentiate into adipocytes, osteoblasts and chondrocytes. MSC have been studied for their clinical application in regenerative medicine and immune regulatory therapy in immune-related disorders (Horwitz et al. 2002; Le Blanc et al. 2005; Kuroda et al. 2007).

Allogeneic Hematopoietic Stem-Cell Transplantation (Allo-HSCT) is an effective therapy for hematologic malignancies and inherited disorders of blood cells. The main complication is Graft-versus-Host Disease (GvHD), a donor T cell-mediated alloreactive inflammatory disease occurring in 20-70% of patients, depending on histocompatibility, with high mortality rate if steroid-refractory (Flowers et al. 2011; Hahn et al. 2008; Lee et al. 2003; Lee et al. 2013). On the basis of clinical manifestations, GvHD can be classified in: i) classic acute GVHD (aGvHD); ii) late-onset aGvHD; iii) classic chronic GvHD (cGvHD); iv) overlap syndrome (Filipovich et al. 2005). Patients developing aGvHD are routinely treated with corticosteroids, which are effective only in 60-70% of cases, whilst 30–40% of patients achieve a partial response or relapse after corticosteroid withdrawal. Thus, second- and third line treatments are required, such as alternative immunosuppressive drugs, anti-lymphocyte serum, anti-inflammatory cytokine antibodies, or extracorporeal photopheresis. Several clinical studies have been performed using MSC as second- or third-line treatment, but standardized protocols are not available so far. Consequently, pre-clinical *in vitro* and *in vivo* studies are required to define a reproducible MSC-based therapeutic protocol for aGvHD.

In this study, we first developed an expansion protocol for human bone marrow derived-MSC (hBM-MSC) using two different supplements for culture media, i.e. fetal bovine serum (FBS) or human platelet lysate (hPL). Interestingly, hPL supplement was more effective than FBS in expanding MSC. Afterwards, we characterized MSC and confirmed their genome stability through karyotype analysis and real time-PCR. MSC were then assessed *in vitro* for their ability to acquire the anti-inflammatory phenotype necessary for avoiding immune rejection and modulating host immune effector cells. MSC priming with TNF-α and IFN-γ led to increased ability in preventing NK cell-mediated lysis. Moreover, using standardized proliferation assays, MSC displayed strong immune suppressive activity towards T, B and NK cells.

We then obtained a reproducible xenogeneic mouse model of aGvHD that was used to assess in vivo the efficacy of hPL-expanded MSC-based immunotherapy with different schedules of MSC administration.

SOMMARIO

Le cellule stromali mesenchimali (MSC), descritte per la prima volta da Friedenstein come cellule aderenti non emopoietiche derivanti da midollo osseo in grado di formare tessuto osseo *in vitro* (Friedenstein et al. 1966; 1968; 1974), sono cellule staminali multipotenti simil-fibroblastiche con tre diverse caratteristiche, come stabilito dalla Società Internazionale di Terapia Cellulare (ISCT) (Horwitz et al. 2005; Dominici et al. 2006): i) capacità di crescere aderenti alla plastica; ii) espressione delle molecole di superficie CD73, CD90 e CD105 in assenza di CD14, CD31, CD34, CD45 e HLA-DR; iii) capacità di differenziare in adipociti, osteoblasti e condrociti. Grazie alla loro plasticità, le MSC sono state oggetto di molti studi in medicina rigenerativa e immunoregolatoria nel campo delle malattie infiammatorie e autoimmuni (Horwitz et al. 2002; Le Blanc et al. 2005; Kuroda et al. 2007).

Il trapianto allogenico di cellule staminali ematopoietiche (Allo-HSCT) è una terapia efficace per alcune malattie ematologiche maligne e non. La principale complicanza dell'Allo-HSCT è la malattia da trapianto verso l'ospite (GvHD), una malattia infiammatoria causata dalle cellule T alloreattive del donatore che insorge nel 20-70% dei pazienti, a seconda del grado di istocompatibilità, e gravata da un'alta mortalità se resistente al trattamento con steroidi (Flowers et al. 2011; Hahn et al. 2008; Lee et al. 2003; Lee et al. 2013). In base alle manifestazioni cliniche, si riconoscono 4 tipi di GvHD: i) GvHD acuta (aGvHD) classica; ii) aGvHD tardiva; iii) GvHD cronica (cGvHD) classica; iv) sindrome sovrapposta (Filipovich et al. 2005). I pazienti che sviluppano aGvHD sono trattati con corticosteroidi, efficaci nel 60-70% dei casi; infatti, il 30-40% dei pazienti mostra una risposta parziale o una ricaduta dopo la sospensione del trattamento. Pertanto sono necessari trattamenti di seconda e terza linea, con farmaci immunosoppressori non convenzionali, siero antilinfocitario, anticorpi monoclonali anti-citochine pro-infiammatorie, o fotoferesi extracorporea.

Diversi studi clinici sono stati eseguiti utilizzando MSC come trattamento di seconda o terza linea, ma non ci sono ancora protocolli standardizzati. Di

conseguenza, sono necessari studi pre-clinici sia *in vitro* che *in vivo* per definire un protocollo terapeutico per aGvHD basato su MSC.

In questo studio abbiamo sviluppato un protocollo di espansione per le MSC umane derivanti dal midollo osseo (hBM-MSC) utilizzando due diversi supplementi nel terreno di coltura: il siero bovino fetale (FBS) o il lisato piastrinico umano (hPL). hPL è risultato più efficiente rispetto a FBS nell'espandere le MSC. E' stata fatta poi una caratterizzazione delle MSC, confermando la loro stabilità genomica attraverso analisi del cariotipo e PCR quantitativa. Le hBM-MSC sono state valutate per la loro capacità di acquisire *in vitro* il fenotipo anti-infiammatorio, resistendo al rigetto mediato dall'immunità innata e modulando le cellule immunitarie effettrici. Il trattamento con TNF-α e IFN-γ ha conferito alle hBM-MSC un'aumentata capacità nell'evitare la lisi mediata da cellule NK; inoltre, le hBM-MSC hanno mostrato una forte attività antiproliferativa nei confronti dei linfociti T, B e NK.

Abbiamo infine sviluppato un modello murino xenogenico riproducibile di aGvHD, in cui abbiamo testato diverse modalità di somministrazione *in vivo* di hBM-MSC espanse in hPL.

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1 INTRODUCTION

1.1 GRAFT-VERSUS-HOST DISEASE (GvHD)

GvHD Clinical Classification

Allogeneic Hematopoietic Stem-Cell Transplantation (Allo-HSCT) is an effective therapy for hematologic malignancies and inherited disorders of blood cells. The main Allo-HSCT complication that can occur is Graft-versus-Host Disease (GvHD), a donor T cell-mediated alloreactive inflammatory disease characterized by 20-70% incidence, depending on histocompatibility degree, age of the recipient, and intensity of the conditioning regimen (Flowers et al. 2011; Hahn et al. 2008; Lee et al. 2003; Lee et al. 2013). Moreover, high mortality is associated to steroid-refractory GvHD (Jamani et al. 2013; Xhaard et al. 2012; Weisdorf et al. 1990; Arai et al. 2002). GvHD was initially classified as acute (aGvHD) or chronic (cGvHD), according to the onset time after Allo-HSCT (aGvHD <100 days, cGvHD >100 days). However, first in the 2005, and then in 2014, the National Institutes of Health revised the classification of acute and chronic GvHD on distinctive features and introducing a new scoring system based on clinical outcomes: number of organs involved, severity, and functional disability (Pavletic & Vogelsang 2015; Filipovich et al. 2005). Clinical manifestations of aGvHD involve skin, gastrointestinal tract and liver, showing in particular maculopapular erythema, gastrointestinal symptoms (abdominal cramps and diarrhea) and cholestatic hepatitis. Since some of these features can be found also in cGvHD, aGvHD diagnosis is based on the presence of these outcomes with the absence of the distinctive cGvHD features (Vigorito et al. 2009; Filipovich et al. 2005). Differently from acute disease, cGvHD involves more target organs including mouth, female genitalia, musculoskeletal system and lungs. cGvHD is clinically characterized by lichen planus-like changes or hyperkeratotic plaques in the mouth; cicatricial conjunctivitis, dry and gritty eyes; lichen planus-like features or stenosis in female genitalia and other clinical outcomes in musculoskeletal system and lungs. Moreover, cGvHD shows peculiar clinical manifestations in target organs involved as well in aGvHD (skin, gastrointestinal tract and liver), for

example, poikiloderma, lichen planus-like eruption or lichen sclerosus-like lesions, depigmentation of skin and esophageal web, stricture, or concentric rings in the gastrointestinal tract. Nevertheless, some clinical outcomes are characteristic both of acute and chronic GvHD, such as anorexia, nausea, vomiting, diarrhea, weight loss and rising serum bilirubin concentration (Filipovich et al. 2005). Thus, based on the clinical manifestations described above, GvHD has been classified in 4 classes: i) classic acute GVHD ii) late onset acute GvHD iii) classic chronic GvHD and iv) overlap syndrome. The first class includes GvHD that occurs within 100 days of hematopoietic cell transplant and displays features of acute GvHD, whereas diagnostic and distinctive features of chronic GvHD are absent. The second class includes GvHD with the same characteristics of the first one occurring after 100 days from the transplant. The third class includes GvHD that shows diagnostic and distinctive features of chronic GvHD at any time, with no features of acute GvHD. Lastly, the fourth class includes cases of GvHD that occurs at any time and presents both acute and chronic features (Filipovich et al. 2005).

Biological basis of GvHD

GvHD has a genetic predisposition and many genes are thought to be involved in its onset, particularly the genetic system of the human major histocompatibility complex (MHC) located on chromosome 6. MHC region is composed of hundreds genes, among which the best known and characterized are those of Human Leukocyte Antigens (HLA) (Petersdorf 2013). The HLA region is located on the short arm of chromosome 6 and includes high polymorphic loci encoding for different peptides involved in the immune response as antigen presenting molecules. These molecules are classified in two distinct groups: HLA-class I antigens (HLA-A, HLA-B and HLA-C) and HLA-class II antigens (HLA-DP, HLA-DQ and HLA-DR).

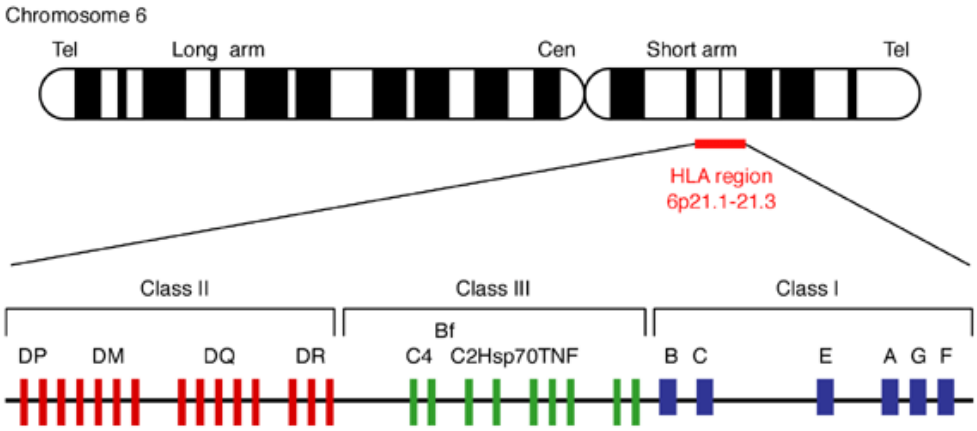

Figure 1. Gene map of the human leukocyte antigen (HLA) region. (Figure from Expert Reviews in Molecular Medicine 2003, Vol.5, Cambridge University Press). The HLA region is located on chromosome 6p21.1 with Class II, III and I genes placed between the centromeric (Cen) and the telomeric (Tel) ends.

Despite both complexes are involved in immune response, they show structural and functional differences. HLA-class I antigens are glycoproteins present on the surface of all nucleated cells as a complex of two polypeptide chains: the light chain and the heavy chain. The light chain is produced by the b2-microglobulin gene located on chromosome 15 and consists of the constant non-polymorphic region of the complex. B2-microglobulin (b2m) is a membrane protein that binds a non-covalent manner the heavy chain maintaining their structure. The heavy chain, composed by three α domains (α1, α2, and α3), a transmembrane segment and a short cytoplasmic tail, is encoded by HLA genes and represents the polymorphic region of the MHCI complex, for this reason held responsible of MHC variability (Bodmer 1987). HLA-class II complex is constituted by a α-chain and a β-chain both composed of two domains (respectively α1, α2 and β1, β2), a connecting peptide followed by a transmembrane region and a cytoplasmic tail. In this case, all peptides are encoded by HLA genes and are expressed on the membrane of some specific antigen presenting immune cells (macrophages, dendritic and B cells) (Bodmer 1987).

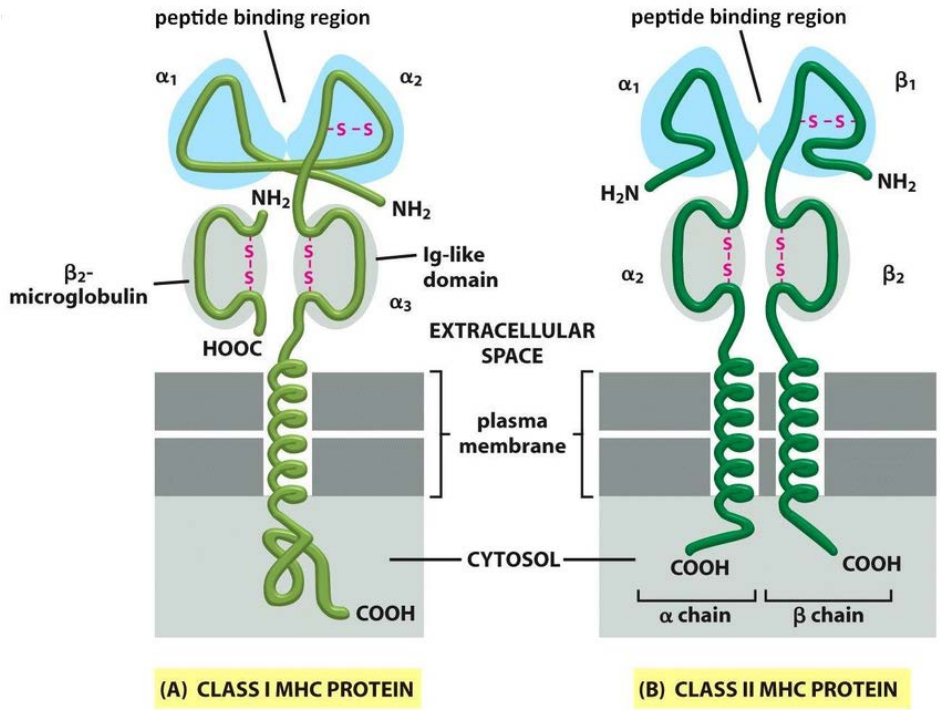

Figure 2. Structures of MHC Class I (A) and Class II (B). (Figure adapted from Alberts et al. 2008). Highlighted in this picture are the two globular domains forming the peptide binding region (PBR) and the two Ig-like domains, characteristic of class I and II MHC proteins.

As mentioned before, the two complexes act in different ways on the triggering of immune cells response. HLA-class I complex binds self-proteins or exogenous proteins (such as viral protein) produced within the cell. MHC-I antigen presenting pathway begins with the intracellular degradation of target proteins followed by the consequent targeting by MHC-I proteins on the endoplasmic reticulum. The pathway continues with the migration through the Golgi apparatus, ending on the cellular surface where the antigen is presented to T $CD8^+$ "cytotoxic" lymphocytes. On the contrary, HLA-class II complex is involved in the binding of exogenous protein deriving from the extracellular environment (such as bacteria) after endocytosis. These proteins are degraded by antigen presenting cells in the acidic endosome compartment, bound by MHC-II proteins and displayed at the cellular surface for the recognition by T $CD4^+$ "helper" lymphocytes.

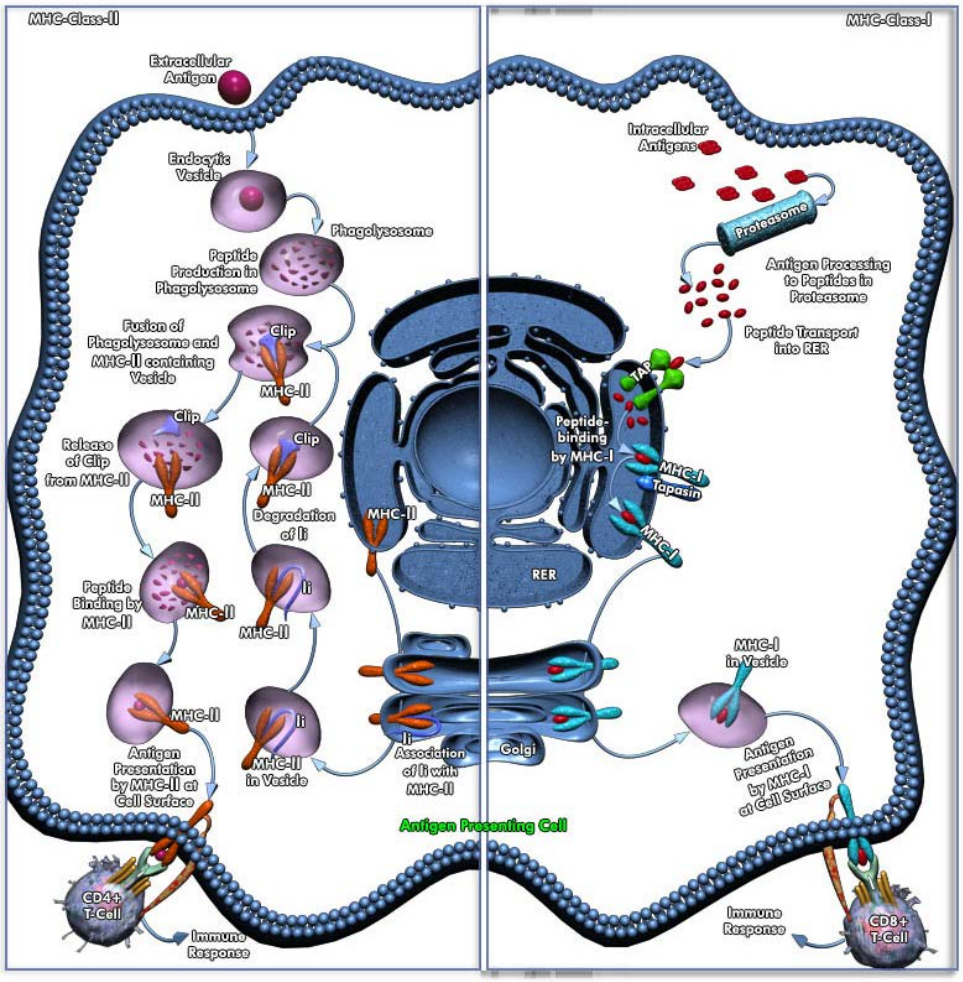

Figure 3. MHC I and MHC II antigen presenting pathways. (Figure from Qiagen website. © 2009 QIAGEN, all rights reserved). Intracellular and extracellular antigens are processed into peptides, then bound by MHC I and II respectively and presented to $CD8^+$ and $CD4^+$ T cells.

The activation of both MHC pathways, due to a mismatch in HLA proteins between hematopoietic stem cells from donor and recipient, leads to an immune response and represents the basis for GvHD onset. Thus, HLA haplotype evaluation before Allo-HSCT is a mandatory step to reduce the risk of GvHD. For instance, Allo-HSCT performed from a HLA-identical sibling (genetically identical) represents the best chance to reduce the risk of GvHD. However, even HLA-haploidentical siblings, related member or unrelated HLA-matched donor are viable alternatives, but in this cases the risk to develop GvHD is higher (Caillat-Zucman et al. 2004). Even though MHC antigens are strongly associated to the related risk to develop GvHD, also the minor histocompatibility antigens

(mHAs) seem to be involved (Dzierzak-Mietla et al. 2012; Chao 2004; Turpeinen et al. 2013). mHAs are immunogenic non-HLA related peptides derived from a non-immunogenic proteins, encoded by polymorphic genes. Following a change in the aminoacids sequence due to a polymorphism or gene deletion, these molecules can be bound by both MHC-I and MHC-II and displayed on cell membrane surface. Once recognized as non-self by T cells, immune response activation is triggered (Dzierzak-Mietla et al. 2012). The involvement of mHAs was firstly discovered thanks to experiments in which graft rejection and GvHD occurred in HLA-matched recipient (Goulmy et al. 1976; Goulmy et al. 1983; Goulmy et al. 1996). Nowadays, a lot of mHAs have been identified originating from both Y and autosomal chromosomes (Linscheid & Petroff 2013). For their implications on Allo-HSCT and GvHD incidence, current research is focused on identification of additional immunogenic non-MHC antigens.

Acute GvHD Physiopathology

For the occurrence of GvHD three conditions have to be satisfied: i) the graft must contain immunocompetent cells, ii) the recipient must express tissue antigens that are not present in the transplant donor iii) the recipient must be unable of mounting an effective response against the transplanted cells (Billingham 1966). In Allo-HSCT these requirements are fully satisfied, as patients receive a transplant with HSC and immunocompetent cells (mainly constituted by T cells) (first requirement); moreover, there is often a HLA-mismatch or mHAs mismatch between the donor and the recipient (second requirement); and finally, patients receive a myeloablative treatment with or without total body irradiation before the transplantation (third requirement). The characteristic phases of acute GvHD development have been largely described by Ferrara and colleagues (Ferrara et al. 1996; Ferrara et al. 1999; Ferrara et al. 2003). Essentially, there are 3 phases: afferent, efferent and effector phase. The afferent phase starts because of the damaging of host tissues by conditioning regimen (chemo or radiotherapy), leading to host cell activation followed by the release of pro-inflammatory cytokines (TNF-alpha, IL-1, IL-6) and danger-associated molecular pattern

(DAMPs) or pathogen-associated molecular pattern (PAMPs) molecules (Xun et al. 1994; Nassereddine et al. 2017). This massive cytokine storm has effects on both the up-regulation of MHC and adhesion molecules and on the activation of host antigen presenting cells (APCs). Hence, this first phase occurs before the transplantation, while the second phase starts after the infusion of immune competent donor cells. The efferent phase is characterized by events occurring after the recognition of antigens displayed on host APCs membrane surface by T donor cells. However, it has been demonstrated that also donor APCs are involved in GvHD progression through a cross-priming of $CD8^+$ T cells (Matte et al. 2004). The activation and expansion of $CD4^+$ T cells occurs after the antigen recognition on MHC-II; on the contrary, the antigen presentation to $CD8^+$ T cells is made by MHC-I. In this second phase, T $CD4^+$ T cells proliferate and differentiate in T helper type 1 (Th1) thanks to the higher level of IL-12 than IL-4. A pivotal role is played by Th1 lymphocytes thanks to the production of IL-2 and IFN-γ (Krenger & Ferrara 1996). The pro-inflammatory cytokines IL-2 and IFN-γ are very important in the maintaining of GvHD progression exerting a positive feedback on Th1 cells, inducing $CD8^+$ T cells to become effector cells and activating macrophages and NK cells (Hill & Ferrara 2000; Krenger & Ferrara 1996). The third phase or effector phase is characterized by the combined action of effector cells and cytokines, such as TNF-α and IL-1. The effector cells involved are not only $CD8^+$ cytotoxic T cells, but also NK cells and macrophages. Macrophages are mostly important as first cell type involved in the production of TNF-α. Thus, host target cells destruction occurs, inducing apoptosis by cytotoxic T cells through perforin/granzyme B mechanism and Fas-Fas ligand interactions, and by the secreted cytokine TNF-α through the activation of TNF-receptor and caspase cascade (Jacobsohn & Vogelsang 2007). The ensemble of this amplified immune response results in a multi-organ failure.

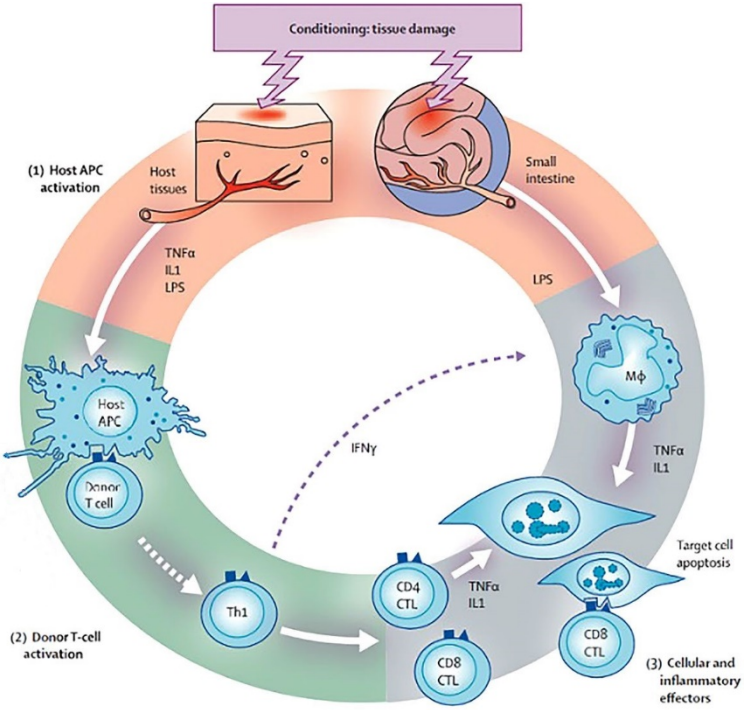

Figure 4. The three-phase model of acute graft-versus-host disease pathogenesis. (Figure adapted from Ferrara et al. 2009). The onset of aGvHD is characterized by tissue damage due to conditioning regimen leading to host APC activation, with the consequent donor T cell activation and the involvement of cellular and inflammatory effectors.

Acute GvHD Prevention and Therapy

GvHD remains the second cause of death in Allo-HSCT patients, nevertheless new findings in immunology have led to novel conditioning regimens, availability of different stem cell sources and new advances in HLA-typing for donor selection (Nassereddine et al. 2017). Considering the possibility to develop moderate or severe aGvHD after Allo-HSCT and the risk that once GvHD occurs patients may not respond to the treatment, prophylaxis is mandatory. The incidence of GvHD is variable depending on the degree of HLA mismatch and the type of transplantation. However, Sullivan and colleagues have shown that GvHD incidence could increase up to 100% without prophylaxis (Sullivan et al. 1986). In aGvHD, prophylaxis involves donor T cell depletion or immunosuppression by pharmacologic strategies. Even though T cell depletion of donor transplant is possible, the risk of graft rejection or leukemic relapse is increased (Patterson et

al. 1986; Kernan et al. 1989; Blaise et al. 1993). For this reason, pharmacological immunosuppression is preferentially used. Methotrexate (MTX) is an antagonist molecule of folic acid and is involved in the inhibition of dihydrofolate reductase and therefore in purine synthesis pathway blockade. Thanks to this mechanism, MTX is used in GvHD prevention leading to T cell inhibition (Huang et al. 2005). Calcineurin inhibition is another mechanism to block T cell proliferation after transplantation. In fact, cyclosporine and tacrolimus are two pharmacological inhibitors used in GvHD prevention; in particular, a combination of cyclosporine or tacrolimus with low doses of methotrexate leads to a major prevention of aGvHD (Storb et al. 1986; Storb et al. 1987; Mrsic et al. 1990; Przepiorka et al. 1996). Recently, a new approach of GvHD prophylaxis has been used, consisting in the administration of high dose of cyclophosphamide and resulting in an effective tolerance after transplantation (Luznik et al. 2008; Luznik & Fuchs 2010; Raiola et al. 2013). Another strategy for GvHD prophylaxis employs monoclonal or polyclonal antibodies directed against T cells (Anti-thymocyte globulin (ATG)) or against interleukin receptors, such as IL-2 receptor (IL-2R). ATG molecule is directed against T cell receptors (i.e. CD3/TCR, CD152) inducing an *in vivo* depletion mainly through apoptosis (Mohty 2007). Randomized control trials, meta-analysis and scientific papers reveal the ability of ATG in reducing the incidence of grade 3-4 aGvHD. However, a retrospective trial published by the Centre for International Blood and Marrow Transplant Research (CIBMTR) showed no differences in aGvHD incidence after ATG treatment (Bacigalupo et al. 2001; Finke et al. 2009; Pidala et al. 2011; Kumar et al. 2012). Differently from ATGs, anti-interleukin receptor monoclonal antibodies, such as murine anti-IL-2 receptor, acts as antagonist. This therapy is based on the evidence that IL-2 is a T cell proliferation-inducing cytokine; thus, after IL-2R/antagonist binding, IL-2 production is blocked and T cell proliferation is impaired. Some studies on this antibody shown an effectiveness on prevention of aGvHD (Anasetti et al. 1990; Anasetti et al. 1991; Chen et al. 2003). Usually, GvHD prophylaxis is lasted even up to 12 months after the Allo-HSCT, therefore in conjunction with treatments. The first line treatment involved glucosteroid such as metilprednisolone. This drug acts reducing the inflammatory response in

various ways: inhibiting the activation of T cells, inducing apoptosis and decreasing the inflammatory effects of pro-inflammatory cytokines. Nevertheless, organs toxicity and side effects were found (Oblon et al. 1992). To date, corticosteroid therapy remains the first-line treatment. Unfortunately, this therapy is successful only in 60-70% of aGvHD patients, whilst 30–40% of patients achieve a partial response or relapse after corticosteroid withdrawal. Thus, second or third-line treatments are required. Different strategies were used to find the best therapeutic agent against GvHD and some of these were already in use as prophylaxis. As described before, monoclonal antibodies, such as Inolimomab and Infliximab, are used in GvHD treatment, the first one directed against IL-2 receptor, while the second one is an anti-TNFα agent. Another biopharmaceutical product that interferes with TNFα is the fusion protein Etanercept. A synergic effect in GvHD treatment has been found between Etanercept and metilprednisolone (Uberti et al. 2005; Levine et al. 2008). The use of Pentostatin, a nucleoside analogue, is an additional strategy that involves the accumulation of 2-deoxyadenosine 5-triphosphate by adenosine deaminase inhibition leading to T cell death. Moreover, extracorporeal photopheresis (ECP), developed by *Therakos Inc.* (Westchester, PA, USA), has been proposed as GvHD treatment. ECP employs ultraviolet A (UVA) irradiation of autologous peripheral blood mononuclear cells (PBMCs) collected by leukapheresis and exposed to the photosensitizing drug 8-methoxypsoralen (8-MOP) (Foss et al. 2002). The photoactive buffy coat is subsequently re-infused into the patient. 8-MOP covalently binds and cross-links DNA of PBMCs upon exposure to UVA light irradiation, resulting in apoptosis. ECP presumably mediates its function by reducing T-cell response, not only inducing apoptosis, but also through phagocytosis of apoptotic lymphocytes by APCs, inducing these cells to produce anti-inflammatory cytokines and regulatory T cells (Hart et al. 2013). Thus, this procedure has been considered a potential tool in acute steroid refractory GvHD treatment (Greinix et al. 2006; Perfetti et al. 2008; Greinix et al. 2010).

In the last decade, cell therapy has become as new strategy for aGvHD treatment. In particular, the use of regulatory T cells and mesenchymal stromal cells (MSC)

has been associated to good results, but more studies are required to define the treatment schedule (Le Blanc et al. 2004; Brunstein et al. 2011).

1.2 MESENCHYMAL STROMAL CELLS (MSC)

MSC discovery, localization and definition

Mesenchymal Stromal Cells (MSC), firstly described by Friedenstein as bone marrow-derived non-hematopoietic adherent cells with the ability to form bone tissue *in vitro* (Friedenstein et al. 1966; 1968; 1974), are multipotent fibroblast-like stem cells. MSC reside in bone marrow, particularly in the extracellular matrix that acts as a structural scaffold with other cell types (bone marrow stromal microenvironment) for the hematopoietic stem cells niche. MSC control the balance between the quiescent and proliferative state of Hematopoietic Stem Cells (HSC) by specific interactions and release of soluble factors. Despite the successful *in vitro* identification and isolation of MSC, the *in vivo* native MSC localization remains still unclear. Recent studies have shown that native MSC in bone marrow could be located in the vascular niche, among vascular smooth muscle cells, in contact with endothelial cells on the abluminal side of the marrow sinuses (Sacchetti et al. 2007; Pontikoglou et al. 2008). Nevertheless, the discovery in different organs of perivascular cells expressing mesenchymal markers and showing multipotent capacity, led to speculate that MSC can be pericytes or derive from these cells (Crisan et al. 2008) and, therefore, are ubiquitous in all tissues.

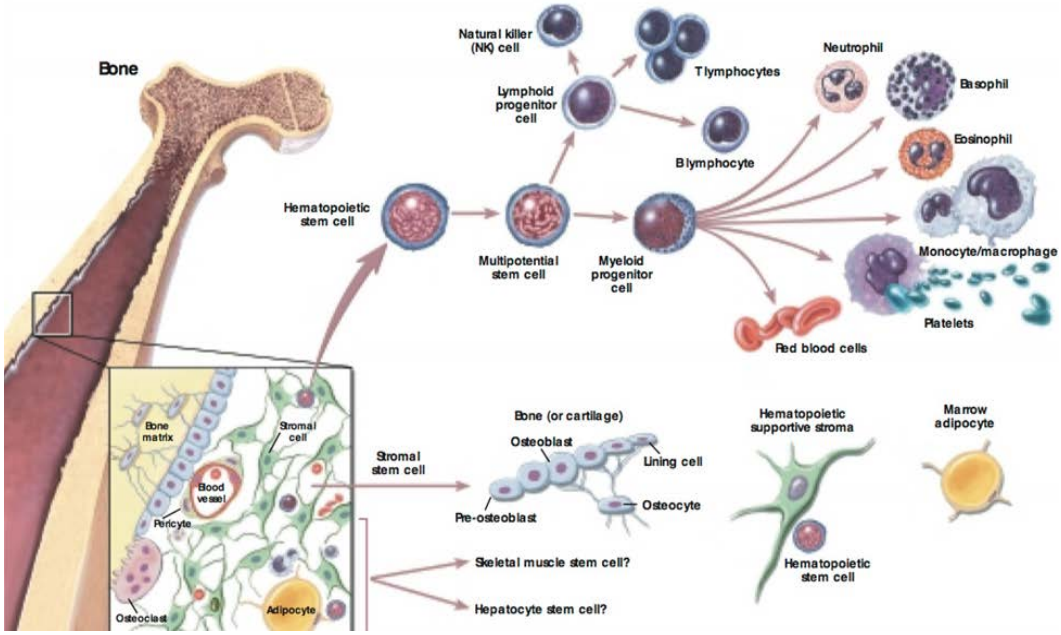

Figure 5. Bone Marrow microenvironment. © 2001 Terese Winslow (assisted by Lydia Kibiuk). Different connective tissue cells origin from a common stem cell population lying within the bone marrow.

In fact, MSC can be isolated not only from adult bone marrow but from a lot of other tissues, both of fetal and adult origin, such as bone marrow, blood, lung, liver and spleen, adipose tissue, peripheral blood, synovial fluid, dental tissues, amniotic fluid and amniotic membrane, endometrium, limb bud, cord blood and Wharton's jelly (Wagner et al. 2005; Cai et al. 2010; Huang et al. 2009; Schüring et al. 2011; Morito et al. 2008; Wang et al. 2004; Jiao et al. 2012; Roufosse et al. 2004; In ’t Anker et al. 2003). Considering the two peculiar characteristics of self-renewal and multipotency, MSC have been classified as adult stem cells (Friedenstein et al. 1966; Friedenstein et al. 1974; Sacchetti et al. 2007). In the 90’s, on the basis of their multi-lineage differentiation capacity, these cells were named Mesenchymal “Stem” Cells (MSC) (Caplan 1991; Pittenger 1999). However, this term caused confusion in the scientific community. Nevertheless, the plastic-adherence cell isolation method, largely used, leads to obtain a heterogeneous cell population, in which only few cells possess the specific properties linked to the term “stem” (i.e. multipotency and long term self-renewal *in vivo*). For these reasons, in 2005 the ISCT committee proposed to refer to this

isolated population with the more generic name of multipotent Mesenchymal Stromal Cells (MSC) (Horwitz et al. 2005).

Furthermore, in 2006 the ISCT suggested the minimal criteria of MSC definition largely accepted until now. Thus, MSC possess three main characteristics: i) the ability to grow adherent to plastic, ii) the membrane surface expression of CD73, CD90 and CD105 molecules together with the lack of CD14, CD31, CD34, CD45 and HLA-DR expression and iii) the ability to differentiate in adipocytes, osteoblasts and chondrocytes (Dominici et al. 2006).

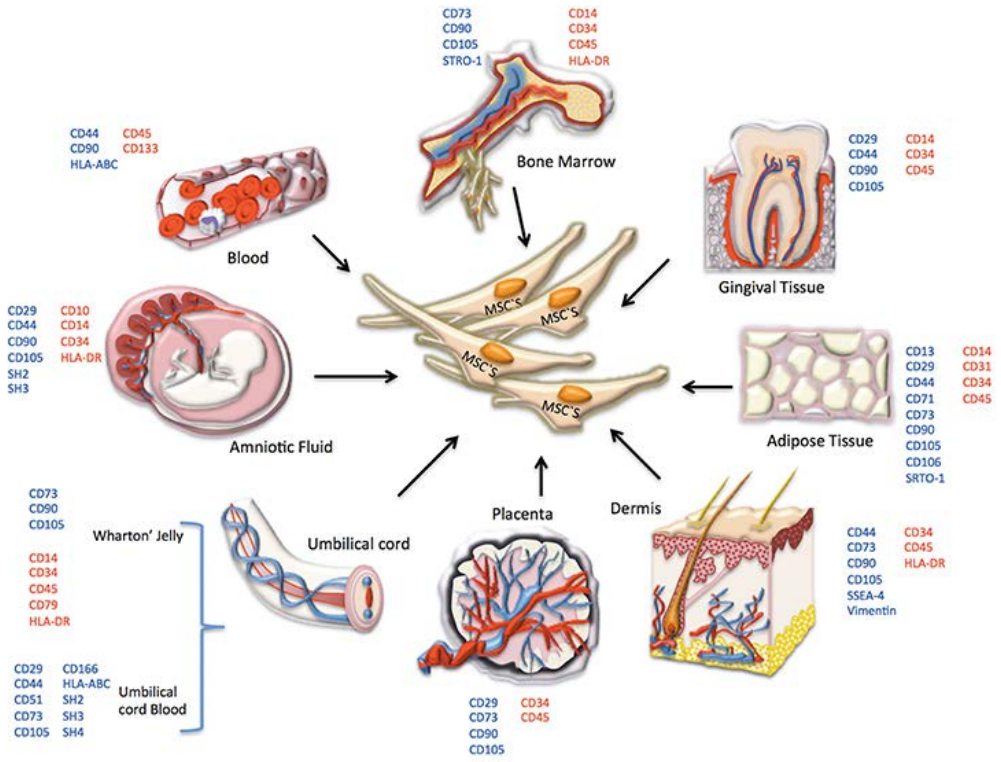

Figure 6. MSC phenotype and tissues origin. (Front. Physiol. Consuelo Merino González et al. 2016). MSC could be identified both in fetal tissues (such as in the placenta, in the amniotic fluid, in the umbilical cord blood) and in adults, particularly in bone marrow and other different tissues (such as dermis, gingival and adipose tissue).

MSC immune modulatory properties

In 2002, Bartholomew at al. firstly described the capability of allogeneic baboon MSC to influence immune responses *in vitro* and to prevent allogeneic skin graft rejection *in vivo* (Bartholomew et al. 2002). Since then, several studies were focused on understanding the immune modulatory property of MSC. In this regard, several works have shown MSC immunosuppressive property towards many immune cells (Di Trapani et al. 2013; Jiang et al. 2005; Benvenuto et al. 2007; Corcione et al. 2006; Tse et al. 2003), even though MSC exert different functions depending on the microenvironment in which they reside. In particular, they can acquire a pro-inflammatory phenotype (MSC1) or an anti-inflammatory phenotype (MSC2) depending on inflammatory cytokine levels (Waterman et al. 2010). Thus, MSC required a "licensing" or "priming" (pMSC) phenotype to exert their immune-modulatory activity; otherwise, at resting conditions (rMSC), they have anti-apoptotic and supporting properties (Krampera 2011). IFN-γ, TNFα, IL-1α and IL-1β are mainly involved in MSC inflammatory priming. However, TNFα, IL-1α and IL-1β seem to exert only a supportive role in MSC2 polarization. In fact, the combination of IFN-γ with TNFα, IL-1α or IL-1β enhances MSC inhibitory effect. However, inhibiting singularly these three cytokines with blocking antibodies does not completely revert T cell inhibition (Ren et al. 2008). On the other hand, IFN-γ is the main mediator of MSC "licensing", as anti-IFN-γ receptor blocking antibodies may revert completely MSC inhibitory effect on T cells (Krampera et al. 2006). In addition, Ren et al. have shown that MSC lacking in IFN-γ receptors were completely unable to inhibit T cells (Ren et al. 2008). However, as mentioned above, MSC polarization depends on inflammatory cytokine concentration; thus, the same pro-inflammatory cytokine can play a dualistic role. Indeed, at low levels, IFN-γ triggers MSC to become pro-inflammatory and to behave as APC by inducing immune response (Stagg et al. 2006; Chan et al. 2006). Similarly, Toll-Like Receptors (TLRs) expressed by MSC play an important role in MSC1 or MSC2 polarization. The activation of TLR3 and TLR-4 on MSC, due to the binding of their specific ligands, results in an impairment of the MSC immune modulatory activity by inhibiting Notch1-mediated T cell activation through Jagged1

downregulation (Liotta et al. 2008). However, when TLR3 and TLR4 are activated for a short time with low dose of ligands, opposite effects on MSC polarization have been shown. TLR4 priming led MSC to acquire a pro-inflammatory phenotype by inducing the production of pro-inflammatory cytokines, such as IL-6, IL-8 and TGF-β. On the contrary, TLR3 priming led MSC to acquire the anti-inflammatory phenotype by producing IL-4, IL-1RA, IDO and PGE2 (Waterman et al. 2010). Furthermore, MSC immunophenotype is strongly modified by inflammatory cytokines; therefore, once MSC become MSC2, they overexpress on cellular surface MHC-class I, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), programmed death ligand 1 (PD-L1), and show a *de novo* expression of MHC-II (Sheng et al. 2008; Ren et al. 2010; Chan et al. 2006). Thus, the acquisition of anti-inflammatory properties by MSC depends on the balance of opposite stimuli in the microenvironment that influences not only MSC functions, but also the immunophenotype (Krampera 2011). Even though the immune modulatory effect of MSC was largely demonstrated, the mechanisms wherewith they act is not completely understood. Anti-inflammatory MSC act both by cell-to-cell contact and paracrine mechanisms. In particular, after the interaction between MSC and immune effector cells occurs, T and B cell proliferation is inhibited through PD-L1, PD-L2 and programmed death-1 (PD-1) pathway activation (Augello et al. 2005; Gu et al. 2013). Other studies have shown the involvement of the Fas-L/Fas pathway in the MSC immune-modulation. Indeed, MSC can induce activated T cell apoptosis through the interaction between Fas-L (express on MSC) and Fas receptor (express on T lymphocytes) (Mazar et al. 2009; Akiyama et al. 2012; Gu et al. 2013). After MSC2 polarization, MSC produce a large amount of soluble molecules that are involved in their immunosuppressive property (Shi et al. 2012) with differences between MSC deriving from different species (Ren et al. 2009; Ma et al. 2014). In mouse MSC, inducible NO synthase (iNOS) expression has been found after MSC “priming” (Ren et al. 2008). As a consequence, nitric oxide is produced by MSC at high concentrations, leading T cell apoptosis *in vitro* (Sato et al. 2007). Moreover, the inhibition of iNOS reverts benefits of MSC treatment on GvHD mouse model (Ren et al. 2008). However, this mechanism does not

occur in human pMSC. On the contrary, indoleamine 2, 3-dioxygenase (IDO) is involved in immune suppression of various immune cell populations only in primed human MSC. IDO is an enzyme that catalyses a rate-limiting step in the kynurenine pathway and is involved in tryptophan degradation into kynurenine, a metabolite with immune modulatory effects on T and NK cells (Frumento et al. 2002; Meisel et al. 2004; Krampera et al. 2006). In particular, IDO acts by blocking T cell proliferation and inducing apoptosis by tryptophan deficit and kynurenine formation, involved in regulatory T cell induction (Mezrich et al. 2010). Prostaglandin E2 (PGE2) is another anti-inflammatory molecule involved in MSC-mediated immune-modulation on different immune cells. PGE2 production is enhanced in primed MSC and PGE2 synthesis blockade with specific inhibitors restores T cell proliferation (Aggarwal & Pittenger 2009). However, PGE2 is not a pivotal factor to suppress T cell proliferation and NK cells activity, but it acts in combination with IDO in human or iNOS and NO in mice (Spaggiari et al. 2008; Matysiak et al. 2011; Sato et al. 2007). MSC, through PGE2 secretion, make macrophages produce IL-10, an anti-inflammatory cytokine (Németh et al. 2009). Another molecule produced by both human and mouse MSC after inflammatory stimulation is TGF-β. This molecule is involved in the direct inhibition of peripheral blood lymphocytes, but also in the induction of $FoxP3^+$ regulatory T cells, as demonstrated by both *in vitro* and *in vivo* experiments (Di Nicola et al. 2002; Nemeth et al. 2010; Yoshimura & Muto 2011). In addition, Human Leukocyte Antigen-G (HLA-G) plays important roles in immune response modulation and graft rejection. A soluble isoform, HLA-G5, is secreted by activated MSC. HLA-G5 contributes to the suppression of T cell proliferation and NK cell activity, as shown by blocking experiments with neutralizing anti-HLA-G antibodies, and to $FoxP3^+$ regulatory T cell induction (Selmani et al. 2008). Moreover, HLA-G5 plays a role in allograft acceptance (Lila et al. 2002; Le Rond et al. 2006). Lastly, MSC can also exert their immune modulatory effects in a paracrine way, though the production of extracellular vesicles (EVs), in particular microvesicles and exosomes, which may inhibit directly B, NK and indirectly T cell proliferation (Di Trapani et al. 2016).

Thus, due their immunological properties, MSC represent a potential therapeutic tool in different clinical applications (i.e. tissue would healing, regenerative medicine, inflammatory and autoimmune diseases, GvHD).

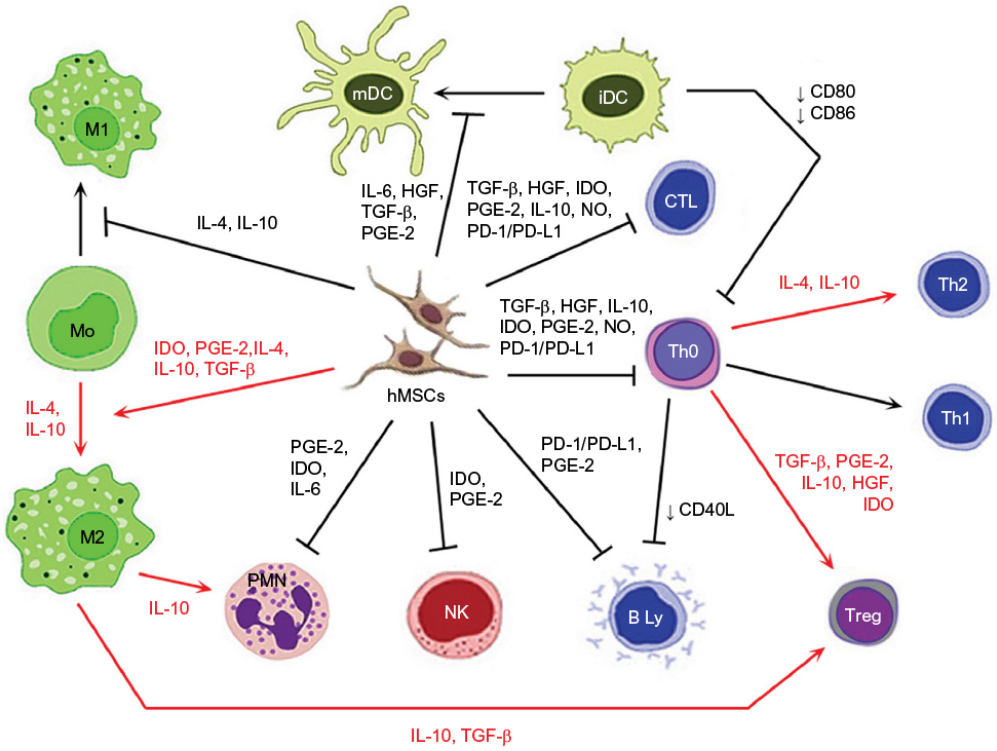

Figure 7. Immune-modulatory action of activated MSC. (Figure from Zachar et al. J Inflamm Res. 2016). MSC display broad immune modulatory properties; in the picture, red arrows indicate stimulation, black arrows show suppression and blunt-ended arrows mean direct inhibition.

MSC application in regenerative medicine

MSC are defined as multipotent cells thanks to their ability to differentiate into different tissues of mesodermal origin, including osteocytes, chondrocytes, adipocytes, but also cardiomyocytes (Xu et al. 2004) and smooth and skeletal muscles (Dezawa 2005). However, the interest for MSC in regenerative medicine was boosted by some controversial evidence of *in vitro* pluripotency, including trans-differentiation into cells belonging to ectodermal and endodermal tissues, such as neurons (Tropel et al. 2006), hepatocytes (Schwartz et al. 2002) and insulin-producing cells (Xie et al. 2009).

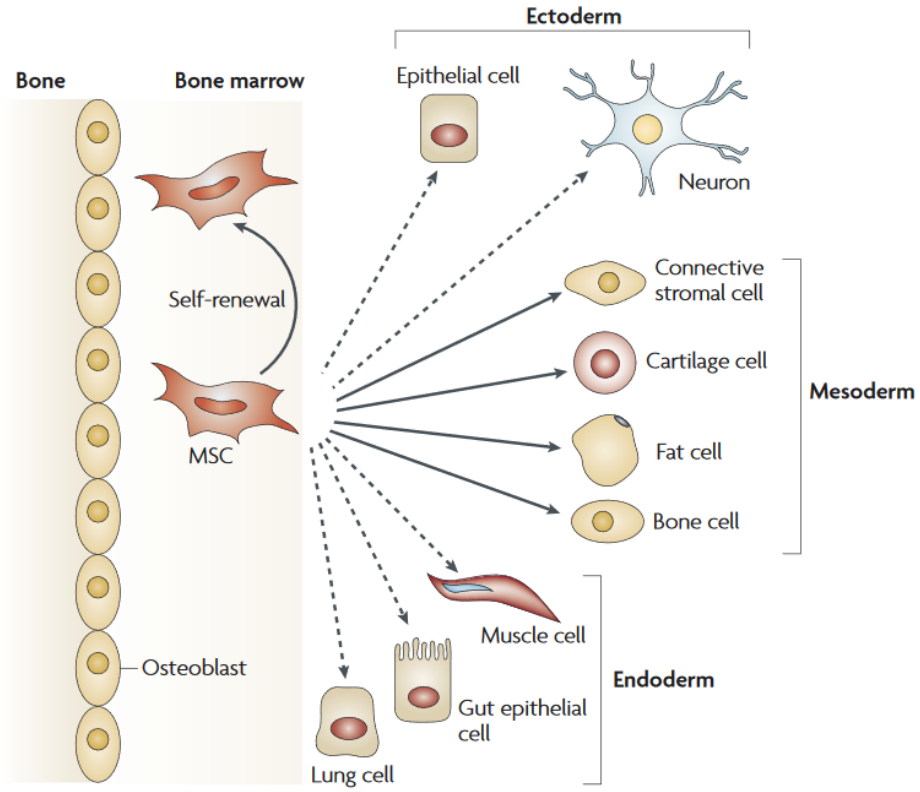

Figure 8. The multipotential properties of MSC. (Figure from Uccelli et al. 2008 Nat Rev Immunol.). Mesenchymal stem cells in bone marrow cavity are capable to self-renew (curved arrow), to differentiate towards mesodermal lineages (straight arrows), and to transdifferentiate into ectodermal and endodermal lineages (dashed arrows).

In the last decades, *in vivo* data have highlighted the potential use of MSC therapy in regenerative medicine. One of the most reliable MSC applications is bone regeneration; in particular, autologous or xenogeneic MSC have been used with a hydroxyl-apatite matrices to repair sheep, canine, rat and also human segmental bone defects (Bruder et al. 1998; Kon et al. 2000; Arinzeh et al. 2003; Quarto et al. 2001; Burastero et al. 2010). Another application in bone regeneration involves the treatment of the osteogenesis imperfecta (OI), due to genetic defects in collagen deposition (Otsuru et al. 2012). After 3 months from bone marrow cells infusion, 3 children with OI showed new dense bone formation with an increased total body bone mineral content that led to a reduced frequencies of bone fracture (Horwitz et al. 1999). Moreover, the same benefits on OI were observed with isolated allogeneic MSC (Horwitz et al. 2002). Cartilage repair was also

investigated: the main strategy adopted in the treatment of cartilage defects is based on tissue engineering to induce *in situ* differentiation of MSC in chondrocytes. Different studies were carried out on both small and large animals (rabbit, sheep, and horse). In 2004, a study suggested the possibility to repair articular cartilage defects by inducing *in situ* differentiation after transplantation of a beta-tricalcium phosphate (beta-TCP) bio-ceramic scaffold with autologous bone marrow MSC in a sheep model (Guo et al. 2004). Other studies were performed using different carriers for MSC, such as Hyaluronic Acid (HA) (Lee et al. 2007), poly-lactic-glycolic acid (PLGA) (Uematsu et al. 2005), fibrin (Dragoo et al. 2007) or collagen (Qi et al. 2012). Moreover, Dragoo et al. used MSC from adipose tissue instead of MSC derived from bone marrow (Dragoo et al. 2007). For knee osteoarthritis treatment, some clinical studies have been performed using autologous or allogeneic MSC both from adipose tissues and bone marrow (Centeno et al. 2008; Pak 2011; Vega et al. 2015). As far as cardiac regeneration after infarction is concerned, there is some evidence that MSC implanted into murine myocardium are capable of differentiating into cardiomyocytes and inducing angiogenesis, but this phenomenon is scarce and controversial (Toma et al. 2002). Animal studies showed improvements in cardiac function, but a few cardiomyocytes-differentiated MSC were evident inside the infarcted tissue and the main putative mechanism was the induction of angiogenesis through soluble factors (Orlic et al. 2001; Min et al. 2002; Miyahara et al. 2006). Based on these assumptions, clinical studies were carried out. In 2004, 69 patients with acute myocardial infarction were treated with an autologous BMSC suspension containing 8 to 10×10^9 cells/ml (not *ex-vivo* expanded MSC). After a direct injection of this suspension into the infarcted tissue, no deaths occurred and several improvements in cardiac functions were observed (Chen et al. 2004). In the same year, Wollert et al. performed a randomized clinical study on 60 patients. In this study, the ability of autologous bone-marrow cells (again, not *ex-vivo* expanded MSC) to improve global left-ventricular ejection fraction (LVEF) was assessed at 6 month-follow-up after intracoronary transfer. Evaluating the variation of global left-ventricular ejection fraction (LVEF) from baseline to 6 months, bone-marrow cells transfer seemed to

enhance left-ventricular systolic function in myocardial segments adjacent to the infarcted area. On the contrary, no significant changes were observed in the infarcted region compared to control group (Wollert et al. 2004). In 2017, a phase I/II randomized controlled trial (RIMECARD Trial) was in favour of the efficacy and safety of intravenous injection of umbilical cord MSC in the treatment of patients with heart failure (Bartolucci et al. 2017), but further studies are necessary to consider MSC as an effective strategy for heart function impairment.

Evidence has been provided for MSC trans-differentiation into ectodermal lineages, such as neurons and Schwann-like cells (Safford et al. 2002; Anghileri et al. 2008; Datta et al. 2011; Tomita et al. 2013). Thus, a potential therapeutic application of MSC for brain injuries and neurological disorders has been suggested. Pre-clinical studies in mice and rats displayed the MSC capability to migrate, survive and improve functional recovery in brain or spinal cord injury (Kang et al. 2003; Naghdi et al. 2009; Pavlova et al. 2012). Another approach was used in Parkinson Disease (PD) and in amyotrophic lateral sclerosis (ALS) based on MSC differentiation into specific soluble factor-producing cells (i.e. neurotrophic factors (NTF)-MSC), thanks to genetic modifications (Barzilay et al. 2009; Ratcliffe et al. 2013). They all are preliminary results that need to be confirmed.

Lastly, MSC application in liver injuries and diabetes was investigated. Several studies have reported the possibility to induce the differentiation of MSC derived from different sources in functional hepatocyte-like cells (endodermal trans-differentiation), in which the hepatocyte nuclear factor 4 alpha (HNF4α) has a pivotal role. Hepatocyte-like cells were transplanted into mice with acute liver injury, showing a regenerative supporting activity and prevention of injury progression (Stock et al. 2014). In the last few years, *in vitro* and *in vivo* MSC trans-differentiation in functional pancreatic cells was tested (Phadnis et al. 2011; Tang et al. 2012), leading to several studies to set up MSC-based cell therapies. A pre-clinical study performed on nude diabetic mice, transplanted with human insulin-producing cells derived from *in vitro* differentiation of MSC, has pointed out that these stem cells may control the diabetic status for 3 months (Gabr et al. 2013). Moreover, nude mice with streptozotocin (STZ)-induced diabetes and

transplanted with insulin-producing cells obtained *in vitro* from MSC, showed improvement in glycemia levels (Xin et al. 2016).

MSC application in neurodegenerative diseases, autoimmune diseases and GvHD

MSC could be a therapeutic tool in several diseases involving immune system. As for neurodegenerative diseases, Alzheimer disease (AD) is one of the most common neurodegenerative diseases and it could represent a target for MSC based therapy. Indeed, MSC may modulate the inflammatory environment acting on different cells population in the brain. In particular, MSC regulate microglia-dependent production of pro-inflammatory cytokines and Aβ-degrading enzymes, and induce regulatory T cells (Yang et al. 2013; Ma et al. 2013). In addition, MSC promotes amyloid plaque clearance and neuronal survival, enhancing cell autophagy pathway (Shin et al. 2014). Through the development of the experimental autoimmune encephalomyelitis mouse model, the therapeutic effect of MSC in multiple sclerosis (MS) has been evaluated (Zappia et al. 2005, Constantin et al. 2009; Constantinescu et al. 2011). MSC of both bone marrow and adipose origin ameliorate experimental autoimmune encephalomyelitis by decreasing B, T cells and macrophage infiltration in the central nervous system (CNS) and inducing T cell anergy (Zappia et al. 2005, Constantin et al. 2009; Constantinescu et al. 2011).

Despite these interesting results in neurodegenerative diseases, systemic inflammatory and autoimmune diseases still represent the main MSC application fields. Rheumatoid arthritis (RA) is a T- and B-cell dependent autoimmune disease characterized by joint inflammation due to loss of immunological self-tolerance. Different preclinical studies on mouse model of collagen-induced arthritis (CIA) have shown benefits after MSC treatment. MSC can improve CIA both if administrated at the time of arthritis induction (Day 0) and after 21 days, when CIA is boosted (Augello et al. 2007). However, it was reported that in adjuvant-induced and spontaneous arthritis model, MSC showed an effect only if injected before the onset of the disease (Papadopoulou et al. 2012) . Another

preclinical study has demonstrated that infusion of human MSC significantly ameliorates the severity of experimental arthritis (González et al. 2009). In this study, the beneficial effects of MSC involved the reduction of pro-inflammatory cytokines, such as IL-17, IFN-γ, IL-2, TNFα, RANTES and macrophage inflammatory protein-2, as well as the induction of anti-inflammatory cytokines, such as IL-10 and TGF-β (González et al. 2009). Moreover, the induction of regulatory T cells by MSC was involved in the improvement of CIA in mouse models (Augello et al. 2007; González et al. 2009). In a recent *in vitro* co-culture study, the production of pro-inflammatory cytokines by peripheral blood mononuclear cells isolated from patients with RA was inhibited by MSC (Baharlou et al. 2017).

The efficacy of MSC transplantation was assessed also in patients with Systemic Lupus Erythematosus (SLE). Four SLE patients with glucocorticoid-refractory disease and treated with MSC displayed improvement in terms of SLE disease activity index and renal function, leading to stable disease remission (Sun et al. 2009). The efficacy of MSC was tested in inflammatory bowel diseases (IBD), such as ulcerative colitis (UC) and Crohn's disease (CD), two progressively fatal diseases often without a curative treatment. A mouse model for IBD was obtained by administrating both dextran sodium sulfate (DSS) and 2, 4, 6-trinitrobenzene-sulfonate acid (TNBS) (Chinnadurai et al. 2015). Thus, different studies have pointed out the efficacy of MSC in improving IBD in mice, showing a down-regulation of pro-inflammatory cytokines and improving stool condition, weight gain and histopathology (Abdel Salam et al. 2014; He et al. 2012). The safety and the therapeutic effect of MSC in severe ulcerative colitis were also demonstrated in a clinical study, in which 34 patients were treated with MSC in addition to conventional treatment. Among this population, 30 patients had an improvement of the disease and no side effects were found in any of the 34 patients compared to control group (Hu et al. 2016). Moreover, several prospective, retrospective and clinical studies have revealed the potential application of MSC in ameliorating Crohn's disease fistulas (Garcia-Olmo & Schwartz 2015).

In 2004, the first successful use of MSC in severe aGvHD treatment was reported. Haplo-identical bone marrow-derived MSC were administrated twice in a 9-year

old patient with severe steroid-refractory aGvHD, resulting in a rapid and progressive improvement of the disease until 1 year after transplant (Le Blanc et al. 2004). Since then, several pre-clinical studies were performed in mouse models, with controversial results. The ability of mouse adipose tissue-derived MSC to control the disease progression was shown in a mouse model of GvHD (Yañez et al. 2006). In another mouse model of GvHD, human MSC were capable of attenuating T cell proliferation, reducing inflammation and improving mouse survival (Auletta et al. 2015). However, the evidence of efficacy of mouse MSC in inhibiting T cell proliferation *in vitro* was not always associated to GvHD prevention in mice (Sudres et al. 2006). MSC require the presence of IFN-γ to be effective in mice and IFN-γ-pre-activated MSC are more efficient in suppressing GvHD (Polchert et al. 2008). Using humanized a NSG mouse model, a single MSC injection at day 7 from allo-HSCT reduced liver and gut GvHD and increased mouse survival, while no beneficial effect was observed if MSC were administrated at day 0. However, MSC pre-treated with IFN-γ efficiently suppressed GvHD even if administrated at day 0 (Tobin et al. 2013). In NOD/SCID mice, human cord blood-derived MSC could prevent GvHD if administered in multiple doses at weekly intervals starting at day 0; however, they failed to prevent GvHD if administered in a single dose or at the onset of the disease (Tisato et al. 2007). Therefore, the timing of MSC administration represents a critical issue for MSC effectiveness.

In 2008, a successful phase II clinical trial was published with 55 steroid-refractory aGvHD patients treated with different doses of MSC. Of them, 27 patients received one infusion, while 28 patients received two or more doses up to five: 39 of 55 patients responded to MSC treatment, 30 of them achieving complete response with a 2-year survival of 52% compared to controls (10%). No acute or late side effects were observed following MSC infusion (Le Blanc et al. 2008). Other studies have shown the safety and efficacy of MSC treatment for steroid-refractory aGvHD in pediatric patients (Introna et al. 2014; Erbey et al. 2016). A private company (Osiris therapeutic inc.) has recently developed a commercial MSC product (Prochymal®) for steroid refractory aGvHD treatment. In a compassionate use study, 12 children were treated with Prochymal®,

achieving an overall response rate of 100% with a 58% of complete remission and survival at 100 days (Prasad et al. 2011). In addition, in a phase II clinical trial, 32 patients with grade II-IV GvHD were treated with Prochymal® in combination with corticosteroids. In this study, an overall response rate of 94% and a complete remission in 77% of patients were reported, revealing the efficacy of this therapy (Kebriaei et al. 2009). Nevertheless, in 2009 Osiris therapeutic inc. announced that a phase III trial with Prochymal® failed to achieve the primary endpoint. Other phase III trial are in progress, whose results will be crucial to have adequate cues for the clinical use of MSC to treat aGvHD patients.

1.3 MOUSE MODELS

Humanized mouse model for GvHD

The first studies on GvHD were carried out in mouse models leading to the characterization of the typical physiopathological stages of this immune disorder. As GvHD is based on MHC mismatch between recipient and donor, GvHD mouse models were obtained thanks to the transplantation of mismatched splenocytes or purified T cells to a sub-lethally irradiated murine host. The most commonly studied aGvHD mouse model was obtained using C57BL/6 mouse ($H2^b$) donor lymphocytes transplanted into sub-lethally irradiated BALB/c mouse ($H2^d$) (Schroeder & DiPersio 2011). The reproducibility of this murine aGvHD models was used for the development of a standardized and well-defined scoring system that allows to define the severity of aGvHD in mice (Tobin et al. 2013). However, there are some limiting factors associated with these murine models. First, the progression and the pathology of aGvHD are similar but not identical in mice and humans. Moreover, murine immunology is characterized by different immune cells subsets, lymphocyte differentiation, and MHC molecule and cytokine expression (Mestas & Hughes 2004). The mechanisms occurring in mouse cells and human cells are quite different. Thus, in mouse models, the efficacy of human cell therapies cannot be established. The possibility to use humanized mouse model of different diseases arose from the identification of *scid* (severe combined immunodeficiency) mutation in CB17 mice by Bosma in 1983. In the following years, *scid* mutation was further investigated by different groups leading to the discovery that *scid* gene encodes a trans-acting factor involved in Ig gene rearrangement re-joining event and that the mutation of this gene leads to a failure in V(D)J rearrangement, causing mature T and B cell lack (Schuler et al. 1986; Hendrickson et al. 1988; Lieber et al. 1988). In 1995, *PRKDC* (protein kinase, DNA activated, catalytic peptide) gene was identified as the human homologous of the mouse SCID gene and *scid* mutation in PRKDC gene was found also in mice (Kirchgessner et al. 1995; Miller et al. 1995). CB17*scid* model was suitable for studies on the engraftment of human lymphocytes and hematopoiesis because of the lack of mature murine T and B cells. However, there were still two major

problems, i.e. the low rate of engraftment and proliferation of human lymphocytes (Mosier et al. 1988; Lapidot et al. 1992) and the age-related development of some functional T and B cell clones (Bosma et al. 1988; Nonoyama et al. 1993). The persistence of innate immune system was responsible for the scarce human cell engraftment in *scid* model; consequently, new strains with deficiency in innate immune system were obtained. In 1980, Makino et al. discovered NOD (non-obese diabetic) mouse strain. This strain spontaneously develops diabetes associated with a rapid weight loss (Makino et al. 1980). Thanks to the development of a stable NOD strain, its immunological background was studied. NOD mice strain displayed some immunity deficiencies, including a decrease of T cell number and, even more interestingly, a reduction in NK cells activity (Kataoka et al. 1983). These findings allowed to introduce *scid* mutation into NOD background to obtain an improved model for the study of human diseases. Thus, the NOD-scid mouse was developed by coupling CB17*scid* mice and NOD mice (Shultz et al. 1995). This strain is characterized by a lack of mature T and B cells, innate immunity defects, including NK cell activity deficiency, and a diabetes-free condition. Compared to CB17*scid* mice, only about 10% of NOD-scid mice showed autologous lymphocyte recovery after 6 months (Shultz et al. 1995); moreover, a higher engraftment of human lymphocytes was observed (up to 10 fold) (Greiner et al. 1998). NOD-SCID model had been considered the best model for the study of human diseases for several years. However, this model showed some pitfalls, such as the short lifespan (8 months) due to thymic lymphomas occurring in about 70% of mice, and the low engraftment of human lymphocytes probably due to residual NK cell activity (Prochazka et al. 1992; Shultz et al. 1995; Greiner et al. 1998). In 2002 NOD-scid IL-2rγnull mouse model was developed (Ito et al. 2002) obtained thanks to a mutation in the interleukin-2 receptor gamma chain (IL-2R gamma) discovered in X-linked severe combined immunodeficiency in humans (Noguchi et al. 1993). As IL-2R gamma chain is a common component of receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 and these interleukins are involved in T, B and NK cell development, the loss-of-function mutation in this gene leads to an impairment of lymphocyte development, especially T and NK cells (DiSanto et al. 1995; Sugamura et al.

1996; Kovanen & Leonard 2004). Thus, the introduction of IL-2rγnull mutation in NOD-scid mice led to a humanized mouse model devoid of T, B and NK cells. In 2005, the administration of cord blood hCD34^{+} purified cells in NOD-scid IL-2rγnull mice led to the development of a complete human immune system, demonstrating the suitability of this model for studying human immune system (Ishikawa et al. 2005). In the last few years, several laboratories have developed different immune deficient mouse strains based on IL-2rγ mutation. These models differ for mouse background and/or type of IL-2rγ mutation. In NOD-scid background there are two distinct humanized models according to IL-2rγ mutation, i.e. NSG and the NOG mouse models. NOG mice express a truncated/inactive form of the γ chain, whereas NSG mice do not express any γ chain (Koboziev et al. 2015); both are the most suitable humanized mouse models to perform pre-clinical studies for the treatment of GvHD (Ito et al. 2009; King et al. 2009; Ali et al. 2012).

1.4 HUMAN PLATELT-LYSATE AND FETAL BOVINE SERUM AS CULTURE SUPPLEMENT

Human Platelet Lysate (hPL) vs. Fetal Bovine Serum (FBS)

The use of animal serum-supplemented media for cell culture is a debated issue. Fetal bovine serum (FBS) is still the most common culture supplement worldwide since it was introduced for the first time in 1958. However, several concerns are associated to the use of FBS for scientific purpose. The ethical issue has the greatest impact, as FBS is obtained from blood of calf fetuses collected by syringing their hearts without any form of anesthesia, after slaughtering the pregnant cows (Jochems et al. 2002). Some procedural safeguards were adopted to avoid the risk of extracting blood from vital fetus (Van Der Valk et al. 2004), but they are not followed by some producers due to commercial interests. In addition, to satisfy the high FBS request, some producers may sophisticate serum composition by adding other substances, such as growth-promoting additives, bovine serum albumin and water (Van Der Valk et al. 2017). Two cases of FBS abuse or alteration were reported. The first case occurred in 1994, when about 30,000 liters of New Zealand serum were sold despite the annual production of FBS was of about 15,000 liters (Hodgson 1995). The second was reported in 2013, when the U.S. Food and Drug Administration (FDA) discovered approximately 280,000 liters of adulterated FBS (Gstraunthaler et al. 2014). For all these reasons, FBS composition is not always fully known and some major changes can be observed within different batches (Baker 2016). This content variability determines significant differences in cell culturing, thus hampering the definition of standardized protocols and affecting reliability of scientific data. FBS-mediated suppression of glycosaminoglycan and type II collagen production has been described in fibroblast-like-type-B synoviocytes, leading to the inhibition of TGF-β1-dependent chondrogenesis (Bilgen et al. 2007). In addition, human MSC cultured in FBS have a more differentiated transcription profile compared to MSC cultured in autologous human serum (Shahdadfar et al. 2005), as well as being a possible vehicle for mycoplasma, viral contaminant, endotoxins or prions and therefore representing a major problem for both *in vitro* cell

culturing and *in vivo* pre-clinical and clinical studies. In particular, the risk of unknown infections or adverse reactions due to FBS components is a significant concern for clinical application. Thus, xeno-free supplement for cell culture is more and more required in clinical trials to improve patient's safety.

Human platelet lysate (hPL) represents a potential candidate to replace FBS as supplement for cell culture. hPL is a blood product deriving from platelet concentrates (PC) that are typically used for transfusion purposes and are clinically tested for safety by specialized blood donation centers. Even though the ethical issue on animal use is completely fixed, the use of PC for cell culturing instead of human transfusion may rise some ethical concerns as well. However, PC are no more suitable for transfusion after 4-5 day at 22±2°C, so they can be used for hPL preparation. As 50-60% of PC stored units expire and have to be discarded, hPL could be easily available (Astori et al. 2016). An additional issue is represented by potential bacterial contamination of PC due to the storage at room temperature, in addition to the presence of blood-borne pathogens, such as viruses. Thanks to the modern viral diagnostic assays, the risk of most common virus transmission (i.e. HIV, HCV and HBV) is dramatically reduced (Busch et al. 2005; Dodd 2007; Zou et al. 2012), even though other emerging viruses might be not detected. To this aim, different methods for viral inactivation of PC can be used. The first one is based on a photo-activation process leading to viral nucleic acid strand breakage (Ruane et al. 2004) or transcription and replication blockade (Klein 2005). The second method employs a solvent/detergent treatment that acts by destroying viruses envelop (Horowitz et al. 1992). As for bacteria and parasite contamination, they can be more easily removed during platelet lysate (PL) production by freeze-thaw step or by using 0,2 μm pore filters just before its addition to the culture medium.

In 2005, the use of hPL for human MSC culture was suggested for the first time (Doucet et al. 2005). hPL is a supplement containing a wide variety of growth factors, i.e. PDGF-AA, -AB and -BB, TGF-β1 and -β2, EGF, VEGF, b-FGF, BDNF, and HGF (Shih & Burnouf 2015). TGF-β 1, 2 and 3 induce MSC proliferation and chondrogenic differentiation (van der Kraan et al. 2009; Ogawa et al. 2010; Weiss et al. 2010). Similarly, BMP-2, -4, -6 and -7 induce MSC

osteogenic differentiation *in vitro* and *in vivo* (Lou et al. 1999; Luu et al. 2007). In addition, FGF, EGF and VEGF promote MSC expansion and survival (Pons et al. 2008). Although hPL is better characterized and enriched in growth factors as compared to FBS, its content differs in terms of cytokine levels amongst donors; consequently, hPL has the same concerns of FBS in terms of data reproducibility. However, this problem can be minimized by pooling PC from different donors, thus lowering batch-to-batch content variability (Fekete et al. 2012). Moreover, the presence of dedicated facilities for PC production and a broad characterization of hPL can help to standardize the methods for hPL production. Thus, hPL seems to be a suitable substitute of FBS, in terms of reproducibility and safety, for *ex vivo* MSC production for clinical purposes.

2 RATIONAL HYPOTHESIS AND AIMS

The development of new treatments for steroid-refractory aGvHD represents one of the most important challenge to improve the efficacy of Allo-HSCT. MSC therapy is an emerging treatment for aGvHD, but safe and reproducible *ex vivo* MSC expansion protocols are required. Several MSC-based clinical studies have been performed, leading to controversial results due to the lack of standardized methods. Consequently, to define a reproducible MSC-based therapeutic protocol for aGvHD, further pre-clinical *in vitro* and *in vivo* studies are required.

In this study, we have tried to reach several goals:

- Developing an efficient human bone marrow MSC (hBM-MSC) *ex vivo* expansion protocol for clinical application, comparing FBS with hPL as culture supplement:
- Demonstrating hBM-MSC phenotype and genome stability following *ex vivo* expansion in hPL;
- Confirming the maintenance of hBM-MSC immunological properties following *ex vivo* expansion in hPL;
- Developing a reproducible aGvHD xenogeneic mouse model suitable for pre-clinical studies;
- Defining a hPL-expanded hBM-MSC-based immunotherapy for aGvHD.

3 MATERIALS AND METHODS

3.1 HUMAN PLATELET LYSATE (HPL) PRODUCTION AND QUALITY CONTROL

Platelet Rich Plasma (PRP) pool obtained from 15 healthy donors was stored at -80°C for 16 hours. Afterwards, it was thawed in a water bath at 37°C for 2 hours to perform platelet lysis by thermal shock. hPL was obtained with two consecutive centrifugations at 4000 g for 20' followed by a filtration with a 0,65 μm filtration system (Macopharma). hPL obtained was stored at -20°C until use. All the steps were performed maintaining sterile condition. Levels of PDGF-AB and TGF-β were evaluated by ELISA assay following manufacturer's instruction (R&D System) (Laboratorio di Terapie Cellulari Avanzate, Vicenza).

3.2 CELL ISOLATION AND CULTURE

3.2.1 Human Bone Marrow-Derived Mesenchymal Stromal Cells (hBM-MSC)

Human BM-MSC from 5 healthy donors were isolated and expanded, in parallel, with two different culture media: FBS-supplemented medium and hPL-supplemented medium. The procedure of hBM-MSC isolation and expansion is illustrated below (**Figure 9**). Briefly, bone marrow samples from healthy donors were seeded in 5-layer flask (875 cm^2) at mononuclear cells (MNC) density of 50.000/cm^2 using αMEM 5% hPL (hPL-BM-MSC) or αMEM 10% FBS (FBS-BM-MSC) media. After 72 hours, cells were washed with PBS 1X and culture medium was replaced. Once confluence was reached, at day 12 ± 2, cells were harvested using Tryple Select 1X (Gibco) and re-seeded at a cellular density of 4.000/cm^2 in αMEM 8% hPL or αMEM 10% FBS. Afterwards, culture medium was replaced once (day 15 ± 2) until day 19 ± 2, when cells were harvested and frozen at -80°C using the appropriate freezing solution: 10% Human Albumin + 10% DMSO + NaCl or 90% FBS + 10% DMSO for hPL-hBM-MSC and FBS-hBM-MSC, respectively. After 24 hours, cells were transferred in liquid nitrogen for long term storage. At the end of P0 (day 12 ± 2) and P1 (day 19 ± 2), cellular

phenotype was evaluated by flow cytometry and Population Doubling (PD) was calculated, both in hPL and FBS expansion conditions. For *in vitro* and *in vivo* experiments hPL-BM-MSC and FBS-BM-MSC were thawed in a heated water bath set at 37°C; subsequently a pre-warmed thawing solution (10% Human Albumin + 0,6% ACD-A + NaCl or 40% RPMI + 60% FBS respectively) was slowly added to cell suspension. Finally, cells were centrifuged at 400 g for 5' at room temperature and resuspended in the respective culture medium. hBM-MSC were used in the experiments from P2 to P5.

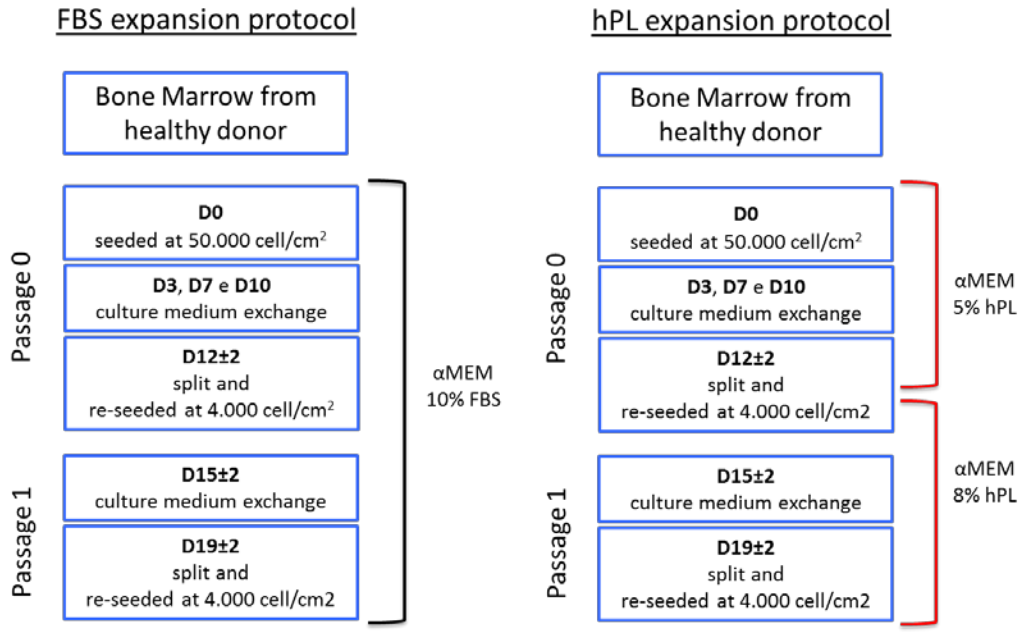

Figure 9. FBS and HPL-expansion protocol for hBM-MSC. Schematic representation of the expansion protocols used for *in vitro* hBM-MSC expansion.

3.2.2 Peripheral Blood Mononuclear Cells And T, B, NK cell Isolation

Peripheral Blood Mononuclear Cells (PBMC) were obtained from buffy coat through density gradient stratification: peripheral blood was diluted 1:2 with sterile PBS 1X and then it was carefully layered on to the top of the Lymphoprep™ solution (StemCell Technologies) with a ratio of 4:3 (diluted blood: Lymphoprep™) in a 50 ml tube. Samples were centrifuged at 800 g for 30' at room temperature in order to stratify the blood. After that, the middle white ring (PBMC) was collected in a new 50 ml tube and washed with PBS 1X. PBMC were washed two more time with PBS 1X by centrifugation at 400 g for 5' at room temperature. In some experiments, sorted T, B or NK cells were used, so a negative selection using immune-magnetic beads was performed according to the manufacturer's instructions (MACS cell separation columns and T, B, NK isolation kit Miltenyi Biotec). Before immunomagnetic separation, PBMC lymphocyte populations were characterized by cytofluorimetric analysis using anti-CD3-FITC, anti-CD16/56-PE, anti-CD45-PerCP, anti-CD19-APC, anti-CD4-APC-H7 and anti-CD8-PECy7 (BD-bioscience). After the separation, sorted lymphocytes were assessed for purity using anti-CD3-FITC, anti-CD16/56-PE, anti-CD45-PerCP anti-CD19-APC, anti-CD4-APCH7 and anti-CD8-PECy7 (BD-bioscience) and viability using TO-PRO-3-iodide (Life Technologies). PBMC and purified immune effector cells were frozen at -80°C in a freezing solution (90% FBS + 10% DMSO) and after 24 hours they were transferred in liquid nitrogen for long-term storage. For *in vitro* and *in vivo* experiments, PBMC and purified immune effector cells were thawed in a heated water bath set at 37°C, subsequently a thawing solution (40% RPMI + 60% FBS) was slowly added to the cells. Finally, cells were centrifuged at 400 g for 5' at room temperature and re-suspended in the respective culture medium.

3.2.3 Cell Culture Media - Summary

Cell Type	Culture Medium Components
hPL-hBM-MSC	Minimum Essential Medium Eagle α-modification (αMEM) Human platelet lysate (5% or 8% v/v) Pen/Strep (1% v/v) L-Glutamine (1% v/v) Heparin (3UI/ml)
FBS-hBM-MSC	Minimum Essential Medium Eagle α-modification (αMEM) Heat Inactivated FBS (10% v/v) Pen/Strep (1% v/v) L-Glutamine (1% v/v)
PBMC	RPMI 1640 Heat Inactivated FBS (10% v/v) Pen/Strep (1% v/v) L-Glutamine (1% v/v)
T cells	RPMI 1640 Human Serum (10% v/v) Pen/Strep (1% v/v) L-Glutamine (1% v/v)
B cells	RPMI 1640 Heat Inactivated FBS (10% v/v) Pen/Strep (1% v/v) L-Glutamine (1% v/v
NK cells	Iscove's Modified Dulbecco's Media (IMDM) Human Serum (10% v/v) Pen/Strep (1% v/v) L-Glutamine (1% v/v)

3.2.4 Freezing and Thawing Solutions - Summary

Cell Type	Freezing Solution	Thawing Solution
hPL-hBM-MSC	NaCl solution (0,9 w/v) Human Albumin 200g/L (10% v/v) DMSO (10% v/v)	NaCl solution (0,9 w/v) Human Albumin 200g/L (10% v/v) ACD-A (0,6% v/v)
FBS-hBM-MSC	FBS (90% v/v) DMSO (10% v/v)	FBS (60% v/v) MEM α-modification (40% v/v)
PBMC T cells B cells NK cells	FBS (90% v/v) DMSO (10% v/v)	FBS (60% v/v) RPMI 1640 (40% v/v)

3.3 MESENCHYMAL STROMAL CELL CHARACTERIZATION

3.3.1 Total Cell Count, Population Doubling and Clonogenic Assay

The total amount of hPL- or FBS-hBM-MSC obtained was evaluated at the end of the expansion protocol (day 19 ± 2). The clonogenic ability of hPL- and FBS-hBM-MSC was evaluated culturing 100.000 cells at P0 for 14 days in T25 flask. Cumulative population doubling (cPD) was obtained by adding the population doubling calculated at the end of P0 and at the end of P1. PD was calculated using the following formula:

$$\boldsymbol{PD} = \frac{(\,log10\ n°\ of\ cells\ at\ the\ end\ of\ passage\ -\ log10\ n°\ of\ seeded\ cells)}{log10^2}$$

3.3.2 MSC Integrity

3.3.2.1 MSC Karyotypic Analysis

hBM-MSC at P3 were seeded in amnio-dishes (Euroclone) at cellular density of 1200/cm^2 and incubated at 37°C, 5% CO_2 for 24 hours. To block cells in metaphase, MSC were treated overnight with colcemid (10 µg/ml). Afterwards, cells were treated with a hypotonic solution (KCl 0.075M), fixed and stained with a solution of Quinacrine (100mg) in McIlvaine buffer (100ml) for Q-Fluorescence-Quinacrine (QFQ) banding. Karyotype was analyzed by fluorescence microscopy by two genetists (Struttura Semplice Genetica Medica, AOUI, Verona).

3.3.2.2 Real-Time Polymerase Chain Reaction (PCR)

Poly(A) RNAs were isolated and reverse-transcripted from hBM-MSC using MultiMACS M96thermo Separator (Miltenyi Biotec) and MultiMACS cDNA synthesis kit (Miltenyi Biotec) following the manufacturer's instructions. Then, 10 ng of cDNA were analyzed by real-time PCR in a final volume of 25 µl using PowerUp SYBR Green Master Mix (Thermo Scientific) containing primers for

the amplification of P53, P21, c-myc and hTERT (0,2 μM). β-2M was used as housekeeping control (see all primers sequences in **Table 2**). PCR conditions were set as follow: 2' at 50°C, 2' at 95°C, followed by 50 cycles of 15 seconds at 95°C and 15 seconds at 60°C. Real-time PCR assays were run on Cobas Z480 instrument (Roche). Relative gene expression was calculated by the ΔCT method and normalized to housekeeping control genes. Absolute quantification was calculated by the ΔΔCT method.

PCR Reaction Mix	Final Concentration	Volume
Master mix (2X)	1X	12,5 μl
FWD primers (10μM)	0,2 μM	0,5 μl
REV primers (10μM)	0,2 μM	0,5 μl
cDNA (10ng)	5 ng/μl	2 μl
H_2O		9,5 μl
		25 μl

Table 1. qRT-PCR reaction mix content.

p53	**FWD** GTCTGGGCTTCTTGCATTCT
	REV AATCAACCCACAGCTGCAC
p21	**FWD** CTGGAGACTCTCAGGGTCGAAAA
	REV TGTAGAGCGGGCCTTTGAGG
c-Myc	**FWD** CTCCTGGCAAAAGGTCAGAG
	REV TCGGTTGTTGCTGATCTGTC
hTERT	**FWD** CAGGCTCTTTTTCTACCGGAAGA
	REV AGTGCTGTCTGATTCCAATGCTT
β-2M	**FWD** TCTCGCTCCGTGGCCTTA
	REV AATCTTTGGAGTACGCTGGATAGC

Table 2. Summary of FWD and REV primers used in qRT-PCR.

3.3.3 MSC Immunophenotyping

MSC identity was checked according to the ISCT guidelines. Briefly, hBM-MSC were detached using Tryple Select 1X (Gibco) and washed with PBS. At least $1x10^5$ cells for each condition were labeled for 15' at room temperature in the dark with fluorescent monoclonal antibodies or isotype control for endothelial and hematopoietic markers (IgG1κ-PE, CD14-PE, CD31-PE, CD34-PE and CD45-PE, BD-bioscience), immunological markers (HLA-ABC-PE and HLA-DR-PE, BD-

bioscience) and MSC markers (CD73-PE, CD90-PE and CD105-PE, BD-bioscience). To select only viable cells, the viability marker TO-PRO-3-iodide (Life Technologies) was added in all tubes. After incubation, cells were washed with PBS, centrifuged at 400 g for 5' and analyzed by flow cytometry (BD FACSCanto™ II). Data collected were analyzed with FlowJo software.

3.3.4 MSC Differentiation Assays

3.3.4.1 Osteogenic Differentiation

At the end of the expansion protocol, 50.000 cells/well of hPL- or FBS-BM-MSC were seeded in a 24-well plates in αMEM 2% FBS + 1% Glutamine. After 24 hours, when 70% confluence was reached, the culture medium was removed and the StemMACS OsteoDiff Media (Miltenyi Biotec) was added. Until day 15, culture medium was replaced every 3-4 days. To assess the osteogenic differentiation, cells were stained with Alizarin Red. Briefly, cells were washed twice with PBS and then fixed with PFA 4% for 5' at room temperature. Afterwards, cells were washed with H_2O deionized and stained with Alizarin Red 2% for 5' at room temperature. Finally, calcium accumulation was evaluated by microscopy.

3.3.4.2 Adipogenic Differentiation

At the end of the expansion protocol, 50.000 cells/well of hPL- or FBS-hBM-MSC were seeded in a 24-well plates in αMEM 10% FBS + 1% Glutamine. After 24 hours, when 70% confluence was reached, the culture medium was removed and a medium containing a-MEM 10% FBS + 1% Glutamine + IBMX 100 μg/ml + Dexamethasone DXM 1μM + Humulin R 10 μg/ml was added. Until day 15, culture medium was replaced every 3-4 days. To assess the adipogenic differentiation, cells were stained with Oil-Red-O. Briefly, cells were fixed with isopropanol 60% for 1' at room temperature. Afterwards, cells were stained with Oil-Red-O for 10' at room temperature. Lastly, cells were washed with isopropanol 60% and the presence of lipidic vacuoli was evaluated by microscopy.

3.4 MSC IMMUNOLOGICAL ASSAYS

3.4.1 MSC Inflammatory Immunophenotyping

hBM-MSC were stimulated (primed) or not (resting) with pro-inflammatory cytokines TNF-α (15 ng/ml) and IFN-γ (10 ng/ml) (R&D Systems) for 48 hours. Cells were detached and washed with PBS by centrifugation at 400 g for 5'. At least $1x10^5$ hBM-MSC per condition were re-suspended with PBS in 5 ml polystyrene round-bottom tubes. Then, cells were stained for 15' at room temperature with isotype controls (IgG1κ-PE, BD bioscience), (IgG1κ-PE, IgG2b-PE and IgG2a-APC, Biolegend) or with the following monoclonal antibodies: CD54-PE, CD80-PE, CD86-PE, CD-106 PE, HLA-ABC-PE, HLA-DR-PE (BD-bioscience), CD273-APC, CD-274-PE, CD279-PE (Biolegend). To select only viable cells during the analysis, the viability marker TO-PRO-3-iodide (Life Technologies) or Propidium Iodide (PI) was added to the tubes. To evaluate the viability of MSC, cells were stained with Annexin V-FITC and PI. After incubation, cells were washed with PBS, centrifuged at 400 g for 5' and analyzed by flow cytometry (BD FACSCanto™ II). Data collected were analyzed with FlowJo software.

3.4.2 MSC Immunogenicity

The ability of MSC to elude the innate immune system was checked by cytotoxicity assay according to the manufacturer's instructions (DELFIA® cell cytotoxicity kit, Perkin Elmer) using NK cells as effectors. Briefly, $1x10^6$ NK cells/well were seeded and activated in a 24-well plate with rhIL-2 (100U/ml) for 48 hours at 37°C. At day +2, hBM-MSC were labeled with BATDA for 30' at 37°C and then washed 3 times with PBS+HEPES 20 mM by centrifugation at 300 g for 10'. Activated NK cells were co-cultured with BATDA-labeled MSC target cells in a V-bottom 96-well plate at the Effector (NK):Target (MSC) ratios of 1:1, 5:1, 15:1, 25:1 at 37°C for 3h. After the incubation, 20 µl of supernatant were transferred in a flat-bottom 96 well plate and 200 µl of Europium solution were added. After 15' of incubation in the dark, fluorescence was measured at 615 nm

using VICTOR™X4. The specific MSC lysis was calculated with the following formula:

$$\textbf{\textit{Specific Lysis}}\ (\textbf{\textit{SL}}) = \frac{Experimental\ release\ (counts)\ -\ Spontaneous\ release\ (counts)}{Maximum\ release\ (counts)\ -\ Spontaneous\ release\ (counts)}\ x\ 100$$

3.4.3 CFSE Proliferation Assay

To assess the ability of hBM-MSC to modulate the proliferation of Immune Effector Cells (IECs), co-cultures with purified lymphocytes were performed. IECs were labeled with carboxyfluorescein-succinimidyl-ester 5 µM (CFSE, Life Technologies) in pre-warmed PBS-BSA 0.1% and activated with the proper stimuli (see Table 3). Afterwards, the co-cultures with resting or primed MSC with purified T, B or NK cells at different MSC:IECs ratio (1:10, 1:1 and 1:1 respectively) were set up. In some experiments, to assess the involvement of MSC soluble factors, the co-cultures were performed with Transwell® plate system with a 0.4 µm pore size membrane (Corning) at the MSC:IECs ratio of 1:5 and 1:10. The IECs proliferation was evaluated after 4 days (for B cells) or 6 days (for T and NK cells) by flow cytometry FACSCanto™II (BD bioscience). The percentage of relative proliferation was calculated using the following formula:

$$\%\ Rel.\ Pr. = 100 + \frac{[(\text{GeoMean CFSE Sample} - \text{GeoMean CFSE not stimulated IECs}) - (\text{GeoMean CFSE stimulated IECs} - \text{GeoMean CFSE not stimulated IECs})]}{(\text{GeoMean CFSE stimulated IECs} - \text{GeoMean CFSE not stimulated IECs})}\ x\ 100$$

IECs	Stimuli	Final Concentration	
T	**anti-CD3**	0.5 µg/ml	PeliCluster
	anti-CD28	0.5 µg/ml	PeliCluster
B	**CPG ODN**	2.5 µg/ml	InvivoGen
	CD40L	50 ng/ml	R&D systems
	MAB 50	5 µg/ml	R&D systems
	IL-2	20 U/ml	Miltenyi Biotec
	FAB (IgG,IgM, IgA)	2 µg/ml	Jackson Immunoresearch
NK	**IL-2**	100 U/ml	Miltenyi Biotec

Table 3. Summary of lymphocytes activating stimuli used in proliferation assays.

3.5 *IN VIVO* EXPERIMENTS

3.5.1 Mice

NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug}/JicTac (NOG) from 8- to 12-week-old female mice were purchase from Taconic Biosciences and housed in CIRSAL animal facility, University of Verona. Ethical approval for all work was granted from Italian Ministry of Health.

3.5.2 aGvHD Humanized Mouse Model

NOG mice were irradiated with a total dose of 1.2 Gy (TBI) as conditioning regimen before the intravenous injection of 1x10^6 cells/g of thawed human PBMC or PBS in control mice. All mice were monitored until the end of the experiments (30 days) evaluating the onset of aGvHD clinical symptoms (**Table 4**) and the mouse weight loss. Mice were ethically sacrificed when the loss of weight was over of 20% or at the cumulative clinical score of 6, or at the end of the experiment.

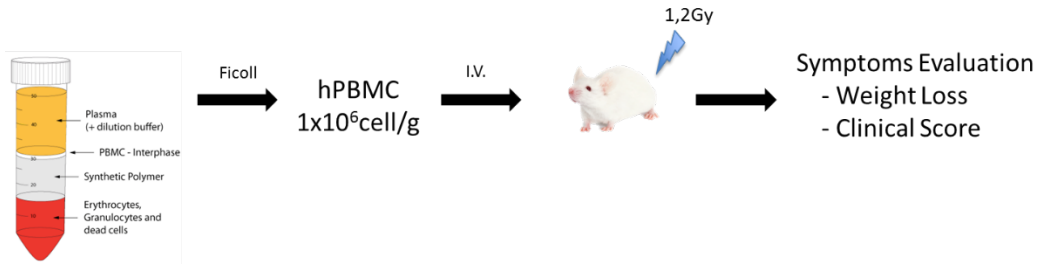

Figure 10. Experimental plan for aGvHD induction and evaluation in NOG mice.

Clinical Score	0	0.5	1	1.5	2
Posture	Normal	Slight hunching	Moderate hunching but correct movement	Strong hunching and slightly impaired movement	Strong hunching and distinct impaired movement
Activity	Normal	Less movement than normal, more easier to catch	Very little movement	Animal stay still but will move when touched	Animal has no activity also when touched
Fur	Normal	Slight ruffling on the neck	Slight ruffling on the neck, belly and back	Moderate ruffling all over the body	Matted Fur and color changing (yellowing)

Table 4. Clinical scoring system used for aGvHD onset evaluation in NOG mice.

3.5.3 aGvHD Evaluation

The disease was evaluated at cellular level assessing the percentage of circulating human CD45+ cells and human T cells in different target organs by flow cytometry. Moreover, tissues damaging and T cell infiltration were evaluated by immunohistochemistry and histological analysis.

3.5.3.1 aGvHD Flow Cytometric Analysis

Lung, liver, spleen and kidney were collected in gentleMACS tubes (Miltenyi Biotec) with PBS EDTA 1 mM and organs dissociation was obtained using gentleMACS Dissociator (Miltenyi Biotec). Bone marrow was collected washing mice femurs and tibiae with a 1 ml sterile syringe (26 G x 1/2" 0.45 x 12.7mm) containing PBS. Single cell suspension was obtained after dissociation by filtration with 40 μm cell strainer. Lastly, cells were counted and washed with PBS by centrifugation at 400 g for 5'. Peripheral blood was collected from mice retro-orbital vein in collection tube with heparin; $5x10^5$ cells in 100 μl of blood or cell suspensions were transferred in 5 ml round bottom tube, stained and incubated for 15' in the dark at room temperature with the following antibodies: anti-CD3-FITC, anti-CD56-PE, mouse anti-CD45-PerCP-Vio700 (Miltenyi Biotec), anti-CD19-APC, anti-CD4-APCH7, anti-CD8-PECy7, anti-HLA-DR-

V450, anti-CD45-V500 (BD-bioscience). Afterwards, 2 ml of lysis solution 1X (Stock 10X: NH_4Cl 44.3g, $KHCO_3$ 5g, 0.3g EDTA in dH_2O) were added to the tubes and incubated for 20' to eliminate red blood cells. Finally, cells were washed with PBS by centrifugation at 400 g for 5', re-suspended and analyzed by flow cytometry FACSCanto^{TM}II (BD bioscience).

3.5.3.2 aGvHD Histopathology and Immunohistochemistry

Liver, lung, spleen, kidney, intestine and skin were collected and fixed in formalin (Mondial). Samples were embedded in liquid paraffin at 58-60°C followed by a solidification step. Slices of tissues were obtained with the microtome and then samples were treated with xylene to eliminate the paraffin and with sequential immersions in ethylic alcohol (from 100% to 70%) to rehydrate tissues. Afterwards, tissues were stained with hematoxylin/eosin and anti-CD3 to evaluate tissue damages and T cell infiltration. Then, relying on the scores reported in table 5, two pathologists assigned the histopathological score. Images were taken using objective magnifications of 10X and 40X by optical microscope (Axio Observer Z.1, Zeiss) (Laboratorio di Anatomia e Istologia Patologica, AOUI, Verona).

Organs	Damage	Score	
LUNG SPLEEN KIDNEY	INFLAMMATION	0	No infiltration
		1	Sporadic or <5% infiltration
		2	Mild infiltration of 5%-25%
		3	Moderate infiltration of 25%-50%
		4	Severe infiltration of >60%
LIVER	PORTAL INFILTRATE	0	None
		1	Mild, some or all portal areas
		2	Moderate, some or all portal areas
		3	Moderate/marked, all portal areas
		4	Marked, all portal areas
	BILIARY DAMAGE	0	Absent
		1	Minimal
		2	Mild and diffuse
		3	Moderate
		4	Severe with new small bile duct present in all portal area
	CENTRILOBULAR VEIN ENDOTHELIITIS	0	Normal (occasional lymphocytes around portal triads are acceptable)
		1	Rare (1 to 2/0.5 cm) focal collections of mononuclear cells in parenchyma)
		2	Endotheliitis present in one vessel/0.5 cm (Sub-endothelial infiltrate of a depth at least 2 cells in 1 vessel)
		3	Endotheliitis present in >3 vessels/0.5 cm with the infiltrating depth >3 cells
		4	Endotheliitis as above present in virtually all vessels
	APOPTOSIS	0	Absent
		1	Minimal (< 2 foci 10x)
		2	Moderate (2-4 foci 10x)
		3	Severe (> 5 foci 10x)
SKIN	INFLAMMATION	0	None
		1	Focal infiltrates
		2	Widespread infiltrates
SMALL INTESTINE	INFLAMMATION	0	None
		1	Mild
		2	Moderate
		3	Severe without ulceration
		4	Severe, with ulceration

Table 5 Histopathological scoring system used to define aGvHD severity in mouse organs.

3.5.4 aGvHD Treatment with hBM-MSC

hPL-BM-MSC were cultured until passages 3 or 4, detached with Tryple, counted and re-suspended in PBS. aGvHD NOG mice were treated with intravenous injections of 1 or 2 x 10^6 cells/mouse of resting or primed hPL-BM-MSC, while control NOG mice were injected with PBS at different time point after the PBMC injection. All mice were monitored until the end of experiments (30 days) evaluating the onset of aGvHD clinical symptoms and their weight loss. The effect of treatments was evaluated as survival rate. Mice were ethically sacrificed when the loss of weight was over 20%, at the cumulative clinical score of 6, or at the end of the experiment.

3.6 STATISTICAL ANALYSIS

GraphPad Prism 5 software was used for statistical analysis. For nonparametric analysis, Mann-Whitney and Wilcoxon test were used. For parametric analysis, paired T test was used where applicable. Kaplan-Meier survival curves were established for each group and Mantel-Cox test was used. P-value is indicated when differences between two groups were statistically significant.

4 RESULTS

4.1 MSC CHARACTERIZATION

4.1.1 hPL ameliorates hBM-MSC expansion maintaining cell genome integrity

Bone marrow from 5 healthy donors was expanded with 2 different expansion protocols using either FBS- (standard) or hPL-supplemented culture media. Before use, hPL was tested by ELISA assay for the content of PDGF-AB and TGF-β1 (**Figure 11**). At the end of cell expansion (day 19 ± 2), a higher number of cells were obtained in all the 5 donors with hPL-based medium compared to the FBS-based medium (**Figure 12A**). This effect was explained by the clonogenicity assay (**Figure 12B**) that showed the ability of hPL-hBM-MSC to form more colonies, suggesting a better efficiency of hPL-based medium in enhancing the adhesion and expansion of hBM-MSC. Moreover, the evaluation of cumulative population doubling revealed that hPL-hBM-MSC had more division cycles compared to FBS-hBM-MSC (**Figure 12C**). To exclude neoplastic transformation of cells during expansion, hBM-MSC karyotype was checked. At passage 3, FBS- or hPL-hBM-MSC were analyzed after a treatment with colchicine, showing a normal karyotype in all cases (**Figure 13**). In addition, the expression of a number of oncogenic genes (c-Myc, hTERT), onco-suppressor genes (p53) and cell cycle regulator genes (p21) was evaluated between passage 0 and passage 1 by quantitative real time PCR. A significant difference in gene expression was found for p53, but not for p21 and c-Myc in FBS-hBM-MSC. Nevertheless, p53 was never found down-regulated, thus excluding a situation favoring neoplastic transformation. Of note, no significant differences were found in p21, p53 and c-Myc gene expression in hPL-hBM-MSC. In addition, hTERT expression was undetectable in all cases (**Figure 14**).

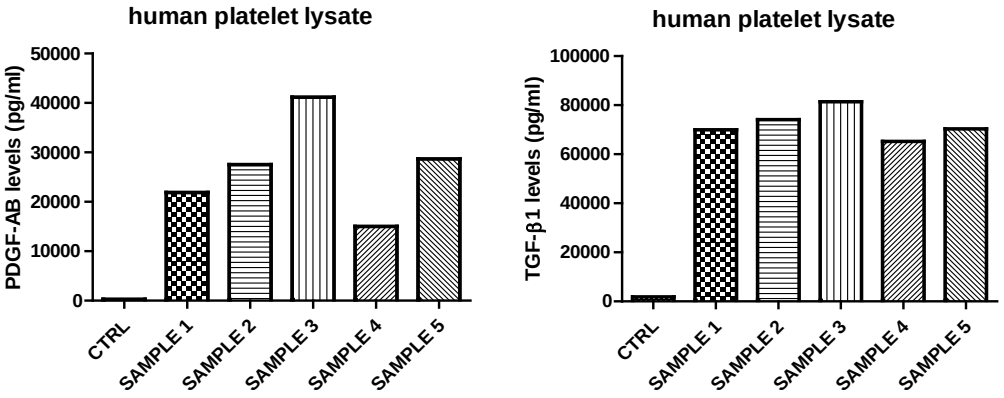

Figure 11. Growth factors levels in hPL. Five samples of different hPL pool were assessed for PDGF-AB and TGF-β content. A plasma pool sample was used as control.

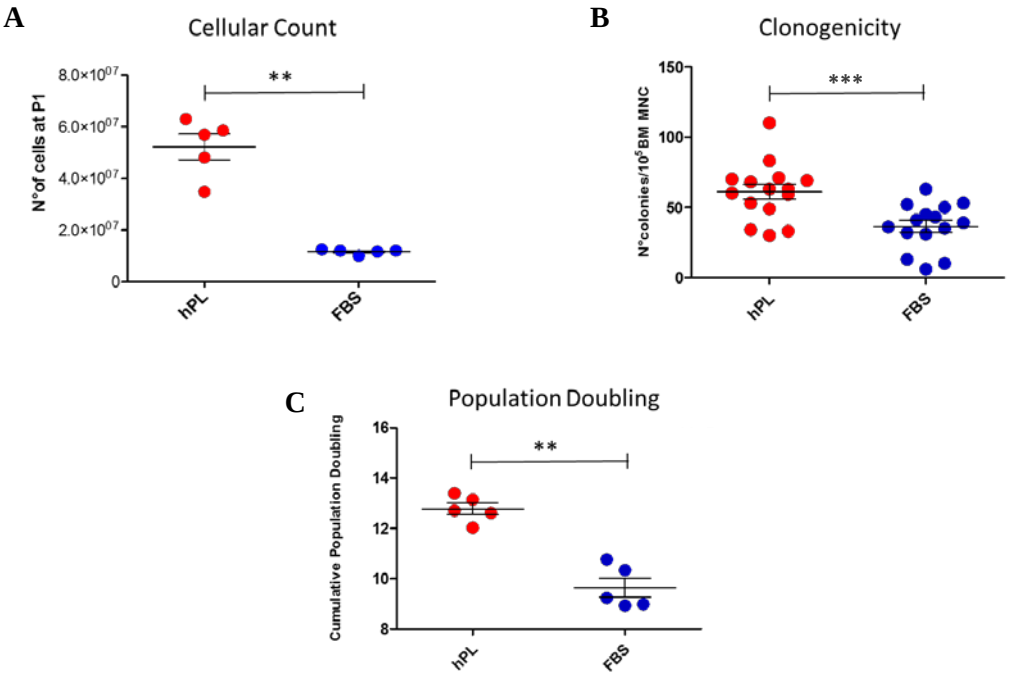

Figure 12. Evaluation of MSC expansion. A: The total amount of cells was evaluated at the end of expansion protocol (day 19 ± 2) in both hPL and FBS condition. Error bars represented mean ± SEM of five independent experiments. **B:** 100.000 hPL- or FBS-hBM-MSC were seeded at P0 in T25 flasks and the number of colonies was evaluated after 14 days. Error bars represented mean ± SEM of five independent experiments. **C:** The cumulative population doubling (cPD) was calculated adding the PD at the end of P0 and P1. Error bars represented mean ± SEM of five independent experiments. **A-C** Mann-Whitney test was used for statistical analysis ******P<0.005; **B** paired t-test was used for statistical analysis *******P<0.0001.

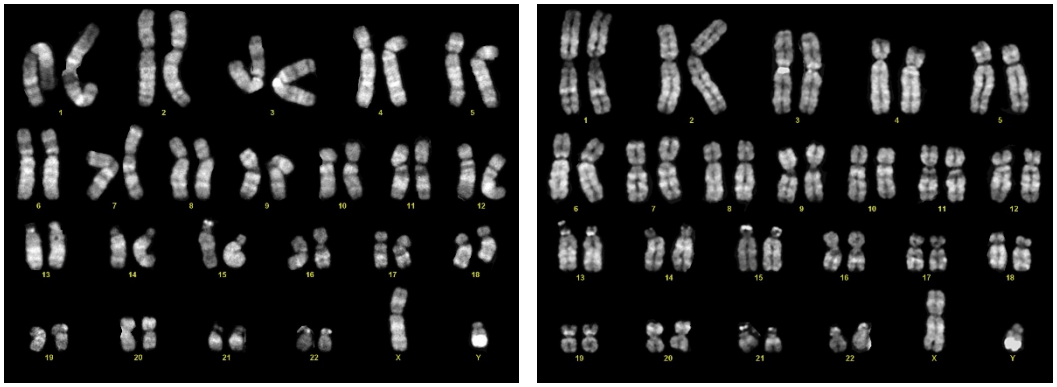

Figure 13. MSC Karyotype. Representative images of karyotype asset of hBM-MSC donor expanded in FBS (left) or hPL (right). In all 5 hBM-MSC samples, chromosomes number was evaluated analyzing at least 30 metaphases after treatment with colchicine for 16 hours.

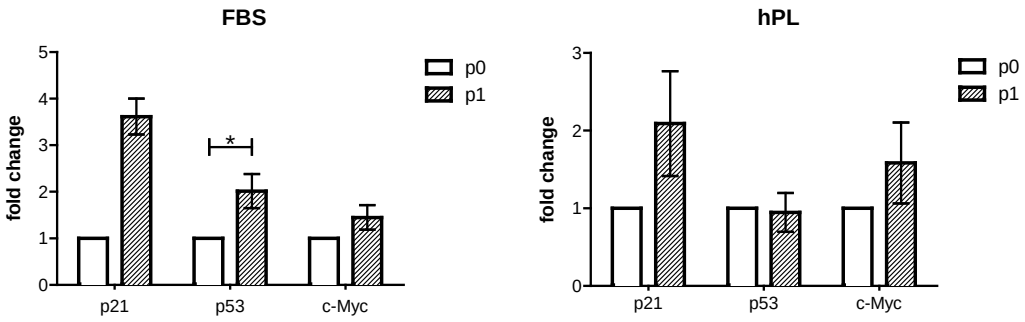

Figure 14. Oncogeneic and Onco-suppressor gene evaluations. The expression of p21, p53, c-Myc and hTERT was evaluated by Real Time PCR at the end of P0 and P1. Data are expressed as fold change of the relative expression at P1 on the relative expression at P0. Error bars represent mean ± SEM of six independent experiments. Wilcoxon test was used for statistical analysis *****P<0.05.

4.1.2 MSC identity is not affected by expansion protocol with hPL-supplemented medium

Cells were characterized according the ISCT guidelines. hPL- and FBS-hBM-MSC were detached at the end of expansion and checked for hematopoietic, immunological and mesenchymal markers by flow cytometry. The lack of expression of CD14, CD31, CD34, CD45 and HLA-DR and the expression of CD73, CD90, CD105 and HLA-ABC demonstrated the typical mesenchymal phenotype (**Figure 15**). Noteworthy, hPL-BM-MSC showed a significant higher expression of mesenchymal markers (except for CD105) and HLA-ABC (MHC-I). Furthermore, we also confirmed the mesodermal differentiation potential showing the ability of hBM-MSC to differentiate in adipocytes and osteoblasts (**Figure 16**).

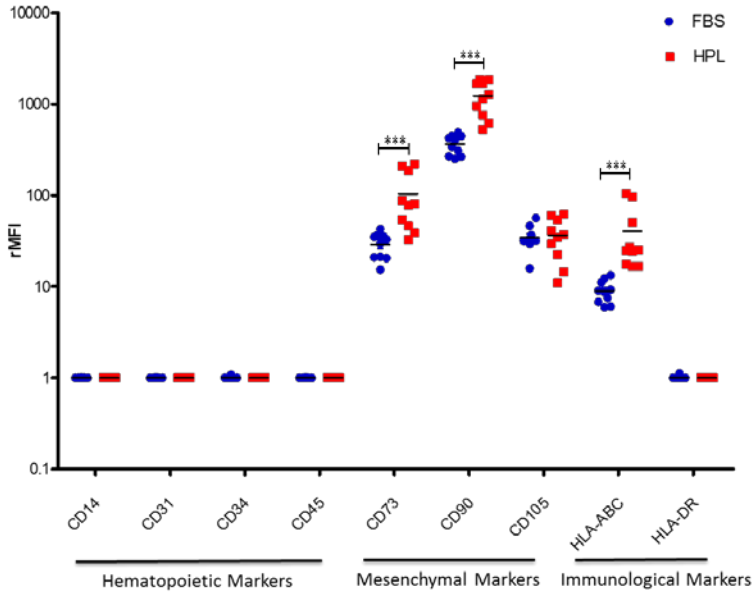

Figure 15. MSC immune phenotype. The expression of hematopoietic, immunological and mesenchymal markers was evaluated at the end of expansion (day 19 ± 2) by flow cytometry. Data are represented as relative median fluorescence intensity. Error bars represented mean ± SEM of ten independent experiments. Wilcoxon test was used for statistical analysis ***P≤0.001.

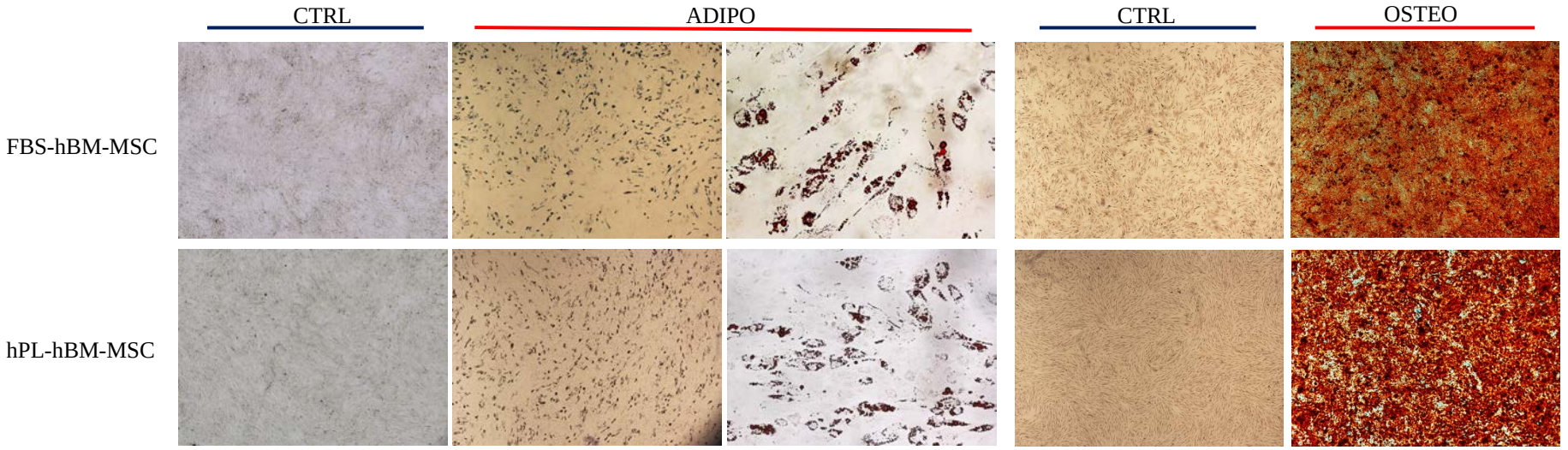

Figure 16. MSC differentiation. FBS- or hPL-hBM-MSC were seeded and cultured in standard culture medium (CTRL), in adipogenic medium (ADIPO) or in osteogenic medium (OSTEO) for 15 days. For adipocytic differentiation detection, CTRL cells and ADIPO cells were stained with Oil-Red-O. For osteoblastic differentiation detection, CTRL cells and OSTEO cells were stained with Alizarin Red. For CTRL, images were taken at 2.5x magnification. For ADIPO differentiation at 2.5x and 20x magnification, for OSTEO differentiation 2.5x of magnification.

4.2 MSC IMMUNOLOGICAL ASSAYS

4.2.1 hPL-hBM-MSC acquire the anti-inflammatory phenotype after treatment with IFN-γ and TNF-α

MSC can exert their anti-inflammatory activity once they undergo the "licensing" or "priming" process that occurs when high levels of pro-inflammatory cytokines, such as IFN-γ, TNF-α or IL-1, are present in the microenvironment. Therefore, to evaluate whether hPL-hBM-MSC could acquire the anti-inflammatory phenotype, as previously described for FBS-hBM-MSC, we treated cells with IFN-γ and TNF-α. Overexpression of different cell markers was evaluated by flow cytometry after 48 hours. The increase of the adhesion molecules CD54 (I-CAM) and CD106 (V-CAM), the anti-inflammatory molecules CD273 (PD-L2) and CD274 (PD-L1) and the immunological molecule HLA-DR (MHC-II) was observed in both cell types (**Figure 17**). Unexpectedly, hPL-hBM-MSC showed a higher expression of HLA-ABC (MHC-I) compared to FBS-hBM-MSC in primed condition. Moreover, similar MHC-I expression was observed in both resting hPL-hBM-MSC and primed FBS-hBM-MSC.

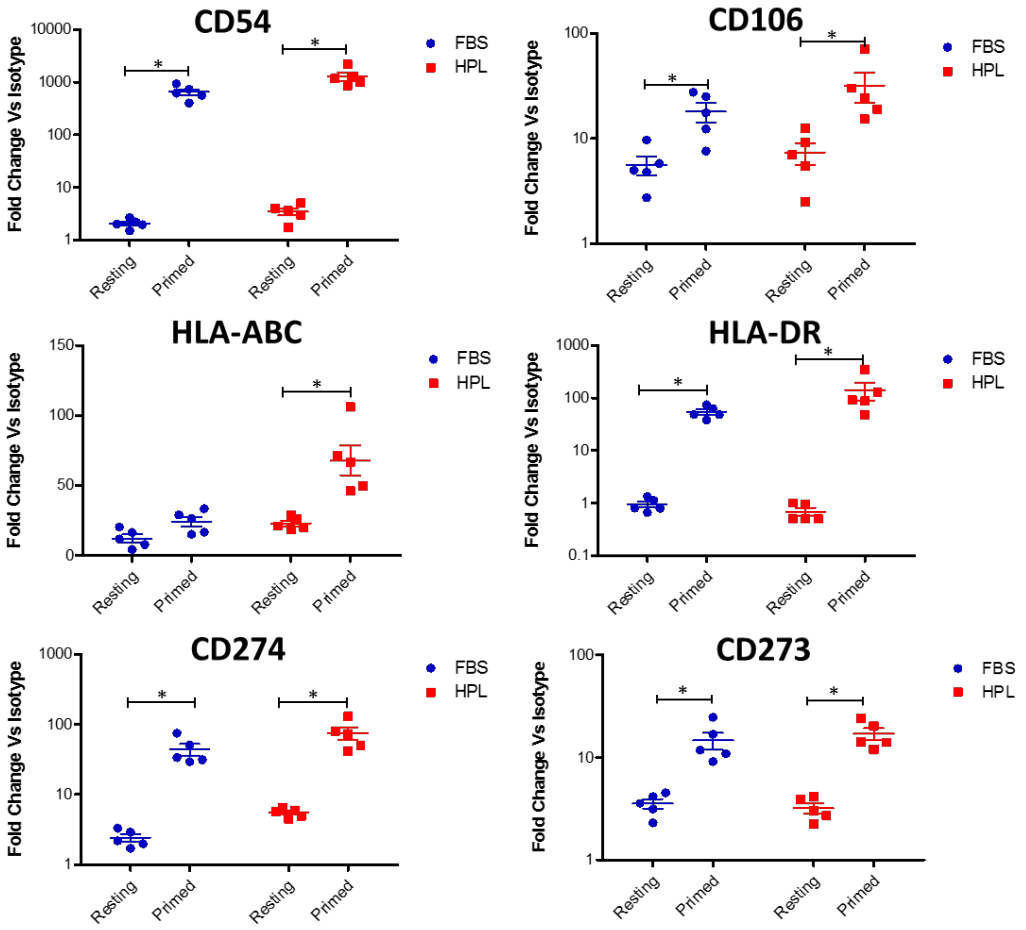

Figure 17. MSC priming. FBS-hBM-MSC and hPL-hBM-MSC were treated or not with IFN-γ (10 ng/ml) and TNF-α (15 ng/ml) for 48 hours. Evaluation of markers expression was performed by flow cytometry. Results are represented as fold change expression of specific marker compared to isotype control. Error bars represented mean ± SEM of 5 independent experiments. Wilcoxon test was used for statistical analysis P*<0.05

4.2.2 Primed-MSC are capable of eluding NK cell control

The innate immune system plays a role in the immune surveillance recognizing exogenous agents and activating the immune response. Cell immunogenicity is one of the main issues in cell therapy, since allogeneic cells could be recognized and destroyed by patient's immune system, especially NK cells, one of the main effector cells of the innate immune system. To measure this phenomenon, we used a NK cell-based cytotoxicity assay by co-culturing hBM-MSC with activated NK cells, thus finding that resting hBM-MSC had an intrinsic ability to resist to NK cell-mediated lysis. Pre-priming with IFN-γ and TNF-α further increased hBM-MSC refractoriness to NK cell-mediated lysis (**Figure 18A and 18B**). No significant differences were found comparing primed FBS-hBM-MSC and hPL-BM-MSC (**Figure 18D**). However, resting hPL-hBM-MSC showed a slightly higher ability to elude NK cell surveillance compared to resting FBS-hBM-MSC (**Figure 18C**).

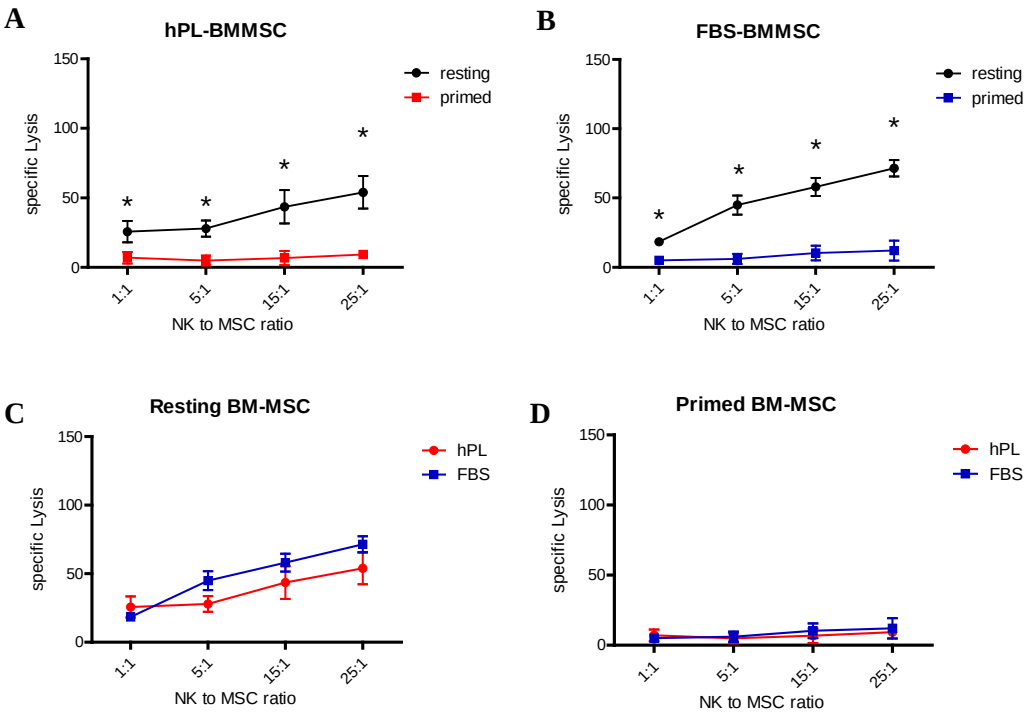

Figure 18. MSC immunogenicity assay. A-B: hPL-hBM-MSC and FBS-hBM-MSC were treated or not with IFN-γ (10 ng/ml) and TNF-α (15 ng/ml) for 48 hours and incubated with pre-stimulated NK cells (rhIL-2 100 U/ml) for 3 hours. **C:** comparison of resting hPL- and FBS-hBM-MSC. **D:** comparison of primed hPL- and FBS-hBM-MSC. Data are represented as NK cell-

specific lysis. Error bars represented mean ± SEM of 5 independent experiments. Wilcoxon test was used for statistical analysis P*<0.05.

4.2.3 hPL-BM-MSC, similarly to FBS-hBM-MSC, suppress T, B and NK cell proliferation

A proliferation assay was carried out to evaluate the ability of hPL- and FBS-hBM-MSC to inhibit purified T, B and NK cell proliferation. We confirmed the natural ability of resting hBM-MSC to inhibit T and NK cells. On the other hand, resting hBM-MSC showed a trophic effect towards B cells. Moreover, we found that primed hBM-MSC had a higher inhibiting activity towards T and NK cells and acquired immune suppressive activity also towards B cells (**Figure 19**). The percentage of T cell inhibition by resting FBS-hBM-MSC and hPL-hBM-MSC was 75.7% and 81.1%, respectively, while by primed FBS-hBM-MSC and hPL-hBM-MSC was 85.6% and 84.8%, respectively. The inhibition of B cells by primed hBM-MSC was very similar to that of T cells (83.7% and 85.2% for FBS-hBM-MSC and hPL-hBM-MSC, respectively). Finally, NK cell inhibition by primed hBM-MSC was lower compared to T and B cells (79.9% and 59% for FBS-hBM-MSC and hPL-hBM-MSC, respectively) but significant. Therefore, hPL-expansion protocol did not affect hBM-MSC inhibitory functions.

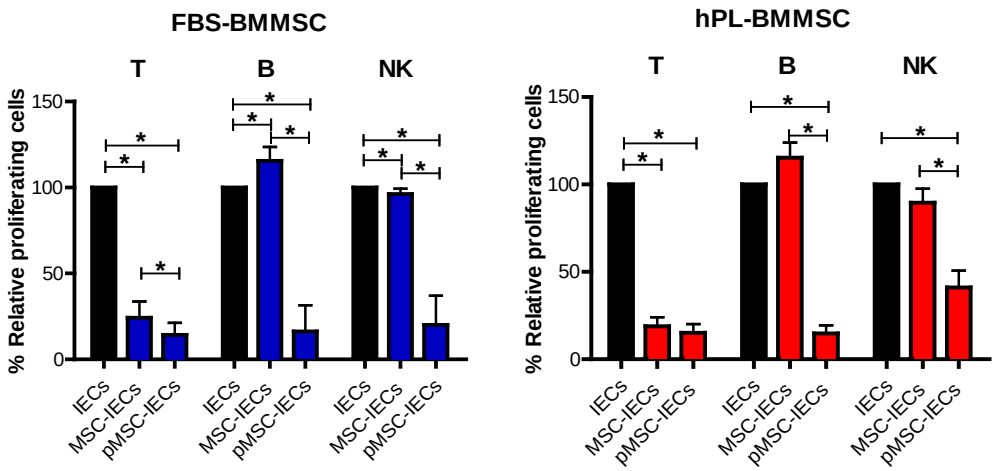

Figure 19. hBM-MSC immune modulatory activity. Sorted T, B or NK cells were activated with proper stimuli and cultured alone or in presence of resting or primed hBM-MSC for 4 days (B

cells) or 6 days (T and NK cells) at the MSC:IEC ratio of 1:10 for T cells, and 1:1 for B and NK cells. Data are represented as percentage of relative proliferating cells. Error bars represented mean ± SEM of 5 independent experiments. Wilcoxon test was used for statistical analysis P*<0.05.

4.2.4 Soluble factors are involved in T cell immunosuppression

We assessed the ability of soluble factors derived from resting or primed hBM-MSC to inhibit T cells. Therefore, we performed co-cultures using Transwell® system to avoid cell-to-cell contact. We found that soluble factors are highly involved in hBM-MSC-mediated T cell immunosuppression. T cells were strongly inhibited at both resting and primed conditions at the 1:5 ratio (for FBS-hBM-MSC: 76% and 80%, for hPL-hBM-MSC: 65% and 64%, respectively) and at the 1:10 ratio (for FBS-hBM-MSC: 66% and 76%, for hPL-hBM-MSC: 43% and 38%, respectively). In the latter case, hPL-hBM-MSC showed lower inhibitory activity towards T cells as compared to FBS-hBM-MSC (**Figure 20**).

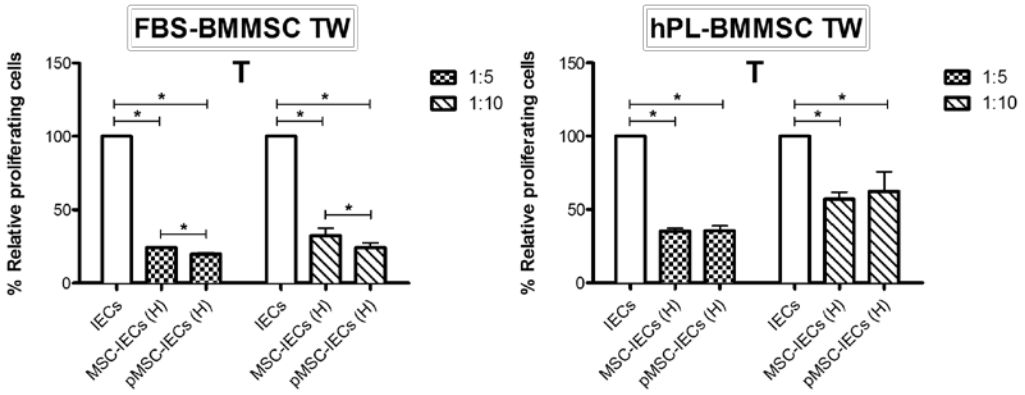

Figure 20. Soluble factor-mediated immune modulatory activity. Purified T, B or NK cells were activated with proper stimuli and cultured alone or in presence of resting or primed, FBS- or hPL-expanded, hBM-MSC for 4 days (B cells) or 6 days (T and NK cells) in a Transwell® system at the E:T ratio of 1:5 and 1:10. Data are represented as percentage of relative proliferating cells. Error bars represented mean ± SEM of 5 independent experiments. Wilcoxon test was used for statistical analysis P*<0.05

4.3 XENOGENEIC aGvHD MOUSE MODEL

4.3.1 Characterization of Peripheral Blood Mononuclear Cells for aGvHD Induction

PBMC characterization was important to determine whether each PBMC batch was suitable for "*in vivo*" aGvHD induction. Thus, through flow cytometry we identified the composition of lymphocyte subsets of every PBMC batch as quality control assay before usage (**Figure 21A**). We found that at the end of purification protocol, PBMC were composed of 72-90% of T cells, 2-9% of B cells and 5-13% of NK cells (**Figure 21B**). Moreover, we checked CD4:CD8 ratio in T cell population, and we found about of 2:1 ratio as expected in healthy donors (**Figure 21C**). The, we evaluated though CFSE proliferation assay the activation ability of thawed PMBC, particularly T cells (**Figure 21D**), and we found that all PBMC stimulated with αCD3 and αCD28 showed a proliferation rate over 90% (**Figure 21E**).

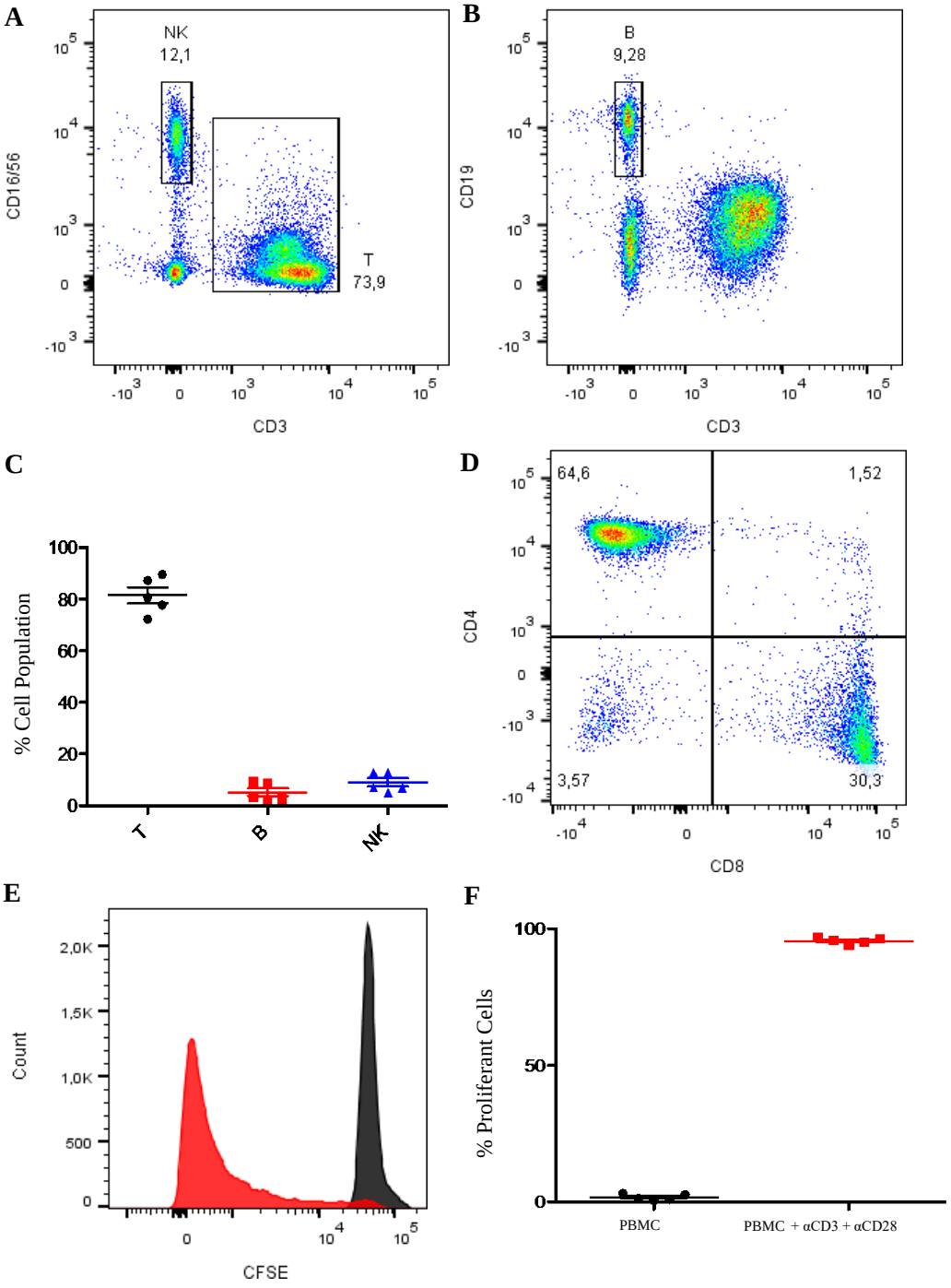

Figure 21. PBMC characterization. A-B: Representative plots of PBMC composition in terms of T, B and NK cells. **C:** T, B and NK cells in isolated PBMC. Data are represented as percentage of cell populations. Error bars represented mean ± SEM of 5 independent experiments. **D:** Representative plot of T cell composition in terms of CD4+ and CD8+ T cells in isolated PBMC. **E:** Representative histograms of CFSE proliferation assay on PBMC. **F:** CFSE proliferation assay of unstimulated or CD3-CD28-stimulated PBMC. Data are represented as percentage of proliferating cells. Error bars represented mean ± SEM of 5 independent experiments.

4.3.2 Severe Systemic aGvHD Xenogeneic Mouse Model

To obtain a reproducible aGvHD mouse model, NOG mice were sub-lethally irradiated with 1.2 Gy and intravenously injected with $1x10^6$ PBMC. The percentage of weight loss was evaluated until day 30, taking into consideration that at the beginning a weight loss was observed in all mice due to the irradiation. The onset of aGvHD occurred at day 9, when mice started to lose weight more rapidly, whereas control mice gained weight and at the end of the experiment (Day 30) recovered completely their initial weight (**Figure 22A**). Death rate in PBMC-injected mice was 100% (**Figure 22B**). After death, different organs were analyzed, thus finding high degree of human lymphocyte infiltration in bone marrow, peripheral blood, kidney, lung, liver and spleen (**Figure 22C**). Amongst infiltrating human CD45+ cells in mouse tissues, the vast majority was represented by T cells in all organs (**Figure 22D**). Of note, a few B cells were found in the enlarged spleen of some PBMC-injected mice in comparison to control mice (1 cm and 1.4 cm size, respectively) (**Figure 22E**). To confirm the aGvHD onset, we evaluated tissue damages and T cell infiltration also through immunohistochemistry. Hematoxylin/Eosin (HE) and CD3 staining were performed on lung, liver, spleen, kidney, skin and small intestine (**Figure 23 and 24**). We confirmed the broad infiltration in the main target organs and a slight infiltration in skin and small intestine. Finally, we scored aGvHD severity in mouse organs (**Table 6**) on the basis of the parameters described in **Table 5** (Materials & Methods). All these data confirmed the presence of a reproducible, systemic and severe aGvHD xenogeneic mouse model.

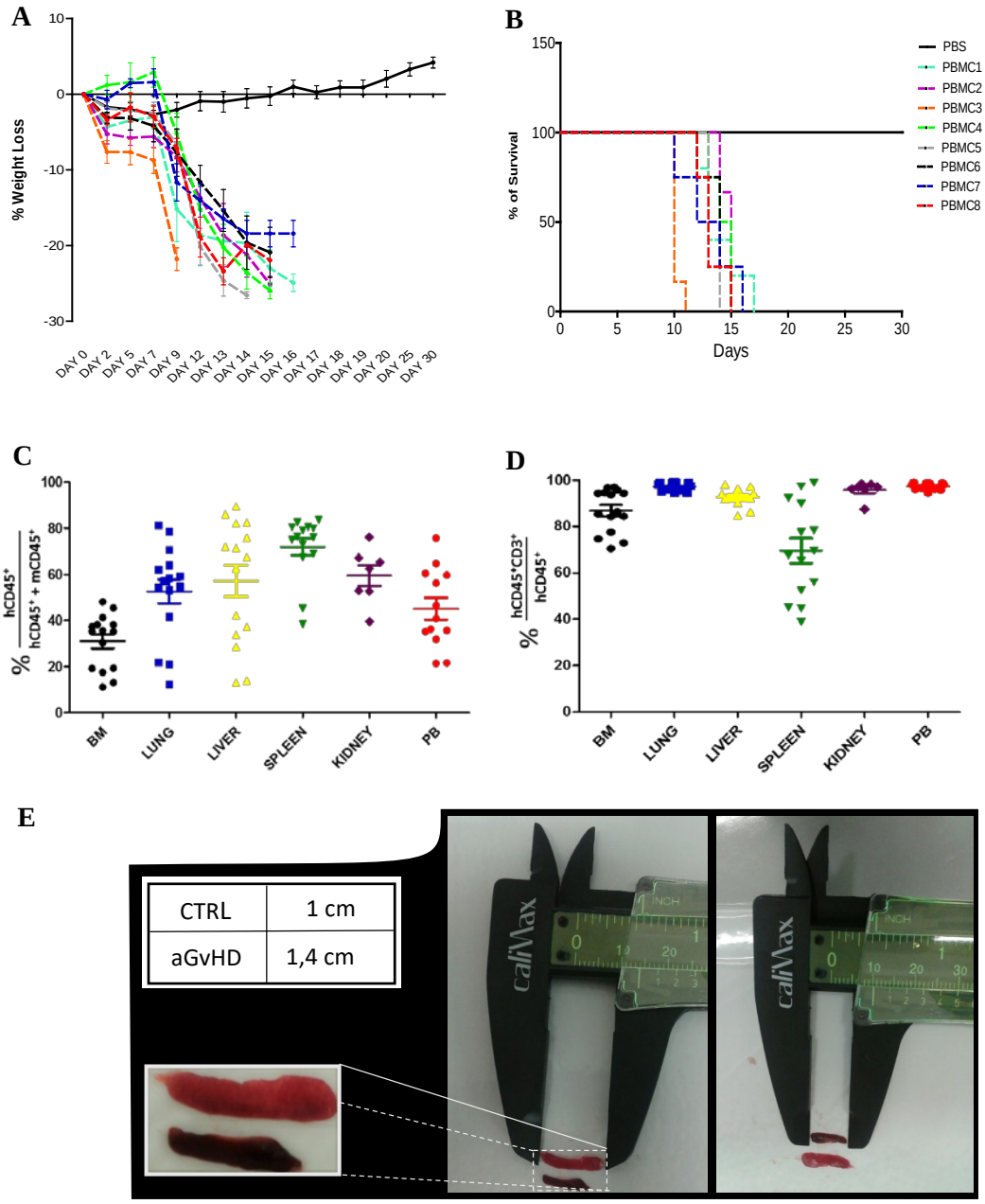

Figure 22. aGvHD mouse model. A: Weight change curves of aGvHD mice (PBMC) and control mice (PBS). **B:** Kaplan-Meier survival curves of aGvHD mice (PBMC) and control mice (PBS). Log-rank (Mantel-Cox) test was used for statistical analysis P***<0.001**. C:** human infiltrating cells in different mouse target organs. Data are represented as percentage of human CD45+ cells on total CD45+ cells. Error bars represented mean ± SEM of 15 independent experiments**. D:** human infiltrating T cells in different mouse target organs. Data are represented as percentage of human CD3+ cells on human CD45+ cells. Error bars represented mean ± SEM of 15 independent experiments. **E:** Splenomegaly in aGvHD mice.

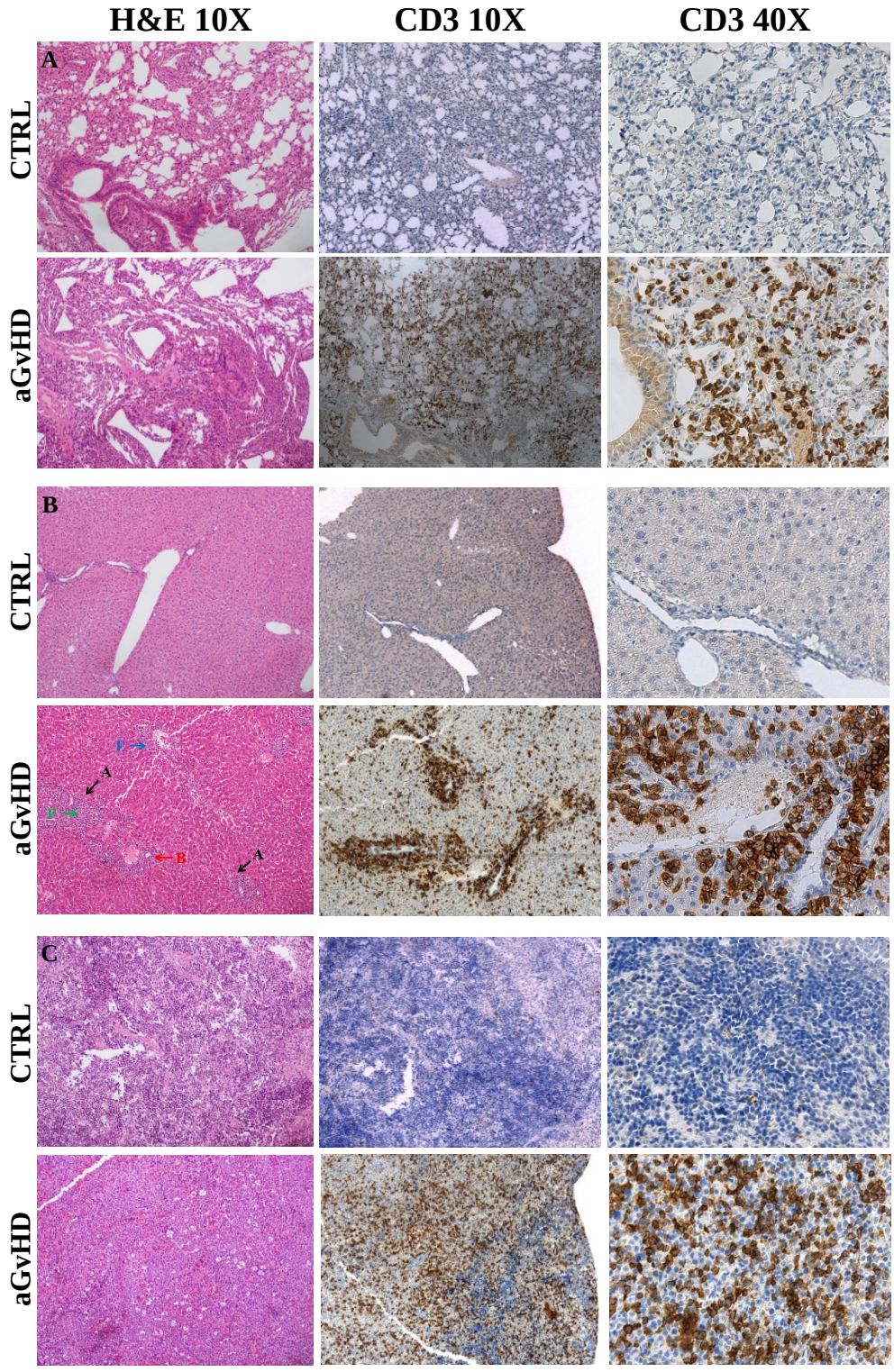

Figure 23. Histopathological evaluation of mouse tissues. Tissues from aGvHD or control mice (lung (**A**), liver (**B**), spleen (**C**)) were fixed and stained with Hematoxylin and Eosin or anti-hCD3 to evaluate tissue damage and T cell infiltrates. In figure **B**, black arrow A = Apoptosis, red arrow B = Biliary damage, arrow green P = Portal infiltrate and blue arrow E= Endotheliitis.

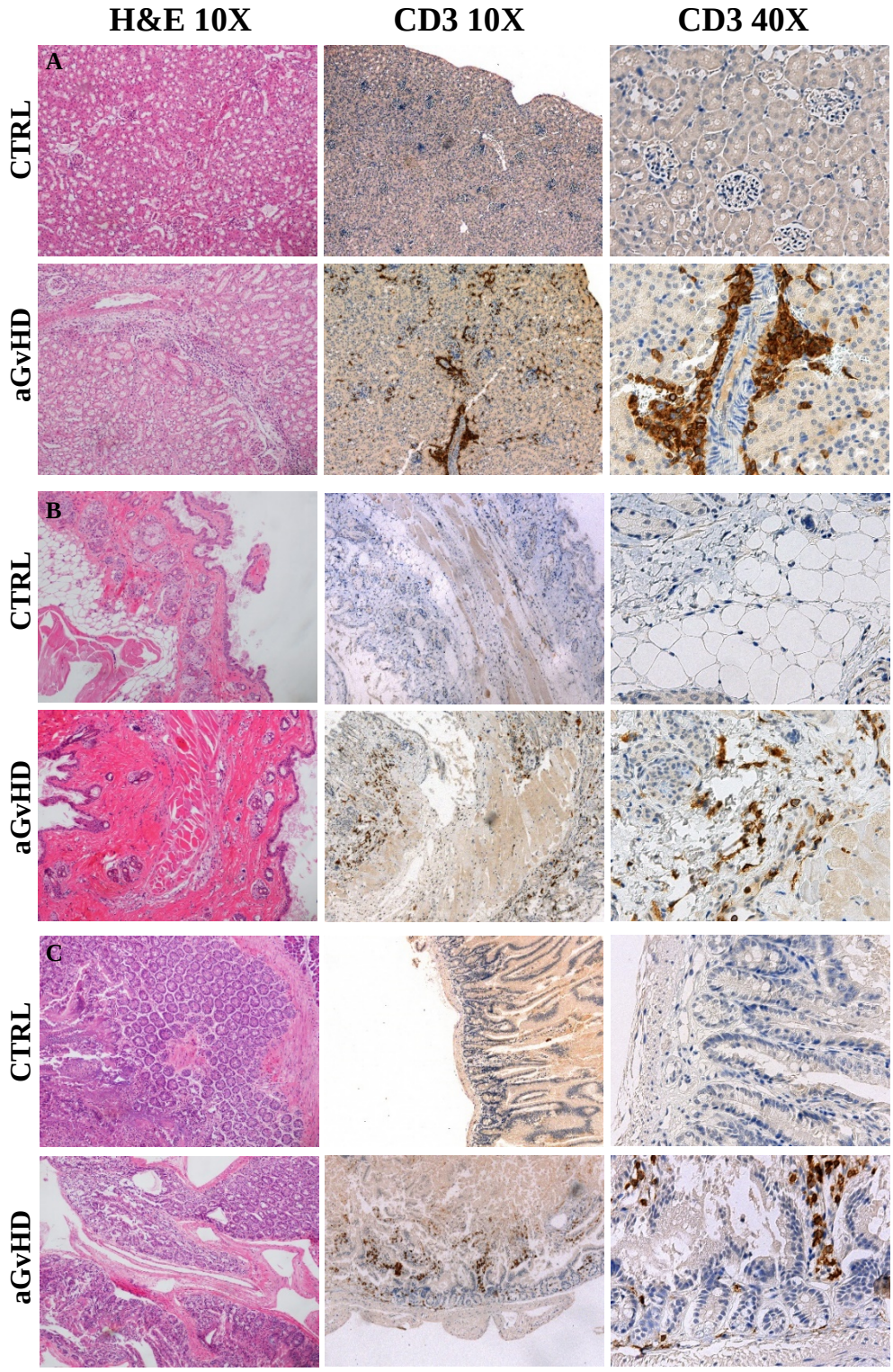

Figure 24. Histopathological evaluation of mouse tissues. Tissues from aGvHD or control mice (kidney (**A)**, skin (**B)**, small intestine (**C**)) were fixed and stained with Hematoxylin and Eosin or anti-hCD3 to evaluate tissue damage and T infiltrates.

	LUNG	LIVER				SPLEEN	KIDNEY	SKIN	SMALL INTESTINE
	INFLAMMATION	INFLAMMATION	BILIARY DAMAGE	CENTROLOBULAR VEIN ENDOTHELIITIS	APOPTOSIS	INFLAMMATION	INFLAMMATION	INFLAMMATION	INFLAMMATION
A	2	3	3	1	3	2	3	1	1
B	4	4	4	2	3	4	4	2	3
C	0	0	0	0	0	0	0	0	0
D	0	0	0	0	0	0	0	0	0
E	1	1	0	0	0	2	0	0	1
F	2	2	1	0	1	3	1	0	1
G	1	1	1	0	0	3	1	0	0
H	2	2	2	1	1	3	2	0	1
I	4	3	3	3	3	3	3	1	2
J	4	4	4	3	3	4	3	1	1
K	3	3	2	3	1	2	1	1	1
L	4	3	3	3	3	3	2	1	1
M	3	3	2	2	3	3	3	1	1

Table 6. Histopathological score of mouse organs. A, B, E, F, G, H, I, L and M: aGvHD mice; C and D: control mice.

4.3.3 hPL-hBM-MSC treatment of aGvHD mice has no significant effect on mouse survival

Different schedules of treatments with hPL-hBM-MSC were tested in aGvHD mice, i.e. three administrations of $1x10^6$ hPL-hBM-MSC and two different time points of administration (Day +2, +5, +7 vs. Day +5, + 7, +9). We did not find significant differences in mouse survival according to the administration schedules. Moreover, no differences were found in survival between aGvHD mice and hBM-MSC-treated mice (**Figure 25A**). Hypothesizing that $1x10^6$ hBM-MSC dose was probably not enough for a therapeutic effect, we decided to increase the dose. Thus, $2x10^6$ hPL-hBM-MSC were intravenously injected at Day +5, +7, +9. In this case, probably due to pulmonary embolism for high dose of MSC (data not shown), we had 50% of mouse mortality during the intravenous injection with resting hBM-MSC and 100% with primed hBM-MSC. To prevent this problem, we performed intraperitoneal injection of $1x10^6$ resting or primed hBM-MSC for 10 days (from day +1 to day +10 vs from day +7 to day +16). Again, no differences in mouse survival were found with this administration route (**Figure 25B and 25C**). Finally, we used two different hBM-MSC doses ($0.5x10^6$ and $2x10^6$ hPL-hBM-MSC) and 4 administrations with larger time interval amongst injections (day +4, +8, +12, and +16). We found a slight, but not significant improvement of survival curve of hBM-MSC-treated aGvHD mice as compared to aGvHD mice (**Figure 25D**). None of the mice died of embolism, but a few mice survived until day +16 to conclude the hBM-MSC administration schedule.

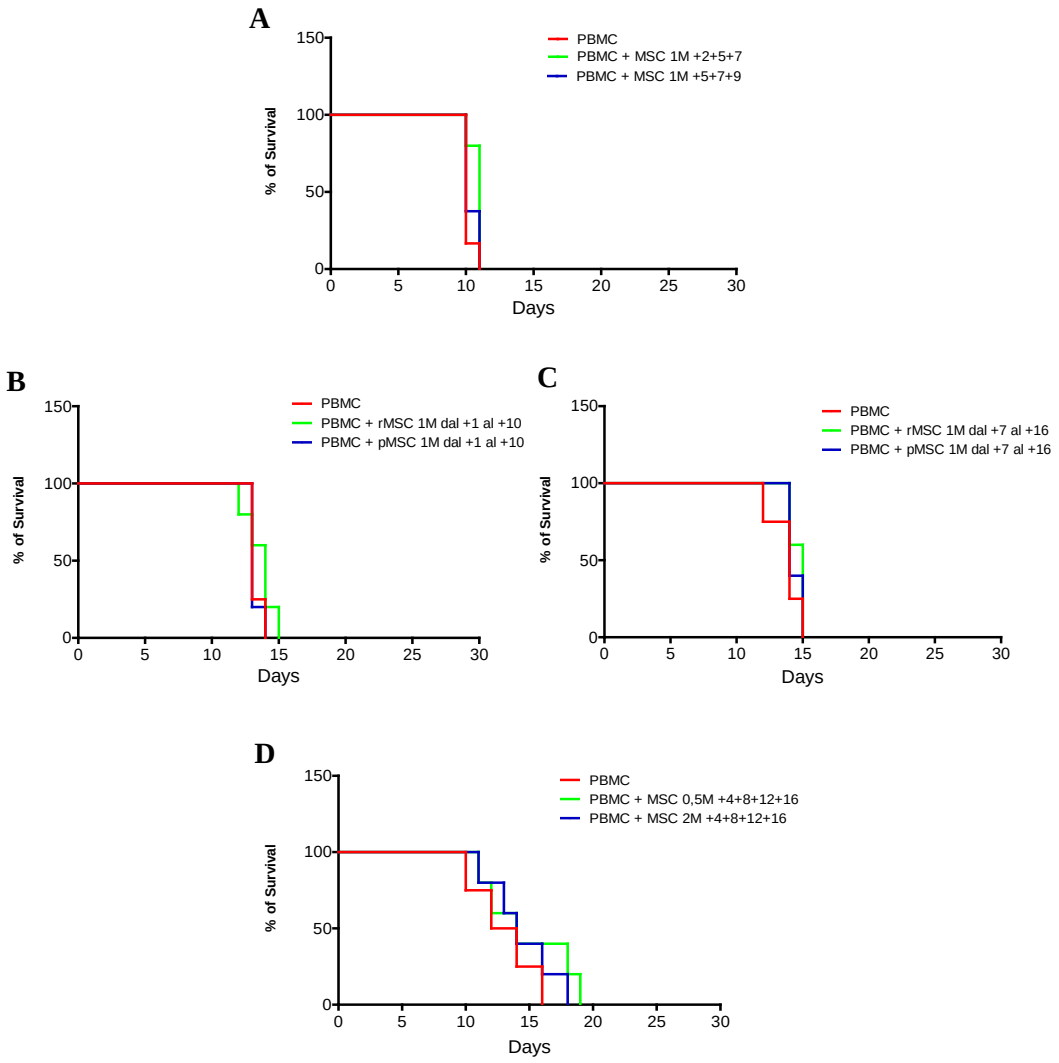

Figure 25. hPL-hBM-MSC treatment of aGvHD mice. A: Kaplan-Meier survival curves of aGvHD mice treated or not with intravenous injection of $1x10^6$ resting hBM-MSC at day +2, +5, +7 or day +5, + 7, +9 after PBMC injection (Day 0). **B and C:** Kaplan-Meier survival curves of aGvHD mice treated or not with intraperitoneal injection of $1x10^6$ resting or primed hBM-MSC starting at day +1 up to day +10 or starting at day +7 up to day +16 after PBMC injection (Day 0). **D:** Kaplan-Meier survival curves of aGvHD mice treated or not with intravenous injection of 0.5 or $2x10^6$ resting MSC at day +4, +8, +12, +16 after PBMC injection (Day 0).

5 DISCUSSION AND CONCLUSIONS

After Allo-HSCT, GvHD occurs in 20-70% patients. This donor T cell-mediated alloreactive inflammatory disease is depending on histocompatibility degree between donor and recipient and is characterized by high mortality in case of steroid-refractoriness (Flowers et al. 2011; Hahn et al. 2008; Lee et al. 2003; Lee et al. 2013). Thus, second- or third-line therapies are required when the first line corticosteroids treatment fails. For this purpose, MSC can represent a suitable tool for the treatment of inflammatory diseases, including GvHD, as suggested by the growing literature published on MSC biology starting from 2002 (Di Nicola et al. 2002; Bartholomew et al. 2002; Le Blanc et al. 2004; Krampera et al. 2006; Augello et al. 2007; Ren et al. 2008; Di Trapani et al. 2016).

Patient's safety in the clinical setting of cell therapies is a major concern, so the use of xeno-free product is always preferred. Human platelet lysate (hPL) a is potential alternative to FBS as culture supplement for clinical grade-MSC expansion (Doucet et al. 2005).

Here, we assessed the *in vitro* immunological properties and *in vivo* application of hBM-MSC in aGvHD, showing the safety and the advantages in using hPL-expanded hBM-MSC. The cell number required for clinical application is a critical point. Indeed, the number of MSC necessary for a single patient in aGvHD cell therapy is very high and ranges from about 0.4 to $9x10^6$ per kg body weight (Le Blanc et al. 2004; Kebriaei et al. 2009; Dominici et al. 2006; Le Blanc et al. 2008; Introna et al. 2014; Erbey et al. 2016). In addition, more than one MSC infusion is usually required. Our results showed that the hBM-MSC expansion protocol adopted led to a greater number of cells when using the hPL-supplemented medium as compared to the FBS-supplemented medium. Indeed, hPL contains several growth factors, such as PDGF-AA, -AB and -BB, TGF-β1 and -β2, EGF, VEGF, b-FGF, and HFG that support MSC proliferation (Pons et al. 2008; Shih & Burnouf 2015; Astori et al. 2016). In addition, we showed that hPL-hBM-MSC possess a greater adhesion capability to the culture flasks at the first seeding (P0) as well as proliferation rate than FBS-hBM-MSC. Although a higher proliferation rate correlates with a more rapid cell senescence, we found

that at the end of culture the genome integrity was maintained, both in terms of karyotype and cell cycle-related gene expression. Moreover, no evidence of cell senescence, in terms of transcriptional profile and *in vitro* functional properties, was observed. According to the ISCT minimal criteria for MSC definition, the isolated hBM-MSC population was characterized with standardized methods. In fact, the achievement of a homogeneous MSC population is essential for clinical application. Thus, the ability to differentiate into adipocytes and osteocytes and the expression of specific cell markers confirmed the MSC identity of the isolated cell populations. Our results were in line with the ISCT criteria, showing the presence of the MSC markers (CD73, CD90 and CD105), the absence of hematopoietic markers (CD14, CD31, CD34, CD45) and the capability of adipogenic and osteogenic differentiation *in vitro* (Dominici et al. 2006). Moreover, we showed that hPL increased significantly the presence of CD73 and CD90 molecules on hBM-MSC as compared to FBS. These findings suggest that hBM-MSC expanded in hPL may have a stronger immature phenotype, as CD90 modulation correlates with the enhancement of osteogenic and adipogenic differentiation *in vitro* (Moraes et al. 2016). In addition, hPL-hBM-MSC may exert a major immune modulatory activity, as the inhibition of CD73 expression on MSC restores T cell proliferation in a co-culture system and reverts their therapeutic effect on experimental autoimmune uveitis (EAU) (Chen et al. 2016); consequently, the use of hPL-hBM-MSC for clinical application in inflammatory diseases could have some advantages.

However, several mechanisms are involved in MSC immune suppression, and the acquisition of the anti-inflammatory phenotype (MSC2) is mandatory to exert immune modulatory functions (Krampera 2011). It is well known that IFN-γ and TNF-α play a role in MSC2 differentiation. We found, after the treatment with IFN-γ and TNF-α, the overexpression on hBM-MSC of CD54 (I-CAM), CD106 (V-CAM), MHC-I (HLA-ABC), MHC-II (HLA-DR), CD274 (PD-L1) and CD273 (PD-L2) molecules, thus suggesting the acquisition of MSC2 phenotype (Menard et al. 2013; Di Trapani et al. 2013; Ren et al. 2010; Chan et al. 2006; Sheng et al. 2008). In addition, we reported the over-expression of PD-L2 in primed hBM-MSC; the function of this molecule in the PD-L/PD1-pathway is still

unclear, but it could act as inhibitory molecule towards immune effector cells (Davies et al. 2017).

Several studies have highlighted the inhibitory effect of MSC on different immune effector cells, but many literature data are still contradictory. In our study, we used standardized protocols (Menard et al. 2013) to quantify the immune suppressive ability of hPL- and FBS-expanded hBM-MSC towards human lymphocytes, both at resting and inflammatory-primed conditions. We found that primed hBM-MSC efficiently inhibited T, B and NK cell proliferation. By contrast, resting hBM-MSC efficiently inhibited T cells and less significantly NK cells, but they were unable to suppress B cell proliferation for the lacking production of IFN-γ that plays a pivotal role in the MSC acquisition of immune-suppressive properties (Krampera et al. 2006; Ren et al. 2008). As B cells are not IFN-γ-producing cells, differently from T and NK cells, they were not inhibited by resting hBM-MSC that, in turn, displayed a supporting role towards B cell survival and proliferation, as previously shown (Krampera 2011). As human MSC exert their immune modulatory activity mainly through paracrine effects, we performed the CFSE-based proliferation assay on T cells in a Transwell® system to prevent cell-to-cell contact. Thus, we confirmed the involvement of soluble factors in hPL-hBM-MSC immune suppressive mechanism, as previously shown for FBS-hBM-MSC (Di Trapani et al. 2016).

The ability of NK cells to recognize foreign antigens, thus hampering allogeneic cell therapies, is mainly based on MHC-I levels present on target cell surface. NK cells activate the immune response and exert their cytotoxic action towards cells that do not express or express MHC-I at low levels (i.e. virus-infected cells or tumour cells) (Moretta et al. 1996). hBM-MSC showed a low immunogenicity towards NK cells, especially after IFN-γ/TNF-α-mediated priming. Interestingly, resting hPL-hBM-MSC had a slightly better ability to elude NK cell-mediated lysis as compared to resting FBS-hBM-MSC, probably due to the higher level of HLA-ABC expression by resting hPL-hBM-MSC. This hypothesis is supported by the ability of MHC-I molecules to interact with the inhibitory KIRs on NK cells (Vély et al. 1996; Lanier 1997; Long 2008).

To achieve a clinical protocol for GvHD treatment, pre-clinical studies in mouse models are required. Several studies were performed on mouse background leading to the current knowledge on MSC functions. However, significant differences were found in GvHD pathology and progression between mouse and human settings. Moreover, different mechanisms occur during the interaction amongst murine cells, murine and human cells or human cells (Mestas & Hughes 2004). Thus, to translate pre-clinical evidence into clinical practice, the use of humanized mouse models is mandatory. Here, we developed a severe aGvHD model in xenogeneic mice through the injection of human PBMC. In our model, differently from other humanized models, aGvHD appears earlier and is rapidly lethal; thus, in a 30 day-experiment the effect of MSC can be reproducibly evaluated. Unfortunately, we found that hPL-hBM-MSC do not ameliorate aGvHD symptoms: we tested different treatment schedules, but only in one of these a slight, but not significant improvement of mouse survival could be achieved. These findings are in line with other studies in which MSC could efficiently inhibit T cells *in vitro*, but failed to suppress aGvHD *in vivo* (Badillo et al. 2008; Sudres et al. 2006). As reported in literature (Lee et al. 2009; Kim et al. 2014; Saat et al. 2016), lung entrapment of intravenously injected MSC could be the main explanation for the inefficacy of MSC treatment, preventing MSC from reaching target organs. Although human MSC exert their anti-inflammatory activity mainly through soluble factors, the short lifespan of MSC after the injection could hamper any beneficial effects (Lee et al. 2009; Kim et al. 2014; Saat et al. 2016). Moreover, at the time of injection, the level of pro-inflammatory cytokines (i.e. IFN-γ) could not be sufficient to prime injected human MSC. In a previous study, the use of IFN-γ-primed murine MSC for aGvHD treatment had beneficial effects (Polchert et al. 2008). In our study, on the other hand, the administration of primed hPL-hBM-MSC was associated to high mortality rate following injection. Therefore, our mouse model, very efficient in determining aGvHD in a reproducible manner, still needs some improvements to assess the efficacy of hPL-hBM-MSC administration to prevent and control aGvHD onset and gravity.

In conclusion, our work pointed out that the clinical use of hPL as supplement for hBM-MSC cultures have several advantages not only for patient's safety, avoiding heterologous compounds, but also in terms of cell production efficiency and maintenance of hBM-MSC phenotype and functions. Furthermore, the development of a reproducible xenogeneic mouse model of severe aGvHD may be useful for additional pre-clinical studies involving hPL-hBM-MSC or MSC of different tissue origin. Our group is currently testing both other MSC administration schedules to improve the pre-clinical *in vivo* model and alternative approaches to infuse biologically active molecules capable of modulating the functions of the immune effector cells involved in aGvHD onset and progression. In particular, as human MSC exert their effects mainly through paracrine mechanisms (Di Trapani et al. 2016), we are currently testing the use of primed hBM-MSC-derived extracellular vesicles (EVs) instead of whole MSC. This approach could have several significant advantages in terms of prompt availability for clinical use (previous production and cryopreservation of primed EVs), precise quantification of the biological efficacy (EV dose expressed as functional units) and reproducibility of the results (depending on the administered EV dose and not on proliferation capability of infused MSC). For all these reasons, hPL-hBM-MSC-derived EVs could represent a potential therapeutic tool for aGvHD that could be tested with our preclinical xenogenic mouse model.

6 REFERENCES

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