Human Mesenchymal Stromal Cells: Biological Characterization and Clinical Application

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Human Mesenchymal Stromal Cells:
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Ai miei genitori

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CHAPTER 1:

GENERAL INTRODUCTION

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MESENCHYMAL STROMAL CELLS

Introduction

In addition to hematopoietic stem cells (HSCs), the bone marrow (BM) also contains mesenchymal stem cells (MSCs). These cells were first recognized more then 40 years ago by Friedenstein *et al.* who described a population of adherent cells from the BM which were non-phagocytic, exhibited a fibroblast-like appearance and could differentiate *in vitro* into bone, cartilage, adipose tissue, tendon and muscle.[1] Moreover, after transplantation under the kidney capsule, these cells gave rise to the different connective tissue lineages.[2]

MSCs have been demonstrated to display chemotactic ability, to migrate to sites of inflammation and injury,[3] as well as to secrete paracrine mediators able to reverse acute organ failure.[4] Indeed, MSC infusions have been successfully used in repairing tissue injury secondary to allogeneic hematopoietic stem cell transplantation (HSCT).[5] In view of their immunosuppressive properties, as well as of their role in tissue repair and trophism, MSCs represent a promising tool in approaches of immunoregulatory and regenerative cell therapy.[6,7]

Recently, a standardized nomenclature for MSCs has been proposed and the term “multipotent mesenchymal stromal cells” (with the acronym MSCs) has been introduced to refer to this population of fibroblast-like plastic-adherent cells.[8]

In this study, we will refer to multipotent mesenchymal stromal cells with the acronym MSCs.

Sources of MSCs

Human MSCs were first identified in postnatal BM[1] and later in a variety of other human tissues, including periosteum, muscle connective tissue, perichondrium, adipose tissue and fetal tissues, such as lung, BM, liver and spleen.[9-13] Amniotic fluid and placenta have been found to be rich sources of MSCs;[14,15] both fetal and maternal MSCs can be isolated from human

placenta.[15] MSCs have been also identified in umbical cord blood (UCB); however, probably as a consequence of their low frequency in UCB, conflicting results in terms of success rate of MSC isolation have been initially reported.[16,17] It is now clear that selection of UCB units to be processed by specific quality criteria, such as volume and storage time, can be considered critical parameters for the successful isolation of MSCs from this source.[17]

In general, MSCs represent a minor fraction in BM and other tissues; the exact frequency is difficult to calculate because of the different methods of harvest and separation. However, the frequency in human BM has been estimated to be in the order of 0.001-0.01% of the total nucleated cells, and therefore about 10 fold less abundant than HSCs.[18] Furthermore, the frequency of MSCs declines with age, from $1/10^4$ nucleated marrow cells in a newborn to about $1/2x10^6$ nucleated marrow cells in a 80-year old person.[18]

Multilineage potential of MSCs

One of the hallmark of MSCs is their multipotency, defined as the ability to differentiate into several mesenchymal lineages, including bone, cartilage, tendon, muscle, marrow stroma and adipose tissue (AT).[18-20] Usually trilineage differentiation into bone, adipose tissue and cartilage is taken as a criterium for multipotentiality.

To induce osteogenic differentiation, cells are cultured in the presence of dexametasone, ascorbic acid and β-glycerophosphate. To detect osteogenic differentiation cells are stained for alkaline phosphatase activity by substrate solution and for calcium depositions with Alzarin Red.[13] Adipogenic differentiation can be induced with dexametasone, insulin, indomethacin and 1-methyl-3-isobutylxantine. Cells containing lipid vacuoles can be stained after 3 weeks with Oil red O.[13] Chondrogenic differentiation is obtained after culturing MSCs in pellets, in the absence of serum and in the presence of Transforming growth Factor-β3 (TGF-β3) and Bone Morphogenetic Protein-6 (BMP-6).[21,22]

Chondrocytes can be stained for extracellular matrix components with Toluidine Blue and/or by PCR for collagen type II, IX and X.

Recently, it has been reported the existance of pluripotent cells that have the ability to differentiate into cells of the mesodermal lineage, but also into endodermal and neuroectodermal cell types, including neurons,[23] hepatocyte[24,25] and endothelium.[26] Such pluripotent stem cells have been identified in BM and referred to as multipotent adult progenitor cells (MAPCs),[27] human BM-derived multipotent stem cells (hBMSCs),[28] marrow-isolated adult multilineage inducible (MIAMI) cells,[29] or very small embryonic-like stem (VSEL) cells.[30] Similar pluripotent cells have been reported by Kogler *et al.* in UCB, and have been referred to as "Unrestricted Somatic Stem Cells" (USSCs).[31]

Immunomodulatory properties of MSCs *in vitro*

MSCs display unique immunological properties that have been demonstrated by several independent groups both *in vitro* and *in vivo*, in animal models and in humans. In the beginning, most studies focused the attention on the effects of MSCs on T-lymphocytes; however, it is now become evident that these cells display their effects on other cells involved in immune responses, including B lymphocytes, dendritic cells and Natural Killer (NK) cells.[32-34] See Figure 1.

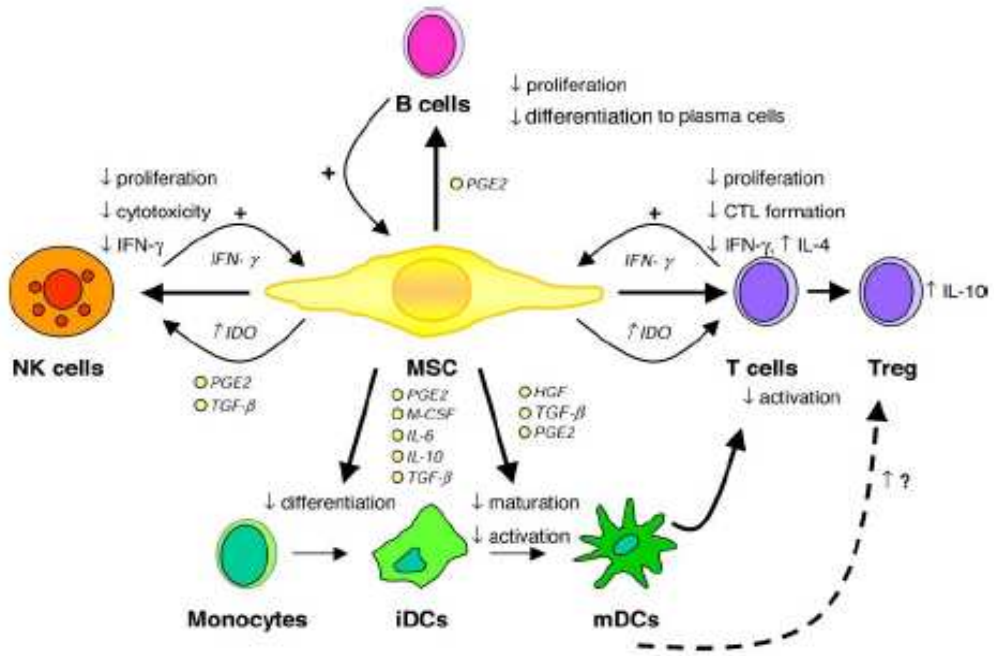

Figure 1. Immunomodulatory effects of MSCs.
CTL indicates cytotoxic T cell; iDCs, immature dendritic cells; mDCs, mature dendritic cells; HGF, hepatocyte growth factor; IDO, indolamine 2,3-dioxygenase; PGE2, prostaglandin E2; IL, interleukin; IFN, interferon and TGF-beta, transforming growth factor beta. *Illustration by Paulette Dennis; modified from Nauta AJ, Fibbe WE, Blood 2007. Copyright: permission.*

MSCs were first demonstrated to suppress *in vitro* T lymphocyte proliferation induced by alloantigens,[35] mitogens,[36] CD3 and CD28 agonist antibodies.[37,38] MSCs have been reported to inhibit the cytotoxic effects of cytotoxic T cells (CTLs), probably due to suppression of CTL proliferation.[39] This inhibition of T-cell proliferation is not HLA-restricted; MSCs are able to induce a similar degree of inhibition in the presence of both autologous and allogeneic responder cells, this supporting the concept that MSCs can be considered *universal suppressors*.[35,38] Since the separation of MSCs and PBMCs by transwell experiments does not completely abrogate the suppressive effect, most human MSC-mediated immune suppression on activated T-lymphocyte

has been attributed to the secretion of anti-proliferative soluble factors, such as TGF-β, hepatocyte growth factor (HGF), prostaglandin E2 (PGE2), indoleamine 2,3-dioxygenase (IDO), nitric oxide and interleukin (IL)-10.[35-44] However, published data do not exclude that a part of the immunosuppressive effect exerted by human MSCs on alloantigen-induced T-cell activation be dependent on cell-to-cell contact mechanisms. Of interest, the calcineurin inhibitors, cyclosporine-A and tacrolimus, currently employed to prevent or treat graft-versus-host disease (GvHD), enhance the immune suppressive effect of human MSCs, in particular for what concerns the *in vitro* activation of alloantigen-specific, T-cell mediated cytotoxic activity.[45] Some authors have shown that the unresponsiveness of T cells in the presence of MSCs is transient and that T cell proliferation can be reinitiated after MSC removal.[32,36,38]

Inhibition of lymphocyte proliferation by MSCs has not been associated with the induction of apoptosis, but it is rather interpreted as due to inhibition of cell division, thus preventing T-lymphocyte capacity to respond to antigenic triggers, while maintaining these cells in a quiescent state.[36,38,46] MSCs have been also reported to induce regulatory T cells (Treg), as demonstrated by the increase in the population of CD4+CD25+FoxP3+ cells in mixed lympocyte cultures in the presence of MSCs.[47]

MSCs have been reported to interfere with dendritic cell (DC) differentiation, maturation and function. Differentiation of both monocytes and CD34+ progenitors into $CD1a^{+}$-DCs is inhibited in the presence of MSCs and DCs generated in this latter condition are impaired in their function, in particular in their ability to induce activation of T cells.[48,49] Transwell experiments have demonstrated that the suppressive effect of MSCs on DC differentiation is at least partly mediated by soluble factors, namely IL-6 and M-CSF, PGE2, IL-10.[49] Alternatively, MSCs might favor the induction of regulatory APCs, through which they could indirectly suppress T cell proliferation.

The ability of MSCs to inhibit B cell proliferation was first reported in murine studies.[46] Thereafter, human MSCs have been demonstrated to suppress the proliferation of B cells activated with anti-Ig antibodies, soluble CD40 ligand and cytokines, as well as to interfere with differentiation, antibody production and chemotactic behaviour of B lymphocytes.[50] Krampera *et al.* have reported that MSCs are able only to reduce the proliferation of B cells in the presence of IFN-gamma, thanks to its ability to induce IDO activity by MSCs.[51] In contrast with these observations, Traggiai *et al.* have recently reported that BM-derived MSCs are able to promote proliferation and differentiation into immunoglobulin secreting cells of transitional and *naive* B cells isolated from both healthy donors and pediatric patients with systemic lupus erythematosus.[52]

It has been reported that MSCs are able to suppress NK cell proliferation after stimulation with IL-2 or IL-15.[39,47,53] Indeed, while MSCs do not inhibit the lysis of freshly isolated NK cells,[39] these latter cells cultured for 4 to 5 days with IL-2 in the presence of MSCs display a reduced cytotoxic potential against K562 target cells.[51] Transwell experiments have suggested that the suppression of IL-15 driven NK cell proliferation as well as of their cytokine production by MSCs, is mediated by soluble factors.[51,53] On the contrary, the inihibitory effect displayed by MSCs on NK cell cytotoxicity required cell-cell contact.[53]

Altough MSCs were initially considered immunoprivileged and therefore capable of escaping lysis by freshly isolated NK cells,[39] recent experiments have demonstrated that IL-2-activated both autologous and allogeneic NK cells are capable of effectively lysing MSCs.[53,54] Although MSCs express normal levels of MHC class I that should protect against NK-mediated killing, they display ligands that are recognized by activating NK receptors that, in turn, trigger NK alloreactivity.[54] Moreover, it has been recently demonstrated that MSCs can be lysed also by cytotoxic T-lymphocytes, when infused into MHC-mismatched mice, resulting in their rejection.[55]

In conclusion, several studies have demonstrated tha MSCs are capable of modulating *in vitro* the function of different cells active in the immune response. Whether this effect is displayed through real suppression of immune responses or to a nonspecific antiproliferative effect is still unclear. The mechanisms by which MSCs display their immunosuppressive effect are largely restricted to *in vitro* studies. The *in vivo* biological relevance of the *in vitro* observations, therefore, represents an important issue that is currently addressed by several research groups.

Ex vivo isolation/expansion and characterization of MSCs

MSCs can be relatively easily isolated from BM and other tissues and display a remarkable capacity for extensive *in vitro* expansion to numbers that allow *in vivo* testing in humans.[20,56-61]

Most of the information available on MSC phenotypic and functional properties are derived from studies performed on cells cultured *in vitro*. To date, MSC isolation/identification has mainly relied on their adherent properties, immunephenotype by flow-cytometry and differentiation potential. In detail, *ex vivo* expanded MSCs have been phenotypically characterized on the basis of the expression of nonspecific markers, including CD105 (SH2 or endoglin), CD73 (SH3 or SH4), CD90 (Thy-1), CD166, CD44, and CD29[18,19] (see Table 1). In addition, culture-expanded cells lack the expression of some haematopoietic and endothelial markers, such as CD14, CD31, CD34 and CD45, at least in case of BM-derived cells, whereas a proportion of adipose tissue-(AT) derived MSCs express CD34.[11,62,63]

Little is known about the characteristics of the primary precursor cells *in vivo*, since it has not yet been possible to isolate the most primitive mesenchymal cell from bulk cultures. One of the hurdles has been the inability to prospectively isolate MSCs because of their low frequency and the lack of specific markers. Recently, the identification and prospective isolation of mesenchymal

progenitors, both in murine and human adult BM, have been reported, based on the expression of specific markers. [64-70]

One group has reported the identification, isolation and characterization of a novel multipotent cell population in murine BM, based on the expression of the stage-specific embryonic antigen-1 (SSEA-1). This primitive subset, that is found both directly in the BM and in mesenchymal cell cultures, can give rise to SSEA-1^+ MSCs and is proposed to be placed at the apex of the hierarchical organization of the mesenchymal compartment.[64]

In human cells, surface markers such as SSEA-4, STRO-1 and the low affinity nerve growth factor receptor (CD271),[65-68] which enrich for MSCs, have been employed with the aim to prospectively isolate MSCs. Moreover, Battula *et al.* have recently isolated by flow cytometry MSCs from human BM using antibodies directed against the surface antigens CD271, mesenchymal stem cell antigen-1 (MSCA-1) and CD56, and identified novel MSC subsets with distinct phenotypic and functional properties.[69] Platelet derived growth factor receptor-beta (PDGF-RB; CD140b) has been also identified as a selective marker for the isolation of clonogenic MSCs[68] and other reports have demonstrated a 9.5-fold enrichment of MSCs in human BM cells with prominent aldehyde dehydrogenase activity[70] (see Table 1). The relevance and usefulness of these markers for the prospective isolation and consequent expansion of MSCs from BM and/or other sources is being evaluated and will possibly allow a more precise definition of the cell products employed both in the experimental and clinical setting.

Antigen	**Expanded/primary MSCs**	**Human/murine MSCs**
CD105 (endoglin, SH2)[18-20]	Expanded	Human, murine
CD73 (ecto-5' nucleotidase, SH3, SH4)[18-20]	Expanded	Human
CD166 (ALCAM)[13]	Expanded	Human
CD29 (β1-integrin)[13,18-20]	Expanded	Human, murine
CD44 (H-CAM)[18-20]	Expanded	Human, murine
CD90 (Thy-1)[13,18-20]	Expanded	Human
TRA-1-81	Expanded	Human (placenta)
Sca-1	Expanded	Murine
STRO-1[66]	Primary	Human (BM)
CD349 (frizzled-9)[68]	Primary	Human (BM, placenta)
SSEA-4[64,65]	Primary	Human +/- (BM, placenta)
Oct-4[30]	Primary	Human +/- (BM, placenta, fetal tissues)
Nanog-3	Primary	Human +/- (BM, placenta)
SSEA-1[30,64,65]	Primary	Murine (BM)
CD271 (low-affinity nerve growth receptor)[67,69]	Primary	Human
MSCA-1[69]	Primary	Human (BM)
CD140b (PDGF-RB)[68]	Primary	Human

Table 1. Antigens expressed on culture-expanded and primary MSCs

MSCs can be expanded *in vitro* to hundreds of millions of cells from a 10 to 20 ml BM aspirate.[60,61] The cell yield after expansion varies with the age and condition of the donor and with the harvesting technique. Therefore, differences in isolation methods, culture conditions, media additives greatly affect cell yield and possibly also the phenotype of the expanded cell product.[61,71,72] For these reasons, efforts have been made within the European Group for Blood and Marrow Transplantation (EBMT) MSC expansion consortium for the standardization of MSC isolation and expansion procedures. This organization, including European centers interested in the biology and clinical application of MSCs, has defined common protocols, in order to facilitate comparisons between cell products generated at different sites and to run large-scale clinical studies.

In this regard, MSCs are currently expanded *in vitro*, either under experimental or clinical grade conditions, in the presence of 10% fetal calf serum (FCS)[71,72] and serum batches are routinely pre-screened to guarantee both the optimal growth of MSCs and the bio-safety of the cellular product. Despite this, the use of FCS has raised some concerns when utilized in clinical grade preparations, because it might theoretically be responsible for the transmission of prions and still unidentified zoonoses. It may also cause immune reactions in the host, especially if repeated infusions are needed, with consequent rejection of the transplanted cells.[73] In view of these considerations, serum-free media, appropriate for extensive expansion and devoid of the risks connected with the use of animal products, are under investigation. The possibility of using autologous or allogeneic human serum for *in vitro* expansion of MSCs has been tested and autologous serum has proved to be superior to both FCS and allogeneic serum in terms proliferative capacity of the expanded MSCs.[74] The reduction of bovine antigens by a final 48-hour incubation with medium supplemented with 20% human serum, to prepare hypoimmunogenic MSCs, has also been proposed.[75] Human platelet-lysate (PL), which consists of human

platelet growth factors (GFs) in a small volume of plasma, has been recently demonstrated to be a powerful substitute for FCS in MSC expansion.[76,77] Indeed, the high concentration of natural GFs contained in platelets may offer a significative advantage in terms of proliferative capacity of MSCs, providing high numbers of cells in a short culture-period.[76,77] However, further studies are needed to better understand the behaviour of PL-expanded MSCs (MSCs-PL), as compared to those cultured in FCS-based medium. In particular, a comprehensive characterization of the biological and functional properties of MSCs-PL, in comparison with FCS-expanded MSCs, needs to be performed before introducing this culture supplement in the routine preparation of cellular products for clinical application. Moreover, clinical data on the safety and efficacy of MSCs have been mainly obtained with cells expanded in the presence of FCS, whereas relatively little *in vivo* experience is available with MSCs cultured in PL.

Another issue related to the *ex vivo* expansion of MSCs is that the manipulation may alter the functional and biological properties of the cells, leading to the accumulation of genetic alterations, as already shown by few groups.[62,78,79] The use of MSCs in clinical applications requires that the bio-safety of these cells be carefully investigated through appropriate and sensitive tests. In particular, the absence of transformation potential in cultured cells has to be documented before infusion into patients, particularly into immune-compromised subjects where failure of immune surveillance mechanisms might further favor the development of tumors *in vivo*. The possibility that karyotyping on expanded cells be included in the release criteria for MSC administration into patients is currently being discussed. A precise characterization of the genetic profile of MSCs could allow to identify phenomena of senescence, developing in cells at the end of their life-span, versus transformation of cells, due to the occurrence of genetic alterations.

In vivo animal models to test the properties of MSCs

The immunomodulatory and reparative/anti-inflammatory properties of MSCs have been tested in a variety of animal models (see Table 2).

Both in a sheep model and in non-human primates, it has been shown that MSCs can engraft and distribute to a number of tissues after systemic infusion.[80,81] A number of studies have documented that marrow stroma remains of host origin after allogeneic HSCT in the majority of the patients,[82-86] although others have shown the contrary.[87-89] Therefore, the transplantability of MSCs in humans remains controversial.

Systemic infusion of allogeneic BM-derived MSCs from baboons has been demonstrated to prolong the survival of allogeneic skin grafts, as compared to animals not receiving MSCs.[90] Moreover, human MSCs have been shown to promote engraftment of UCB-derived HSCs in NOD-SCID mice and in fetal sheep[80,91,92] and this enhancing effect was particularly prominent when relatively low doses of HSCs were transplanted.[91]

For years, MSCs have been considered cells which could be potentially ignored by the immune system. However, it has been recently demonstrated that allogeneic MSCs are not intrinsically immunoprivileged, as, under appropriate conditions, they can induce an immune response, resulting in their rejection when infused into MHC-mismatched mice.[55] On the contrary, the infusion of syngeneic host-derived MSCs resulted, in the same model, in enhanced engraftment of allogeneic stem cells.[55] These observations are interpreted to indicate that MSCs may promote engraftment, provided that they survive *in vivo* and are not rejected as the result of an allo-immune response.

Several animal studies have addressed the issue of the suppressive effect of MSCs in the context of GvHD prevention/treatment, with the aim of elucidating whether it can be ascribed to a true 'immune' effect, rather than a nonspecific antiproliferative function of the cells. However, conflicting results have been published, in particular on the role of MSCs in GvHD prevention. In one study,

AT-derived MSCs have been infused systemically in mice early after transplantation of haploidentical hematopoietic stem cells and were able to rescue the animals from lethal GvHD.[93] Sudres *et al.* have reported that a single dose of BM-derived MSCs at time of allogeneic BM transplantation did not affect the incidence and severity of GvHD in mice,[94] whereas UCB-derived MSCs administered at weekly intervals were able to prevent GvHD development after allogeneic transplantation of human peripheral blood mononuclear cells in NOD/SCID mice.[95]

Thanks to their capacity to modulate immunoresponses and/or to promote tissue repair, MSCs are considered a potential novel treatment modality for autoimmune diseases.[5,7,33,34] In this context, studies on the effects of MSCs in animal models of autoimmunity have been recently reported. Murine MSCs have been demonstrated to ameliorate experimental autoimmune encephalomyelitis (EAE), a model of human multiple sclerosis, through the induction of peripheral T-cell tolerance against the pathogenic antigen.[96,97] In contrast, infusion of MSCs had no beneficial effects on collagen-induced arthritis (CIA) when tested in a murine model of rheumatoid arthritis.[98] In mouse models, MSCs have been used also for the treatment of diabetes and their infusion led to an increase in the number of pancreatic islets and insulin-producing β cells.[99] In a murine model of systemic lupus erythematosus, MSCs were able to inhibit autoreactive T and B cells ameliorating the signs and sympthoms of the disease.[100] Moreover, the infusion of rat MSCs in a rat experimental model of glomerulonephritis was able to stimulate glomerular healing, probably due to the secretion of soluble factors.[101] Besides EAE, infusion of MSCs has been thought to play a role in the protection of neurons from damage occurring in other conditions, such as spinal cord injury, stroke and amyotrophic lateral sclerosis.[102-104] Very recently, the topical implantation of BM-derived MSCs has been demonstrated to be beneficial also in the healing process of experimental colitis in rats, confirming the ability of these cells to

modulate immune-responses and to promote tissue repair through their trophic activity.[105] Other studies in animal models of organ injury (heart, lung, kidney and liver) have suggested similar results in terms of MSCs ability to promote an anti-inflammatory effect and, thus, to protect against tissue injury.[4,106-109]

In most of the reported studies, the therapeutic effect of MSCs does not seem to be associated with their differentiation into the resident cell-types, but appears to be mostly related to their anti-proliferative and anti-inflammatory effect, as well as to their capacity to stimulate survival and functional recovery in injured organs, likely through paracrine mechanisms.

Table 2. MSC therapy in experimental disease models

Animal, model	Outcome	Reference no.
Baboon, skin graft Tx	Prolonged skin graft survival	90
NOD-SCID mouse, HSCT	Promoted engraftment	91,92
Fetal sheep, HSCT	Promoted engraftment	80
Mouse, graft rejection	Decresed graft rejection (syngeneic MSCs)	55
Mouse, GvHD	Prevention of GvHD	93
Mouse, GvHD	No effect on development of GVHD	94
Mouse, GvHD	Prevention of GvHD, unefficacious for GvHD treatment	95
Mouse, EAE	Prevention of EAE development	96,97
Mouse, CIA	No effect	98
Mouse, STZ diabetes	Ameliorated diabetes and kidney disease	99
Mouse, SLE	Ameliorated signs and sympthoms of SLE	100
Rat, glomerulonephritis	Stimulated glomerular healing	101
Rat, experimental colitis	Stimulated intestinal mucosa healing	105
Rat, heart transplantation	Migrated to the heart during chronic rejection	106
Rat, myocardial infarction	Promoted an anti-inflammatory effect	107
Mouse, lung injury	Protected from bleomycin-induced injury	108
Mouse, ischemia /riperfusion kidney injury	Protected against ischemia/riperfusion injury	109
Mouse, acute hepatic failure	Protected against hepatic injury	4

Tx, transplantation; HSCT, hematopoietic stem cell transplantation; GvHD, graft-versus-host-disease; EAE, experimental autoimmune encephalomyelitis; CIA, collagen-induced arthritis; STZ, streptozotocin; SLE, systemic lupus erythematosus.

Clinical applications of MSCs

The immunesuppressive capacity and the regenerative/reparative potential of MSCs have generated clinical interest in the field of HSCT, in order to prevent graft rejection and to prevent/control GvHD, as well as in the context of Regenerative Medicine with the aim of facilitating tissue repair (see Table 3).

The ability of MSCs to enhance the engraftment of HSCs after transplantation has been demonstrated both in animal models,[80,91,92] as previously mentioned, and in clinical trials. The experimental data *in vivo*, together with the known physiological role played by MSCs in sustaining haematopoiesis, have provided the rationale for testing the capacity of these cells to facilitate haematological recovery after HSCT in humans. The first clinical trial on the use of MSCs for accelerating haematological recovery was performed in 28 breast cancer patients given autologous transplantation and co-infused with $1–2\times10^6$ MSCs/kg body weight. No MSC-related toxicity was registered, whereas a rapid haematopoietic recovery was noted.[110] After this study, a multicenter trial aimed at evaluating the safety of MSC infusion was conducted in 46 patients receiving allogeneic HSCT from an HLA-identical siblings.[111] MSC co-infusion was not associated with adverse events; haematopoietic recovery was prompt for most patients and moderate to severe acute GvHD was observed in 28% of the patients.

The most impressive clinical effect of MSCs *in vivo* has been observed in the treatment of acute GvHD developing after allogeneic HSCT. The first striking report of this effect was reported by Le Blanc *et al.* who described a pediatric patient experiencing grade IV acute GvHD of the liver and gut after allogeneic HSCT from an unrelated volunteer and resistant to multiple lines of immune suppressive therapy. The child was rescued by the infusion of BM-derived MSCs isolated from the mother.[112]

In view of the promising experimental results on the use of MSCs for the treatment of autoimmune diseases,[96-101] their role in the clinical setting is now

beginning to be explored. MSCs isolated from patients with systemic sclerosis have been reported to be functionally impaired *in vitro*;[113] while, other reports have documented that these latter cells, as well as MSCs from patients with variuos autoimmune diseases, exhibit the same phenotypical and functional properties as their healthy counterparts.[114,115]

Both in animal models and in patients, it has been shown that BM-derived cells play a role in the healing process following intestinal injury and in the regeneration of various cellular components of the mucosa.[105,116-118] Recently, in a phase-I clinical trial, autologous, AT-derived MSCs have been successfully employed for the treatment of 4 patients with fistulizing Crohn's Disease (CD).[119] Based on these encouraging results, a phase-II trial on autologous AT-derived MSCs[120] and a phase-III trial on third-party, BM-derived MSCs[121,122] in CD patients refractory to conventional therapies, are underway.

Therapeutic infusion of MSCs has been employed in the context of *Osteogenesis Imperfecta (O.I.),* a genetic disease characterized by production of defective type I collagen which is responsible for the occurrence of fractures, retarded bone growth with progressive bone deformation and premature death. Horwitz *et al*. first reported that transplantation of BM cells from an HLA-identical sibling could, at least transiently, ameliorate the clinical conditions of patients with *O.I.,* as donor-derived, mesenchymal progenitors contained in transplanted BM could migrate to the bones and give rise to osteoblasts. However, so far no clear demonstration that MSCs were responsible for the claimed improvement of bone structure and clinical condition has been provided. [123,124] The same group has subsequently reported that the infusion of purified allogeneic MSCs might have been capable of enhancing the therapeutic benefits of allogeneic BM transplantation in *O.I.* patients.[73]

Since MSCs express high levels of arylsulfatase A and alfa-L-iduronidase, MSC treatment has been proposed also for patients affected by metachromatic leukodystrophy (MLD) and Hurler disease, 2 inherited diseases caused by the

deficiency of the above mentioned enzymes. In a report from Koc *et al*., donor-derived MSCs were infused in 11 patients with MLD and Hurler disease after allogeneic HSCT. Although there was no major improvement in the overall health of the patients, in 4 of 5 children with MLD an improvement in nerve-conduction velocity was observed.[125]

Although, little is known on the engraftment and *in vivo* survival of MSCs, the few clinical studies performed so far suggest that their clinical use is feasible and safe. To date, no severe adverse reactions have been recorded in humans after MSC administration, both in terms of immediate, infusional toxicity and of late effects. These observations might also be due to the limited survival of MSCs *in vivo* or to short follow-up time of the patients treated. Another important clinical safety concern is the possibility of ectopic tissue formation after MSC treatment. Recently calcifications were observed in the infarcted hearts of mice that received local MSC treatment.[126]

The mechanisms by which MSCs exert their immunomodulatory and reparative effects *in vivo* are still poorly understood and require extensive *in vitro* and *in vivo* testing. The possibility that allogeneic MSC may be rejected due to recognition by the immune cells of non immune-ablated hosts, as already suggested in an animal model,[55] or due to sensitization to bovine proteins remaining in the cell product after *ex vivo* expansion, deserves further investigation.

Moreover, concerns remain over the potential systemic immunosuppression mediated by MSCs after *in vivo* administration. In immunocompetent mice, Djouad *et al*. demonstrated that local as well as systemic infusion of MSCs suppressed the host antitumor immune response, thus favoring allogeneic tumor formation.[127] In humans, recent data suggest that the co-transplantation of MSCs and HSCs may result in increased risk of relapse in hematologic malignancy patients, as compared to patients receiving standard HSCT.[128]

Finally, *ex vivo* expanded MSCs should be properly studied to demonstrate their genetic stability before administration into patients, to avoid any possible risk related to the infusion of transformed cells, bearing genetic alterations developed during *in vitro* culture.

In conclusion, while MSC treatment represents a promising and novel modality for the treatment of many disorders, concerns remain over the potential of systemic immune suppression, ectopic tissue formation and malignant transformation of MSCs. These concerns apply, in particular, to the use of autologous and expanded MSCs. Long term follow-up studies are required to address these issues.

To completely exploit the potentiality of this new treatment modality more *in vivo* work is required to increase our knowledge on how MSCs mediate their suppressive effect and reduce inflammatory responses. Moreover, *in vivo* tracking studies to examine the survival, distribution and homing of MSCs after infusion in humans are necessary. The identification of a universal MSC marker is warranted both to dissect the hierarchy of the different MSC subsets and to facilitate the generation of homogenous cell products at different sights. Once more largely defined, these *in vivo* biological activities of MSCs could be properly employed as a novel therapeutic strategy to stimulate tissue repair and modulate immune responses in a variety of immune-mediated and inflammatory diseases.

AIMS OF THE PRESENT STUDY

In this thesis we will focus on the biological and functional characterization of human MSCs and on the role of these cells in the context of hematopoietic stem cell transplantation. The ability to differentiate into cell-types of mesodermal origin, in partcular into the chondrogenic lineage, the immune regulatory effect of BM- and UCB-derived MSCs expanded in medium supplemented with either FCS or PL and the potential susceptibility to undergo malignant transformation

after long-term *in vitro* culture are evaluated. Moreover, preliminary results on the use of BM-derived MSCs in different clinical context of HSCT, such as haploidentical T-cell depleted HSCT from a partially matched family donor, UCB transplantation and steroid-resistant, severe acute GvHD are reported.

A lot of interest has recently emerged in techniques for cartilage tissue engineering where mesenchymal progenitor cells can be delivered within an appropriate carrier system to repair and regenerate pathologically altered cartilage.[26,56] In **chapter 2** the ability to diffentiate into the chondrogenic lineage of MSCs isolated from different fetal and adult tissue sources is studied and compared. For this purpose, MSCs are isolated and expanded from fetal lung and BM, as well as from maternal placenta and adult BM, with the aim of investigating which is the preferred MSC source to be employed for cartilage repair. The influence of the cell passage on the ability of MSCs to differentiate into chondrocytes is also evaluated.

Table 3. Clinical experience with MSCs (published reports)

Disease, setting	MSC therapy	N. of patients	Outcome	Reference N.
Breast cancer, autologous HSCT	Autologous MSCs	28	Accelerated hematological recovery	110
Hematological malignancy, allogeneic HSCT	Allogeneic MSCs	46	Prompt haematopoietic recovery, GvHD prevention	111
Acute GvHD, allogeneic HSCT	Haploidentical MSCs	1	Resolution of grade IV acute GVHD	112
Fistulizing Crohn's disease, local infusion	Autologous MSCs	4	Repair of fistulas	119
O.I., allogeneic HSCT	Allogeneic MSCs	6	Improvement of bone structure and clinical condition (?)	73
Inborn errors of metabolism, allogeneic HSCT	Allogeneic MSCs	11	No major improvement in overall health. (MLD:improvement in nerve-conduction velocity)	125
Hematological malignancy, allogeneic HSCT	Allogeneic MSCs	10	Increased risk of relapse	128
Complications after allogeneic HSCT	Third party MSCs	10	Tissue repair (hemorrhagic cystitis, pneumomediastinum)	5

HSCT, hematopoietic stem cell transplantation; GvHD, graft-versus-host-disease; O.I., Osteogenesis Imperfecta; MLD, metachromatic leukodystrophy.

MSCs are mainly expanded *in vitro*, either under experimental conditions or in clinical grade preparations, in the presence of FCS.[59,61] However, the use of FCS raises some concerns when utilized in the clinical setting.[71] For this reason, the identification of a serum-free medium that allows extensive expansion of MSCs for clinical application, is warranted. In **chapter 3,** the use of PL as alternative culture supplement for MSC *ex vivo* isolation and expansion is tested. The aim of the study is to characterize MSCs expanded in the presence of PL for their phenotype, differentiation and proliferative capacity, immunoregulatory effect on alloantigen-specific immune responses, as well as genetic stability, as compared to MSC cultured in FCS-based medium.

Concerns that adult human MSCs may be prone to malignant transformation have been recently raised.[62,79] The absence of transformation potential in cultured MSCs has to be documented particularly when considering their infusion into immune-compromised subjects where failure of immune surveillance mechanisms might further favor the development of tumors *in vivo*. **Chapter 4** describes the analysis of the potential susceptibility to malignant transformation of human BM-derived MSCs at different *in vitro* culture time points. The aim of this study was to ascertain whether the biological properties of MSCs after *ex vivo* expansion remain appropriate for their use in cell therapy.

For many children with life threatening hematological diseases stem cell transplantation is the only curative option. In those children lacking a matched related or unrelated donor, haplo-identical peripheral blood stem cell transplantation (PBSCT) from a healthy parent is a feasible alternative. To reduce the risk of fatal GvHD, as a complication of transplantation across major histocompatibility antigens, intense T cell depletion is required. However, this significantly increases the risk of either graft failure or early rejection[129,130] In **chapter 5** we describe the results of a phase I/II pilot study of co-

transplantation of BM-derived, *ex vivo* expanded MSCs of donor origin in children undergoing transplantation of granulocyte colony stimulating factor (G-CSF)-mobilized, CD34+ selected progenitor cells from an HLA-disparate relative. The study is intended to sustain hematopoietic engraftment and reduce graft failure rate by means of the co-infusion of MSCs. Children with hematological malignancies or non-malignant disorders, lacking an HLA-matched donor were enrolled in the study in two participating centers (Leiden University Medical Center and Fondazione IRCCS Policlinico S. Matteo).

GVHD is a potentially life threatening complication of HSCT or the infusion of donor lymphocytes (DLI).[131,132] The mainstay of treatment for established GvHD is cortico-steroids with a response rate in the order of 30-50%. In those patients resistant to steroid treatment, second line therapy remains unsatisfactory and overall survival is poor.[131] Several studies indicate that MSCs have immunosuppressive and reparative properties; for these reasons these cells are proposed as new terapeutic tool in GVHD management.[32-34,41] In **chapter 6** the results of a multicenter, phase II study on the use of BM-derived MSCs to treat steroid-resistant, severe acute GvHD after allogeneic HSCT in 55 pediatric and adult patients, affected by either malignant or non-malignant disorders, are reported. This study addresses the issue of safety and efficacy of the infusion of HLA-identical or disparate, *in vitro* expanded, BM-derived MSCs for the treatment of severe, steroid-resistant acute GvHD.

CD is a chronic inflammatory enteropathy in which a dysregulation of the immune response towards intestinal bacteria in genetically susceptible individuals plays a pathogenetic role.[134,135] Despite the large number of therapeutic options available,[136] there is a growing number of CD patients with refractory/recurrent disease and alternative strategies are needed both to increase the proportion of patients achieving remission and to improve their

quality of life. Thanks to their capacity to modulate immune response and promote tissue repair, MSCs represent a potential novel treatment for autoimmune/inflammatory diseases, including CD. **Chapter 7** describes the phenotypical and functional characterization of *in vitro* expanded MSCs from CD patients, in view of their future clinical application. The aim of the study was to evaluate the feasibility of isolating and expanding *ex vivo* MSCs from BM of CD patients with active disease, and to carry out a phenotypical and functional characterization of these cells in comparison with BM-MSCs isolated from healthy donors.

The frequency of MSCs in UCB is very low and the presence of mesenchymal progenitors in full-term UCB has been questioned in recent years. The attempts of many groups to obtain MSC from this source, employing FCS-based media, have either failed[137,138] or yielded low numbers.[16,139] In **chapter 8** we test the ability of a PL-supplemented medium to support the generation and *ex vivo* expansion of MSCs from full-term UCB (UCB-MSCs), as well as characterize these latter cells for their biological and functional properties, in comparison to PL-expanded BM-MSCs. In particular, we focus on the investigation of both the genetic stability and the immunoregulatory function, exerted on alloantigen-specific immune response, of UCB-MSCs.

In **chapter 9** the results and conclusions of these studies are summarized and the future clinical applications of MSCs in the context of HSCT and Reparative/Regenerative Medicine are discussed.

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CHAPTER 2

Human mesenchymal stem cells derived from bone marrow display a better chondrogenic differentiation compared with other sources

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Summary

Mesenchymal stem cells are multipotent cells capable of differentiation into several mesenchymal lineages. These cells have been isolated from various tissues such as adult bone marrow, placenta and fetal tissues. Since a specific phenotypical marker for MSCs is lacking, MSCs are currently characterized on the basis of phenotype and capacity to differentiate into multiple mesenchymal lineages. However, how the potential of these cells to differentiate into the chondrogenic lineage is influenced by the tissue of origin has not been examined. The aim of this study was to investigate whether MSCs isolated from different sources exhibit differential multilineage differentiation potential.

Design: MSCs from fetal and adult tissues were phenotypically characterized and examined for their differentiation capacity, based on morphological criteria and expression of extracellular matrix components.

Results: Our results show that both fetal and adult MSCs undergo chondrogenesis under appropriate conditions. Nevertheless, MSCs of bone marrow origin, either fetal or adult, exhibit a higher chondrogenic potential than fetal lung and placenta derived MSCs, as demonstrated by the appearance of typical morphological features of cartilage, the intensity of Toluidine Blue staining and the expression of collagen type II, IX and X after culture under chondrogenic conditions. In addition, the capacity of MSCs to differentiate into chondrocytes was reduced upon passaging of cells.

Conclusions: MSCs are an attractive source for cartilage tissue engineering strategies. Hence, exploring the chondrogenic potential of different sources is of great interest for such a purpose. Our study indicates that bone marrow is to be considered as the preferred MSC source for cartilage engineering.

Introduction

Articular cartilage has a limited capacity of healing after injury. Traumatic damage and degenerative diseases of the cartilage such as osteoarthritis or rheumatoid arthiritis are common health problems worldwide. Therefore a lot of interest has recently emerged in techniques for cartilage tissue engineering. The current strategies of cartilage repair, based on the use of autologous chondrocytes, have some limitations including the small number of cells available with restricted proliferative capacity and the further damage at donor site of harvest. For these reasons, new techniques are now focusing on the use of mesenchymal progenitor cells to be delivered within an appropriate carrier system to repair and regenerate pathologically altered cartilage [1,2]. In fact, mesenchymal stem cells (MSCs) play a role in bone and cartilage homeostasis and it has been shown that the chondrogenic activity of these cells is reduced in patients with advanced osteoarthritis (OA) [3]. One of the mechanisms involved in the repair of damaged articular cartilage may be the *de novo* chondrogenesis from MSCs [4,5]. Therefore, based on the *in vitro* observation of the differentiation into chondrocytes, on their expandability and availability, MSCs can be considered as an attractive candidate for purposes of cartilage engineering [6,7].

MSCs are multipotent cells with the ability to differentiate into several mesenchymal lineages, including osteoblasts, adypocytes and chondrocytes [8-10]. Although MSCs were originally isolated from bone marrow [11], they have also been isolated from other tissue sources. MSCs have been identified in fetal tissues such as lung, bone marrow, liver and spleen in first- and second-trimester [12,13]. Placenta has been shown to be another rich source of MSCs of both fetal and maternal origin [14]; and MSCs have been also isolated from umbical cord blood, although in low frequency, and adipose tissue. At present no unique phenotype has been identified for MSCs; therefore the isolation and characterization of MSCs relies on the expression of a number of characteristic

markers on culture expanded cells and on their ability to differentiate into the various mesenchymal differentiation lineages.

In the present study, we investigate the multilineage differentiation potential of MSCs derived from 4 different sources and whether this capacity is influenced by the tissue of origin.

Materials and methods

Isolation and culture of human MSCs

Fetal tissues

Fetal lung (fL) and fetal bone marrow (fBM) were obtained from the same fetus from women undergoing elective termination of pregnancy between 15 and 22 weeks of gestation. The study was approved by the hospital ethical committees and informed consent was obtained. Single cell suspensions of fetal lung were made by mincing and flushing the organ through a 100 μm nylon filter with IMDM medium (Cambrex, Verviers, Belgium) containing 1% penicillin/streptomycin (P/S; Cambrex, Verviers, Belgium) and 2% heat-inactivated fetal calf serum (FCS; Cambrex, Verviers, Belgium), i.e. washing medium. Single cell suspensions of fetal bone marrow were obtained by penetrating the long bones with a needle (23 gauge) and flushing the bones with washing medium. After washing, the cell suspension was depleted of red cells by incubation for 10 minutes in NH4Cl (8,4 g/L)/KHCO3 (1/g) buffer at 4 °C. The cells were subsequently plated at 160.000/cm² in culture medium consisting of M199 (Gibco, Paisley, Scotland) supplemented with 10% FCS, P/S, Endothelial Cell Growth Factor (ECGF) 20 μg/ml (Roche Diagnostics GmbH, Mannheim, Germany) and heparin 8 U/ml in tissue culture flasks (Greiner Bio-One GmbH, Mannheim, Germany) previously coated with 1% gelatin for 30 minutes at room temperature.

Placenta

Placentas (PL) were derived from second- and third-trimester pregnancies after informed consent and approval by the hospital ethical committees. Tissue specimens of placenta (maternal origin) were first washed with PBS; single cell suspensions were made by mincing and flushing the tissue through a 100 μm nylon filter with washing medium. The cells were subsequently cultured as described above. To confirm the maternal origin of these cells, molecular HLA-typing was performed on the PL-MSCs cultures.

Adult bone marrow

Adult bone marrow (aBM) samples were obtained from healthy donors for allogeneic stem cell transplantation under a protocol approved by the Ethical Review Board. Mononuclear cells were isolated by density gradient (Ficoll, 1.077 g/ml) and plated at 160.000/cm² in culture medium consisting of DMEM-Low Glucose (Gibco, Paisley, Scotland) supplemented with 10% FCS and P/S.
All cells were kept in a humidified atmosphere at 37 °C with 5% CO2. Three to five days after plating, non-adherent cells were removed and the medium was refreshed. When grown to confluency, adherent cells were detached with trypsin/EDTA (Cambrex, Verviers, Belgium) for 5 minutes at 37 °C and reseeded for expansion or differentiation.

Flow cytometric analysis

Culture expanded fL-, fBM-, aBM- and PL-MSCs were phenotypically characterized by flow cytometry (FACSScan, Becton Dickinson). Fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated antibodies against CD166 (CLB, Amsterdam The Netherlands), CD105 (Ancell Corporation, Bayport, MN, USA), CD90 (Pharmingen, San Diego, CA), CD34, CD45 and CD80 (Beckton Dickinson, San Jose, CA), CD31 (DAKO, Glostrup, Denmark),

HLA-ABC (Instruchemie, Hilversum, The Netherlands), HLA-DR (Beckton Dickinson, San Jose, CA) were used, as well as isotype controls.

Osteogenic and adipogenic differentiation

The adipogenic and osteogenic differentiation capacity of MSCs from the 4 different sources was determined as previously described [13]. In short, to induce osteogenic differentiation, cells were cultured in α-MEM supplemented with 10% FCS, P/S, dexametasone (10^{-7} M) and ascorbic acid (50 μg/ml). β-glycerophosphate (5 mM) was added from day 7 onwards. For adipogenesis, insulin (10 mg/ml), indomethacin (0,25 M) and 1-methyl-3-isobutylxantine (IBMX, 50 mM) were added to this medium. Cells were incubated in differentiation medium for 3 weeks, with medium replacement twice a week, at 37°C with 5% CO_2. To detect the osteogenic differentiation cells were stained for alkaline phosphatase (AP) activity using Fast Blue and for calcium depositions with Alzarin Red. The adipogenic differentiation was evaluated through the morphological appearance of fat droplets.

Chondrogenic differentiation

For chondrocyte differentiation, 200,000 MSCs were placed per well in 96-well suspension culture plates, U-shape, (Greiner Bio-One GmbH, Mannheim, Germany) and centrifuged at 1200 rpm for 4 minutes to a pellet. Pellets were cultured at 37 °C with 5% CO_2 in 200 μl of serum-free chondrogenic medium consisting of DMEM-High Glucose (Gibco, Paisley, Scotland), 40 μg/ml proline (Sigma, USA), 100 μg/ml sodium pyruvate (Sigma, USA), 50 mg/ml ITS+Premix (BD Biosciences, Bedford, MA), 1% Glutamax (Gibco, Paisley, Scotland), P/S, 50 μg/ml ascorbate-2-phosphate (Sigma, USA), 10-7 dexametasone (Sigma, USA), 10 ng/ml Transforming growth Factor-β3 (TGF-β3, R&D Systems, Minneapolis) and 500 ng/ml Bone Morphogenetic Protein-6

(BMP-6, kindly provided by dr. S. Vucikevic) The medium was refreshed every 3 days for 21 days. We investigated at least three samples per source and 3 different passages per sample.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from 1 million undifferentiated MSCs and from pellets at day 21 of the differentiation period, from each source, using RNAeasy kit (Quiagen Gmbh, Hilden). RNA was reverse transcribed into cDNA using First Strand cDNA Synthesis kit for RT-PCR (Roche Diagnostics Gmbh, Mannheim, Germany) according to the manifacturer's instructions. cDNA was amplified using a GeneAmp PCR System 2400 (Applied Biosystems, Foster City, CA). Expression of collagen type II, IX and X mRNA was quantified by real-time quantitative PCR using the Bio-Rad iCycler with SYBR Green. Data were corrected for β_2-microglobulin expression. The following oligonucleotide primers were used:

collagen type II: forward 5'-CCCTCTCCCACACCTTCCTC-3' and reverse 5'-GGGTGAGGGATTCCAGGGAAA-3';

collagen type IX: forward 5'-AGGACACAAGGGTGAAGAAGGT-3' and reverse 5'-TTTTCCCCTTTGTCCCCAACTATG-3';

collagen type X: forward 5'-TTTTGCTGCTAGTATCCTTGAACTT-3' and reverse 5'-AGGAGTACCTTGCTCTCCTCTTACT-3';

β_2-microglobulin: forward 5'-TGCTGTCTCCATGTTTGATGTATCT-3' and reverse 5'-TCTCTGCTCCCCACCTCTAAGT-3'.

All PCR reactions were performed with 5 ng cDNA and according to the manufacturer's protocol of the qPCR Core Kit (Eurogentec, Southampton, UK) in a final volume of 25 μl.

The cDNA was amplified using the following thermal cycling conditions: one cycle at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Each sample was assayed in triplicate and water was

used as a negative control. Fluorescence spectra were recorded and the threshold cycle number (Ct) was read. For each source mean Ct was calculated and from this value the fold difference from expression in the human growth plate according to the equation $2^{-\Delta\Delta Ct}$. For visualization, this value was log-transformed and expressed in figure 2C.

Histological analysis

After 21 days of culture in chondrogenic medium, pellets were fixed in 10% formalin, dehydrated by treatment with graded ethanols and incubated in butanol overnight. Thereafter pellets were embedded in paraffin and cut into 5 µm sections using a Reichert Jung 2055 microtome (Leica, Rijswijk, The Netherlands). The sections were then mounted on glass slides and stained with Toluidine Blue.

The immunohistochemical staining for collagen type X was performed on deparaffinized sections. To block non-specific activity, sections were pretreated with hydrogen peroxidase. Sections were stained with mouse monoclonal antibody against collagen type X (Quartett, Berlin, Germany). The binding of mouse IgG was detected by biotinylated rabbit-anti-mouse IgG (DAKO, Glostrup, Denmark), followed by incubation with horseradish-peroxidase-conjugated-streptavidine (Amersham Biosciences, UK). The peroxidase activity was revealed using 3-amino-9-ethylcarbazole (AEC) substrate. After sections were washed, they were counterstained with hematoxylin.

Results

Morphology and immunophenotypic characterization of MSCs

MSCs isolated from all four different sources displayed the characteristic MSC-like spindle-shape, however subtle differences in morphology were present (Figure 1A). fL- and PL-MSCs showed a more elongated and thin shape compared to the rounder and thicker shape of BM-MSCs.

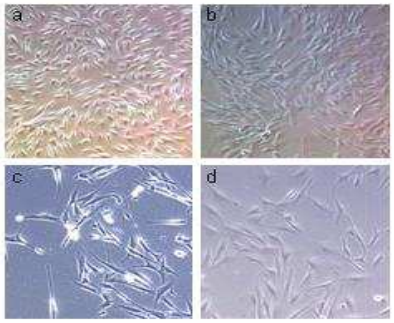

Figure 1A. Morphology of culture-expanded MSCs. MSCs from the four different sources display the characteristic spindle-shaped morphology, however subtle differences are present. a, b: Morphology of fBM- and aBM-MSCs respectively, showing a splindle-round shape. Magnification x5. c, d: Morphology of fL- and PL-MSCs respectively, displaying a spindle-thin shape. Magnification x10.

MSCs from all samples were immunophenotypically analyzed at passage 2 or 3. The phenotypes of fL-, fBM-, aBM- and PL-MSCs were similar and in agreement with previous publication [9,13,14], i.e. CD90, CD105, CD166, HLA-ABC positive and CD34, CD45, CD31, CD80, HLA-DR negative (Figure 1B).

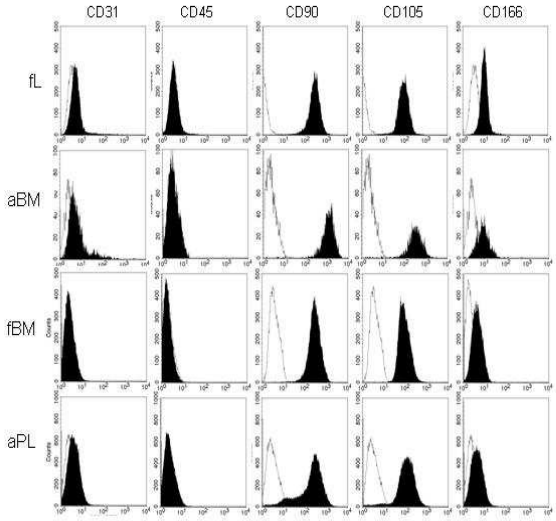

Figure 1B. Immunophenotype of culture-expanded MSCs. Immunophenotypic characterization of MSCs from a representative sample for each source of MSCs. MSCs of all origin were positive for CD90, CD105, and CD166 surface antigens and negative for CD45 and CD31 molecules.

Osteogenic and adipogenic differentiation

To examine the differentiation capacity of MSCs from the different sources, cells were induced into osteoblasts and adipocytes and examined by histological stainings. fL-, fBM-, aBM-MSCs and PL-MSCs were all able to differentiate into osteoblasts as demonstrated by the histologic detection of alkaline phosphatase activity and calcium depositions, and into adipocytes as revealed by the formation of lipid droplets (data not shown). However, PL-MSCs showed a lower ability to form both osteoblasts and adipocytes, while fL-MSCs were less capable to differentiate into the adipogenic lineage, as compared to bone marrow sources (Table 1).

Table 1. Differentiation potential of MSCs derived from different tissue sources

	Adipocyte-like morphology	Alzarin Red staining Intensity	Toluidine Blue Intensity	Chondrocyte-like morphology	N. of positive pellets/total N. of pellets evaluated
fL-MSC	+	++	+/-	+/-	0/4
fBM-MSC	++	++	++	++	3/3
aBM-MSC	++	++	+	++	4/4
PL-MSC	+/-	+/-	-	-	0/4

The presence of adipocyte-like morphology, the intensity of Alzarin Red and Toluidine Blue staining and the chondrocyte-like morphology are scored as: -, +/-, +, ++. The adipocyte-like morphology is defined by the appearance of fat droplets. The chondrocyte-like morphology is defined by the following features: decrease in cell density with distance between cells, rounded morphology of cells and nuclei, deposition of extracellular matrix, presence of chondrocytic lacunae. Only pellets that are scored + or ++ for both the Toluidine Blue staining and the chondrocyte-like morphology are considered positive for cartilage formation (last column on the right).

The differentiation capacity into osteoblasts and adipocytes was maintained, unmodified, untill passage 7 (P7).

The chondrogenic differentiation capacity of MSCs is influenced by the tissue of origin

Next, the chondrogenic differentiation capacity of MSCs of different origin was examined and compared. FL- (n= 4 samples), fBM- (n= 3 samples, derived from the same fetus), aBM- (n= 4 samples) and PL-MSCs (n= 4 samples, maternal origin) were centrifuged into micromasses, cultured as pellets and differentiated in serum-free medium containing ascorbate-2-phosphate, dexamethasone, TGF-β3 and BMP-6.

MSCs from all sources formed a pellet after centrifugation, although pellets consisting of fL- and PL-MSCs were frequently less stable and showed a more irregular shape compared to the pellets from fBM- and aBM-MSCs that were firm and spherical. Pellets derived from fBM- and aBM-MSCs were larger in size after 21 days of culture compared to those derived from fL- and PL-MSCs. In particular, fBM- and aBM-MSCs showed an increase in both the diameter and the area of the pellets after 3 weeks of culture in chondrogenic medium (data not shown). Furthermore, a 4.2- and a 2.9-fold increase in weight was observed in pellets formed by fBM and aBM-MSCs respectively at day 21 of culture, whereas fL- and PL-MSCs exhibited a decrease in weight of pellets over the culture period (Figure 2A). These observations suggest that cartilage extracellular matrix has been syntesized and deposited in pellets from bone marrow cells, leading to the increase in size and weight.

To more specifically analyze the process of chondrogenesis, the expression of collagen type II, IX and X was measured by Q-PCR on undifferentiated cells and on pellets 3 weeks after induction (Figure 2B). Pellets formed by fBM- and aBM-MSCs showed a marked increase in the expression of collagen type II, IX and X after the induction period, compared to fL- and PL-MSCs pellets. These data were confirmed by the histological analysis: fBM- and aBM-MSCs produced more proteoglycans, hence more extracellular matrix, and expressed more chondrocyte-like *lacunae*, as indicated by the intensity of the Toluidine

Blue staining (Figure 2C). Moreover, the immunohistochemical staining for collagen type X was positive only in sections derived from fBM and aBM samples, suggesting that only these cells started to terminally differentiate into hypertrofic chondrocytes (Figure 2D). A scoring system was used to compare the different degrees of chondrogenic differentiation between the 4 sources; evaluating the intensity of the Toluidine Blue staining and the appearance of the typical chondrocyte-like morphology, both scored from – to ++. The stained sections from both fBM- and aBM-MSCs obtained the highest score with 100% of the samples evaluated demonstrating differentiatiation into chondrocytes; in fact, fBM- and aBM- cells showed the most intense Toluidine Blue staining and the typical morphological features of differentiated cartilage such as the decrease in cell density with distance between cells, the rounded morphology of cells, the deposition of extracellular matrix stained in purple and the presence of chondrocytic lacunae, whereas samples from fL- and PL-MSCs didn't (Tab. 1).

The chondrogenic differentiation capacity of MSCs decreases with passage number. In order to examine whether the number of cell passages has an influence on the chondrogenic differentiation capacity of MSCs, different passages of fL-, fBM-, aBM- and PL-MSCs were evaluated. When considering fBM- and aBM-MSCs, a passage-dependent decrease in cartilage formation was observed (Table 2). In particular, the early passages (P2-3) displayed the most intense Toluidine Blue staining and all the typical features of differentiated cartilage, whereas the later passages (P4-5 and P6-7) showed only a moderate or mild staining with less evidence of cartilage differentiation (data not shown). Moreover, the capacity of BM-MSCs to differentiate into chondrocytes declined between passage 6 and 8. For fL- and PL-MSCs, the influence of cell passage was not evident since little or no differentiation towards the chondrogenic lineage was observed overall.

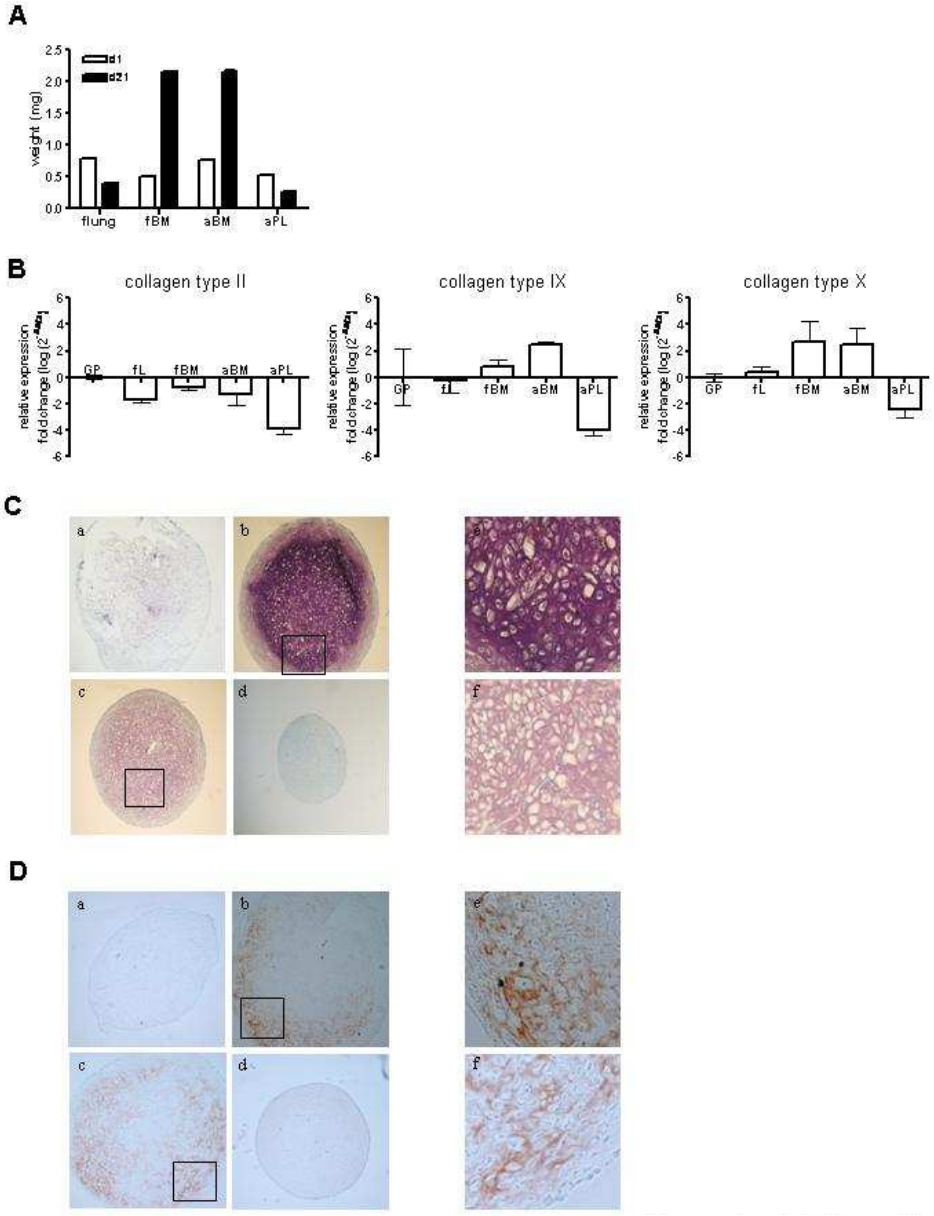

Figure 2. Chondrogenic differentiation of MSCs from different sources. (A) Wet weights of the pellets from the four different sources at day 1 and 21 of the induction period (n = 3). (B) Real-time RT-PCR analysis of collagen type II, IX and X during chondrogenic differentiation corrected for the housekeeping gene β2 microglobulin. Values are fold difference compared to expression in human growth plate (GP) and expressed as log ($2^{-\Delta\Delta Ct}$) (C) Toluidine Blue staining on paraffin embedded sections

after 3 weeks of differentiation. Representative pictures from each source at passage 3 are shown: a) fL-MSCs; b) fBM-MSCs; c) aBM-MSCs; d) PL-MSCs; e) and f) are enlargements of figure 2C b) and c) respectively, for detailed morphology. (D) Immunohistochemical staining for collagen type X on deparaffinezed sections after the induction period. Representative pictures at passage 3 are depicted: a) fL-MSCs; b) fBM-MSCs; c) aBM-MSCs; d) PL-MSCs; e) and f) are enlargements of figure 2D b) and c) respectively, for detailed morphology.

Table 2. Influence of cell passage on the chondrogenic capacity of MSCs from different tissue sources.

	P2-3	P4-5	P6-7
fL-MSC	0/4	0/4	0/4
fBM-MSC	3/3	3/3	1/3
aBM-MSC	4/4	4/4	2/4
PL-MSC	0/3	0/3	0/4

The differentiation capacity is expressed as number of positive samples on the total number of samples evaluated per passage-group. A sample is scored positive when satisfying the criteria described in Table 1.

Discussion

Skeletal defects resulting from disease, malformation or injury are an interesting area of application for stem cell therapy. The therapeutic effect of the transplantation of MSCs in children with *Osteogenesis Imperfecta*, a genetic disorder resulting in the abnormal production of collagen type I [15-17], suggests the potential of MSCs to ameliorate bone disorders.

MSCs are an ideal candidate for strategies of tissue engineering since they are easily isolated and can be rapidly expanded to numbers that are required for clinical application [10,18,19].

In this study we compared the chondrogenic differentiation potential of culture expanded MSCs derived from fetal BM and lung, placenta and adult BM. In comparison with fetal lung and placenta, fetal BM derived MSCs exhibited a significant enhanced capacity to differentiate into chondrocytes, as evidenced

by the increase in weight, diameter and area of pellets formed by bone marrow cells over the differentiation period, the increased levels in the expression of mRNA of extracellular matrix components, such as collagen type II, IX and X, the positivity of the immunohistochemical staining for collagen type X and the marked intensity of the Toluidine Blue staining. A similar prefered chondrogenic potential was observed for adult BM-derived MSCs, showing that BM as a source of MSCs was responsible for this differentiation potential, rather that the fetal developmental stage of the tissue. The chondrogenic differentiation was reduced and ultimately lost, after prolonged passages of the cultures. Although in vivo experiments are required to further substantiate the biological significance of these findings, our results suggests BM as the preferred source for cartilage tissue engineering.

MSCs have been shown to require specific culture conditions to induce differentiation towards the chondrogenic lineage. These requirements include a high cell density facilitating cell-cell contact and the use of serum-free medium with the addition of bioactive factors [20]. In particular BMP-6, a member of the TGF-β superfamily of growth factors, has been demonstrated to enhance the chondrogenic differentiation of human MSCs in a pellet culture system [21,22]. Our results confirm these findings and add that not only aBM-MSCs but also MSCs isolated from fetal tissues, namely lung and bone marrow, can undergo chondrogenesis under the same conditions.

In a study from *in't Anker* and colleagues [13] it was shown that MSCs isolated from second-trimester fetal bone marrow, lung, liver and spleen exhibit a different potential to differentiate into osteoblast and adipocytes, despite a similar immunophenotype. Moreover, a recent study from *Im et al* [23] demonstrated that adipose tissue-derived MSCs possess a lower osteogenic and chondrogenic potential than BM-derived MSCs. Also in our experiments, despite MSCs derived from the four different sources showed a comparable phenotypic characterization and morphology, the capacity to form cartilage was

more expressed in fBM- and aBM-MSCs. Moreover, we found that PL-MSCs were less capable to form both osteoblasts and adipocytes, while fL-MSCs showed to have a lower ability to differentiate into the adipogenic lineage compared to bone marrow sources. These differences in the differentiation potential might reflect some intrinsic diversities of MSCs residing in the various tissues, suggesting that the relation between immunophenotype and function of MSCs needs to be further investigated. Alternatively, the frequency of cells with lineage-specific differentiation capacity may differ between tissue sources. Indeed, the identification of specific markers enabling to distinguish between different populations of MSCs would be an important tool in the understanding and employment of these cells in the clinical setting.

We found that the chondrogenic potential of MSCs decreases with the increase of the cell passage, as shown by the inferior growth in size and the mild intensity of the Toluidine Blue staining in pellets from the later passages (P6-7). These findings are in agreement with the study from *Sekiya et al* [22] in which a decrease in the chondrogenic potential of adult bone marrow-derived MSCs was seen after each consecutive passage under very similar culture conditions. In *Sekiya* experience, only a selected population of MSCs, the so called small and rapidly self-renewing cells (RS cells), retained the ability to form cartilage at P5. This loss of chondrogenic potential may be due to the graduate elimination of MSCs with chondrogenic potential and overgrowth by MSCs that lack differentiation potential, or may be due to functional alterations in chondrogenic MSCs.

In conclusion, our results show that: I) MSCs of both fetal and adult origin undergo chondrogenesis under appropriate culture conditions; II) fBM- and aBM-MSCs express a higher chondrogenic ability than fL- and PL-MSCs, based on morphological, molecular, histochemical and immunohistochemical criteria; III) an inverse correlation between passage number and chondrogenic differentiation capacity of MSCs is present. Based on these observations and

considering the attractive role that MSCs could play in strategies of cartilage tissue engineering, our data suggest that bone marrow cells are to be considered as the preferred MSC source to be employed for cartilage repair.

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CHAPTER 3

Optimization of *in vitro* expansion of human multipotent mesenchymal stromal cells for cell-therapy approaches: further insights in the search for a fetal calf serum substitute

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Summary

There is great interest in mesenchymal stromal cells (MSCs) for cell-therapy and tissue engineering approaches. MSCs are currently expanded *in vitro* in the presence of fetal calf serum (FCS); however, FCS raises concerns when used in clinical grade preparations. The aim of this study was to evaluate whether MSCs expanded in medium supplemented with platelet-lysate (PL), already shown to promote MSC growth, are endowed with biological properties appropriate for cell-therapy approaches. We confirm previously published data showing that MSCs expanded in FCS/PL display comparable morphology, phenotype and differentiation capacity, while PL-MSCs were superior in terms of clonogenic efficiency and proliferative capacity. We further extended these data by investigating the immune-regulatory effect of MSCs on the alloantigen-specific immune response in mixed lymphocyte culture (MLC). We found that MSCs-PL are comparable to MSCs-FCS in their capacity to: i) decrease alloantigen-induced cytotoxic activity; ii) favor differentiation of $CD4^+$ T-cell subsets expressing a Treg phenotype; iii) increase early secretion of IL-10 in MLC supernatant, as well as induce a striking augmentation of IL-6 production. As compared with MSCs-PL, MSCs-FCS were more efficient in suppressing alloantigen-induced lymphocyte subset proliferation and reducing early IFNγ-secretion. Resistance to spontaneous transformation into tumor cells of expanded MSCs was demonstrated by molecular karyotyping and maintenance of normal morphology/phenotype after prolonged *in vitro* culture. Our data support the proposed immunological functional plasticity of MSCs and suggest that MSCs-PL can be used as an alternative to MSCs-FCS, although these latter cells might be more suitable for preventing/treating alloreactivity-related immune complications.

Introduction

Mesenchymal stromal cells (MSCs) are multipotent progenitors with the ability to differentiate along multiple cell lineages, such as osteoblasts, adipocytes and chondrocytes.[1-3] Bone marrow (BM) is the most common source of MSCs; however, MSCs have been isolated from various other sources, namely placenta, amniotic fluid, cord blood, adipose tissue and fetal lung.[4-11] Today MSCs are considered a useful tool for cell therapy and tissue engineering approaches;[12-14] in fact, these cells can be relatively easily isolated, mainly from BM, and display a remarkable capacity for extensive *in vitro* expansion. Indeed, MSCs have been already employed in clinical trials in a number of contexts, such as the facilitation of hematopoietic and immune reconstitution after hematopoietic stem cell transplantation (HSCT),[15,16] prevention and treatment of acute and chronic graft versus host disease (GvHD), [16,17] treatment of children with *Osteogenesis Imperfecta* (*O.I.*)[18-20] and metabolic disorders,[21] as well as for regeneration of bone and cartilage in degenerative disorders using tissue engineering techniques.[14,22,23].

MSCs are currently expanded *in vitro,* either under experimental or clinical grade conditions, in the presence of 10-20% fetal calf serum (FCS), which is considered crucial for the *ex-vivo* expansion of MSCs.[24,25] FCS characteristics are routinely pre-screened to guarantee both the optimal growth of MSCs and the bio-safety of the cellular product. Despite this, the use of FCS raises some concerns when utilized in clinical grade cellular preparations, since the administration of animal products to humans might theoretically cause the transmission of prions and still unidentified zoonoses. Moreover, bovine proteins or peptides might be incorporated by MSCs during culture procedures[26,27] and cause immune reactions in the host, especially if repeated infusions are needed, with consequent rejection of the transplanted cells.[19] As a results, several countries have legislated warnings and restrictions on the clinical use of cell therapy products prepared in the presence of FCS. In view of

these considerations, the identification of a serum-free medium appropriate for both the extensive expansion necessary to reach the large numbers of MSCs required for clinical application, and the exclusion of risks connected with the use of animal products, is warranted.

Recently, platelet-derived products have gained clinical interest due to their efficacy in enhancing bone regeneration and soft tissue healing.[28-30] Platelet-lysate (PL) is a concentration of human platelet growth factors in a small volume of plasma, obtained by lysing the platelet bodies through temperature-shock; therefore, PL contains all the fundamental growth factors that are secreted by platelets to initiate wound healing, including platelet-derived growth factors (PDGFs), basic fibroblast growth factor (b-FGF), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1) and transforming growth factor-β (TGF-β).[30,31]

As Doucet et al.[31] have recently demonstrated, PL is a powerful substitute for FCS in MSC expansion, thus we carried out a study aimed at evaluating whether human MSCs expanded *in vitro* in PL-supplemented medium are endowed with biological properties appropriate for their use in cell therapy approaches. In particular, we focused on the investigation of the immune regulatory effect of MSCs on alloantigen-specific immune response and the evaluation of MSC resistance to spontaneous transformation into tumor cells, a potential risk related to expansion procedures.

Materials and methods

Bone marrow donors

Bone marrow aspirates were harvested from eight healthy hematopoietic stem cell donors (median age 13.5 years), after obtaining written informed consent. Thirty ml of bone marrow (BM) from each donor were assigned to MSC generation; heparin was added as anticoagulant. The Institutional Review Board of Pediatric Hematology-Oncology approved the design of this study.

PL preparation

Apheresis procedures were performed at the Transfusion Service of our Hospital, collecting platelets (PLTs) from ten healthy volunteers, using the Trima Cobe (Lakewood, Co, USA) cell separator device. Written informed consent was always obtained. All apheresis products contained $5x10^{11}$ PLTs and were qualified according to Italian legislation. Immediately after collection, PLT apheresis products were frozen at -80°C and subsequently thawed at 37°C to obtain the release of PLT-derived growth factors. Heparin (5000 UI) was added to PLT bags to avoid gel formation. Apheresis were centrifuged at 900 g for 30 minutes, three times to eliminate platelet bodies. Finally, PL preparations obtained through this procedure were pooled in a single culture supplement to be used for the generation and expansion of MSCs from all BM donors enrolled in the study.

Isolation and culture of BM-derived MSCs

Mononuclear cells were isolated from BM aspirates (30 ml) by density gradient centrifugation (Ficoll 1.077 g/ml; Lymphoprep, Nycomed Pharma, Oslo, Norway) and plated in non-coated 75-175 cm^2 polystyrene culture flasks (Corning Costar, Celbio, Milan, Italy) at a density of 160,000/cm^2. Four different culture conditions, based on the basal medium (Mesencult, StemCell Technologies, Vancouver, Canada) supplemented with 2mM L-glutamine and 50 µg/ml gentamycin (Gibco-BRL, Life Technologies, Paisely, UK) were tested: I) 10 % FCS (Mesenchymal Stem Cell Stimulatory Supplements, StemCell Technologies); II) 5% PL; III) 2,5% PL; IV) 1% PL. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. After 48 hour adhesion, non-adherent cells were discarded and culture medium was replaced twice a week. MSCs grown under the four different culture conditions, were harvested after reaching ≥ 80% confluence, using Trypsin (Sigma-Aldrich, Milano, Italy), and re-plated for expansion at 4,000 cells/ cm^2 until passage (P)

5. MSCs from four donors were cultured until P10. The same approach was employed for eight different BM donors from whom MSCs were generated and cultured in parallel under the four conditions mentioned.

The colony-forming unit-fibroblast assay (CFU-F) was performed as described previously.[32,33] CFU-F formation was examined under the four culture conditions after incubation for 12 days in a humidified atmosphere (37°C, 5% CO_2); the clonogenic efficiency was calculated as the number of colonies per 10^6 BM mononuclear cells seeded. According to the International Society for Cellular Therapy on the nomenclature of mesenchymal progenitors, the cells cultured for this study were defined as multipotent stromal cells.[34]

MSC Multilineage differentiation potential

The adipogenic and osteogenic differentiation capacity of MSCs was determined at P2-3 for all BM donors as previously described (in't Anker et al, 2003), utilizing the respective supplement (10% FCS, 5% PL, 2.5% PL, 1% PL) for each culture condition. To detect the osteogenic differentiation, cells were stained for alkaline phosphatase (AP) activity using Fast Blue (Sigma-Aldrich) and for calcium deposition with Alzarin Red (Sigma-Aldrich). Adipogenic differentiation was evaluated through the morphological appearance of fat droplets with Oil Red O (Sigma-Aldrich).

Flow cytometry

FITC, PE, PerCP, or PerCPCy5.5 monoclonal antibodies (MoAb) specific for the following antigens were employed: 1) CD45, CD14, CD34, CD13, CD80, CD31, HLA A-B-C, HLA-DR, CD90, CD73, CD62L, CD11a, CD11c, CD18, CD49d, anti-human integrin β7 (BD PharMingen, San Diego, CA, USA), CD105, CD166, CD44, CD29 (Serotec, Kidlington, Oxford, UK) for the assessment of MSC surface phenotype; 2) CD3, CD4, CD8, CD56, CD25, CD152 (CTLA4), CD27 (BD PharMingen), Foxp3 (eBioscience, San Diego,

CA, USA), for evaluation of lymphocyte subsets. Appropriate isotype-matched controls (BD Bioscience, eBioscience) were included. Intracellular staining for CD152 (CTLA4) and Foxp3 was performed following the manufacturer's instructions. In brief, cells were stained with MoAbs to surface antigens (CD4 and CD25), washed, fixed, permeabilized and stained for intracellular antigens with specific anti-CD152 or anti-Foxp3 MoAb. Two-color or three-color direct immune fluorescence cytometry, with FACScalibur flow cytometer (BD Biosciences), was performed according to a previously described method.[35]

Mixed lymphocyte cultures (MLCs) and cytotoxicity assay

Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque density gradient from healthy volunteer's heparinized PB samples and employed on the same day of collection. Primary MLCs were performed according to previously described methods;[35,36] the only difference with previously employed methodological approaches was the use of 10% FCS supplemented RPMI 1640, instead of RPMI 1640 supplemented with 5% pooled human serum in order to avoid any interference with human cytokines measurement. Briefly, non-irradiated "third-party" MSCs, allogeneic to both responder (R) and stimulator (S) PBMCs, were added at the R to MSC ratio of 10:1. The immune regulatory effect of MSCs cultured with either 5% PL (MLC+MSC-PL) or 10% FCS (MLC+MSC-FCS) was compared, using MLC performed in the absence of MSC as a control (ctrl-MLC). T and NK-lymphocyte subset expansion was evaluated by counting $CD3^{+}CD4^{+}$ or $CD3^{+}CD8^{+}$ T-cells and $CD3^{neg}CD56^{+}$ NK-cells per ml of culture, recovered after 10-days MLC and comparing those with the initial number of cells (day 0). Differentiation of regulatory T cells (Treg) was evaluated by the detection of the percentage of $CD4^{+}CD25^{+}$ and $CD4^{+}CD25^{bright}$ T lymphocytes, together with the expression of CD152, CD27 and Foxp3 on CD4+CD25+ lymphocytes.

Alloantigen-induced cell-mediated cytotoxic activity was tested in a 5-hour ^{51}Cr-release assay as previously described.[35,36] Results are expressed as % of specific lysis of target cells. ^{51}Cr-labeled target cells included PHA-activated S-PBMCs (S-PHA) and the same lots of MSC-PL or MSC-FCS added to MLCs. The only difference with previously employed methodological approaches[35] was that adherent MSCs ^{51}Cr-labeled over-night were the target for the cytotoxicity assay.

Measurement of growth factors and cytokines by ELISA

The concentration of PDGF-AB, TGF-β1, b-FGF, IGF-1, VEGF in PL and FCS was evaluated using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA), following the manufacturer's instructions. The concentration of IFN-γ, IL10, IL6, IL12, IL7, IL2, IL15 and TGFβ in supernatant of MLC after 12, 24, and 48 hours, was quantified by ELISA using monoclonal antibody pairs (Pierce Endogen, Rockford, IL, USA). Briefly, plates (Corning Costar) were coated with purified antibodies at the appropriate concentrations. Standard curves were prepared with recombinant human cytokine (Pierce Endogen). Biotin-labelled antibodies (Pierce Endogen) were added and HRP-conjugated streptavidine (Pierce Endogen) was used to develop the reactions. Plates were read at 450 nm (Titertek Plus MS 212M).

Molecular karyotyping

Molecular karyotyping was performed through array comparative genomic hybridization (array-CGH) with the Agilent kit (Human Genome CGH Microarray, Agilent Technologies, Santa Clara, CA, USA) on MSCs cultured either in 10% FCS or 5% PL. Cultures from four BM donors at different passages (from P2 to P7) were analyzed. The array-CGH platform is a 60-mer oligonucleotide-based microarray that allows a genome-wide survey and molecular profiling of genomic aberrations with a resolution of ~35 kb (kit 44B). DNA was extracted with GenElute blood genomic DNA kit (Sigma-

Aldrich) according to the manufacturer's protocol. DNA (7μg) from MSCs and controls of the same sex (control DNA, Promega, Madison WI, USA) were double-digested with RSAI and AluI (Promega) for 3 hours at 37 °C. After column purification, 2 μg of each digested sample were labeled by random priming (Invitrogen, Carlsbad, CA, USA) for two hours using Cy5-dCTP (Amersham, GE Healthcare, UK) for the MSCs DNA and Cy3-dCTP (Amersham) for the control DNA. Labeled products were column purified (CyScribe GFX Purification Kit, Amersham). After probe denaturation and pre-annealing with 50 μg of Cot-1 DNA (Invitrogen), hybridization was performed at 65 °C with shaking for 40 hours. After two washing steps, the array was analyzed through the Agilent scanner and the Feature Extraction software (v8.1). Graphical overview was obtained using the CGH analytics software (v3.2.32).

Statistical analysis

CFU-F numbers and cumulative cell counts obtained under the four different culture conditions were compared through analysis of variance followed by *post hoc* comparisons between each possible pair condition (10% FCS, 5% PL, 2.5% PL, 1% PL) applying the Bonferroni correction for multiple tests. The non parametric Kruskall-Wallis test was performed for the comparison of the expansion time from P0 to P5; *post hoc* comparisons between each possible condition pair applying the Bonferroni correction for multiple tests were performed.

Results

MSC CFU-F frequency and proliferative capacity

In order to compare the effect of FCS with that of decreasing PL concentrations on the proliferative capacity of MSCs, BM-derived mononuclear cells from eight different donors were plated in parallel cultures utilizing the four different

culture conditions (I, II, III, IV, see “Materials and Methods” section). BM samples were assayed for CFU-F frequency after 12-days culture and the results were as follows: condition I (10% FCS) showed a mean value of 15.75 ± 2.06 CFU-Fs per 10^6 mononuclear cells plated, whereas the mean value of MSC cultured in medium II (5% PL), III (2.5% PL) and IV (1% PL) was 28.50 ± 3.61 ($P<0.00001$), 17.33 ± 6.21 ($P=0.5$), 2.25 ± 1.22 ($P<0.00001$) CFU-Fs, respectively. As compared to MSCs cultured in the presence of 10% FCS, the calculated cumulative cell counts (Figure 1) were significantly higher when MSCs were cultured in the presence of 5% PL ($P<0.00001$), comparable in the presence of 2.5% PL ($P=0.13$) and significantly lower when MSCs were grown in the presence of 1% PL ($P<0.00001$). Indeed, a clear dose-dependent effect of PL on the proliferative capacity of MSC was present in all cultures; this effect was also noted in the capacity to form CFU-F in the presence of 5% PL, as this condition yielded the highest frequency of units that were also the largest in size (data not shown). Moreover, the median time to reach 80% confluence was shorter for all passages from 1 to 5 in the presence of 5% PL (5.5 days) than for all other culture conditions. In fact, the required median time to approach 80% confluence was 7.5, 8, and 10 days for condition III (2.5% PL), I (10% FCS), and IV (1% PL) respectively. In particular, when cultured in the presence of 5% PL, MSC reached P5 in 39.5 ± 1.2 days ($P<0.00001$), whereas it took 50 ± 2.5 and 66.5 ± 2.3 days for conditions III ($P=0.23$) and IV ($P<0.00001$) respectively. The time needed for MSCs to reach P5 in the presence of FCS was 51.7 ± 2.9 days. Therefore, condition II was associated with an advantage in terms of time, approximately 10 days for a complete cycle of expansion, yielding a number of cells that was more than 1 logarithm superior when compared to condition I. Condition II was the only one employed in the comparison with FCS in the analysis of immune regulatory effect and molecular karyotyping (see below).

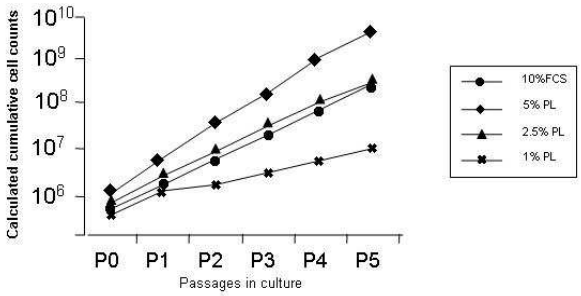


Figure 1. Calculated cumulative cell counts of MSCs cultured from passage (P) 0 to 5, in the presence of 10 % FCS; 5 % PL; 2,5 % PL; 1% PL. Results are expressed as the mean calculated from data obtained from eight BM donors.

MSC Morphology, surface phenotype and differentiation capacity

MSCs isolated in the presence of either FCS or one of the three PL concentrations displayed the characteristic MSC-like spindle-shape; however, subtle differences in morphology were observed (Figure 2A). In fact, MSCs cultured in PL-containing media (condition II, III and IV) showed a thinner shape compared to the thicker MSCs expanded in the presence of FCS; moreover, PL-MSC frequently tended to grow in clusters rather than a uniform distribution in the flask. MSCs cultured in the presence of each concentration of PL required only 2-3 minutes incubation with trypsin at room temperature to obtain their complete detachment from the plastic, whereas 5-8 minutes at 37°C were necessary to harvest MSCs supplemented with 10% FCS. In this regard, the expression of surface adhesion molecules and integrins (CD166, CD62L, CD44, CD49d, CD29, CD11a, CD18, CD11c, anti-human integrin β7) was comparable on MSCs expanded both in FCS and 5% PL (data not shown).

The surface phenotype of MSCs cultured under the four different conditions was analyzed by flow cytometry at P1, P3 and P5; the phenotypes were similar and in agreement with previous reports;[3,9,10] (data not shown). In particular, by the third passage, contamination with hematopoietic cells was no longer detectable for all four culture conditions and greater than 98% of cells expressed

the MSC-typical surface marker pattern. In detail, MSCs were positive for CD90, CD73, CD105 and CD13 surface antigens and negative for CD34, CD45, CD14, CD80, CD31 molecules. The expression of HLA-DR was always below 2% under all culture conditions, whereas HLA-class I was uniformly present on MSCs (>98% of positive cells).

MSCs expanded under the four culture conditions were induced into osteoblasts and adipocytes and examined for their differentiation capacity by histological staining. Results demonstrated that MSCs cultured in medium I, II, III and IV were all comparably able to differentiate into osteoblasts (Figure 2B) and adipocytes (Figure 2C). In accordance with previously reported data[31], no macroscopic differences were detected in the capacity to form both osteoblasts and adipocytes under the four culture conditions for the eight donors studied.

MSC cultures expanded from four donors, in the presence of either FCS or 5% PL, were prolonged until P10 without observing any alteration in their morphology and surface phenotype.

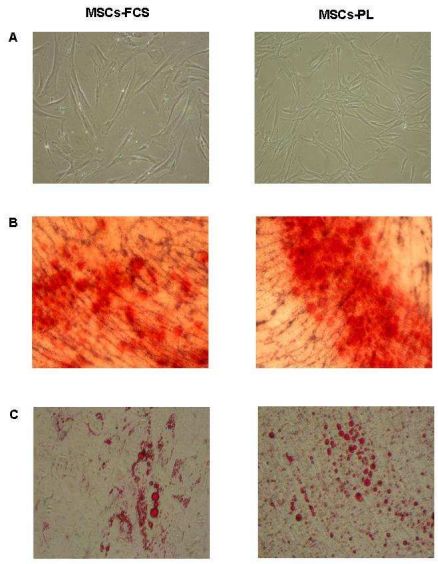


Figure 2. MSC morphology and differentiation capacity. A) Representative photographs of MSCs expanded in the presence of 10% FCS (MSCs-FCS) and 5% PL (MSCs-PL) from donor 5 are presented. The morphology of MSCs expanded in the presence of

2.5% and 1% PL was similar to cells cultured with 5% PL. MSCs-PL display the characteristic spindle-shaped morphology, however cells tend to be thinner compared to MSCs-FCS and to grow in clusters. Magnification x10. B) Osteogenic differention capacity of MSCs-FCS and MSCs-PL (+5% PL). The differentiation into osteoblasts is demonstrated by the histological detection of AP activity (purple reaction) and calcium deposition stained with Alzarin Red staining. Shown are representative photographs from donor 2. Magnification x 20. C) Adipogenic differentiaton capacity of MSCs-FCS and MSCs-PL. The differentiation into adipocytes is revealed by the formation of lipid droplets (stained with Oil Red O staining). Shown are representative photos from donor 2. Magnification x 20. For B) and C) results for MSCs expanded in 2.5% and 1% PL were comparable.

Ex-vivo expanded MSC immune regulatory effect

As mentioned above, for this set of experiments, MSCs cultured in the presence of FCS (MSCs-FCS) were compared with MSCs expanded in the presence of 5% PL (MSCs-PL).

The immune regulatory capacity of *ex-vivo* expanded MSCs was evaluated by assessing the *in vitro* interaction between MSCs and the alloantigen-specific immune response elicited in primary MLC in two independent experiments. In agreement with several previously reported studies, we observed that the addition of both MSCs-FCS and MSCs-PL were able to inhibit alloantigen-induced lymphocyte proliferation, even though MSCs-FCS apparently displayed a stronger inhibitory effect than MSCs-PL (Figure 3A).

The stronger inhibitory effect of MSCs-FCS, as compared to MSCs-PL, was evident on total $CD3^+$ cells (Figure 3B), on $CD4^+$ (Figure 3C) and $CD8^+$ (Figure 3D) T lymphocytes, as well as on $CD3^{neg}CD56^+$ NK cells (Figure 3E). In particular, the inhibitory effect of MSCs-PL on $CD4^+$ T lymphocyte proliferation was almost negligible in both experiments.

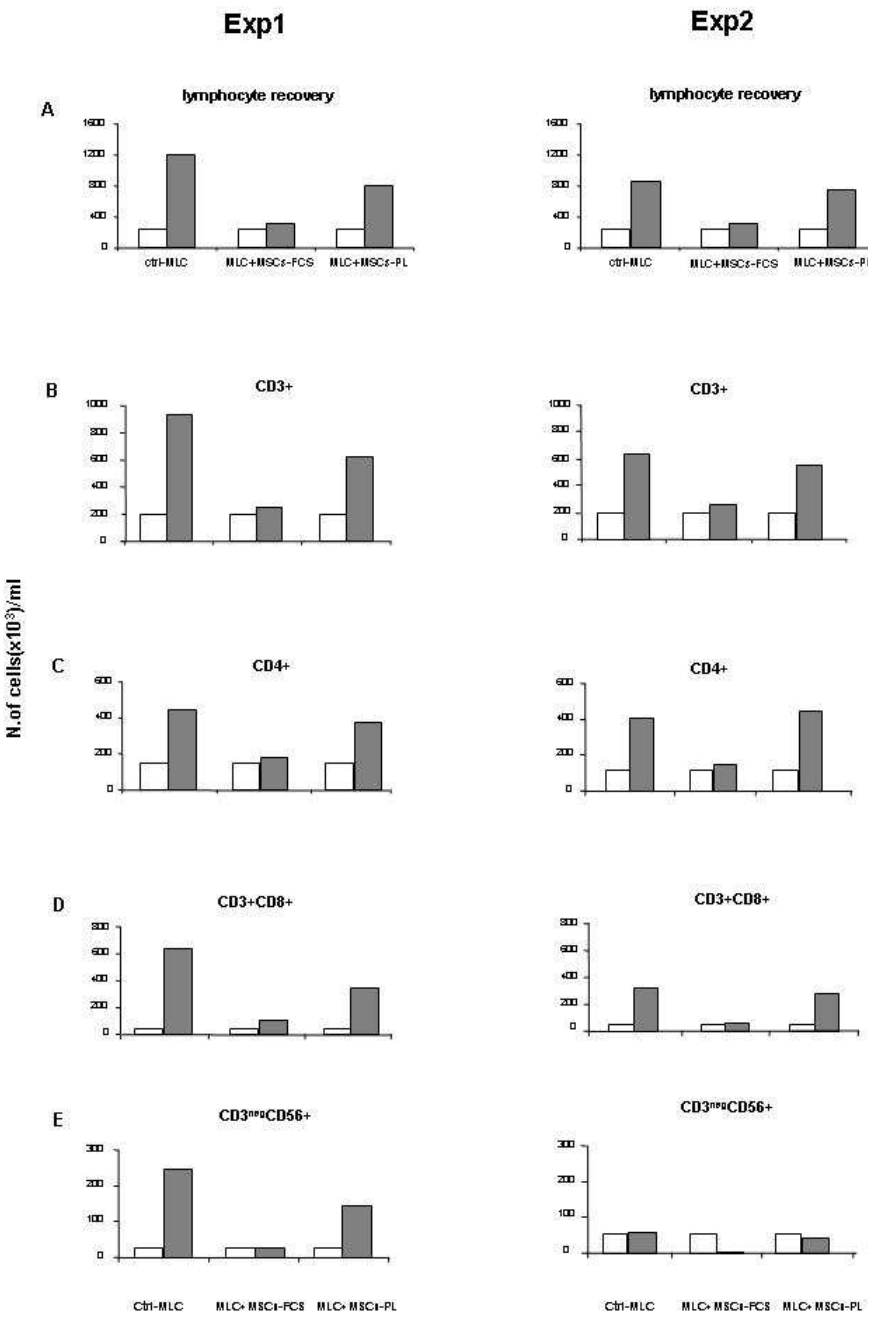
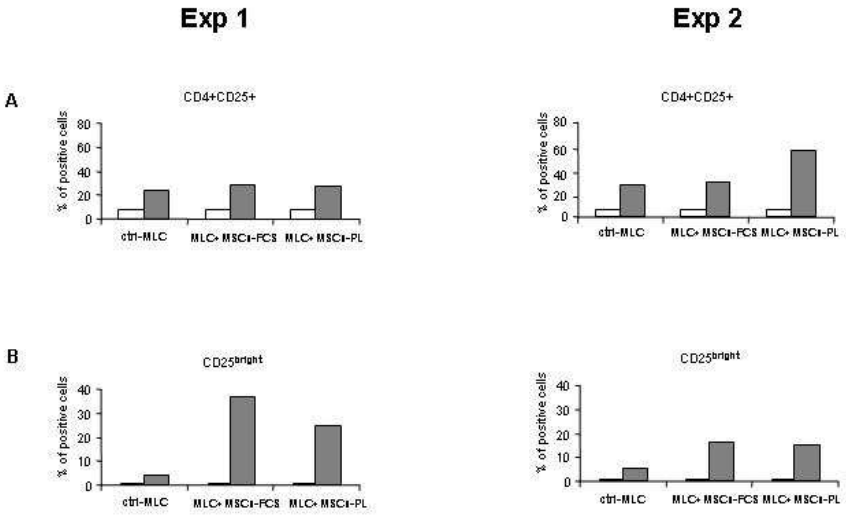


Figure 3. Effect of MSCs on T and NK-lymphocyte subset expansion induced by allogeneic stimulus. Recovery of total number of lymphocytes (A), CD3+ (B), CD4+ (C), CD3+CD8+ (D) and $CD3^{neg}CD56+$ (E) T-lymphocytes subsets, with respect to the

initial number (white columns), was assessed after 10-days primary culture (gray columns). MLC was performed in the absence (Ctrl-MLC) or presence of third-party MSCs cultured in 10% FCS (MLC+MSCs-FCS) or 5% PL (MLC+MSCs-PL). The MSCs were added at a R-PBMC/MSC ratio of 10:1; results are expressed as number of cells/ml of culture. Two independent experiments (Exp 1, Exp 2) are presented.

The percentage of $CD4^+CD25^+$ T cells considerably increased after 10-days primary ctrl-MLC in both experiments, as compared to day 0 (Figure 4A); a comparable increase was observed in MLC supplemented with either MSC-FCS or MSC-PL in experiment 1, while a higher percentage of this cell subset was observed after addition of MSC-PL, as compared to ctrl-MLC, in experiment 2. In an attempt to discriminate $CD4^+CD25^+$ Tregs from conventional early activated $CD4^+CD25^+$ T lymphocytes, expression of the level of CD25 ($CD4^+CD25^{bright}$ T cells, Figure 4B), as well as CD27, CTLA4 and FoxP3 molecules (Figure 4C) was evaluated within the $CD4^+CD25^+$ T cell subset. We found a higher percentage of $CD4^+CD25^{bright}$ and an augmented percentage of FoxP3+ cells in the presence of either MSCs-FCS or MSCs-PL as compared to ctrl-MLC, while CTLA4 and CD27 were variably expressed in the two experiments.



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	CTLA4	CD27	FoxP3
Ctrl-MLC	99	86	20
MLC+MSCs-FCS	100	87	52
MLC+MSCs-PL	100	81	36

	CTLA4	CD27	FoxP3
Ctrl-MLC	24	57	25
MLC+MSCs-FCS	62	75	30
MLC+MSCs-PL	42	52	43

Figure 4. Effect of MSCs on differentiation of $CD4^+CD25^+$ T-lymphocyte subsets induced by allogeneic stimulus. Percentages of $CD4^+CD25^+$ cells (A) and $CD25^{bright}$ (on gated CD4+ cells) (B) were calculated on effectors recovered after 10 days (gray columns) and compared to the initial cell counts (white columns). Percentages of $CTLA4^+$, $CD27^+$ and $Foxp3^+$ cells (C) were calculated on gated $CD4^+CD25^+$ cells. MLC was performed in the absence (Ctrl-MLC) or presence of third-party MSCs cultured in 10% FCS (MLC+MSCs-FCS) or 5% PL (MLC+MSCs-PL). The MSCs were added at a R-PBMC/MSC ratio of 10:1; results are expressed as percentage of positive cells. Two independent experiments (Exp 1, Exp 2) are presented.

Evaluation of the cytokine production kinetics, documented that: i) MSCs-FCS are able to inhibit, while MSCs-PL increase early IFNγ secretion in primary MLC; ii) both types of MSCs increase early secretion of IL-10 in primary MLC; iii) a remarkably high production of IL-6 can be observed in MLC grown in the presence of both MSCs-FCS and MSCs-PL, as compared with ctrl-MLC or

MSCs alone (see Table 1). IL-12, IL-7, IL-2 and IL-15 were undetectable in all culture conditions, while results of TGFβ secretion were considered unreliable, due to the high concentration of this cytokine in the FCS supplemented medium employed for MLC experiments.

Table 1. Kinetics of cytokine secretion in culture supernatants

		Exp1			Exp2		
		12h	24h	48h	12h	24h	48h
IFNγ	ctrl-MLC	14	43	506	<0.21	<0.21	12
	MLC+MSCs-FCS	9	32	172	<0.21	<0.21	2
	MLC+MSCs-PL	14	43	514	<0.21	18	78
IL-10	Ctrl-MLC	251	303	174	5	39	29
	MLC+MSCs-FCS	296	416	404	22	192	170
	MLC+MSCs-PL	338	391	417	13	237	222
IL-6	Ctrl-MLC	3174	2870	1475	<1.9	624	<1.9
	MLC+MSCs-FCS	53000	75000	64000	2093	15900	64400
	MLC+MSCs-PL	49000	55000	77415	1107	12600	27370

Concentration of IFN-γ, IL-10, IL-6 and TGFβ was quantified in MLC-supernatants collected after 12, 24, 48 hours culture in the absence (ctrl-MLC) or the presence of MSCs-FCS or MSCs-PL. IFNγ, IL-10 and IL-6 were undetectable in the supernatant of MSCs simultaneously cultured in the absence of PBMCs. Results are reported as pg/ml. Two independent experiments (Exp 1, Exp 2) are presented.

In order to assess the effect of MSCs on alloantigen-specific cytotoxic lymphocytes, effector cells recovered from MLC were tested for their cytotoxic capacity towards allogeneic target cells (S-PHA blasts), that were stimulator cells in MLC. Results shown in Figure 5A demonstrate that both MSC-FCS and MSC-PL are endowed with the capacity to inhibit alloantigen-induced cell-mediated cytotoxicity. Alloantigen-induced cytotoxic capacity was also tested towards the same lot of "third-party" MSC-FCS or MSC-PL, added to MLC at day 0. This part of the experiments was planned to investigate the ability of lymphocytes activated by alloantigens, in the presence or absence of "third-

party" MSCs, to mediate cell lysis of MSCs themselves. Results demonstrate a low level of MSCs lysis in both ctrl-MLC experiments; the presence of either MSC-FCS or MSC-PL only marginally affected this type of cytotoxic activity (Figures 5B and 5C).

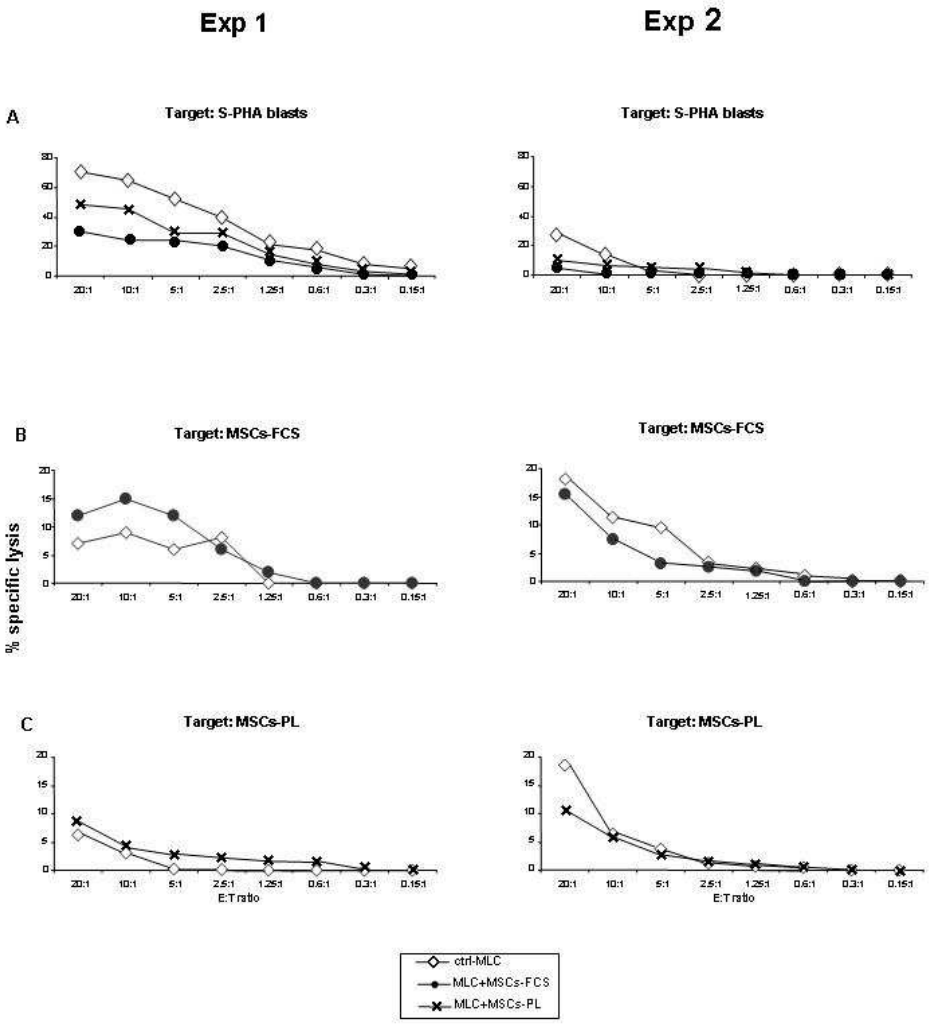

Figure 5. Effect of third-party MSCs on cell-mediated cytotoxic activity induced by allogeneic stimulus. ^{51}Cr-labeled target cells included S-PHA (A) and the same lots of MSCs-FCS (B) or MSCs-PL (C) added to MLCs. Effector to target (E:T) ratios ranged between 20:1 to 0.15:1. Results are expressed as % specific lysis of target cells. Two independent experiments (Exp 1, Exp 2) are presented.

Molecular karyotyping

Also for this set of experiments, MSCs-FCS and MSCs-PL were chosen and tested for their genomic situation; in particular four of the eight BM donors were studied at baseline (BM mononuclear cells) and at different passages in culture by means of array-CGH. In order to avoid false positive results, we performed the array-CGH on BM mononuclear cells against control DNA and by mixing MSCs-PL with MSCs-FCS. In the latter case, duplications/deletions that could be found in MSC-FCS or MSC-PL against control DNA but not in MSC-FCS against MSC-PL could be safely considered polymorphisms, whereas duplications present in MSC-FCS against control DNA that corresponded to deletions in MSC-PL against MSC-FCS or viceversa were considered indicative of a true imbalance.

The results of array-CGH demonstrated that MSCs expanded *in vitro*, both in the presence of PL and FCS, do not show imbalanced chromosomal rearrangements; indeed we could not detect any, deletion or duplication of material in the samples studied even at a submicroscopic level. However, array-CGH is not able to unravel balanced chromosomal rearrangements; this has to be properly excluded by an assessment with classic cytogenetics.

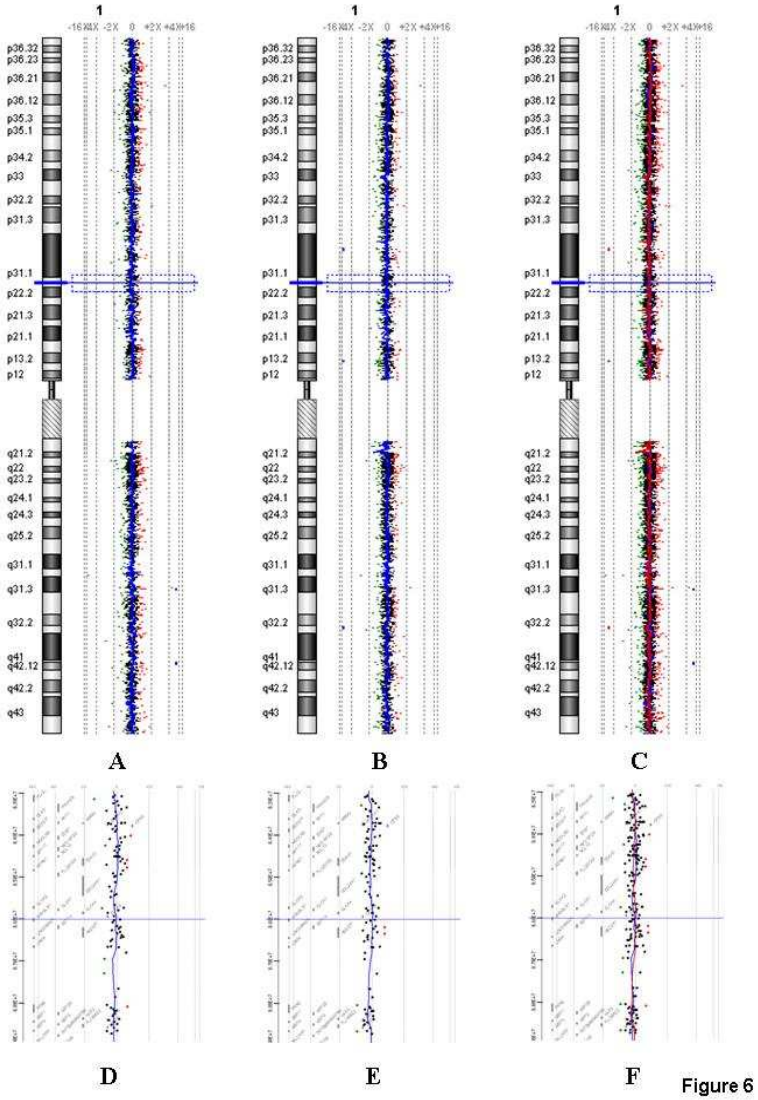

Figure 6. Representative array-CGH profiles of chromosome 1 of the same MSCs donor. A) MSCs cultured in the presence of 5% PL at P1; B) MSCs cultured in the presence of 10% FCS at P7; C) The two experiments are superimposed: blue and red lines apply to MSCs cultured in 5% PL and in 10% FCS respectively; D,E,F) Enlargements of the regions indicated by the blue panels in every experiment shown above. The array-CGH profiles of MSCs cultured in 5% PL and 10% FCS are linear and perfectly overlapped. This demonstrates that *in vitro* expanded MSCs do not show unbalanced chromosomal rearrangements.

Discussion

Many insights in the MSC biology, as well as of their immune regulatory properties and regenerative potential, have been obtained in the last few years and these have provided the support for considering MSCs today as an attractive and powerful tool for cell therapy-based approaches.[12-23] FCS is currently utilized to supplement culture medium in protocols designed to generate and expand *in vitro* MSCs to be employed for clinical use.[25] However, for cell therapy-based approaches, MSCs should be expanded according to Good Manufacturing Practice (GMP) procedures that require very stringent quality criteria for sterility and the utilization of specific reagents, preferably devoid of heterologous proteins (see European Commission-Health and Consumer Protection Directorate-General. Technical requirements for the coding, processing, preservation, storage, and distribution of human tissues and cells. Directive 2004/23/EC). Alternatives to the use of FCS might be autologous serum

PL preparations have already been demonstrated to be a powerful source of growth factors, useful in the treatment of a variety of soft and hard-tissue surgical conditions and in the management of non-healing wounds.[28-30] The utilization of PL as a culture supplement for MSC expansion in cell therapy-based protocols has been recently suggested as a promising alternative to FCS.[31]

In this study, we have tested three different concentrations of PL, and compared them with FCS, for *in vitro* expansion of human MSCs, in particular focusing on the immune regulatory activity of the different types of MSC and the maintenance of their karyotype stability at the end of the expansion procedure.

Our data demonstrate that 5% PL is superior to 10% FCS in terms of clonogenic efficiency and proliferative capacity, therefore providing more efficient expansion, together with significant a time saving. When lower concentrations of PL were employed, the clonogenic efficiency was either comparable (2.5% PL) or inferior (1% PL) to that of MSCs cultured in the presence of FCS.

Altogether these data suggest that PL preparations exert a dose-dependent effect on MSC expansion. The expansion promoting effect is likely due to the high concentration of natural growth factors contained in PL. Indeed, we have measured the concentrations of PDGF-AB, TGF-β1, b-FGF, IGF-1 and VEGF in the pooled PL and all values resulted remarkably superior to the concentrations of the same growth factors present in our lot of FCS (data not shown).

Our findings are in keeping with those published by Doucet et al.[31] demonstrating that growth factors contained in PL are able to promote MSC expansion in a dose-dependent manner. However, while Doucet and colleagues[31] showed that 5% PL was able to increase the size of CFU-F but not their number; we found that the clonogenic efficiency of MSCs generated in the presence of 5% PL was significantly superior ($P<0.00001$) as compared to 10% FCS. This discrepancy might be explained by differences in the MSC isolation procedure (spongious bone fragment supernatant vs BM aspirates as a starting material, plating concentrations) or in the assessment time of CFU-F (10 vs 12 days).

Moreover, in contrast with the observations of Doucet et al.[31], we noted a subtly different morphology in our MSCs cultured in the presence of each concentration of PL, as compared to cells grown in 10% FCS. In fact, MSCs expanded in PL, although maintaining a spindle-shape, resulted finer/thinner in width and tended to grow in clusters in the flasks. Moreover, MSCs cultured in PL behaved differently from those grown in 10% FCS in the trypsinization phase, requiring a very short time for detachment from the plastic. In a recent study from Shahdadfar et al.[37], in which autologous serum (AS) was compared to FCS for efficiency in supporting the expansion of MSCs, a similar phenomenon was described, the use of AS providing a very rapid detachment of MSCs from the flasks. In agreement with their results, also in our experiments, the expression of some adhesion molecules and integrins tested on the surface

of MSCs expanded both in 10% FCS and 5% PL could not account for this different behavior.

As compared to MSCs-FSC, the different concentrations of PL altered neither the purity nor the phenotype of the expanded MSCs and the expression of the typical MSC markers was maintained unmodified until P5 in all donors tested. In keeping with Doucet et al.[31], MSCs expanded in the presence of PL retained their ability to differentiate into osteogenic and adipogenic lineages, demonstrating that PL do not affect the multipotency of these cells.

Four MSC samples, expanded in the presence of either FCS or PL until P10 (in a time-frame of around 14 weeks) demonstrated a progressive decrease in the expansion capacity together with the maintenance of their original surface phenotype and spindle shape morphology. These data suggest that BM-derived MSCs do not display an aptitude for spontaneous transformation, in contrast to what has been recently described by Rubio et al.[38] for MSCs derived from adipose tissue.

The bio-safety of BM-derived MSCs was further confirmed by karyotype analysis performed by means of array-CGH; in fact, the results of these experiments demonstrated that MSCs expanded *in vitro* both in the presence of PL and FCS are devoid of genomic imbalances. Recently, array-CGH has been introduced as a rapid and high-resolution method for the detection of both benign and disease-causing genomic copy-number variations.[39] This technique has been successfully used for analysis of tumor samples and cells lines[40,41], and more recently also used to test cultured embryonic stem cells[42]. The relevant interest emerging regarding the utilization of MSCs in clinical approaches in several fields in medicine, requires that their karyotype be tested after prolonged *in vitro* culture in order to guarantee their bio-safety. In fact, detection of cytogenetic aberrations arising during the expansion period in cells would obviously represent a strong and clear contraindication for their clinical use. Presently, array-CGH, thanks to its high genomic resolution, may be considered

the method of choice to test the genetic situation of MSCs expanded *in vitro*, although balanced chromosome rearrangements should contemporarily be excluded by traditional cytogenetics.

Our study demonstrates that the immune regulatory properties of MSC-PL are comparable to those of MSCs-FCS in terms of their capacity to decrease alloantigen-induced cytotoxic activity, favor the differentiation of $CD4^+$ T cell subsets expressing the Treg phenotype[43], increase the early secretion of IL-10 in MLC supernatant, as well as induce a striking augmentation of IL-6 production. On the contrary, the suppressive effect on alloantigen-induced lymphocyte subset proliferation and early IFNγ-secretion was more evident with MSCs-FCS, as compared to MSCs-PL. Both MSCs-FCS and MSCs-PL are susceptible to partial lysis by cytotoxic cells emerging from MLC. Taking into account that both alloantigen-specific cytotoxic T lymphocytes and alloreactive NK or NK-like cells are able to mediate alloantigen-induced cell-mediated cytotoxic activity[36,44,45], these results are in keeping with two recently published studies documenting that *in vitro* activated NK cells are able to lyse either autologous or allogeneic human MSC[46,47].

The immune regulatory function of human MSC has been extensively investigated[48]. While there is general agreement on the fact that human MSCs are able to impair alloantigen-induced lymphocyte proliferation[48,35], conflicting results on other immune properties of MSCs have been reported and these discrepancies might perhaps be explained by the functional plasticity of these cells. For instance, several studies have demonstrated that the immune suppressive activity of MSCs is related to their capacity to alter dendritic cell function and to impair antigen-presenting cell (APC) maturation[35,49,50,51], while even more recent reports documented antigen-presenting properties of these cells.[52,53] Taking into account that IL-6 is a pro-inflammatory cytokine involved in the regulation of several immune functions including the enhancement of APC function and cytotoxic lymphocyte activity[54], our observation reported

here on the production of large quantities of IL-6 in MLC supernatants, related to the presence of MSCs, should be in line with the antigen-presenting properties of these cells. On the other hand, in the same MLC we also observed MSC capacity to decrease alloantigen-specific cytotoxic activity.

Altogether, our data support the hypothesis of a remarkable immunological functional plasticity of human MSCs and suggest that the use of MSCs-PL, which seem to be endowed with a relatively low immune suppressive activity, could be more appropriate in reparative/regenerative cell-therapy approaches or in strategies aimed at improving hematopoietic/immune recovery after HSCT. On the contrary, as MSCs-FCS seem to display a more pronounced immune suppressive function, they might be more suitable for preventing or treating alloreactive-related immune complications, such as severe GvHD in HSCT and graft rejection in HSCT and solid organ transplantation.

In view of our results, we propose that 5% PL may replace FCS in the generation and expansion of MSCs in some cell-therapy protocols. Indeed, in the clinical setting where at least $1x10^6$ MSC/Kg are required, the use of 5% PL appears to provide very efficient expansion in a time-frame of 2-3 weeks, instead of 4-5 weeks necessary with current protocols. In this regard, further studies are necessary to precisely characterize the growth factor composition of PL, considering its variability from donor to donor, to optimize the preparation procedure for PL and the MSC expansion protocol with this supplement.

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CHAPTER 4

Human bone marrow-derived mesenchymal stem cells do not undergo transformation after long-term *in vitro* culture and do not exhibit telomere maintenance mechanisms

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Summary

Significant improvement in the understanding of mesenchymal stem cell (MSC) biology has opened the way to their clinical use. However, concerns regarding the possibility that MSCs undergo malignant transformation have been raised. We investigated the susceptibility to transformation of human bone marrow (BM)-derived MSCs at different *in vitro* culture time points. MSCs were isolated from BM of 10 healthy donors and propagated *in vitro* until reaching either senescence or passage (P) 25. MSCs in the senescence phase were closely monitored for 8-12 weeks before interrupting the cultures. The genetic characterization of MSCs was investigated through array-comparative genomic hybridization (array-CGH), conventional karyotyping and subtelomeric fluorescent *in situ* hybridization (FISH) analysis both before and after prolonged culture. MSCs were tested for the expression of telomerase activity, hTERT transcripts and alternative lengthening of telomere (ALT) mechanism at different passages. A huge variability in terms of proliferative capacity and MSCs life-span was noted between donors. In eight of ten donors, MSCs displayed a progressive decrease in proliferative capacity until reaching senescence. In the remaining two MSC samples, the cultures were interrupted at P25 to pursue data analysis. Array-CGH and cytogenetic analyses demonstrated that MSCs expanded *in vitro* did not document chromosomal abnormalities. Telomerase activity and hTERT transcripts were not expressed in any of the examined cultures and telomeres shortened during the culture period. ALT was not evidenced in the MSCs tested. BM-derived MSCs can be safely expanded *in vitro* and are not susceptible to malignant transformation, thus rendering these cells suitable for cell-therapy approaches.

Introduction

In recent years, a significant improvement in the understanding of multipotent mesenchymal stromal cell (MSC) biology[1] has opened the way to the clinical use of these adult stem cells. MSCs have been employed in several approaches for reparative/regenerative cell therapy, as well as in the perspective of modulating immune response against alloantigens.[2-13]

MSCs have the ability to differentiate into multiple lineages, such as osteoblasts, tenocytes, adipocytes and chondrocytes[14-16] and may be identified by both their capacity to adhere to plastic and their phenotypic characterization through a panel of cell surface molecules including CD90, CD105 and CD13. However, a unique and specific MSC marker, which would allow their exclusive identification, has not yet been found.

The large interest in MSC applicability for clinical approaches relies on the ease of their isolation from several human tissues such as bone marrow (BM), adipose tissue, placenta and amniotic fluid[17-19], on their extensive capacity for *in vitro* expansion and on their functional plasticity.

Concerns that adult human MSCs may be prone to malignant transformation have been recently raised. In fact, human adipose tissue-derived MSCs have been shown to undergo spontaneous transformation after long-term *in vitro* culture.[20] The same phenomenon was also noted in murine BM-derived MSCs[21], which, after numerous passages in culture, increased telomerase activity and proceeded to malignant transformation. A previously published study[22] has also documented that murine gastric epithelial cancer originates from BM-derived cells, presumably MSCs, after recruitment of these cells to the chronically injured mucosal site.

The use of MSCs for clinical approaches in many fields of medicine first requires that the bio-safety of these cells be carefully investigated through appropriate and sensitive tests. Indeed, the absence of transformation potential in cultured MSC has to be documented before considering infusion of these

cells into patients, particularly into immune-compromised subjects where failure of immune surveillance mechanisms might further favor the development of tumors *in vivo*.

The aim of this study was to investigate the potential susceptibility of human BM-derived MSC to malignant transformation at different *in vitro* culture time points and to ascertain whether the biological properties of these cells after *ex vivo* expansion remain appropriate for cell therapy approaches.

Materials and methods

Bone marrow donors

BM cells were harvested, under local or general anesthesia, from 10 healthy hematopoietic stem cell donors (median age 18 years), after obtaining written informed consent. Twenty-thirty ml of heparinized BM from each donor were employed for MSC generation and expansion. The Institutional Review Board of Pediatric Hematology-Oncology approved the design of this study.

Isolation and long-term culture of BM-derived MSCs

Mononuclear cells were isolated from BM aspirates by density gradient centrifugation (Ficoll 1.077 g/ml; Lymphoprep, Nycomed Pharma, Oslo, Norway) and plated in non-coated 75-175 cm^2 polystyrene culture flasks (Corning Costar, Celbio, Milan, Italy) at a density of 160,000/cm^2 in complete culture medium: Mesencult (StemCell Technologies, Vancouver, Canada) supplemented with 10 % FCS (Mesenchymal Stem Cell Stimulatory Supplements, StemCell Technologies), 2 mM L-glutamine and 50 µg/ml gentamycin (Gibco-BRL, Life Technologies, Paisely, UK). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. After 48 hour adhesion, non-adherent cells were removed and culture medium was replaced twice a week. MSCs were harvested after reaching ≥ 80% confluence, using Trypsin (Sigma-Aldrich, Milano, Italy), and propagated at 4,000 cells/cm^2

continuously until reaching a senescence phase or passage (P) 25. The senescence phase was defined as a decrease in MSC proliferative capacity, finally leading to cell cycle arrest. MSCs in the senescence phase were closely monitored for an additional 8-12 weeks before interrupting the cultures, in order to look for the appearance of a crisis phase defined as uncontrolled cell proliferation. Post-senescence clones were isolated by limiting dilution: to obtain single cell-derived clones, MSCs were seeded at 1 cell/well in a 96-well culture plate (Corning Costar) and cultured as described above. The cells were observed daily for 4-6 weeks to examine colony formation.

Flow cytometry

To phenotipically characterize MSCs and to define their purity, fluorescein isothiocyanate (FITC) or phycoerithrin (PE)-conjugated monoclonal antibodies specific for CD45, CD14, CD34, CD13, CD80, CD31, HLA A-B-C, HLA-DR, CD90 (BD PharMingen, San Diego, CA), CD73, CD105 (Serotec, Kidlington, Oxford, UK), CD133 (Miltenyi Biotec S.r.l., Bologna, Italy), VEGFR2 (Sigma-Aldrich) were used. MSCs expanded from the 10 BM donors were analyzed every three passages, starting from P3 (P3, P6, P9, P12, etc.) Appropriate, isotype matched, non-reactive fluorocrome-conjugated antibodies were employed as controls. Analysis of cell populations was performed by means of direct immunofluorescence with a FACScalibur flow cytometer (BD BD PharMingen) and data calculated using CellQuest software (BD Pharmingen).

Multilineage differentiation potential of MSCs

To assess their differentiation capacity, MSCs cultured from all BM donors were induced into adipocytes and osteoblasts at P3, P6 and at later passages whenever possible, employing a method previously described.[23,24]

To detect osteogenic differentiation, cells were stained for alkaline phosphatase (AP) activity using Fast Blue (Sigma-Aldrich) and for calcium deposition with

Alzarin Red (Sigma-Aldrich). The adipogenic differentiation was evaluated through the morphological staining of fat droplets with Oil Red O (Sigma-Aldrich).

Telomerase activity detection assay

Telomerase activity was measured by the polymerase chain reaction (PCR)-based telomeric-repeat amplification protocol (TRAP).[25] Samples containing 0.1, 0.5 and 1 μg of protein were analyzed by the TRAPeze kit (Intergen Company, Oxford, UK) according to the manufacturer's protocol. After extension of the substrate TS (5'-AATCCGTCGAGCAGAGTT-3') oligonucleotide by telomerase, the telomerase products were amplified by PCR in the presence of a 5'[^{32}P]-end-labeled TS primer for 28 cycles and resolved in 10% polyacrylamide gels. Protein extract (0.5 μg) from the telomerase-positive tumor cell line (JR8) was used as a positive control sample in each TRAP assay. Each reaction product was amplified in the presence of a 36-bp internal TRAP assay standard (ITAS). A sample was scored as telomerase activity-positive when positive TRAP results were obtained from at least one protein concentration.

RNA extraction and reverse transcriptase (RT)-PCR analysis of the human telomerase reverse transcriptase (hTERT)

Total cellular RNA was extracted from frozen samples with RNeasy micro kit (QUIAGEN GmbH, Hilden, Germany) according to the manufacturer's protocol. Total RNA (0.5 μg) from each sample was reverse-transcribed by using the RT-PCR Core kit (Applied Biosystems, Branchburg, NJ) with random hexamers, and the resultant cDNA was then amplified with the same kit. Amplification of full length and alternatively spliced hTERT cDNA was obtained using TERT-2164S (5'-GCCTGAGCTGTACTTTGTCAA-3') and TERT-2620AS(5'-CGCAAACAGCTTGTTCTCCATGTC-3') oligonucleotides

with initial heating at 95°C for 3 min, followed by 35 cycles at 95°C for 30 s, 62°C for 50 s, 72°C for 50 s, and 72°C for 5 min. The amplification was performed in a mixture containing 0.3 μCi [α-32p]deoxycytidinetriphosphate (300 Ci/mmol; Amersham Pharmacia Biotech, Cologno Monzese, Milan, Italy). Primers for the internal β-actin control were added during cycle 15 at 72°C. Amplified products were electrophoresed on a 5% non-denaturing polyacrylamide gel in 1X Tris-borate EDTA buffer. The gel was dried and autoradiographed.

Detection of alternative lengthening of telomere (ALT)-associated promyelocytic leukemia (PML) bodies (APB*s)*

Cells were fixed in 1:1 methanol:acetone and processed to detect APBs by combined PML immunofluorescence and telomere FISH according to Henson et al.[26] Images were captured on a Nikon Eclipse E600 fluorescence microscope using ACT-1 (Nikon, Tokyo, Japan) image analysis software and processed using Adobe Photoshop Image Reader 7.0 software. APB status was determined according to previously defined criteria. The presence of an APB was defined by the localization of a telomeric DNA focus within a nuclear PML body; samples were scored as APB-positive if they contained APBs in ≥0.5% of cells. To avoid false positives, an APB was considered to be present only when the telomeric DNA fluorescence within a PML body was more intense than that of telomeres, and a cell was not considered to contain APBs if >25% of the co-localized *foci* occurred outside the nucleus. To avoid false negatives, at least 2,000 nuclei were examined, and the assay was repeated in the presence of negative results. Samples from ALT-positive (IIICF/c-EJ-*ras*) or telomerase-positive (JR8) tumor cell lines were used as positive and negative controls for the APB assay.

Telomere length analysis

Total DNA was isolated using QuicKpicK genomic DNA kit (BioNobile, Medi Diagnostici, Milan, Italy), digested with the *Hinf*I restriction enzyme, electrophoresed using CHEF-DR II Pulsed Field system (BioRad, Hercules, CA), transferred to a nylon membrane, and hybridized with a 5'-end [γ-^{32}P]dATP-labeled telomeric oligonucleotide probe $(TTAGGG)_4$ as previously reported.[27.] Autoradiographs were scanned (ScanJet IIcx/T, Hewlett Packard, Milan, Italy) and digitalized by Image Quant (Molecular Dynamics, Sunnyvale, CA). Each gel was standardized by inclusion of DNA from GM847 (ALT-positive) and HeLa (telomerase-positive) cell lines. ALT status was determined by calculating whether the mean, variance and semi-interquartile range of the terminal restriction fragment (TRF) length distribution was greater than 16 kb, 1,000 kb, and 4 kb, respectively. Samples were classified as ALT-positive when 2/3 or 3/3 of these criteria were met for unimodal or bimodal TRF length distributions, respectively.[26] Statistical analysis of TRF length distributions was performed using the Telometric software.[28]

Molecular karyotyping

Molecular karyotyping was performed through array comparative genomic hybridization (array-CGH) with the Agilent kit (Human Genome CGH Microarray, Agilent Technologies, Santa Clara, CA). The array-CGH platform is a 60-mer oligonucleotide-based microarray that allows a genome-wide survey and molecular profiling of genomic aberrations with a resolution of about 75 kb (kit 44B). The genetic situation of the 10 BM donors was tested before culture (defined as time 0 or T_0), using either BM mononuclear cells (BMMNCs) or peripheral blood lymphocytes (PBLs), and after *in vitro* culture on MSCs at P3 (the passage at which MSCs are usually harvested for clinical use or T_1). Six

MSCs samples were also evaluated at later passages, between P11 and P15 (T_2), after prolonged *in vitro* culture. The method for array-CGH analysis of MSCs has been reported in detail elsewhere.[24]

Cytogenetic analysis (karyotyping and subtelomeric FISH)

Prior to harvest, the cultures of all MSC BM donors at various passages (P2-P11) were incubated at 37°C with colcemid (IrvineScientific, Santa Ana, CA) at 1 µg/ml final concentration for 2 hours. The cells were fixed and spread according to standard procedures. Metaphases of cells were Q-banded and karyotyped in accordance with the International System for Human Cytogenetic Nomenclature recommendations (ISCN, 1995). Fluorescent in situ hybridization (FISH) with chromosome subtelomeric-specific probes (ToTelVysion, Vysis, Downer's Grove, IL) was performed on fixed metaphase chromosomes obtained from three of the ten karyotyped MSC donors, according to manufacturer's instructions and protocol. In total, the kit makes it possible to analyze 36 short and long arms subtelomeres and the 5 long arms subtelomeres of the acrocentric chromosomes.

Results

Characterization of human BM-derived MSCs during long term in vitro culture

MSCs derived from BM of the 10 healthy donors were isolated and propagated in long term *in vitro* culture. MSCs from all donors were characterized by morphology, differentiation capacity and immune phenotype at different culture time points, namely P3, P6, P9, P12, P15 and later, whenever possible, every 2-3 passages (Figure 1A, 1B, 1C and 1D).

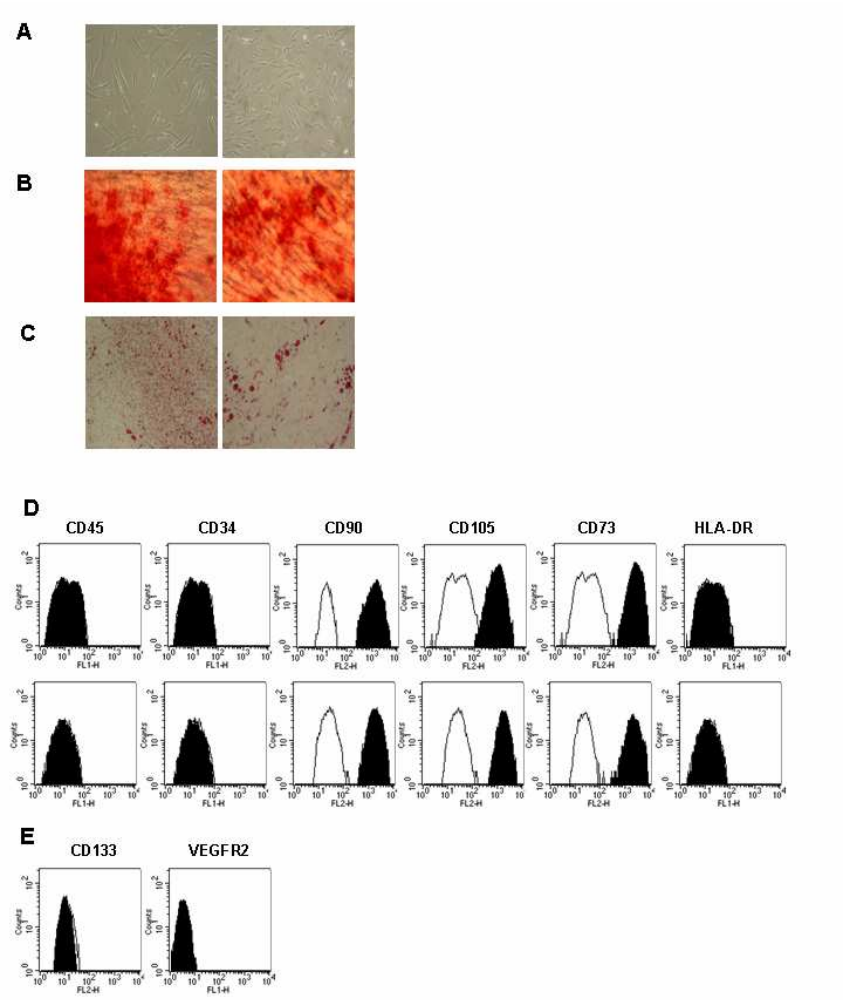

Figure 1. Characterization of human BM-derived MSCs during long term *in vitro* culture. A) Morphological appearance of BM-derived MSCs from DONOR #7 at P3 (*left*) and P15 (*right*). MSCs at P15 are smaller in size as compared to the same cells at P3, although they maintained the typical spindle shape. Magnification x10. B) Osteogenic differentiation capacity of MSCs from DONOR N. 5 at P3 (*left*) and P15 (*right*). The differentiation into osteoblasts is demonstrated by the histological detection of AP activity (purple reaction) and calcium deposition stained with Alzarin Red. Magnification x 20. C) Adipogenic differentiation capacity of MSCs from DONOR #5 at P3 (*left*) and P15 (*right*). The differentiation into adipocytes is revealed by the formation of lipid droplets stained with Oil Red O. Magnification x 20. D) Immunophenotypic characterization of MSCs from DONOR #3 at P3 (*superior row*) and P15 (*inferior row*). E) Expression of CD133 and VEGFR2 molecules on the surface of MSC from DONOR #3 at P15.

A wide variability between donors was noted in terms of proliferative capacity and *in vitro* life span of their cultured MSCs (see Figure 2). The first two donors (donor #1 and #2) showed an early arrest of MSC growth; these two samples entered the senescence phase after 44- and 56-days culture at P4 and P5, respectively. Thereafter, MSCs from both donors were monitored, during their senescence phase, daily for twelve weeks. A crisis phase did not occur in the two samples, even after repeated cryopreservation and thawing procedures. In fact, the MSCs progressively died during the senescence period (characterized by appearance of picnotic bodies within the cells) and detached spontaneously from the flasks. In the case of donor #1, after 8 weeks in the senescence phase, we observed the appearance of a few spindle-shaped cells growing in clones, at a very low rate (post-senescence phase). These cells were analyzed by flow-cytometry and showed the typical MSC markers (CD90, CD105, CD73, CD13: >95% positive cells; HLA-DR: <5% positive cells), whereas they were negative for hematopoietic markers (CD45, CD34) and for both CD133 and VEGFR2 (data not shown). Post-senescence MSCs could not be further propagated; repeated attempts to obtain single cell-derived clones by plating 1 cell/well in a 96-well plate failed.

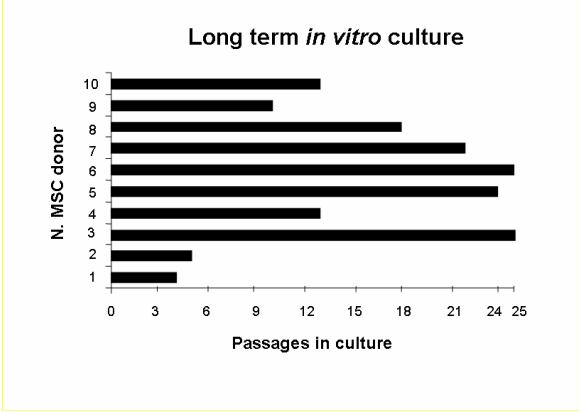

Figure 2. *In vitro* life span of MSC cultures, defined as number of passages before observation of senescence, derived from 10 different donors (#1-10). A large variability between the donors is observed.

Three BM donors (donors #9, #10 and #4) showed an intermediate arrest in MSC growth. These cells displayed a progressive decrease in their proliferative capacity until they reached a senescence phase at P10, P13 and P13, respectively. MSCs from these three donors maintained their typical spindle-shaped morphology, differentiation capacity to form osteoblasts and adipocytes, and their surface markers throughout the culture period.

Donors #8, #7 and #5 showed a late MSC growth arrest, respectively at P18, P22 and P24. Also in this group, MSCs were regularly characterized by morphology, differentiation potential and immune phenotypic analysis every 3 passages and did not display any relevant abnormality (Figure 1B and 1C, pictures on the right; Figure 1D, lower panel). Moreover, when exceeding P10, MSCs were routinely screened for the expression of CD133 and VEGFR2; these markers, which have been found to be expressed on a transformed MSC subpopulation derived from human BM[35], resulted negative (Figure 1E). However, MSCs from donor #7 and #5, when cultured after P13, became smaller in size as compared to the same cells at P3 (Figure 1A, picture on the right), although maintaining the typical spindle shape and a constant growth rate. MSCs reached the senescence phase at 26 and 32 weeks respectively for donor #7 and #5, and required a rather uniform amount of time to reach confluence at 8 to 10 days at every passage. In the case of donor #7, MSCs spontaneously differentiated into adipocyte-like cells when approaching senescence at P22 and could not be further propagated.

MSCs expanded from donor #3 and #6 were trypsinized and replated 25 times continuously with a total culture period of 33 and 44 weeks respectively; thereafter, their long term cultures were interrupted to allow data analysis. The behavior of MSCs expanded from the last two donors was very similar to that of MSCs from the previous couple of donors. With the exception of the acquisition of a smaller cell size, all other phenotypic and functional characterization parameters, including telomerase activity and hTERT expression, were in

agreement with the definition of *in vitro* expanded MSCs.[1,29] In particular, we did not observe any acceleration in cell growth rates.

Lack of expression of telomere maintenance mechamisms in human BM-derived MSCs during long term in vitro culture

MSC cultures obtained from all 10 donors were tested at different *in vitro* passages (from 2 to 6 passages for each culture) for the expression of telomerase catalytic activity (Figure 3A) by TRAP assay. Specifically, in all cultures, an early passage (P1-P3) and later passages (P6-P24) were studied. TRAP results failed to evidence the presence of enzyme catalytic activity in all tested samples, including a post-senescence culture obtained from donor #1.

To gain insights into the molecular mechanisms responsible for the repression of telomerase activity in MSCs, we assessed the expression of the hTERT gene, which codes for the catalytic component of human telomerase[30], in the same cultures screened for telomerase activity. Since it has been demonstrated that alternative splicing of hTERT is involved in the regulation of telomerase activity[31], we analyzed the expression of the different hTERT transcripts (including not only the hTERT full-length transcript, but also three additional splice variants, α^-, β^- and $\alpha^-\beta^-$) through the use of a specific primer set for the reverse transcriptase domain of hTERT. RT-PCR results failed to evidence the expression of any hTERT transcript in all cultures examined (Figure 3B), thus indicating that the absence of telomerase activity in cultured MSCs was ascribable to a lack of hTERT gene transcription.

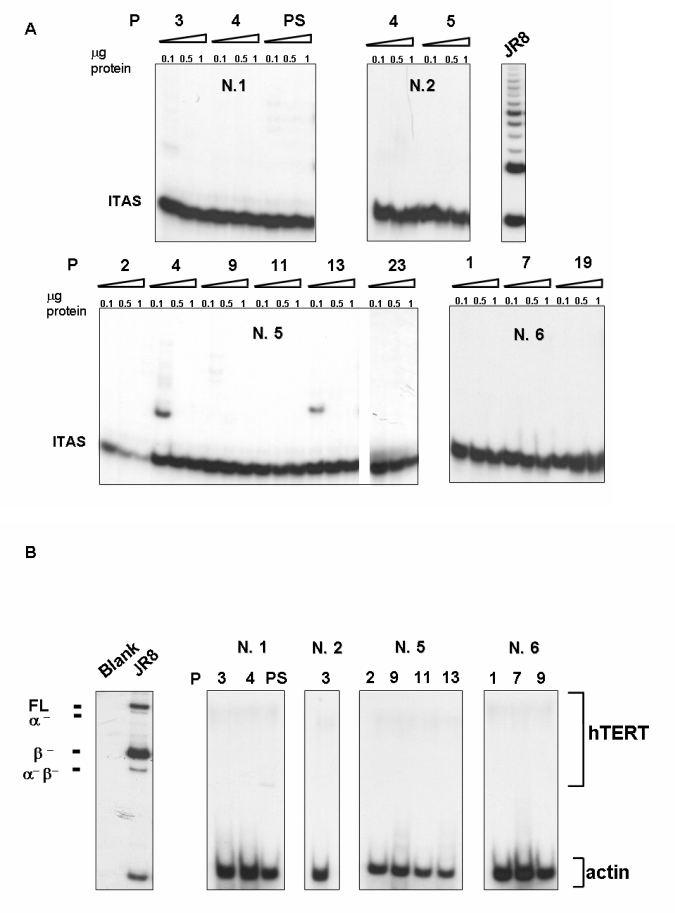

Figure 3. A) Telomerase activity of MSC cultures derived from donors #1,2,5,6 at different passages (P). Telomerase activity was detected by the TRAP assay using different protein concentrations. PS, post-senescence culture. The telomerase-positive cell line JR8 was used as a positive control. The location of the internal amplification standard (ITAS) is reported. B) Expression of h-TERT mRNA transcripts, including the full length (FL) and alternative splicing variants α^-, β^-, and $\alpha^-\beta^-$, as detected by RT-PCR in MSC cultures derived from donors #1,2,5,6 at different passages (P). Telomerase subunits were coamplified with β-actin as the internal standard. The telomerase-positive cell line JR8 was used as a positive control. The blank represents a negative control to which no RNA was added.

Consistent with the lack of telomerase activity, when we analyzed telomere length in cultures obtained from 4 donors (#3-5-6-7) at early (P1-P3) and late (P14-P24) passages, we found evidence of telomere shortening as indicated by a

progressive reduction in the mean TRF length (from 12 Kb to 9.3 Kb in the cultures obtained from donor #6) or appearance of shorter TRFs (<4 Kb in late cultures from donors #3-5-6-7) (Figure 4).

To address the possibility that alternative mechanisms of telomere maintenance referred to as ALT[32] are operating in telomerase-negative MSCs, we screened them for the expression of APBs, which are subnuclear structures containing telomeric DNA, telomere-specific binding proteins and proteins involved in DNA recombination and replication, and represent a peculiar characteristic of ALT cells[33]. However, the results we obtained through a combined immunostaining/FISH approach in the same cultures assayed for telomere length failed to evidence the presence of APBs (data not shown). The absence of an ALT phenotype in these cultures was further confirmed by the pattern of TRF length distribution. In fact, ALT-type telomeres, which are extremely long and heterogeneous[33], were not observed in MSCs (Figure 4).

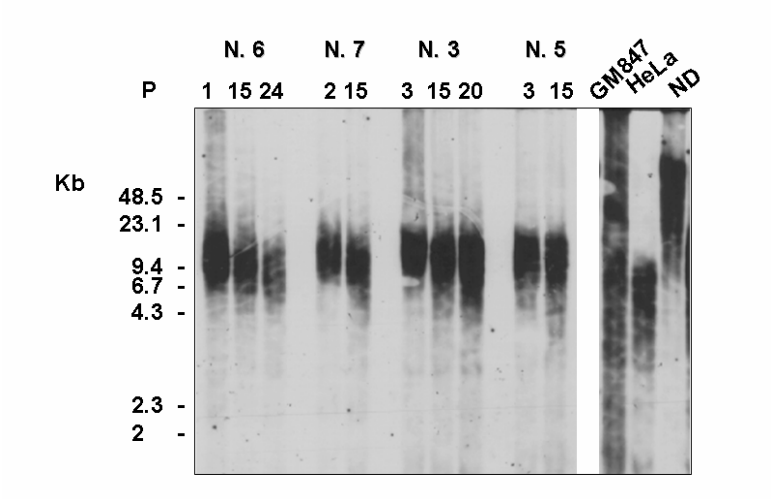

Figure 4. Telomere length distribution of MSC cultures derived from donors #3,5,6,7 at different passages (P). GM847 and HeLa cell lines were used as ALT-positive and telomerase-positive controls, respectively. ND, non-digested DNA.

Karyotype and subtelomeric FISH analysis

BMMNCs/PBLs (T_0) and MSCs at P3 (T_1) derived from all donors were tested for their genetic situation; MSCs at P11-21 (T_2) from six of the ten donors were also studied. In all cases, molecular karyotyping was analyzed by means of array-CGH. The comparison of results from two or three experiments for each donor (T_0, T_1, and T_2), allowed us to distinguish between large copy number variations (LCVs)[34], constitutionally present in donor's genome, and true chromosomal imbalances. Results of the array-CGH analysis demonstrated that, even after long term culture, BM-derived MSCs expanded *in vitro* did not show unbalanced chromosomal abnormalities, as well as submicroscopic rearrangements, considering that the resolution of our approach is about 75 kb (Figure 5). In fact, the array-CGH profiles of the repeated experiments from the same donor were perfectly overlapping and no deletions or duplications were present, besides the LCVs reported in the available databases[1].

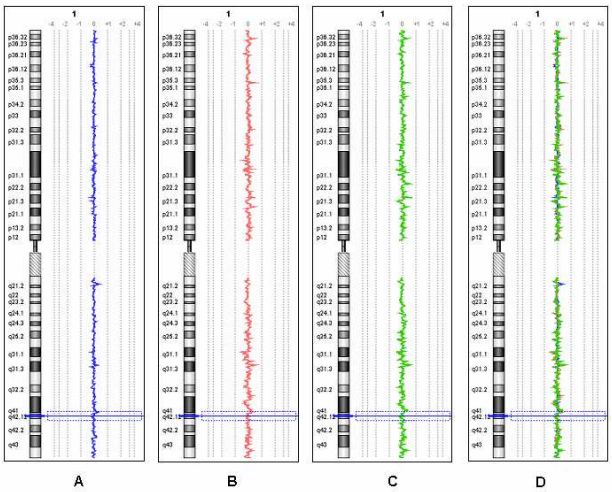

Figure 5. Representative MSC array-CGH profiles of chromosome 1 from donor #6 at: A) T_0: PBLs); B) T_1: MSCs at P3; C) T_2: MSCs at P14. D) Three overlapping experiments: blue line applies to PBLs, red line to P3 and green line to P14. The array-CGH profiles of MSCs are linear and perfectly overlapped, even when there are duplications or deletions caused by LCVs. This demonstrates that *in vitro* expanded MSCs do not show unbalanced chromosomal rearrangements.

[1] http://projects.tcag.ca/variation/

Since array-CGH is not able to unravel balanced chromosomal rearrangements, classical cytogenetics with conventional QFQ banding and FISH analysis with chromosome subtelomeric-specific probes were performed on chromosome metaphases obtained from MSCs at variable passages in culture (P2-11), in order to detect reciprocal translocations. Karyotyping was performed in all donors: only one out of the ten MSC samples was characterized by a pericentric inversion of chromosome 9, which represents a well-known variant without any phenotypic effect. Therefore, all MSC donors were characterized by a normal karyotype (Figure 6A). FISH analysis with chromosome subtelomeric-specific probes was performed on chromosome methaphase of three donors (two at T_1 and one at T_2, Figure 6B) and resulted normal in all cases.

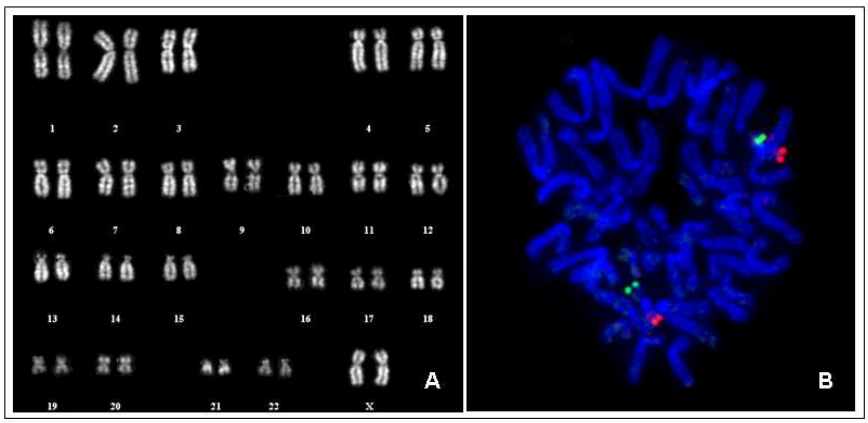

Figure 6. A) Normal Q-banding karyotype (360-400 band) of MSCs from donor #2 at P2. B) FISH analysis with chromosome 16-subtelomeric-specific probes on a metaphase from donor #5 at P18. Green signals represent subtelomeric regions of the short arm of chromosome 16; red signals those of the long arm. The location of signals on chromosome 16 only demonstrates that no translocation involving this chromosome is present.

Discussion

In the present study, we have generated, propagated in long term *in vitro* culture and monitored human MSCs derived from 10 BM donors. Importantly, BM represents the source of MSCs most commonly used in cell-therapy approaches. MSCs from all donors were characterized, at several culture time points, on the basis of the typical parameters for MSC definition (such as morphology, immune phenotype and differentiation capacity), from a genetic point of view and by the expression of telomerase activity and alternative mechanisms of telomere maintenance.

Considering the wide interest in MSCs, in particular for those derived from BM, for clinical approaches, the bio-safety features of these cells need to be carefully investigated, in order to exclude the occurrence of functional or genetic alterations before releasing these cells for clinical use.

Our results demonstrate that human BM-derived MSCs can be cultured long term *in vitro*, without loosing their peculiar morphological, phenotypical and functional characteristics. Moreover, MSCs propagated in culture continuously for up to 44 weeks maintained a normal karyotype, without showing expression of telomere maintenance mechanisms. Consistent with these findings, a progressive reduction in the mean MSC TRF length, or appearance of shorter TRFs, was observed.

In contrast to what has been recently described by Rubio et al.[20] for human, adipose tissue-derived MSCs, none of our MSC samples bypassed the senescence period by developing a crisis phase characterized by a cell cycle rate accelerated compared to pre-senescence MSCs. On the contrary, all BM-derived MSCs demonstrated a progressive decline in their proliferative/expansion capacity mainly resulting into the development of a senescence phase after variable *in vitro* culture times (6-44 weeks; Figure 2). These observations on the proliferative life-span of MSCs are in agreement with previously published studies on cultured MSCs.[19,20] Only in one case (donor #1), after 8 weeks in the

senescence phase, the appearance of a few spindle-shaped cells growing in clones at a very low rate was observed (post-senescence phase). However, these post-senescence MSCs could not be propagated further and never developed a crisis phase. Repeated attempts to obtain single cell-derived clones by plating 1 cell/well in a 96-well plate failed; this cell behavior is very different from that described by Wang *et al*.[35] on a subpopulation of human MSCs derived from one single BM sample (named 'huBM020') that showed very rapid population doubling and could be easily cloned in a single-cell assay. Post-senescence MSCs were analyzed by flow-cytometry and showed the typical MSC markers at high levels (including CD90 and CD105), whereas they were negative for hematopoietic markers (CD45, CD34) and for CD133 and VEGFR2. On the contrary, the transformed cells described by Wang and colleagues[35] were shown to express the endothelial markers CD133 and VEGFR2, as well as low levels of CD90, and they were CD105 negative. The same down-regulation of the MSC membrane markers CD90 and CD105 was noted in the post-crisis cells described by Rubio *et al*[20], which were derived from adipose tissue. Furthermore, the TRAP assay performed on post-senescence MSCs derived from donor N.1 failed to exhibit the expression of telomerase catalytic activity. All these findings demonstrate that our post-senescence cells are not different from normal MSCs, since they display the same particular MSC characterization parameters.

Regarding morphology, we noticed that MSCs from four donors (#3-5-6-7) became smaller in size when cultured over P13-15, although they maintained the typical spindle shape and a constant, growth rate (Figure 1A, right). On the contrary, the cells described by both Wang and Rubio[35,20] resulted in cells morphologically distinct from typical MSCs; they were round or cuboidal, and, in the paper published by Wang and colleagues[35], also exhibited contact-independent growth and formed *foci* with cells released into suspension. In our study MSCs from all ten donors, tested both at early (P3, infusion passage) and

late passages (P9-25) did not show immune phenotypic abnormalities and maintained a high level of purity throughout the culture period (Figure 1D). Moreover, all cultures exceeding P10 were analyzed for the expression of CD133 and VEGFR2 and resulted negative. Also the ability to differentiate into osteoblasts and adipocytes was preserved throughout the culture period in all MSC donors (Figure 1B and 1C).

In agreement with previous reports[36], all our MSC samples lack expression of telomerase activity both at early passages and after long term *in vitro* culture (up to P24). This finding was confirmed by the absence of hTERT transcript expression and reflected by a progressive telomere shortening in cultured MSCs. On the contrary, the transformed mesenchymal cells described by other authors[20,35] exhibited telomerase activity. This phenomenon was also observed by Miura *et al.*[21] in murine BM-derived MSCs that, after numerous passages in culture, gradually increased telomerase activity and proceeded to a malignant state, resulting in fibrosarcoma formation *in vivo*. Some human tumors, mainly those of mesenchymal origin including soft-tissue and osteogenic sarcomas and glioblastomas[26], maintain their telomeres by the ALT mechanism. Telomere dynamics in ALT cells are consistent with a recombination-based mechanism, and characteristics of ALT cells include unusually long and heterogeneous telomeres, as well as the presence of peculiar subnuclear structures termed APBs. In all MSC samples tested, the presence of APBs failed to be evidenced. Moreover, the pattern of TRF distribution that we observed in the cultures was not consistent with the ALT phenotype.

The bio-safety of BM-derived MSCs was further investigated by molecular karyotyping performed by array-CGH, classical cytogenetics and subtelomeric FISH analysis. Array-CGH is a rapid and high-resolution technique useful for the detection of both benign and disease-causing genomic copy-number variations in tumors and genetic disorders, and also for testing cultured embryonic stem cells.[34,37-39] For its high-resolution capacity and in view of the

difficulty in obtaining cultured MSC metaphases, array-CGH may be considered the method of choice for characterizing the genomic situation of MSCs expanded *in vitro* (Figure 5).[24] However, this technique is unable to detect balanced chromosomal rearrangements, that have been excluded in our MSC samples by performing in parallel classical karyotype and subtelomeric FISH analysis. Altogether, the karyotype analysis experiments, performed before culture, as well as at early and late passages, demonstrated that extensively *in vitro* expanded human BM-derived MSCs are devoid of chromosomal abnormalities, as well as of unbalanced submicroscopic rearrangements. These findings are different from what is observed in embryonic stem cells maintained *in vitro* for a high number of passages.[39] Of course, we cannot completely exclude that point mutations or other subtle molecular events, affecting oncogenes or tumor suppressor genes, might have occurred in cultured cells predisposing them to transformation. Likewise, mechanisms of oncogenesis facilitated by infusion into a immune compromised host are not explored by our approach.

In conclusion, our data indicate that human BM-derived MSCs do not display an aptitude for spontaneous transformation and can be safely expanded *in vitro* without any sign of immortalization or development of chromosomal abnormalities. The susceptibility to malignant transformation described in murine BM-derived MSCs by Miura and colleagues[21] might be related to the animal origin of the cells, which display a high degree of chromosome instability, characterized by the development of both structural and numerical aberrations even at early culture passages. The same authors could not demonstrate a similar behavior in human BM-derived MSCs which were propagated *in vitro* under similar culture conditions. Also in the case of human adipose tissue-derived MSCs[20], the susceptibility to malignant transformation might be strictly connected with the origin of the tissue; indeed, in comparison with BM which is very rich in stem cells, fat tissue contains mainly

differentiated cells, and it generates MSCs that are immune phenotypically slightly different from those derived from BM. Finally, in the study by Wang and colleagues[36], it is very likely that the *in vitro* culture of BM-derived MSCs caused the transformation of a subpopulation of cells capable to express endothelial markers. This is further demonstrated by the fact that the same cells, thawed a few months later, propagated normally in culture and did not give rise to any transformed population.

Our results provide support to the concept that the biological properties of human BM-derived MSCs after *ex vivo* expansion remain suitable for use in cell-therapy approaches; however, considering the relevant interest in the utilization of MSCs in several fields of medicine and the potential risk of developing alterations during the expansion period, it is strongly recommended that phenotype, functional and genetic characteristics of MSCs after *in vitro* culture are tested, to further guarantee safety for the patient.

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CHAPTER 5

Co-transplantation of ex-vivo expanded mesenchymal stem cells accelerates lymphocyte recovery and may reduce the risk of graft failure in haploidentical hematopoietic stem cell transplantation

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Summary

Haploidentical hematopoietic stem cell transplantation (HSCT) is associated with an increased risk of graft failure. Adult bone marrow-derived mesenchymal stromal cells (MSCs) have been shown to support *in vivo* normal hematopoiesis and to display potent immune suppressive effects. We co-transplanted donor MSCs in 13 children undergoing transplantation of HLA-disparate CD34+ cells from a relative. While we observed a graft failure rate of 20% in historical controls, all patients given MSCs showed sustained hematopoietic engraftment, without any adverse reaction. In particular, children given MSCs did not experience more infections as compared to controls. These data suggest that MSCs, possibly thanks to their potent immunosuppressive effect on alloreactive host T lymphocytes escaping the preparative regimen, reduce the risk of graft failure in haploidentical HSCT recipients.

Introduction

T-cell–depleted hematopoietic stem-cell transplantation (HSCT) from an HLA-haploidentical relative is a feasible option for children needing an allograft and lacking an HLA-compatible donor.[1] However, both primary (defined as lack of hematologic recovery or absence of donor chimerism) and secondary (defined as loss of donor chimerism after initial engraftment)[2] graft failure, mainly mediated by host alloreactiveT cells escaping the preparative regimen, have been reported in up to 15% to 18% of children given mismatched HSC transplants,[3] despite the infusion of large numbers of hematopoietic stem cells.[4] Recipients of T-cell–depleted HSC transplants from an HLA-disparate relative are also exposed to an increased risk of life-threatening infections, especially of viral origin, due to the delay in reconstitution of adaptive immunity.[1,3]

Bone marrow (BM) contains pluripotent mesenchymal stromal cells (MSCs), which form cartilage, fat, bone, and muscle.[5] MSCs have been shown to modulate the function of T lymphocytes,[6] including that of alloreactive T cells involved in graft-versus-host disease (GvHD) pathophysiology.[7] In adult patients undergoing transplantation from an HLA-identical sibling,MSCinfusion was shown to be safe and possibly to accelerate hematopoietic recovery, as well as to reduce the incidence of both acute and chronic GvHD.[8] However, it is still unknown whether co-transplantation of MSCs in haploidentical HSC transplant recipients can reduce graft failure.

We carried out a phase 1/2 pilot study of co-transplantation of BM-derived, ex vivo–expanded MSCs of donor origin in children undergoing transplantation of granulocyte colony stimulating factor (G-CSF)–mobilized, CD34-selected progenitor cells from an HLAdisparate relative. The procedure was intended to reduce graft failure rate compared with historic controls.

Patients, materials, and methods

Patients

Children with hematologic malignancies or nonmalignant disorders, including primary immune deficiencies, lacking an HLA-matched donor were enrolled in the study by the 2 participating centers (Leiden University Medical Center and Fondazione IRCCS Policlinico San Matteo). Institutional Review Board approval was provided by the 2 participating centers.

Parents or legal guardians of patients provided written informed consent for inclusion in the study. Written informed consent in accordance with the Declaration of Helsinki was also obtained from donors by an independent physician trained to explain risks associated with mesenchymal and hematopoietic stem cell donation.

Preparation of MSCs

Approximately 5 weeks before HSCT, mononuclear cells were isolated from 50 to 70 mL donor BM by density gradient centrifugation on Ficoll. These were plated in noncoated 75- to 175-cm2 polystyrene culture flasks at a density of 160 000/cm2 in complete culture medium (LG-DMEM [Invitrogen, Paisley, United Kingdom] supplemented with penicillin and streptomycin [Lonza, Logan, UT] and 10% fetal bovine serum [FBS; HyClone, Verviers, Belgium]). We used characterized and defined FBS batches preselected for their potential to support MSC expansion. All procedures were carried out under strict Good Manufacturing Practic (GMP) conditions. Flasks were incubated at 37°C in a CO2 incubator and culture medium was replaced twice weekly. After reaching at least 70% confluence, MSCs were replated at 4000 cells/cm2 using trypsine/EDTA (Lonza). MSCs were infused, fresh or after cryopreservation, at passage 3 or less to reduce the risk of genetic instability. MSCs release criteria for clinical use were as follows: spindle-shape morphology, absence of contamination by pathogens, viability, and an immune phenotype proving the

expression of CD73, CD90, and CD105 surface molecules and the absence of CD34, CD45, and CD31. The target dose for infusion was 1 x 10^6/kg to 5 x 10^6/kg body weight. MSCs were infused at a final concentration of 1 x 10^6 to 2 x 10^6 cells/mL.

Cotransplantation of MSCs and haploidentical peripheral blood stem cells at day 0, under monitoring of vital signs, patients were given MSCs intravenously via a central venous catheter and 4 hours later received T-cell–depleted, G-CSF+ mobilized CD34+ cells, positively selected using the CliniMacs 1-step procedure (Miltenyi Biotech, Bergisch Gladbach, Germany). The target number of CD34+ cells to be infused was 20 x 10^6 CD34+ cells/kg recipient weight.

Statistics

A Student *t* test, Fisher exact test, and chi-square test with Yates correction were used to assess differences between study and historic control groups. A*P* value of less than .05 was considered to be significant.

Table 1. Characteristics of patients and controls

	Patients (n = 13)	Controls (n = 52)	p value
Transplant years (range)	Oct. 2004 – Feb. 2007	March. 1998- Oct. 2004	
Mean age (range) years	8 (1-16)	8 (1-17)	NS
Patient gender			
Male	8 (61%)	31 (60%)	NS
Female	5 (39%)	21 (40%)	NS
Original diseases			
Haematological malignancies	10 (77%)	40 (77%)	(Distribution) 0.2)
ALL	4 (40%)	21 (52.5%)	
CR1	0	2	
CR 2	3	11	
>CR2	1	8	
AML	6 (60%)	12 (30%)	
CR1	0	2	
CR 2	3	5	
>CR2	0	2	
Secondary	1	1	
Refractory	2	2	
MDS	0 (0%)	5 (12.5%)	
RC		1	
RAEB		1	
RAEBt		2	
Aplastic MDS		1	
CML Chronic phase	0 (0%)	2 (5%)	
Immune deficiencies			
Other non-malignant disorders	2 (15%)	2 (4%)	
Fanconi anemia	1 (8%)	10 (19%)	
Hemoglobinopathies	1	4	
HLH	-	1	
Other	-	4	
Donor gender	-	1	
Male: Female			
Conditioning regimen	7 : 6	29 : 23	0.9
TBI-based vs. Chemotherapy-based			
Graft characteristics	8:5 (62 vs. 38%)	30:22 (58 vs. 42%)	0.8
Number of CD34+ cells infused x 10^6/kg (median, range)	25.2 (11.6 – 38.6)	23.0 (12.1 – 47.5)	NS
Number of CD3+ cells infused x 10^5/kg (mean, SD)	0.3 (0.3)	0.5 (0.7)	NS
Haematopoietic recovery			
Number of days to PMN recovery (median, range)	12 (10-17)	14 (9-28)	0.1
Number of days to PLT recovery (median, range)	11 (10-18)	13 (9-100)	0.15
Number of days to reticulocyte recovery (median, range)	11 (10-31)	23 (9-41)	0.02
Number of days to leucocyte recovery (median, range)	11.6 (9-15)	16.5 (10-26)	0.005
Post-HSCT complications			
Graft failure	0 (0%)	11 (20%)	0.06
Primary	-	7	
Secondary	-	4	
Acute GvHD			0.08
Grade I-II	2 (15%)	12 (23%)	
Grade III-IV	0 (0%)	2 (3%)	
Chronic GvHD	1 (8%)	6 (12%)	0.2
Limited	1	4	
Extensive	0	2	

Table 1 Legend

ALL = acute lymphoblastic leukaemia; AML = acute myeloid leukaemia; MDS = myelodysplastic syndrome; RC = refractory cytopenia; RAEB = refractory anemia with excess of blasts; RAEBt = refractory anemia with excess of blasts in transformation; CML = chronic myeloid leukaemia; HLH = hemophagocytic lymphochistiocytosis; CR = complete remission; TBI = total body irradiation; PMN = polymorphonuclear neutrophils; PLT = platelets; HSCT = hematopoietic stem cell transplantation; GvHD = graft-versus-host disease; SD = standard deviation; NS = non-significant.

Results and discussion

Table 1 shows the characteristics of the 14 study patients compared with 47 historic controls that received transplants in either one of the 2 centers and were selected for an equivalent number of CD34+ cells infused and matched for transplant indication.

There was no significant difference between patients and controls in terms of age, sex, malignant versus nonmalignant disease, method of CD34+ cell selection, and number of CD3+ cells infused. In all donors, both expansion of MSCs and mobilization of CD34+ cells were successful. Patients received a mean of 1.6 x 10^6 MSCs/kg (range, 1 x 10^6 MSCs/kg to 3.3 x 10^6 MSCs/kg). No MSC infusion–related toxicity was observed.

Either primary or secondary graft failure occurred in 7 of the 47 children of the control group, whereas no rejection occurred in children who received cotransplants of haploidentical MSCs (P = .14). The number of CD34+ cells infused was superimposable in the study patients (mean, 21.5 x 10^6/kg; range, 11.6 x10^6/kg to 38.6 x 10^6/kg), in controls with sustained engraftment (mean, 21.2 x 10^6/kg; range, 12.1 x 10^6/kg to 47.5 x 10^6/kg), and in those who experienced either primary (mean, 21.7 x 10^6/kg; range, 14.7 x 10^6/kg to 39.4 x 10^6/kg) or secondary (mean, 21.1 x 10^6/kg; range, 12.4 x 10^6/kg to 26.6 x 10^6/kg) graft failure.

Neutrophil and platelet recovery was comparable in study patients and controls (see Table 1 for definitions and details).

However, patients given MSCs had faster recovery of a total leukocyte count above 1.0 x 109/L in comparison to historic controls (mean, 11.5 days [95% confidence interval [CI] 9.0-14.8] versus 14.9 days [95% CI 10.1-26.0], respectively, P = .009).

Lymphocyte recovery accounted for this finding: the absolute numbers of natural killer (NK) cells 1 month after HSCT being 497/μL (95% CI 347-646) in the study group and 252/μL (95% CI 173-330) in controls (P = .02).

However, at 3 months, NK and T-cell recovery was quantitatively no different between study patients and controls.

Chimerism analysis of ex vivo–expanded MSCs derived from recipient BM at 3-month intervals up to 1 year after HSCT using polymerase chain reaction (PCR) for informative donor recipient polymorphisms9 did not show any evidence of donor cells in the majority of patients. In 3 patients, minimal (1%-2%) transient engraftment of donor MSCs was found at 3 months. Hematopoietic chimerism is detailed in the following Table 2.

Table 2. Patient follow up data

UPN	Sex	Age at HSCT	Donor	Diagnosis	Follow up	Chimerism analysis	Time to last BM chimerism	Time to last PB chimerism	Outcome
1	M	15y 6mo	Mother	Refractory AML	+7 mo✞	100% donor	+6 mo	NE	Died Candida sepsis - CR
2*	Mm	2y	Father	X-LPD	+28 mo	100% donor	+ 24 mo	+26 mo	Alive and well
3*	M	2y 4mo	Father	X-LPD	+24 mo	95% donor granulocyte 88% donor CD3 (BM)	+20 mo	+23 mo	Alive and well
4	M	13y 1mo	Mother	Refractory AML	+4 mo	100% donor	+3 mo	+3 mo	Died relapse
5	F	8y 9mo	Father	Fanconi anemia	+16 mo	100% donor	+14 mo	+11 mo	Alive and well transfusion independent
6	F	3y 8mo	Father	ALL 2CR	+7 mo✞	100% donor	+ 3 mo	+3 mo	Died relapse
7	F	13y 4mo	Father	monosomy 7 refactory AML	+4 mo✞	100% donor	+2 mo	+2mo	GVHD Died adenovirus hepatitis – CR
8	F	7y 1mo	Mother	AML CR 2	+12 mo	100% donor	+7 mo	+10 mo	Alive and well CR
9	M	5y	Father	ALL-T CR4	+10 mo	100% donor	+8mo	+7mo	Alive and well CR
10	M	5y 4 mo	Mother	ALL CR2	+8 mo	100% donor	+7mo	+8mo	Alive and well CR
11	F	8y 5mo	Sister	ALL CR2	+7 mo	100% donor	+5mo	+6mo	Alive and well CR
12	F	8y 2mo	Mother	AML CR2	+6 mo	95% donor BM 80% donor PB Recipient % CD4/CD8 positive	+5 mo	+6mo	Alive and well CR
13	M	16y	Father	AML CR2	+3 mo	100% donor	+3 mo	+3mo	Alive and well CR

Table 2 Legend
UPN = unique patient number; BM = bone marrow; PB = peripheral blood; HSCT = hematopoietic stem cell transplantation; M = male; F = female; y = years; mo = months; ALL= acute lymphoblastic leukaemia; AML= acute myeloid leukaemia; X-LPD = X linked lymphoproliferative disorder; NE = not evaluated; CR = complete remission; ✞ = dead. * Patients UPN 2 and 3 are identical twins transplanted from the same haploidentical father

Four study patients died (Table 2), 2 due to relapse and 2 due to infection, compared with 11 controls (7 relapse, 2 infections, 2 GvHD). Episodes of viral reactivation were common in both patients and controls, occurring in 50% of patients belonging to the study group and in 35% of historic controls. However, only 1 study patient died, as a result of disseminated adenovirus infection complicated by grade 2 acute GvHD requiring steroid treatment, compared with 2 historic controls. Since the follow-up of patients in the study group is shorter (range, 3-28 months) than that of historic controls (range, 32-110 months), both relapse rate and probability of overall survival in the study cohort (18% and 72%, respectively) and in controls (26% and 63%, respectively) are not comparable.

Our results indicate that in patients given a T-cell–depleted, HLA-disparate–related allograft from a relative, expansion of donor MSCs is feasible and their clinical use is safe. Moreover, our data suggest that MSC co-transplantation may modulate host alloreactivity and/or promote better engraftment of donor hematopoiesis, reducing the risk of early graft failure.

A case-controlled study, with longer follow-up to exclude the risk of late rejections, can more precisely define the role played by co-transplantation of haploidentical donor MSCs on the outcome of patients given haploidentical, T-cell–depleted HSCT.

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CHAPTER 6:

Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study

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Summary

Severe graft-versus-host disease (GVHD) is a life-threatening complication after allogeneic transplantation with haemopoietic stem cells. Mesenchymal stem cells modulate immune responses in vitro and in vivo. We aimed to assess whether mesenchymal stem cells could ameliorate GVHD after haemopoietic-stem-cell transplantation.

Patients with steroid-resistant, severe, acute GVHD were treated with mesenchymal stem cells, derived with the European Group for Blood and Marrow Transplantation ex-vivo expansion procedure, in a multicentre, phase II experimental study. We recorded response, transplantation-related deaths, and other adverse events for up to 60 months' follow-up from infusion of the cells.

Between October, 2001, and January, 2007, 55 patients were treated. The median dose of bone-marrow derived mesenchymal stem cells was 1.4×10^6 (min–max range 0.4–9 $\times10^6$) cells per kg bodyweight. 27 patients received one dose, 22 received two doses, and six three to five doses of cells obtained from HLA-identical sibling donors (n=5), haploidentical donors (n=18), and third-party HLA-mismatched donors (n=69). 30 patients had a complete response and nine showed improvement. No patients had side-effects during or immediately after infusions of mesenchymal stem cells. Response rate was not related to donor HLA-match. Three patients had recurrent malignant disease and one developed de-novo acute myeloid leukaemia of recipient origin. Complete responders had lower transplantation-related mortality 1 year after infusion than did patients with partial or no response (11 [37%] of 30 *vs* 18 [72%] of 25; p=0.002) and higher overall survival 2 years after haemopoietic-stem-cell transplantation (16 [53%] of 30 *vs* four [16%] of 25; p=0.018).

Infusion of mesenchymal stem cells expanded in vitro, irrespective of the donor, might be an effective therapy for patients with steroid-resistant, acute GVHD.

Introduction

Allogeneic haemopoietic-stem-cell transplantation is the treatment of choice for many malignant and non-malignant disorders.[1,2] Severe graft-versus-host disease (GVHD) is a life-threatening complication after this treatment and donor lymphocyte infusion is used for treatment or prevention of relapse of leukaemia.[3,4] Steroids are still the first-line treatment for established GVHD with a response rate of 30–50%; however, the outcome for patients with severe, steroid-resistant, acute GVHD is poor, and overall survival is low.[1,3–5]

Mesenchymal stem cells are multipotent bone-marrow cells able to differentiate in vitro and in vivo into tissues of mesenchymal origin.[6,7] Moreover, these cells provide support for the growth and differentiation of haemopoietic progenitor cells in bone-marrow microenvironments and, in animal models, promote engraftment of haemopoietic cells.[8] In co-culture experiments with allogeneic lymphocytes, mesenchymal stem cells do not induce lymphocyte proliferation, interferon-gamma production, or up-regulation of activation markers.[9,10]

Mesenchymal stem cells suppress proliferation of activated lymphocytes in vitro in a dose-dependent, non-HLA-restricted, manner.[9–11] In a baboon skin-graft model, Bartholomew and co-workers11 showed that infusion of ex-vivo expanded donor-derived or third-party cells prolonged the time to rejection of histo-incompatible skin grafts. Furthermore, infused cells improve the outcome of acute renal, neural, and lung injury, possibly by promoting a shift from production of pro-inflammatory cytokines to anti-inflammatory cytokines at the site of injury.[12–14]

In phase I and II trials, HLA-identical mesenchymal stem cells expanded ex vivo have been infused to promote haemopoietic recovery after autologous and allogeneic haemopoietic-stem-cell transplantation and to treat patients with osteogenesis imperfecta.[15–20] So far, neither acute nor long-term adverse events have been reported after infusion of mesenchymal stem cells. Two reports on

the use of in-vitro expanded cells for the treatment of severe, acute GVHD have recently been published.[21,22]

To facilitate large-scale, multicentre trials, the European Group for Blood and Marrow Transplantation Developmental Committee has adopted a common protocol for expansion of mesenchymal stem cells. We report the results of a multicentre, phase II study of the use of these cells in 55 patients with severe and steroid-resistant, acute GVHD.

Methods

Patients

Between October, 2001, and January, 2007, patients of all ages with grade 2–4 GVHD after haemopoietic-stem-cell transplantation, who did not respond to steroid treatment (≥2 mg per kg per day) for at least 7 days, or with progression of at least one grade within 72 h were eligible for the study. 55 patients were treated (Table 1); 48 had developed GVHD after transplantation of haemopoietic stem cells and seven after donor lymphocyte infusion. Most patients had grade 3 or 4 GVHD involving two or three organs, confirmed by biopsy in 36 patients (43 biopsies; table 2).

23 patients were treated at Karolinska University Hospital, Huddinge, Sweden (of whom, eight were previously reported [21,22]), 14 at Leiden University Medical Center, Leiden, the Netherlands, eight at Ospedale San Martino or Gaslini Institute, Genova, Italy, seven at IRCCS Policlinico San Matteo, University of Pavia, Pavia, Italy, and three patients were from Royal Adelaide Hospital, Adelaide, Australia.

This phase II study was a prospective registration study to include all patients consecutively treated with mesenchymal stem cells in the participating centres of the European Group for Blood and Bone Marrow Transplantation mesenchymal stem cell consortium: the study was approved by the local ethics

committees or institutional review boards of the participating institutions. Donors and patients, or their legal guardians, gave written informed consent.

Table 1. Characteristics of patients and treatment

	MEASURE
RECIPIENTS	
Recipient age, years	22 (0·5-64)
Male,female	34,21
	25,30
Child,adult	
DIAGNOSIS	
AML	8
ALL	12
CML	7
CLL	2
JMML	4
Multiple myeloma	2
Myeloproliferative disorder	1
Myelodysplastic syndrome	6
Lymphoma	1
Non malignant disorders	10
Solid tumor	2
Disease early , late*	21.24
DONOR and CELLS	
Female donor to male recipient	10
Male donor to female recipient	9
HLA-identical sibling	19
Unrelated A, B, DRβ1 identical	25
Mismatched donor	6
Unrelated CB (matched/mismatched)	3/2
HLA-identical sibling	19
Stem cell source (BM/PBSC/BM+PBSC/CB)	19/30/1/5
GvHD PROPHYLAXIS	
CsA	4
CsA + MTX	38
CsA + MMF	5
CsA + prednisolone	6
Other	2
ATG/ALG/Campath	30/1/5
DOSE	
Nucleated cell dose x 10^8/kg	5.9 (0·17-20·6)
CD34+ cell dose x 10^6/kg	8 (0·15-28)
CYTOMEGALOVIRUS SEROLOGY	
Negative in donor and recipient	14
Positive in donor and recipient	20
Positive in donor or recipient	21

Abbreviations: ALG = Antilymphocyte globulin; ALL = Acute lymphoblastic leukaemia; AML = Acute myeloid leukaemia; ATG = Antithymocyte globulin; BM = Bone Marrow; CB = Cord blood; CML = Chronic myeloid leukaemia; CLL = chronic lymphocytic leukemia; JMML = Juvenile myelomonocytic leukaemia; CsA = Cyclosporine; HLA = Human leukocyte antigen; MTX = Methotrexate; MMF = Mycophenolate mofetil; PBSC = Peripheral blood stem cell.* Early: non-malignant disease, 1st complete remission, 1st chronic phase; late: beyond these stages at time of transplant

Table 2. GVHD grade and organ involvement

	NUMBER OF PATIENTS
GvHD SEVERITY	
GvHD II/III/IV	5/25/25
ORGAN INVOLVEMENT	
Only one organ S/G/L	3/6/1
Two: G+S/G+L/L+S	15/7/4
Three: G+ S+L	19
GvHD CONFIRMED ON BIOPSY	
S/G/L	10/31/2
GvHD TREATMENT PRIOR TO MSC INFUSION	
Cyclosporine or tacrolimus	55 (53)*
Prednisolone ≥ 2 mg/kg	55 (55)*
MMF	10 (10)*
Daclizumab + Infliximab	4 (-)
Daclizumab alone	1 (3)*
Etanercept and PUVA	1 (-)
Extra-corporeal photochemotherapy	10 (8)*
Cyclophosphamide	3
ATG†	2
Rituximab	(1)*
PREVIOUS FAILED THERAPY	
First line	55
Second line	33
Third line	14
Fourth line	4
Fifth line	2

Abbreviations:
G = Gut; L = Liver; S = Skin.;
ATG=antithymocyte globulin. MMF=mycophenolate mofetil. MSC=mesenchymal stem cell. PUVA=psoralen and ultraviolet-A irradiation.
*Numbers in brackets hadimmunosuppressive therapy at time of MSC infusion. †One Thymoglobulin, Genzyme, USA; one ATGAM, Upjohn, USA.

Procedures and definitions

Before treatment with haemopoietic stem cells, patients received either myeloablative or reduced-intensity conditioning (figure 1). Conditioning was myeloablative in 37 patients, who were given cyclophosphamide (120 mg per kg) combined mainly with busulfan (16 mg per kg), melphalan, or fractionated whole-body irradiation (≥ 12 Gy). 18 patients had low-intensity conditioning regimens with fludarabine phosphate combined with various cytotoxic drugs or 2 Gy whole-body irradiation.

As GVHD prophylaxis, most patients received ciclosporin combined with either four doses of intravenous methotrexate or mycophenolate mofetil. In patients receiving cord-blood transplantation, ciclosporin was combined with prednisolone. Recipients of haemopoietic stem cells from unrelated donors were treated with antithymocyte globulin, anti-lymphocyte globulin, or alemtuzumab.

All patients had been treated with prednisolone 2 mg/kg per day or more as first-line immunosuppressive GVHD therapy and were resistant to this treatment.

33 patients (60%) had failed two or more lines of immunosuppressive therapy before receiving mesenchymal stem cells (table 2). All patients continued treatment with steroids and a calcineurin inhibitor (n=53) or mycophenolate mofetil (n=2) at the time of infusion.

20 patients also continued additional treatments while receiving stem cells (table 2).

We defined resistance to treatment as no improvement in overall grade of GVHD or progression.

Acute GVHD was graded according to internationally accepted criteria by physicians at individual centres.[23] When possible, we confirmed diagnosis with biopsy of the involved organs.

We used best response to define the response to treatment: complete response was loss of all symptoms of acute GVHD; partial response was improvement of

at least one grade; stable disease was no change in GVHD grade; and progressive disease was worsening of GVHD.

Patients were judged to have responded if they had either complete or partial response. Transplantation-related mortality included all deaths associated with transplantation of haemopoietic stem cells except those related to recurrence of underlying disease.

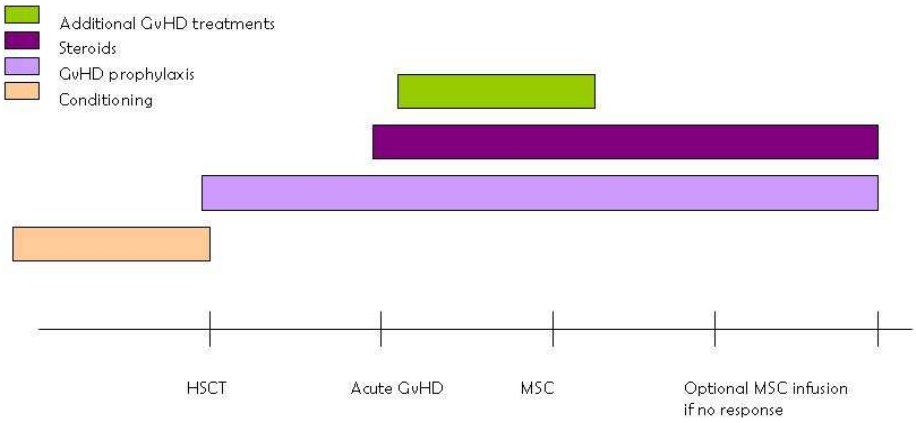

Figure 1: Scheme of mesenchymal stem-cell therapy

Laboratory methods

Table 3 shows the characteristics of the donors and grafts. Mesenchymal stem cells were derived from either HLA-identical stem-cell donors, haploidentical family donors, or unrelated HLA-mismatched donors.

Clinical-grade mesenchymal stem cells were generated under good manufacturing practice conditions according to a common protocol devised by the European Group for Blood and Bone Marrow Transplantation developmental committee and approved by the medicinal-product agencies in the respective countries. Bone-marrow mononuclear cells were separated by density gradient centrifugation as previously described.[7,8,10]

Washed cells were re-suspended in Dulbecco's modified Eagle's medium–low glucose (Life Technologies, Gaithersburg, MD, USA, or Paisley, UK) supplemented with 10% fetal bovine serum (National Veterinary Institute, Uppsala, Sweden, or HyClone, Logan, UT, USA) and plated at a density of 160 000 cells per cm2. Cultures were maintained at 37oC in a humidified atmosphere containing 5% CO□ in 175 cm2 fl asks (Falcon, Franklin Lakes, New Jersey, USA, or Greiner Bio-One, Frickenhausen, Germany). When the cultures were near confluence (>80%), the cells were detached by treatment with trypsin and EDTA (Invitrogen, Grand Island, NY, USA, or Lonza Verviers, Verviers, Belgium) and re-plated at a density of 4000 cells per cm2. When 2×10^6 cells or more were obtained, they were harvested and either cryopreserved in 10% dimethyl sulphoxide (Research Industries, Salt Lake City, UT, USA, or Leiden University Medical Centre Pharmacy, Netherlands) or washed repeatedly and re-suspended to a final concentration of 2×10^6 cells per ml in saline solution according to local guidelines.

Criteria for release of mesenchymal stem cells for clinical use included absence of visible clumps, spindle-shape morphology, absence of contamination by pathogens (as documented by aerobic and anaerobic cultures before release), viability greater than 95%, and immune phenotyping proving expression of CD73, CD90, and CD105 surface molecules (>90%) and absence of CD34, CD45, CD14, and CD3.24

Cells were given as intravenous infusions. Cells for 11 infusions were harvested fresh from culture and given to the patients. In all other cases, frozen cells were thawed and infused.

Table 3. Mesenchymal-stem-cell donor and graft characteristics

	MEASURE
DONORS	
No. of donors	45
Donor sex (male/female)	25/20
Donor age	36 (1-67)
NUMBER OF INFUSIONS BY DONOR TYPE	
HLA-identical sibling	5
HLA-haploidentical donor	18
Unrelated HLA-mismatched donor	69
Volume of bone marrow harvested (ml)	60 (32-220)
Median MSC cell dose (x 10^6/kg, range)	1·4 (0·4-9)
CULTURE PASSAGE AT MSC HARVEST	
Passage 1	14
Passage 2/2+3	42/7
Passage 3/3+4	23/2
Passage 4	4
NUMBER OF MSC INFUSIONS	
One	27
Two	22
Three	4
Four	1
Five	1

Data are number or median (min–max range). MSC=mesenchymal stem cell.

Statistical analysis

Data were analysed as of last data collection in March, 2007. We estimated the probability of survival with the Kaplan-Meier method and significance of differences with the log-rank test (Mantel-Cox). Transplantation-related mortality was estimated non-parametrically. Patients were censored at the time of death or last follow-up. Because relapse and non-relapse mortality are competing events, we estimated their incidence with a non-parametric estimator of cumulative incidence curves.[25,26] All results were expressed as 2-year probability of survival or 1-year cumulative incidence (%) of transplantation-related mortality and 95% CI. We used Fisher's exact test to compare distribution of categorical variables. Analyses were done with the cmprsk package (developed by Gray, June, 2001), Splus 6.2 software, and Statistica software.

Role of the funding source

The sponsors of this study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data and had final responsibility for the decision to submit for publication.

Results

92 infusions of mesenchymal stem cells were given; 27 patients had one infusion, while 28 had two or more (figure 1, table 3). Of the 28 patients treated with multiple infusions, 15 received cells derived from two or more donors. No patients had acute side-effects either during or after infusion; and none have had late side-effects so far. Median time from transplantation of haemopoietic stem cells to infusion of mesenchymal stem cells was 103 days (min–max range 27–533).

Just over half the patients had a complete response, and about a fifth had a partial response; most of the remainder had worsening disease (table 4). A greater proportion of children responded than did adults (p=0.07). Median time from first infusion of mesenchymal stem cells to complete response was 18 days (min–max range 3–63). 30 patients achieving complete response were available for assessment at 6 weeks after infusion. Of these, four had died, 19 still had complete response, one had grade 1 and six had grade 2 acute GVHD.

After one dose, 27 patients had complete response, two had partial response, and 26 did not respond. Of patients who responded to one dose, two were treated with HLA-identical mesenchymal stem cells, three with haploidentical cells, and 24 with third-party cells. The median dose given to patients who responded to the fi rst dose was 1.4×10^6 cells per kg (min–max range $0.8\times10^6$to 9×10^6 cells per kg), which was similar to that given to non-responding patients (1.4×10^6 cells per kg; 0.6×10^6 to1.9×10^6 cells per kg). Six children and one adult who responded to the first infusion were given a second infusion to prevent GVHD recurrence when immunosuppressive drug treatment was reduced.

17 of the patients who did not have sustained complete response after the first dose were treated with subsequent doses. Five patients had complete response but received several doses of mesenchymal stem cells because of GVHD recurrence. Five patients had partial responses and were given multiple doses. One patient did not respond to 0.6×10^6 cells per kg but responded to a second dose of 2×10^6 cells per kg. 12 patients did not respond despite several infusions.

22 of 30 patients with grade 2 or 3 acute GVHD responded, compared with 17 of 25 with grade 4 disease (p=0.77). 28 (78%) of 36 patients with involvement of one or two organs had a response compared with 11 (58%) of 19 patients with involvement of three organs (p=0.21).

The age or HLA-match of the mesenchymal-stem-cell donor had no effect on response rate after infusion (data not shown). In patients receiving HLA-

identical cells, two of five responded to first dose, compared with nine of 13 patients given haploidentical cells and 27 of 37 receiving unrelated cells. Median time from acute GVHD onset to treatment with mesenchymal stem cells was 25 days (min–max range 3–114) in complete responders, compared with 29 (3–116) days among all other patients (p=0.48). We noted variable responses when cells expanded from one donor were given to several recipients. There was no relation between the treatment given before infusion of mesenchymal stem cells and response.

21 patients were alive at the time of analysis (March, 2007) with a median follow-up of 16 months (min–max range 1.5–64 months) after infusion of mesenchymal stem cells (table 4).

Table 4. GvHD response and outcome

	CHILDREN	ADULTS	ALL PATIENTS
	n = 25	n = 30	n = 55
RESPONSE			
Complete	17 (64%)	13 (47%)	30 (55%)
Partial	4	5	9
Stable disease	2	1	3
Progressive disease	2	11	13
Overall response	21 (80%)	18 (60%)	39 (69%)
*SURVIVAL **	13	8	21
CHRONIC GvHD			
Limited	2	0	2
Extensive	4	2	6

*At last data collection, March, 2007.

The estimated probability of survival 2 years after haematopoietic-stem-cell transplantation for the entire cohort of patients was 35% (95% CI 22–38%); in adults, 2-year survival was 26% (10–42%) compared with 45% (23–67%) in children (p=0.06). The 2-year probability of survival in complete responders (52%, 34–70%) was significantly better than that in the patients with partial or no response (16%, 0–32%, p=0.018; figure 2).

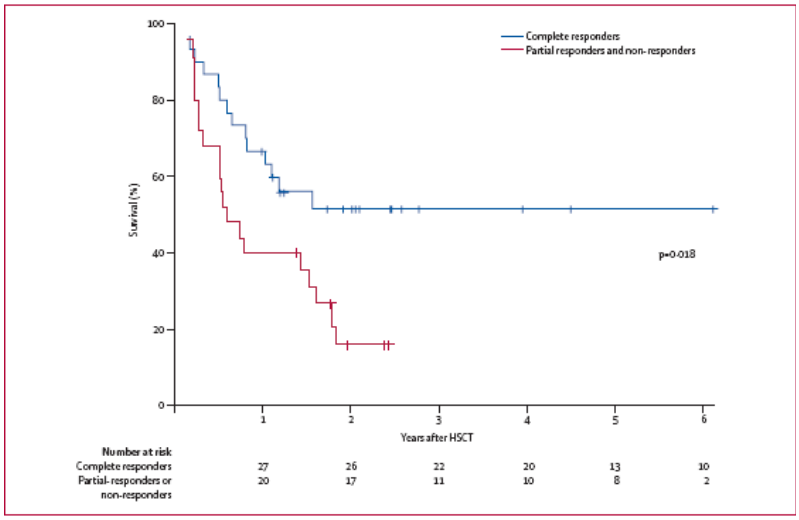

Figure 2: Survival from time of haemopoietic-stem-cell transplantation in patients given mesenchymal stem cells
Survival at the end of follow-up was 52% (95% CI 34–70%) for the 30 complete responders and 16% (0–32%) for the 25 partial responders or non-responders.

The 100-day transplantation-related mortality from time of infusion of mesenchymal stem cells was 13% (0–26%) for patients with complete response compared with 60% (41–79%) in other patients (p=0.002). Transplantation-related mortality 1 year after infusion was 37% (19–55%) in complete responders and 72% (55–89%) in patients with partial or no response (p=0.002; figure 3). Of the survivors, six with complete response developed chronic GVHD, which was limited (mild) in four and extensive (severe or involving several organs) in two patients. Two patients with partial or no response also

developed extensive GVHD. At last follow-up (March 1, 2007), eight with complete response had discontinued all immunosuppressive drugs. Three patients had recurrence of the original disease one with multiple myeloma, one with acute lymphoblastic leukaemia, and one with acute myeloid leukaemia. One patient with Pearson's disease developed acute myeloid leukaemia de novo originating in endogenous haemopoietic cells. All these patients died. Acute GVHD was the most common cause of death (18 patients), with or without concomitant infection. One patient died from chronic GVHD with obstructive bronchiolitis, and one patient died from multi-organ failure after severe haemorrhagic cystitis. Infections in patients who died to acute GVHD included aspergillosis (five), cytomegalovirus (four), and septicaemia caused by *Enterococci* (four), *Klebsiella* sp (one), *Escherichia coli* (one), and an unidentified pathogen (one). Three patients had infection with Epstein-Barr virus, one of whom developed post-transplantation lymphoproliferative disease related to the virus. Of the patients who responded to mesenchymal stem cells, nine died from infections. Pathogens included *Klebsiella* sp, *E coli*, *Pseudomonas*, adenovirus, and varicella zoster virus cinfection.

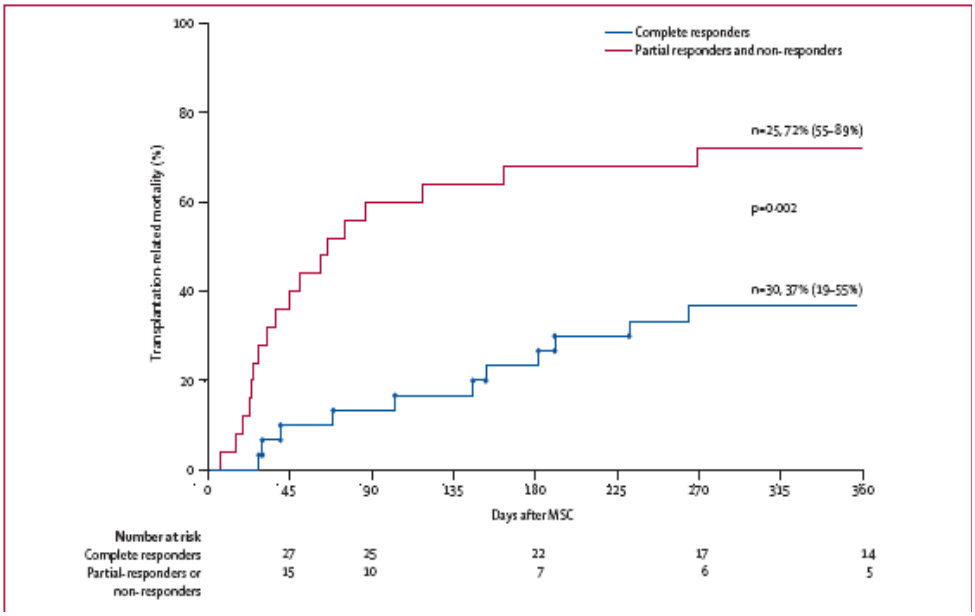

Figure 3: 1-year cumulative incidence of transplantation related mortality from time of infusion of mesenchymal stem cells
Transplantation related mortality was 37% (95% CI 19–55%) among the complete responders and 72% (55–89%) among the partial responders or non-responders.

Discussion

39 of 55 patients with steroid-resistant, severe, acute GVHD responded to treatment with mesenchymal stem cells. Survival in those with complete response was significantly higher and transplantation-related mortality after infusion was significantly lower than in people with partial or no response. The clinical course of the 13 patients with progressive disease despite treatment may represent the natural progression of severe GVHD resulting in death in most patients. No major toxicities were observed, and treatment with mesenchymal stem cells seemed to be safe.

Although various immunosuppressive treatments have been used, there is no established therapy for steroid-resistant, severe, acute GVHD.[1,3–5] The outcome for patients who do not respond to corticosteroids is therefore poor, and survival at 2 years is about 10%.[4,5,27] In this study, survival in people who responded to

mesenchymal stem cells was higher (52%) than previously described for patients with a similar grade of acute GVHD.

The response in patients with grade 2 and 3 acute GVHD was similar to that in patients with grade 4. More patients are needed to investigate responses to the treatment in various subgroups. For example, the difference in response rate between children and adults with acute GVHD of comparable severity was not significant. Because of the low number of patients, the statistical power was insufficient to detect significance for differences the size of that between response rates in adults and in children (estimated power 0.58). At least 80 patients would be needed to detect such a difference with p equal to or less than 0.05.

At present, little is known about mechanisms of suppression of GVHD by mesenchymal stem cells. In-vitro, these cells have various effects on immune cells, including T cells, antigen-presenting cells, natural-killer cells, and B cells.[28–30] The biological relevance of these in-vitro findings is unknown. These cells might suppress donor-T-cell responses to recipient alloantigen. This suppression is probably induced by several mechanisms, including release of soluble factors, induction of regulatory T cells, and repair of damaged target organs.[28–30] Immunological studies specifically addressing this issue are needed to improve our understanding the treatment of acute GVHD.

Our study was designed to assess safety and efficacy of mesenchymal stem cells for refractory acute GVHD. It was not designed to identify the best dose of mesenchymal stem cells. At present we can note only that, on the one hand, clinically meaningful responses were obtained after infusing a dose as low as 0.8×10^6 cells per kg, whereas on the other, doses as high as 1.9×10^6 cells per kg were not successful in all cases. Thus any conclusion as to relevant dose is premature.

In more than half of patients, a single dose produced a response, whereas in a few patients with partial response or with recurrence of GVHD, several doses

were needed to induce a lasting response. The response was not restricted to single organs: skin, gastrointestinal tract, and liver GVHD showed similar responses. How long mesenchymal stem cells survive after injection and to what extent they are able to target tissues are unknown.[22] Thus, whether GVHD multi-organ response to infusion occurs because the cells reach the lymph nodes and inhibit the immunological response that gives rise to GVHD is unclear; they might alternatively or additionally target various organs associated with a tissue-healing effect. Further tracking studies with labelled cells are needed to address these issues. Third-party mesenchymal stem cells were as effective as HLA-identical or haploidentical cells. This finding has practical implications and suggests that third-party cells can be prepared and stored frozen to be used for GVHD therapy. Little is known about whether HLA disparity determines the response to treatment and survival of cells after systemic administration. Most data derived from animals indicate short survival of mesenchymal stem cells after injection in vivo. Clinical benefit t might not require sustained engraftment of many cells but could possibly result from production of growth factors or temporary immunosuppression. With the poor health of patients receiving mesenchymal stem cells, some deaths were expected, despite control of GVHD. Infections are common in severe GVHD because of the state of immunodeficiency that characterizes these patients. Whether treatment can further aggravate immune incompetence or not is unclear. To properly answer the question of whether treatment increases the risk of infections requires a controlled randomized study.

The survival rate for patients with complete response was significantly better than that for those with partial or no response, suggesting that beneficial effects of mesenchymal stem cells are not overridden by a high number of severe infections.

This study was a multicentre collaboration between centres adopting a common protocol for the expansion of mesenchymal stem cells. Because we did not find

significant differences in the response rate to treatment in patients in the different participating centres, we conclude that use of a common protocol affords reproducibility of results. Mesenchymal stem cells derived from bone marrow might be a safe and effective treatment for patients with severe, acute GVHD who do not respond to corticosteroids and other immunosuppressive therapies. The number of infusions needed, the best dose of cells in each infusion, and the possible interactions of cells with other drugs for acute GVHD require further investigation. Although the grim outlook for patients who do not respond to treatment suggests that improved survival rate is probably related to infusions of mesenchymal stem cells, randomised clinical studies are needed to compare this treatment with more conventional approaches.

In summary, this study shows that more than half of the patients with steroid-refractory acute GVHD responded to treatment with mesenchymal stem cells. Whether the cell donor was HLA matched or unmatched did not affect the success of treatment. Just over half of patients with a complete response were alive at 2 years.

Acknowledgments

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CHAPTER 7

Phenotypical/functional characterization of *in vitro* expanded mesenchymal stromal cells from Crohn's disease patients

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Summary

Thanks to their capacity to modulate immune response and promote tissue repair, mesenchymal stromal cells (MSCs) represent a potential novel treatment for autoimmune/inflammatory diseases, including Crohn's disease (CD). The aim of the study was the *in vitro* characterization of MSCs from active CD patients (CD-MSCs) for future clinical application.

MSCs from bone marrow (BM) of 7 CD-patients (median age 32 years) were expanded *ex vivo* in the presence of 5% platelet lysate; cells were investigated for clonogenic efficiency, proliferative capacity, morphology, immunophenotype, differentiation potential, genetic stability and ability to suppress *in vitro* proliferation of both autologous and allogeneic lymphocytes to polyclonal mitogens. Results were compared with those of BM-MSCs of 4 healthy donors (HD).

MSCs were successfully expanded from all patients. Colony-forming unit-fibroblast (CFU-F) frequency and proliferative capacity were comparable in CD- and HD-MSCs. CD-MSCs showed the typical spindle-shaped morphology and differentiated into osteoblasts, adipocytes and chondrocytes. Surface immunological markers did not differ between CD- and HD-MSCs, with the only exception of sizeable levels of HLA-DR at early culture passages (12%-84% at P1) in the former. CD-MSCs ceased their growth at variable passages (from P8 to P25) and entered senescence, without any change in morphology/proliferation rate. Array-Comparative Genomic Hybridization demonstrated that CD-MSCs do not show imbalanced chromosomal rearrangements. CD- and HD-MSCs similarly inhibited *in vitro* proliferation of lymphocytes to mitogens.

CD-MSCs show biological characteristics similar to HD-MSCs and can be considered for approaches of anti-inflammatory and reparative cell therapy in patients with refractory disease.

Introduction

Within the bone marrow (BM) microenvironment, multipotent stromal cells, also referred to as mesenchymal stromal cells (MSCs), are known to be the precursor cells for stromal tissues that support hematopoiesis.[1,2] For many years, MSCs have been considered mainly a component of marrow stroma, without any special function, and endowed only with structural support properties. It is now clear that MSCs give a substantial contribution to the creation of the hematopoietic stem cell (HSC) *niche*, and play a crucial role in the development and differentiation of the lympho-hematopoietic system by secreting a number of growth factors and regulatory cytokines, and by promoting cell-to-cell interactions.[3-5]

After the initial identification in post-natal BM,[1] MSCs have been isolated from a variety of other human tissues, including muscle connective tissue, adipose tissue, foetal tissues, placenta and umbilical cord blood (UCB).[6-10] The isolation and characterization of MSCs rely on their adherence to plastic, the ability to differentiate into the various mesenchymal differentiation lineages and on the expression or absence of a number of surface molecules on culture-expanded cells.[11] MSCs possess unique immunological properties that are displayed on all cells involved in the immune response, including T- and B-lymphocytes, dendritic cells and Natural Killer (NK) cells, as demonstrated by several independent groups both *in vitro* and *in vivo*.[12-16] Based on these biological and functional properties, MSCs have been already successfully employed in the clinical setting, either to enhance hematopoietic stem cell engraftment[17,18] or to treat the most severe form of acute graft versus host disease (GvHD), refractory to conventional treatments.[19,20]

Moreover, MSCs have been demonstrated to display chemotactic ability, to migrate to sites of inflammation and injury,[21] as well as to secrete paracrine mediators able to reverse acute organ failure.[22] Indeed, MSC infusions have been successfully used in repairing tissue injury secondary to allogeneic

hematopoietic stem cell transplantation (HSCT).[23]

Based on these findings, MSCs can be considered as a sort of novel treatment for inflammatory diseases, where cell loss is accompanied by local and systemic inflammatory response.

Crohn's disease (CD) is a chronic inflammatory enteropathy, whose prevalence in Western countries has dramatically increased in the last decade, [24] and in which a dysregulation of the immune response towards intestinal bacteria in genetically susceptible individuals plays a pathogenetic role.[25] Despite the large number of therapeutic options available, *i.e.* anti-inflammatory drugs, antibiotics, immunosuppressant drugs, biological agents and surgical strategies,[26] there is a growing number of CD patients with refractory/recurrent disease. In view of this consideration and of the serious side effects of more aggressive therapies, alternative strategies are needed both to increase the proportion of patients achieving remission and to improve their quality of life.

Very recently, the topical implantation of BM-derived MSCs has been demonstrated to be beneficial in the healing process of experimental colitis in rats, confirming the ability of MSCs to modulate immune-responses and to promote tissue repair through their trophic activity.[27] Moreover, a phase I clinical trial for the treatment of perianal fistulas in 4 CD patients with autologous, adipose tissue derived-MSCs has been reported with promising results.[28]

In this respect, we are currently investigating the potential role of autologous BM-derived MSCs as novel, anti-inflammatory cellular therapy to stimulate tissue repair in CD patients. However, no experimental data have been obtained, so far, on the biological and functional characterization of BM-derived MSCs from these patients. The aim of this study, therefore, was to evaluate the feasibility of isolating and expanding *ex vivo* MSCs from BM of CD patients with active disease, and to carry out a phenotypical and functional characterization of these cells in comparison with BM-MSCs isolated from

healthy subjects. In a perspective of future clinical use, in order to avoid any risk associated with the use of fetal calf serum (FCS), platelet lysate (PL, 5%) was employed as culture supplement to stimulate MSC growth.[29]

Materials and methods

CD Patients and Healthy Donors

Peripheral blood (PB) and BM cells were harvested from 7 patients with active CD (5 males, 2 females, median age 32 years, range 18-59) and 4 healthy HSCT donors (2 males, 2 females, median age 33 years, range 16-47), after obtaining written informed consent. The Institutional Review Board of Fondazione IRCCS Policlinico San Matteo Foundation approved the design of this study.

Peripheral blood mononuclear cells (PBMCs) were isolated from CD patients and healthy donors (HDs) by means of density gradient centrifugation (Ficoll 1.077 g/ml; Lymphoprep, Nycomed Pharma, Oslo, Norway) and cryopreserved for future experiments.

Patient characteristics at time of BM harvest are described in Table 1.

Pt. (*n.*)	Sex	Age (years)	BMI	Disease duration (years)	Behaviour	Location	CDAI	Therapies	Previous therapy
1	F	45	31.0	8	B3	L2	323	MSZ, MTN, anti-TNFa	MSZ, PDN, AZT, AB, anti-TNFa , surg.
2	M	29	19.6	13	B3	L1	197	MSZ, anti-TNFa	MSZ, PDN, AZT, AB, anti-TNFa , surg.
3	M	18	27.0	2	B3	L2	173	MSZ, MTN	MSZ, PDN, AB, surg.
4	F	59	28.0	1.2	B2/B3	L2	172	MSZ	MSZ, PDN, AB, surg.
5	M	37	17.0	12	B2/B3	L3	231	MSZ, AZT, MTN	MSZ,PDN,AZT,AB,surg.
6	M	32	19.0	15	B3	L3	162	MSZ	MSZ, PDN, AB, surg.
7	M	32	22.0	8	B3	L3	242	MSZ	MSZ, PDN, AB

Table 1. Patients' clinical features at time of bone marrow harvest
Pt: patient; F: female; M: male; BMI: body mass index; CDAI: Crohn's disease activity index; MSZ: mesalazine; MTN: Metronidazole; PDN: prednisone; AB: antibiotics; AZT: azathioprine; surg.: surgery.
Disease behaviour and localization are classified according to the Vienna classification (*). Behaviour: B1 nonstricturing, nonpenetrating; B2 stricturing; B3 penetrating; location: L1 terminal ileum; L2 colon; L3 ileocolon; L4 upper gastrointestinal.
A CDAI value ≥ 150 indicates active disease
(*)Gauche C, Scholmerich J, BrynshowJ, et al. A simple classification of Crohn's disease: report of the working party of the world congresses of gastroenterology, Wien 1998. Inflamm Bowel Dis 2000;6:8-15.

Isolation and Culture of BM-derived MSCs

Mononuclear cells were isolated from BM aspirates (20 ml) of CD patients and HDs by density gradient centrifugation (Ficoll 1.077 g/ml; Lymphoprep) and plated in non-coated 75-175 cm^2 polystyrene culture flasks (Corning Costar, Celbio, Milan, Italy) at a density of 160,000/cm^2 in complete culture medium: LG-DMEM (Invitrogen, Paisley, PENN) supplemented with 5% PL,[29] and gentamycin 50 µg/ml (Gibco-BRL, Life Technologies, Paisely, UK). PL was prepared as previously described and used as culture supplement for the generation and expansion of MSCs from all BM samples.[29] Cultures were maintained at 37°C in a humidified atmosphere, containing 5% CO_2. After 48-hour adhesion, non-adherent cells were removed and culture medium was replaced twice a week. MSCs were harvested after reaching ≥ 80% confluence, using Trypsin (Sigma-Aldrich, Milano, Italy), and propagated at 4,000 cells/cm^2 until passage (P) 5. MSCs from 5 CD patients (CD-MSCs) were maintained continuously in culture until reaching senescence. Senescent MSCs were monitored for up to 8 weeks, in order to reveal any change in morphology and/or proliferation rate.[30]

Characterization of ex vivo Expanded MSCs

The colony-forming unit-fibroblast assay (CFU-F) was performed as described previously.[29,31] CFU-F formation was examined after incubation for 10 days; the clonogenic efficiency was calculated as the number of colonies per 10^6 BM mononuclear cells seeded.

CD-MSCs from all 7 patients and MSCs from the 4 HDs (HD-MSCs) were phenotipically characterized by flow-cytometry; fluorescein isothiocyanate (FITC) or phycoerithrin (PE)-conjugated monoclonal antibodies specific for CD45, CD14, CD34, CD13, CD80, CD31, HLA A-B-C, HLA-DR, CD90 (BD PharMingen, San Diego, CA), CD73, CD105 (Serotec, Kidlington, Oxford, UK) were used. Appropriate, isotype-matched, non-reactive fluorocrome-

conjugated antibodies were employed as controls. Analysis of cell populations was performed by means of direct immunofluorescence with a FACSCanto flow cytometer (BD PharMingen) and data were calculated using FlowJo software (Tree Star, Inc. Ashland, OR).

The osteogenic, adipogenic and chondrogenic differentiation capacity of MSCs was determined at P2 or P3 in 4 CD patients and 3 HDs, as previously described.[29,32,33] To detect osteogenic differentiation, cells were stained for calcium deposition with Alzarin Red (Sigma-Aldrich). Adipogenic differentiation was evaluated through the morphological appearance of fat droplets stained with Oil Red O (Sigma-Aldrich). To detect chondrogenic differentiation, cells were stained for proteoglycans Toluidine Blue (Sigma-Aldrich).

In vitro PBMC Proliferation Assay with PHA and OKT3

The proliferation of PBMCs from 6 CD patients and 4 HDs, in response to both phytohemagglutinin (PHA-L; Boehringer, Mannheim, Germany) and anti-CD3 (OKT3; Ortho, Raritan, NJ), in the absence or in the presence of BM-derived MSCs was performed in triplicate in flat-bottom microwells (Corning Costar, Celbio).

In detail, irradiated (30 Gy) autologous or allogeneic MSCs were seeded at MSC:PBMC ratio 1:2, 1:10, 1:20 per well and allowed to attach overnight before adding 10^5 PBMCs per well. PBMCs, in RPMI 1640 medium (Gibco-BRL, Life Technologies) supplemented with 10% FCS (Euroclone, Celbio), were then added with or without PHA (4 μg/ml) and OKT3 (5 ng/ml). After a 3-day incubation at 37°C in a humidified 5% CO_2 atmosphere, ^{3}H-thymidine (^{3}HTdR 0.5 μCi/well; Amersham, Buckinghamshire, UK) incorporation was measured during the last 21 hours by standard procedure. The experiments were performed both in the autologous setting (*i.e.* CD-PBMCs/CD-MSCs;

HD-PBMCs/HD-MSCs) and in the allogeneic setting (*i.e.* CD-PBMCs/HD-MSCs; HD-PBMCs/CD-MSCs).

Molecular Karyotyping

Molecular karyotyping was performed by array-comparative genomic hybridization (array-CGH) with the Agilent kit (Human Genome CGH Microarray, Agilent Technologies, Santa Clara, CA). Since the minimum positive call was considered of three consecutive oligomeres with a $\log_2$ ratio different from zero, the theoretical resolution of the 105kb 60mer oligonucleotide platform was of about 40kb. The genetic profile of 4 CD patients was tested before culture (defined time 0 or T_0) using PBMCs, and after *in vitro* culture on MSCs at P2 or P3 (T_1). CD-MSCs of 2 patients (pt. n. 3 and 4) were also evaluated at later passages (P10 and P13, respectively), after prolonged *in vitro* culture (T_2). DNA extracted from saliva of the 4 CD patients was used as control DNA to test T_0 cells. PBMC DNA of the analyzed patient was used as control DNA in every experiment on CD-MSCs. DNA was extracted using QIAamp DNA Blood Mini Kit (Qiagen, Milan, Italy) according to the manufacturer's protocol. Array-CGH experiments were performed according to the manufacturer's protocol version 5.0. and analyzed through the Agilent scanner and the Feature Extraction software (v9.1). Graphical overview was obtained using the CGH Analytics software (v3.4.27). Quality control parameters for every experiments were valuated using QC metric tool of CGH Analytics Agilent software.

Statistical Analysis

The non parametric Mann-Whitney test for independent samples was performed for the comparison of CFU-F numbers, cumulative cell

counts, inhibition of PBMC proliferation to mitogens. P values less than 0.05 were considered significant.

Results

Characterization of BM-derived MSCs

We have previously demonstrated that, as compared to MSCs cultured in 10% FCS, MSCs of HDs expanded in the presence of 5% PL display comparable morphology, phenotype and differentiation capacity, whereas are superior in terms of clonogenic efficiency and proliferative capacity.[29] Based on these findings, we have chosen 5% PL as culture supplement for the *ex vivo* isolation and expansion of MSCs from BM of all CD patients and HDs evaluated in this study.

As compared to HD-MSCs, BM-derived MSCs from CD patients showed a similar spindle-shape morphology in culture (see Figure 1A). In addition, when BM samples were assayed for CFU-F frequency after 10-day culture the results were as follows: HD-MSCs (obtained from 4 subjects) showed a median value of 24.5 (range 18-31) CFU-Fs per 10^6 mononuclear cells plated, whereas the median value for CD-MSCs (obtained from 7 patients) was 21.5 (range 16-29; P=NS) CFU-Fs.

Similar to HD-MSCs, CD-MSCs were able to differentiate into both osteoblasts, adipocytes and chondrocytes, as demonstrated by the histological detection of calcium depositions positive for Alzarin Red (Figure 1B), the morphological appearance of lipid droplets stained with Oil Red O (Figure 1C) and the histological detection of proteoglycans positive for Toluidine Blue, respectively (Figure 1D), respectively.

No differences were observed in terms of proliferative capacity, calculated as cumulative cell counts from P0 to P5, between HD- and CD-MSCs (P=NS; see Figure 1E).

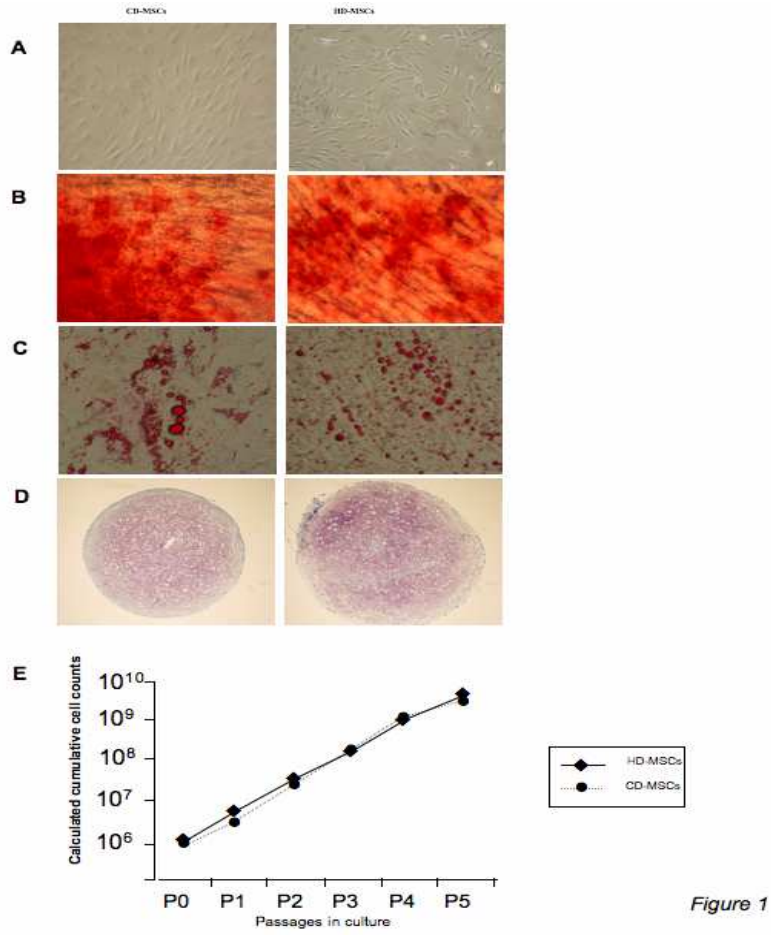

Figure 1. Characterization of Crohn's disease (CD)-MSCs, as compared to healthy donor (HD)- MSCs, expanded in the presence of platelet lysate (PL, 5%)-additioned medium.

A) Morphology of CD-MSCs from patient *n.* 2 at P3, as compared to HD-MSCs at P3. B) Osteogenic differentiation capacity of CD-MSCs from patient *n.* 4 at P3, as compared to HD-MSCs at P3. The differentiation into osteoblasts is demonstrated by calcium deposition stained with Alzarin Red. Magnification x 20. C) Adipogenic differentiation capacity of CD-MSCs from patient *n.* 4 at P3, as compared to HD-MSCs at P3. The differentiation into adipocytes is revealed by the formation of lipid droplets stained with Oil Red O. Magnification x 20. D) Chondrogenic differentiation capacity of CD-MSCs from patient *n.* 4 at P3, as compared to HD-MSCs at P3. The differentiation into chondrocytes is demonstrated by deposition of extracellular matrix stained with Toluidine Blue (proteoglycans). E) Calculated cumulative cell counts from P0 to P5 of CD-MSCs and HD-MSCs cultured in the presence of 5% PL; results are expressed as the mean calculated from data obtained from 7 CD patients and 4 healthy donors, respectively.

Moreover, the median time to reach 80% confluence for all passages from 1 to 5 was 6 days for both HD- and CD-MSCs.

MSCs from 5 CD patients (pt. *n.* 1, 2, 3, 4 and 6) were propagated for long term *in vitro* culture. CD-MSCs from pt. *n.* 1, 2, 3, and 6 ceased their growth at P11, 9, 11 and 8, and entered a senescence phase after 18-, 19-, 20- and 16-week culture, respectively. Patient *n.* 4 showed a late MSC growth arrest (P25), when the cells entered senescence after 28-week culture. Senescent CD-MSCs were monitored in culture daily for up to 8 weeks, without showing any change in morphology, immunophenotype and/or proliferation rate.

CD-MSCs from all 7 patients expressed high levels (>95% positive cells) of CD90, CD73, CD105, CD13 and HLA A-B-C surface antigens, whereas were negative for CD34, CD45, CD14, CD80, CD31 molecules (see Figure 2), this pattern being similar to that observed in HD-MSCs. However, while in HD-MSCs HLA-DR expression was always below 2% even at early passages (data not shown), CD-MSCs at P1-P3 expressed variably measurable levels of this marker (from 12 to 84% at P1; see Figure 2), which tended to decrease during *in vitro* culture and completely disappeared after P4.

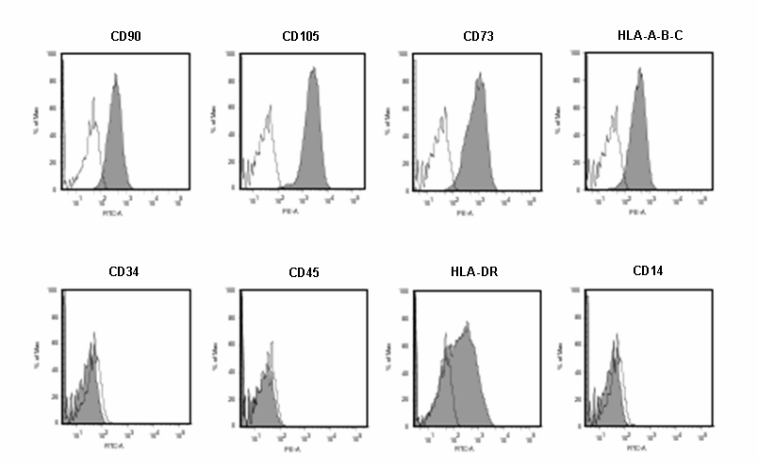

Figure 2. Immunophenotypic characterization of CD-MSCs from patient *n.* 1 at P1 by flow cytometry. CD-MSCs stain positive for CD90, CD105, and HLA A-B-C surface antigens, whereas are negative for CD34 and CD45 molecules. Sixty % of CD-MSCs

from patient n. 1 at P1 are positive for HLA-DR. B) Expression of HLA-DR on CD-MSCs from patient *n.* 1 at P5. Only 2 % of CD-MSCs are positive for HLA-DR.

The expression of HLA-DR was monitored throughout culture for all CD patients (see Table 2 for details).

Pt (*n.*)	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**
1	60	45	22	9	2	0
2	30	30	4	2	ND	0
3	20	10	5	0	0	0
4	17	11	0	0	0	ND
5	84	44	7	1	0	0
6	12	0	0	0	ND	ND
7	13	0	0	0	ND	0

Table 2. Percentages of HLA-DR expression on *ex-vivo* expanded Crohn's disease (CD)-MSCs by flow cytometry. CD-MSCs at early passages (P1-P3) express variable levels of HLA-DR which tend to decrease during *in vitro* culture and disappear after P4.
ND: not determined

Effect of MSCs on PBMC proliferation

The effect of CD- and HD-MSCs on the proliferation of PBMCs stimulated with PHA and OKT3 was evaluated in 6 CD patients and 4 HDs. Results are reported in Figure 3.

As far as the autologous setting (*i.e.* CD-PBMCs/CD-MSCs) is concerned, PHA- and OKT3-stimulated proliferation of CD-PBMCs was reduced by up to 61% ± 9.6% (MSC:PBMC ratio 1:10) and 59% ± 8.1% (MSC:PBMC ratio 1:20), respectively, by the addition of CD-MSCs. However, a direct correlation between the number of CD-MSCs added and the degree of inhibition of CD-PBMC proliferation was not found (see Figure 3A and 3B for the residual percentage of response). A comparable degree of inhibition

was observed when PHA- and OKT3-stimulated HD-PBMCs were tested in the presence of HD-MSCs: 44% ± 12.3% at MSC:PBMC ratio 1:10 for PHA (P=NS); 57% ± 11% at MSC:PBMC ratio 1:20 for OKT3 (P=NS, see also Figure 3C and 3D for the residual percentage of proliferation).

When the allogeneic setting was examined (*i.e.* CD-PBMCs/HD-MSCs), we found that HD-MSCs were comparable to CD-MSCs in the ability to reduce PHA- and OKT3-stimulated proliferation of CD-PBMCs (maximum inhibition for PHA at MSC:PBMC ratio 1:10 = 52.5% ± 21.2%, P=NS; maximum inhibition for OKT3 at MSC:PBMC ratio 1:20 = 45% ± 17%, P=NS). As already shown for the combination CD-PBMCs/CD-MSCs, also in this context a dose-dependent effect of MSC addition was not observed. Figure 3E and 3F report the residual percentage of response.

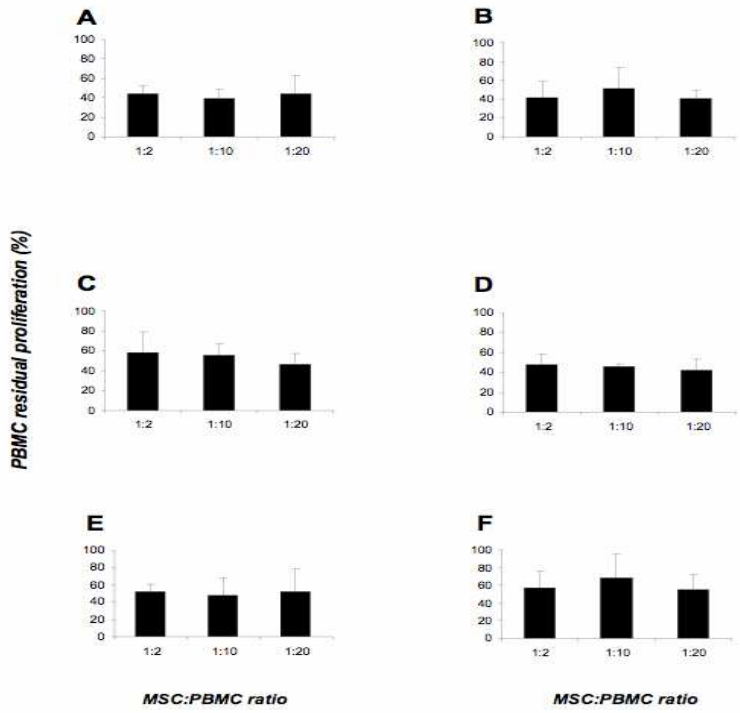

Figure 3. Residual proliferation of CD patient PBMCs (CD-PBMCs) and healthy donor PBMCs (HD-PBMCs), stimulated with PHA and OKT3, in the presence of CD

patient MSCs (CD-MSCs) and healthy donor MSCs (HD-MSCs). Each bar represents the percentage of proliferation of 100.000 PBMCs in the presence of decreasing numbers of MSCs (MSC:PBMC ratio 1:2; 1:10; 1:20). The cpm values at each cell concentration were normalized to the cpm of PBMCs without MSCs in each experiment. Each bar represents the mean ± SD of multiple experiments (4 to 6 MSC samples, each point being in triplicate). A) CD-PBMCs/CD-MSCs, PHA stimulation; B) CD-PBMCs/CD-MSCs, OKT3 stimulation; C) HD-PBMCs/HD-MSCs, PHA stimulation; D) HD-PBMCs/HD-MSCs, OKT3 stimulation; E) CD-PBMCs/HD-MSCs, PHA stimulation; F) CD-PBMCs/HD-MSCs, OKT3 stimulation.

The condition HD-PBMCs/CD-MSCs was also tested: CD-MSCs induced a reduction of HD-PBMC proliferation comparable to that observed when HD-MSCs were tested in the presence of HD-PBMCs (data not shown).
Altogether, we did not find any significant difference between CD- and HD-MSCs in the ability to reduce the proliferation of both autologous and allogeneic PHA- and OKT3-stimulated PBMCs *in vitro*.

Molecular Karyotyping

To test the genetic profile of CD-MSCs, array-CGH experiments were performed on cells from 4 CD patients before culture (T_0), at early passages (T_1) and after prolonged *in vitro* culture (T_2; 2 patients). The comparison of results from two or three experiments for each patient (T_0, T_1 and T_2), allowed us to evidence the possible presence of chromosomal micro-unbalances which could be present only in CD-PBMCs (T_0) or both in CD-PBMCs and in *ex vivo* cultured CD-MSCs (T_1 and T_2). The use of DNA from saliva, as control DNA to test T_0 cells, permitted to exclude copy number variations (CNVs)[34] constitutionally present in the patients' genome and, therefore, to evidence only acquired chromosomal imbalances. Moreover, the DNA from saliva allowed to detect chromosomal deletions typical of T lymphocytes and, thus, present only in PBMCs, but not in MSCs. Results of array-CGH experiments demonstrated that T_0 cells were devoid of chromosomal imbalances, except for micro-deletions of different sizes in *loci* that undergo mitotic recombination

specifically in T cells. These anomalies were not present in CD-MSCs expanded *in vitro*, which did not show imbalanced chromosomal rearrangements even after long term culture (see Figure 4). In fact, the array-CGH profiles of the repeated experiments from the same patient were perfectly overlapping and no deletions or duplications were present, considering the platform resolution of about 40kb.

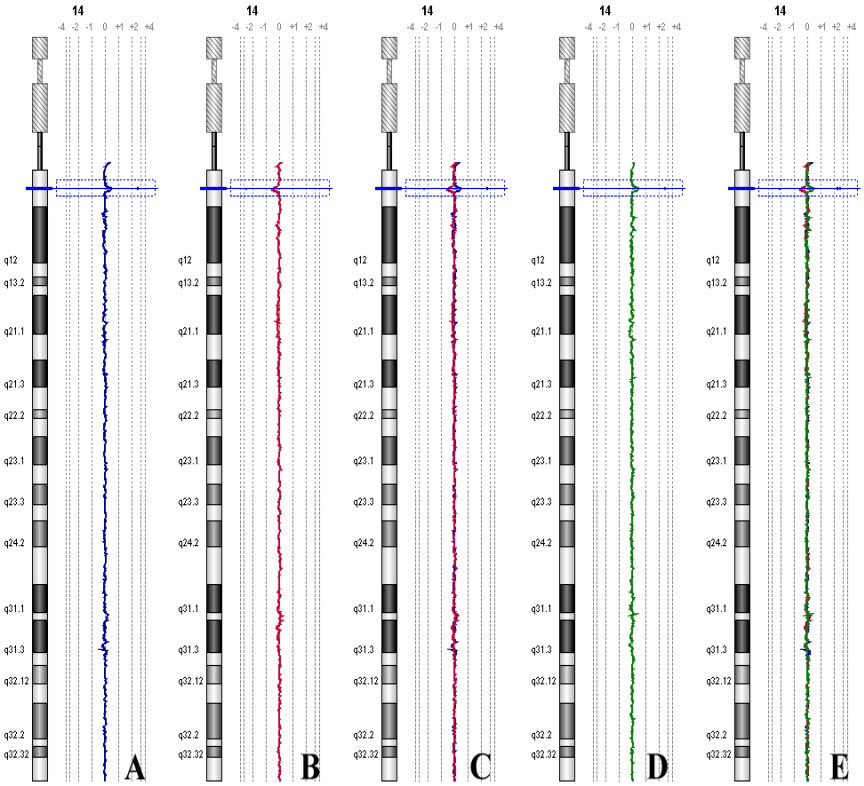

Figure 4. Representative array-CGH profiles of chromosome 14 from patients *n.* 4: A) T_0: PBMCs versus saliva; B) T_1: MSCs at P3 versus PBMCs; C) The two overlapping experiments; red line applies to PBMCs, blue line to MSCs at P3. D) T_2: MSCs at P13 versus PBMCs; E) The three overlapping experiments; red line applies to PBMCs, blue line to MSCs at P3 and green line to MSCs at P13. The first experiment (A) shows the presence of a small deletions in PBMCs in 14q11.2 which involves the TCA (T-cell receptor alpha chain C region) locus. This gene is rearranged specifically in T cells and its organization is similar to an Ig gene, with V, D, J, and C regions. The deletion is present in cellular mosaic, because PBMCs contain all peripheral blood mononuclear cells, a portion of whom is represented by T cells. The somatic deletion of PBMCs is also revealed in experiments on MSCs, where it appears as a duplication, because PBMCs are hybridizated as control DNA. The array-CGH profile of CD-MSCs is linear and no unbalanced chromosomal rearrangements are present.

Discussion

Despite the large number of therapeutic options, disease control in CD remains hard to achieve in many patients.[35] In such patients with refractory disease and in whom progression into an exacerbated form is associated with severe gut fibrosis and formation of strictures and/or fistulas, anti-inflammatory and immunosuppressive therapies, as well as biological agents and surgery, have limited success.[35] In this context, adult stem cells and, in particular, MSCs are under investigation as a novel cell therapy-based approach that could ameliorate the management of CD patients.[36,37]

In the present study, we have isolated and propagated in culture MSCs derived from the BM of 7 patients affected by active CD, with the aim of characterizing their *in vitro* biological and functional properties in view of their possible clinical use in patients refractory to conventional therapies. For this purpose, CD-MSCs have been cultured in the presence of a PL-additioned medium and compared to PL-cultured, BM-derived, MSCs obtained from HDs.

Our data demonstrate that both the isolation and the *ex vivo* expansion of BM-derived MSCs from CD patients are feasible and that this cell population exhibits similar morphology and differentiation potential into osteoblasts, adipocytes and chondrocytes, as compared to HD-MSCs (Figure 1A-D).

Moreover, CD-MSCs display similar CFU-F ability and proliferative capacity (Figure 1E), as compared to HD-MSCs, guaranteeing the possibility of expanding *in vitro* sufficient numbers of cells for clinical application.

As regards the immunophenotype, CD-MSCs displayed the panel of surface markers characteristic of MSC, with the only exception of HLA-DR (Figure 2). The consistent expression of this latter antigen in CD-MSCs at early passages (Table 2) might be related to the condition of active disease found in all patients when their BM was harvested, as demonstrated by the activity indexes (CDAI; see Table 1). In fact, the expression of HLA-DR is lost

shortly after *in vitro* culture, namely when cells are grown in a medium devoid of inflammatory signals/cytokines, which, by contrast, are potentially released by the cells involved in the immune/inflammatory response and present in the extracellular milieu of CD-BM. Support to this interpretation is provided by the observation that BM-derived MSCs from healthy volunteers, expanded in the presence of PL, did not show any expression of HLA-DR,[29] this suggesting that the increased expression of HLA-DR is likely to be attributable to the disease status of the patients. It has been previously shown that *in vitro* exposure of MSCs to IFN-gamma, a cytokine known to be involved in inflammatory processes including those of CD,[38] can induce the expression of HLA-DR on MSC cell surface.[39,40] Moreover, Le Blanc *et al.*[39] have already demonstrated that HLA-DR positive MSCs display comparable immunomodulatory properties as their counterpart not expressing HLA-DR, thus suggesting that this peculiarity of CD-MSCs should not affect their function and does not preclude their possible use in the clinical setting.

We also demonstrated that CD-MSCs are equally effective, as HD-MSCs, in inhibiting *in vitro* polyclonally-induced proliferation of both autologous (*i.e.* derived from the patients themselves) and allogeneic (*i.e.* derived from healthy subjects) PBMCs (Figure 3). In particular, it is noteworthy that CD-MSCs are able to display the same magnitude of inhibition on CD-PBMCs proliferation, as compared to the effect exerted by HD-MSCs on CD-PBMCs. This finding supports the use of patient-derived MSCs, instead of utilizing third party cells. The use of autologous MSCs offers significant advantages in light of the observations that MSCs can be lysed by both allogeneic T cells[41] and NK cells.[15] In the allogeneic setting, it is conceivable that an HLA-disparity possibly resulting in NK alloreactivity[42] could be a condition where NK cells are particularly prone to kill mesenchymal progenitors. Finding an inhibitory effect of MSCs even in the presence of a low MSC:PBMC ratio suggests also

that a meaningful favorable clinical effect could be obtained even after the infusion of a low number of autologous mesenchymal progenitors.

BM-derived MSCs from CD patients could be cultured long term *in vitro,* without loosing their peculiar morphological and phenotypical characteristics and maintaining a normal genetic asset, as demonstrated by array-CGH experiments (Figure 4). In this study, we used for the first time array-CGH with a higher resolution[30] (about 40kb) to test MSCs, in order to have a deeper and more sophisticated evaluation of the genomic situation of *ex vivo* expanded cells, even if this approach is not able to unravel balanced chromosomal rearrangements (detectable only by conventional karyotype) and cell mosaicisms lower than 20%.[43] When studying the genetic stability of *ex vivo,* extensively cultured BM-derived MSCs from HDs, we found that a normal array-CGH profile was associated with the absence of any abnormality in the conventional cytogenetic analysis.[30] Our present results obtained through array-CGH indicate that CD-MSCs do not appear to be susceptible to malignant transformation even after long-term culture, thus rendering these cells suitable for cell-therapy approaches.

Both in animal models and in patients, it has been shown that BM-derived cells play a role in the healing process following intestinal injury and in the regeneration of various cellular components of the mucosa.[44-46] MSCs, through the secretion of soluble factors (such as indoleamine 2,3-dioxygenase, prostaglandin E2, hepatocyte growth factor, etc.) as well as through a direct cell-to-cell contact, have been demonstrated to be able to inhibit T-lymphocyte proliferation to mitogens and allo-antigens, to inhibit cytotoxic T-lymphocytes generation, to influence the secretion of cytokines favouring the anti-inflammatory ones and to promote the differentiation of regulatory T cells.[40,47] Thus, in view of their immunosuppressive properties, as well as of their role in tissue repair and trophism, BM-derived MSCs represent a promising tool in approaches of immunoregulatory and regenerative cell

therapy.[48] Indeed, the potentiality of MSCs in the clinical setting has been already shown both in prevention and in treatment of GvHD occurring after allogeneic HSCT.[17,19,20] In particular, a dramatic effect, in terms of complete resolution of the disease, has been observed in many patients suffering from acute GvHD of the gut refractory to conventional therapy.[19,20] The therapeutic efficacy of BM-derived MSCs on this severe complication of HSCT, as well as the demonstration that MSCs colonize the site of histological injury in patients with gastrointestinal acute GVHD,[19] have suggested their potential use in the treatment of other inflammatory and immune-mediated diseases, including CD. In this regard, recently, the results of a phase-I clinical trial, in which autologous, adipose tissue (AT)-derived MSCs have been used for the treatment of fistulizing CD in 4 patients, have been published. Eight weeks after MSC local infusion, 75% of the fistulas were considered healed and no adverse effect was observed.[28] Based on these encouraging results, a phase-II trial on autologous AT-derived MSCs[49] and a phase-III trial on third-party, BM-derived MSCs (Osiris Therapeutics, Inc. Columbia, MA)[50,51] in CD patients refractory to conventional therapies, are underway.

In this respect, it is worthy to note that *ex vivo* cultured human AT-derived MSCs have been reported to be prone to undergo spontaneous transformation characterized by significant chromosomal instability,[52] while, as mentioned above, BM-derived MSCs do not.[30] The biological and functional characterization of BM-derived CD-MSCs, which we have demonstrated to be comparable to HD-MSCs, confirm the plasticity of these cells, together with their immunomodulatory and differentiation properties, and provide the experimental background to consider their use as innovative therapeutic strategy in the management of CD patients with refractory disease. Moreover, the bio-safety profile of these cells is sustained not only by the genetic stability of their chromosome asset, but also by the fact that they were generated in the presence of PL as culture supplement. In fact, the utilization

of this reagent for the *ex vivo* expansion of MSCs allows to avoid the potential risks associated with FCS, such as the possible transmission of zoonoses and the rejection of the transplanted cells due to immune reactions against animal serum proteins,[53] while providing rapid and efficient expansion.

In summary, this study provides, to our knowledge for the first time, a comprehensive characterization of BM-derived MSCs from CD patients; our results support the potential use of autologous BM-derived MSCs as novel, anti-inflammatory and reparative approach for treating patients with CD refractory to or relapsing after conventional therapies.

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CHAPTER 8

Generation of mesenchymal stromal cells in the presence of platelet lysate: a phenotypical and functional comparison between umbilical cord blood- and bone marrow-derived progenitors

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Summary

Mesenchymal stromal cells (MSCs) are employed in diverse clinical settings in order to modulate immune response. However, relatively little is known about the mechanisms responsible for their immunomodulatory effects, which could be influenced by both cell source and culture conditions.

We tested the ability of a 5% platelet lysate (PL)-supplemented medium to support isolation and *ex-vivo* expansion of MSCs from full-term umbilical-cord blood (UCB). We also investigated the biological/functional properties of UCB-MSCs, in comparison with PL-expanded bone marrow (BM)-MSCs.

Success rate of MSC isolation from UCB was in the order of 20%. UCB-MSCs exhibited the typical morphology, immunophenotype and differentiation capacity. Although they have a low clonogenic efficiency, UCB-MSCs may possess high proliferative potential. The genetic stability of UCB-MSCs was demonstrated by a normal molecular karyotype; in addition, these cells do not express hTERT and telomerase activity, express $p16^{ink4a}$ protein and do not show anchorage-independent cell growth. Concerning alloantigen-specific immune response, UCB-MSCs were able to: i) suppress T- and NK-lymphocyte proliferation, ii) decrease cytotoxic activity and iii) only slightly increase IL-10, while decreasing IFNγ secretion, in mixed lymphocyte culture (MLC) supernatants. While an IDO-specific inhibitor did not reverse MSC-induced suppressive effects, a PGE2-specific inhibitor hindered the suppressive effect of both UCB- and BM-MSCs on alloantigen-induced cytotoxic activity. Both UCB- and BM-MSCs expressed HLA-G.

UCB- and BM-MSCs may differ in terms of clonogenic efficiency, proliferative capacity and immunomodulatory properties; these differences may be relevant for clinical application.

Introduction

In addition to hematopoietic stem cells (HSCs), the bone marrow (BM) also contains mesenchymal stromal cells (MSCs).[1,2] These latter cells exhibit multilineage differentiation potential and are endowed with immunomodulatory properties that have been demonstrated both *in vitro* and *in vivo*.[3,4] In the setting of hematopoietic stem cell transplantation (HSCT), *ex vivo* expanded MSCs have been employed in view of both their immunomodulatory activity and their ability to support hematopoiesis. In particular, in a phase I/II multicenter study, MSCs proved safe and effective when administered to 55 patients with severe, steroid-refractory acute graft-versus-host disease (GvHD).[5] Moreover, in another phase I/II study, co-transplantation of MSCs, together with T-cell depleted peripheral blood stem cells (PBSCs), overcame the problem of graft failure in 14 children given HSCT from an HLA-haploidentical family donor.[6] Similarly, co-transplantation of MSCs and umbilical cord blood (UCB) stem cells is under investigation.[7,8] Recently, in view of their immunosuppressive properties, as well as their role in tissue repair and trophism, MSC infusion has also been proposed as a novel approach for reparative/regenerative medicine in the treatment of autoimmune disorders and chronic inflammatory diseases.[9-11]

Although BM represents the most commonly employed source of MSCs for both experimental and clinical use,[3,12-16] MSCs have been isolated from other sources, including adipose tissue, placenta, amniotic fluid, fetal tissues and UCB.[17-22]

Notably, it has not always been possible to grow MSCs from UCB and in many cases the yield was low. In particular, the presence of mesenchymal progenitors in full-term UCB has been questioned in recent years by many groups, whose attempts to obtain MSC have either failed[23,24] or yielded low numbers.[21,25] In fact, the frequency of MSCs in UCB is very low and, in fetal blood, it has been reported to decline with gestational age from about $1/10^6$

mononuclear cells in first trimester fetal blood to $0.3/10^6$ mononuclear cells in term cord blood.[20] Despite this limitation, Bieback *et al.* have demonstrated that, when critical parameters for the selection of 'good quality' term UCB units are employed, MSCs can be successfully isolated in more than 60% of the processed cord blood units.[22]

MSCs have been mainly expanded *in vitro* in the presence of fetal calf serum (FCS). Cells thus obtained, when infused into patients, may potentially carry the risks for both transmitting zoonoses and causing immune reactions directed against residual animal proteins. For these reasons, culture supplements devoid of animal components, such as platelet lysate (PL), have been tested in recent years for the isolation and expansion of MSCs.[26-28] In particular, our group previously demonstrated that a 5% PL-supplemented medium can support large-scale, *ex-vivo* expansion of BM-derived MSCs (BM-MSCs) and that this medium is superior to 10% FCS in terms of both clonogenic efficiency and proliferative capacity.[27] Conversely, BM-MSCs expanded in PL seem to be endowed with relatively low immunosuppressive activity, as compared with BM-MSCs grown using FCS as culture supplement.[27]

The aim of this study was to test the ability of a PL-supplemented medium to support the generation and *ex vivo* expansion of MSCs from full-term UCB (UCB-MSCs), as well as to characterize these latter cells for their biological and functional properties, in comparison with PL-expanded BM-MSCs. In particular, we focused on the investigation of both the genetic stability and the immunoregulatory function, exerted on alloantigen-specific immune response, by UCB-MSCs. Moreover, we have evaluated the possible mechanisms at the basis of UCB-MSC immunosuppressive effect.

Design and methods

UCB unit collection and selection

UCB units were collected after full-term delivery and stored at the Cord Blood Bank of our Hospital after obtaining signed written informed consent. The Institutional Review Board of the Fondazione IRCCS Policlinico San Matteo approved the study. Citrate Phosphate Dextrose-A, 20 ml, was employed as anticoagulant in the collection bags. Whole UCB units were employed for MSC generation. Ten fresh UCB units (median volume 45 ml, range 40-60) were selected according to the following criteria: 1) total nucleated cell (TNC) count ranging from 500 to 750 $x10^6$; 2) manipulation performed within 24 hours from delivery; 3) overall cell viability greater than 75%, investigated by 7-amino-actinomycin D (7-AAD) and Aldefluor (ALDH). Samples obtained from UCB units before and after mononuclear cell (MNC) separation were analyzed by a Becton Dickinson FACSCanto instrument (BD BioSciences, San Jose, CA, USA), FACSDiva software 5.0, according to the EWGCCA guidelines (European Working Group on Clinical Cell Analysis).[29,30] Cell viability was determined using the 7-AAD dye test (Molecular Probes, Eugene, OR, USA) within the context of expression of surface markers identified by fluorochrome-labeled antibodies. The following monoclonal antibodies were used: anti-CD34 phycoerythrine (PE), anti-CD45 peridinin chlorophyll protein (PerCP), anti-CD133 allophycocyanin (APC) (all from BD BioSciences) and ALDH (StemCell Technologies, Vancouver, Canada). ALDH was detected using the green fluorescence channel following manufacturer's instructions.

PL preparation

PL was prepared as previously described.[27] In brief, aliquots of 50 ml platelet-rich plasma, collected by apheresis, were obtained from ten healthy volunteers at the Transfusion Service of our Hospital. All apheresis

products contained a minimum of $5x10^{11}$ platelets (PLTs). Written informed consent from donors was always obtained and all apheresis products were screened for infectious agents according to National regulations. Immediately after collection, PLT apheresis products were frozen at -80°C and subsequently thawed at 37°C to obtain the release of PLT-derived growth factors. Heparin (5000 UI) was added to PLT bags to avoid gel formation. Apheresis products were centrifuged three times at 900 g for 30 minutes to eliminate PLT bodies. Finally, PL preparations obtained through this procedure were pooled in a single culture supplement to be used for the generation and expansion of UCB-MSCs.

Isolation and culture of UCB- MSCs

MNCs were isolated from the 10 UCB units by density gradient centrifugation (Ficoll 1.077 g/ml; Lymphoprep, Nycomed Pharma, Zurich, Switzerland) after 1:1 dilution with Dulbecco's phosphate buffered saline (D-PBS; Euroclone, Celbio, Milan, Italy) and plated in non-coated 75-175 cm^2 polystyrene culture flasks (Corning Costar, Celbio) at a density of 160,000/cm^2 in complete culture medium: Mesencult (StemCell Technologies) supplemented with 2 mM L-glutamine, 50 µg/ml gentamycin (Gibco-BRL, Life Technologies, Paisely, UK) and 5% PL. This concentration of PL was chosen on the basis of results previously obtained with BM-MSCs.[27] Cultures were maintained at 37°C, in a 5% CO_2 humidified atmosphere. After 48 hours, non-adherent cells were discarded; culture medium was replaced twice a week. Upon the appearance of MSC-like clones, cells were harvested using Trypsin (Sigma-Aldrich, Milan, Italy), re-plated for expansion at a density of 4,000 cells/cm^2 and propagated in culture until reaching a senescence phase. Senescent cells were monitored for up to eight weeks, in order to reveal any change in morphology and/or proliferation rate. Cell growth was

analyzed by direct cell counts and cumulative population doublings (PD) were determined. Number of PD were calculated using the formula $\log_{10}(N)/\log_{10}(2)$ where N=cells harvested/cells seeded and results were expressed as cumulative PD.[31]

UCB-MSC multilineage differentiation potential

The adipogenic and osteogenic differentiation capacity of UCB-MSCs was determined at passage (P) 2, as previously described.[27] To detect osteogenic differentiation, cells were stained for alkaline phosphatase (AP) activity using Fast Blue (Sigma-Aldrich) and for calcium deposition with Alzarin Red (Sigma-Aldrich). Adipogenic differentiation was evaluated through the morphological appearance of lipid droplets stained with Oil Red O (Sigma-Aldrich).

Immunophenotypic characterization of UCB-MSCs

FITC, PE, PerCP-Cy5.5 monoclonal antibodies specific for the following antigens were employed: 1) CD45 (clone HI30), CD14 (clone MΦP9), CD34 (clone 581), CD13 (clone L138), CD80 (clone L307.4), CD31 (clone L133.1), HLA A-B-C (clone G46-2.6), HLA-DR (clone G46-6[L243]), CD90 (clone 5E10), CD73(clone AD2) (all from BD Biosciences), CD105 (clone SN6; Serotec, Kidlington, Oxford, UK), HLA-G (clone MEM-G/9; Exbio, Praha, CZ) for the assessment of MSC surface phenotype; 2) CD3 (clone SK7), CD4 (clone SK3), CD8 (clone SK1), CD56 (clone NCAM16.2), CD25 (clone 2A3), CD152 (CTLA4; clone BNI3), Foxp3 (clone PCH101; eBioscience, San Diego, CA, USA) for evaluation of lymphocyte subsets. Appropriate isotype-matched controls (BD Bioscience, eBioscience) were included. Intracellular staining for CD152 (CTLA4), Foxp3 and HLA-G was performed as previously described.[27,32] Two-color or three-color direct immune fluorescence cytometry was

performed with FACScalibur flow cytometer (BD Biosciences) and data calculated using CellQuest software (BD Biosciences).

Telomerase activity detection assay and reverse transcription (RT)-PCR analysis of the human telomerase reverse transcriptase (hTERT)

Telomerase activity was measured by the polymerase chain reaction (PCR)-based telomeric-repeat amplification protocol (TRAP) using the TRAPeze kit (Intergen Company, FLA, USA) on samples containing 0.6 and 6.0 μg of protein. Protein extract from a telomerase-positive human cell line (JR8) was used as a positive control.[33] A sample was scored as telomerase activity-positive when positive TRAP results were obtained from at least one protein concentration.

For hTERT assessment, total cellular RNA was extracted from frozen samples with the RNeasy micro kit (Qiagen GmbH). A 0.5 μg aliquot from each sample was reverse-transcribed by using the RT-PCR Core kit (Applied Biosystems, Foster City, CA, USA) with random hexamers, and the resultant cDNA was then amplified with the same kit. Amplification of hTERT cDNA was obtained as previously described.[34]

Western immunoblotting

MSCs were lysed on ice in lysis buffer. Total cellular lysates were separated on 15% SDS-polyacrylamide gel and were transferred onto Hybond ECL nitrocellulose membranes (GE Healthcare Europe GmbH, Cologno Monzese, Italy). Nitrocellulose membranes were blocked in PBS-Tween 20 with 5% skim milk, first incubated overnight with the primary antibody specific for $p16^{ink4a}$ (Abcam Inc., Cambridge, MA, USA) and then with the secondary peroxidase-linked whole antibody (GE Healthcare Europe). Bound antibody was detected using the enhanced chemi-luminescence Western blotting detection system (GE Healthcare Europe).

DU145 human prostate cancer and U2OS human osteogenic sarcoma cell lines were used as positive and negative controls for $p16^{ink4a}$ expression, respectively.

Clonogenic assay

Single-cell suspensions of 100,000-1,000 cells/ml in complete medium and 0.3%(w/v) agarose were plated in triplicate in 35 mm culture dishes, over chilled 0.6% agarose feeder layers. Cultures were incubated at 37°C in 5% CO_2 humidified atmosphere and examined at 14 days after plating under an inverted microscope.

Molecular karyotyping

Molecular karyotyping of UCB-MSCs at early (P3) and late (P8-9) passages was performed through array-comparative genomic hybridization (array-CGH) with the Agilent kit 44B (Human Genome CGH Microarray, Agilent Technologies, Santa Clara, CA, USA), as previously described.[27,34] A pool of characterized genomic DNA (Human Genomic DNA Male, Promega, Madison, WI, USA) was used as control DNA for all experiments. Quality control parameters for every experiment were evaluated using the CGH Analytics Agilent software-QC tool.

Mixed lymphocyte cultures (MLCs) and cytotoxicity assay

Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque density gradient from heparinized PB samples from healthy volunteers. Primary MLCs were set up according to previously described methods.[27,32,35] Briefly, non-irradiated, "third-party" UCB-MSCs, allogeneic to both responder (R) and irradiated stimulator (S) PBMCs, were added at a R to MSC ratio of 10:1. Control MLC (ctrl-MLC) was set-

up in the absence of UCB-MSCs. UCB-MSCs employed in MLC experiments had been harvested and cryopreserved at P3. The total number of PBMCs recovered after 10-day MLC was counted in vitality with Trypan Blue (Sigma-Aldrich). Then, recovered cells were analyzed by flow cytometry and percentage of lymphocyte subsets ($CD3^+CD4^+$ and $CD3^+CD8^+$ T-cells, as well as $CD3^{neg}CD56^+$ NK-cells) was determined. Number of lymphocyte subsets per ml culture was, therefore, calculated and compared with the initial number of cells (day 0). Differentiation of regulatory T cells (Treg) was evaluated by measuring the percentage of $CD4^+CD25^+$ and $CD4^+CD25^{bright}$ T lymphocytes, together with the expression of Foxp3 and CTLA4 on $CD4^+CD25^+$ lymphocytes. Alloantigen-induced cell-mediated cytotoxic activity was tested in a 5-hour ^{51}Cr-release assay, as previously described.[27,32,35] Results are expressed as percent specific lysis of target cells. ^{51}Cr-labeled target cells included PHA-activated S-PBMCs (S-PHA) and the same lots of UCB-MSCs that had been added to MLCs.

To evaluate the possible involvement of indoleamine 2,3-dioxygenase (IDO) and/or prostaglandin E2 (PGE2) in the immunosuppressive effect of PL-expanded UCB-MSCs, we set up MLCs in which, in some wells, specific inhibitors of IDO activity (1-Methyl-Tryptophan, 1-M-Trp, Sigma-Aldrich), or of PGE2 (NS-398, Cayman Chemicals, Irvine, CA) were added to MSCs/MLC co-culture at a concentration of 1 mM and 5 μM, respectively.[36] In this set of experiments, BM-MSCs, expanded in 5% PL-supplemented medium,[27] were employed as a control for UCB-MSCs. Cell counts per ml of culture recovered after 10-day MLC as compared to day 0, and alloantigen-induced cell-mediated cytotoxic activity (using ^{51}Cr-labeled S-PHA as target cells) were evaluated for each culture condition. After 72-hour culture, supernatants were collected for the evaluation of IDO activity and PGE2 quantification.

Detection of IDO activity

IDO activity was evaluated by quantifying tryptophan (tryp) and kynurenine (kyn) concentrations in 72-hour culture supernatants with high-performance liquid chromatography (HPLC), by using the HPLC pump, model SCL-10 *VP* (Shimadzu, Kyoto, Japan). For separation, pre-columns (cartridge holder and guard cartridge) from Phenomenex (Torrance, CA, USA) and reverse-phase C18 (octyl) columns (250 mm length, 460 mm internal diameter, 5 micron grain size) from Beckman-Coulter (Milan, Italy), were used. The incorporated UV/VIS detector model UV-SPD-M10 *VP* (Shimadzu) was employed for detection of both kyn and nitrotyrosine at a wavelength of 360 nm. Tryp was detected by a fluorescence detector (Shimadzu, Model RF-535) at 285-nm excitation wavelength and an emission wavelength of 365-nm. Samples were prepared as previously reported.[37] L-tryp, L-kyn, 3-nitro-L-tyrosine, trichloroacetic acid, potassium phosphate and acetonitrile for the HPLC elution buffer were obtained from Sigma-Aldrich. All chemicals used were of analytical grade. Peak area counts were used to calculate concentrations (EZStart software, version 7.3). Tryp and kyn were referred to nitrotyrosine. The reproducibility of the system was controlled by nitrotyrosine counts and variations less than 5% were tolerated.

Measurement of cytokines and HLA-G by ELISA

The concentrations of interferon-gamma (IFN-γ), interleukins (IL)10, IL6, IL12, IL7, IL2, IL15 and transforming growth factor beta (TGFβ) in MLC supernatants after 12, 24, and 48 hours, were quantified by ELISA using monoclonal antibody pairs (Pierce Endogen, Rockford, IL), as previously described.[27] PGE2 levels were evaluated using a commercially available ELISA (R&D System, Minneapolis, MN), according to manufacturer's

instructions. The concentration of soluble HLA-G (sHLA-G) in culture supernatants was quantified by ELISA (sHLA-G ELISA, Exbio); clone MEM-G/9 was employed as anti-HLA-G capture antibody.

Statistical analysis

The non-parametric Kolmogorov-Smirnov test for independent samples was performed for the comparison of cumulative cell counts at P0 and P5 of UCB- and BM-MSCs. Due to the small size of the groups, the maximum significance value obtained was P = 0.1.

Results

Characterization of UCB-MSCs

Ten UCB units obtained at full-term delivery were selected according to the 'quality' criteria described in the Design and Methods section. MNCs were separated, plated as P0 and cultured in medium supplemented with 5% PL. The cultures at P0 were monitored for up to four weeks to allow identification of MSC clones in the flasks. Two out of the ten UCB units (20%; UCB3 and UCB6) gave rise to three and four MSC-like clones after 15- and 14-day culture, respectively. In the remaining 8 UCB units, we did not obtain MSCs, despite the long observation time in culture. MSC clones from UCB3 (UCB3-MSCs) and UCB6 (UCB6-MSCs) were expanded *ex vivo* and characterized for their morphology, differentiation potential, immunophenotype, proliferative capacity, biosafety profile and immunoregulatory properties.

UCB-MSCs displayed the typical spindle-shaped morphology, similar to that of BM-MSCs expanded in the same culture medium (see Supplementary data, Figure 1s A).[27] As already observed for BM-MSCs expanded in the presence of PL,[27] UCB-MSCs required only 2-3 minutes incubation with trypsin at room temperature to obtain the complete

detachment of cells from the flasks, whereas 5–8 min at 37°C are usually necessary to harvest MSCs grown in the presence of 10% FCS.

UCB-MSCs were induced to differentiate into osteoblasts and adipocytes and examined for this capacity by histological staining. The cells were able to differentiate into osteoblasts, as demonstrated by the histological detection of AP activity (purple reaction) and calcium deposition stained with Alzarin Red (Figure 1A), and into adipocytes, as revealed by the formation of lipid droplets, stained with Oil Red O (Figure 1B).

The surface phenotype of UCB-MSCs was analyzed by flow cytometry every two passages (at P1, P3, P5 and so on) and showed the typical panel of MSC markers, in agreement with previous reports.[2,3,20-24,27] In particular, by the second passage, contamination with hematopoietic cells was no longer detectable and more than 98% UCB-MSCs were positive for CD90, CD73, CD105 and CD13 surface antigens and negative for CD34, CD45, CD14, CD80, CD31 molecules. The expression of HLA-DR was always less than 2%, whereas HLA-class I was uniformly present on UCB-MSCs (more than 98% of positive cells). See Supplementary data, Figure 1s B.

Calculated cumulative cell counts from P0 to P5 for UCB3- and UCB6-MSCs, together with counts for BM-MSCs cultured in the presence of 5% PL,[27] for comparison are shown in Figure 1C. UCB-MSCs yielded similar numbers at P5 (1.62×10^9 MSCs for UCB3 and 2.02×10^9 MSCs for UCB6), as compared with BM-MSCs ($3.2 \pm 1.02 \times 10^9$ MSCs as mean ± SD of 8 BM donors; $P>0.1$),[27] even when starting with low cell numbers. In fact, the number of UCB-MSCs collected after trypsinization at P0 was 0.2×10^6 for both units, whereas the mean of MSCs from eight BM donors at the same passage was $2.6 \pm 0.58 \times 10^6$ cells ($P<0.1$). UCB-MSC growth was also evaluated in terms of PD; cumulative PD from P1 to P5 were as follows: 12.9 for UCB3-MSCs, 13.3 for UCB6-MSCs, 10.9 for BM-MSCs (mean of 8 BM donors). Moreover, because of the extremely low

frequency of clones in case of UCB-MSCs, a comparison of the colony-forming unit-fibroblast (CFU-F) assay with BM-MSCs could not be performed. The median time to reach 80% confluence for all passages from P1 to P5 was 6.5 days for both UCB3- and UCB6-MSCs, as compared with 5.5 days in case of BM-MSCs expanded in the presence of 5% PL.[27] Taken together, these results suggest that UCB-MSCs, although displaying a rather low clonogenic efficiency, possess high proliferative potential.

UCB-MSCs were cultured continuously *in vitro* until reaching senescence and monitored daily for up to eight weeks, in order to investigate their propensity to undergo spontaneous transformation *in vitro*. UCB3-MSCs and UCB6-MSCs displayed a progressive decrease in their proliferative capacity until they reached a senescence phase after 73- and 81-days culture at P9 and P10, respectively. The cells maintained their typical spindle-shaped morphology, differentiation capacity and surface markers throughout the culture period.

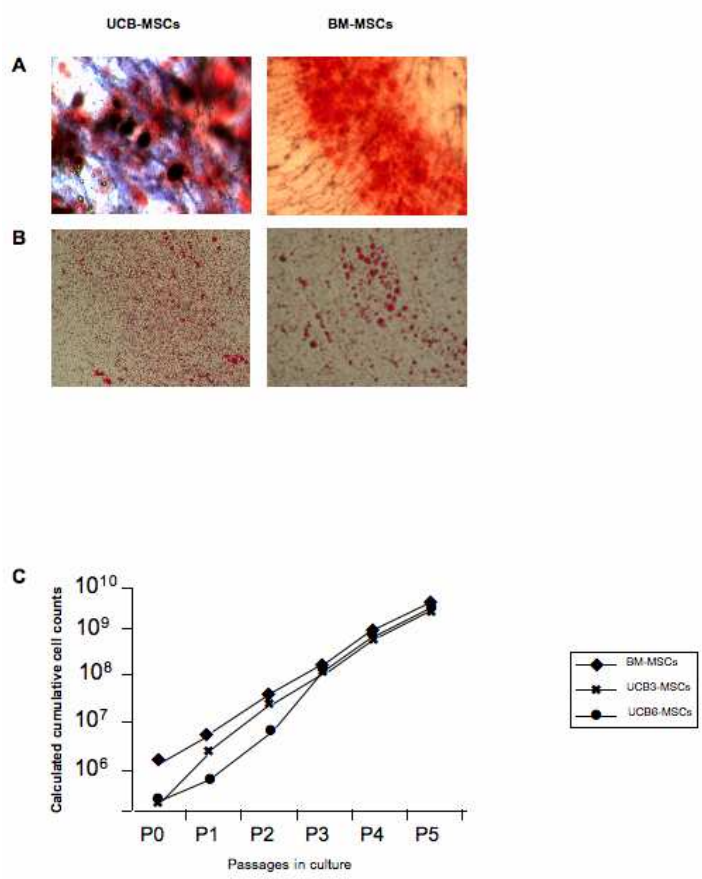


Figure 1. A) Osteogenic differention capacity of UCB-MSCs, as compared with BM-MSCs (right panel). Shown are representative photographs from UCB3-MSCs at P3 and BM-MSCs from donor 2.[27] Magnification x 20. B) Adipogenic differentiaton capacity of UCB-MSCs, as compared with BM-MSCs (right panel). Shown are representative photos from UCB3-MSCs at P3 and BM-MSCs from donor 2.[27] Magnification x 20. C) Calculated cumulative cell counts from P0 to P5 of UCB3- and UCB6-MSCs, as compared with BM-MSCs cultured in the presence of 5% PL-additioned medium (mean of 8 BM-donors) and already reported.[27]

Biosafety profile of UCB-MSCs - Lack of telomerase expression in UCB-MSCs during long-term in vitro culture

MSC cultures from both UCB3 and UCB6 were tested at two different *in vitro* passages (P4 and P8) for the expression of telomerase catalytic activity by the TRAP assay. TRAP results failed to evidence the presence

of enzyme catalytic activity in all tested samples (Figure 2A). To gain insights into the molecular mechanisms responsible for the repression of telomerase activity in UCB-MSCs, we assessed the expression of the hTERT gene, which codes for the catalytic component of human telomerase,[38] in the same cultures screened for telomerase activity. RT-PCR failed to evidence the expression of hTERT mRNA (see Supplementary data, Figure 2s), thus indicating that the absence of telomerase activity in cultured UCB-MSCs was ascribable to lack of hTERT gene transcription.

MSC cultures from both UCB3 and UCB6 were found to express $p16^{ink4a}$ protein, as detected by Western immunoblotting, at all tested *in vitro* passages (Figure 2B). In addition, UCB3-MSCs (P3 and P6) and UCB6-MSCs (P4 and P11) did not show anchorage-independent cell growth, since they failed to generate colonies when plated in double-layer agarose (data not shown).

UCB-MSCs were also tested for their genomic assets; in particular UCB3- and UCB6-MSCs were investigated at early passages (P3) and at later passages in culture (P8 and P9, respectively) by means of array-CGH (see Figure 2C for UCB6-MSCs at P3 and P9). The results of array-CGH experiments revealed that UCB-MSCs expanded *in vitro* do not show unbalanced chromosomal rearrangements; in fact, deletions or duplications of genomic material, excluding Copy Number Variations (CNVs) constitutionally present, were absent in the UCB-MSCs studied.

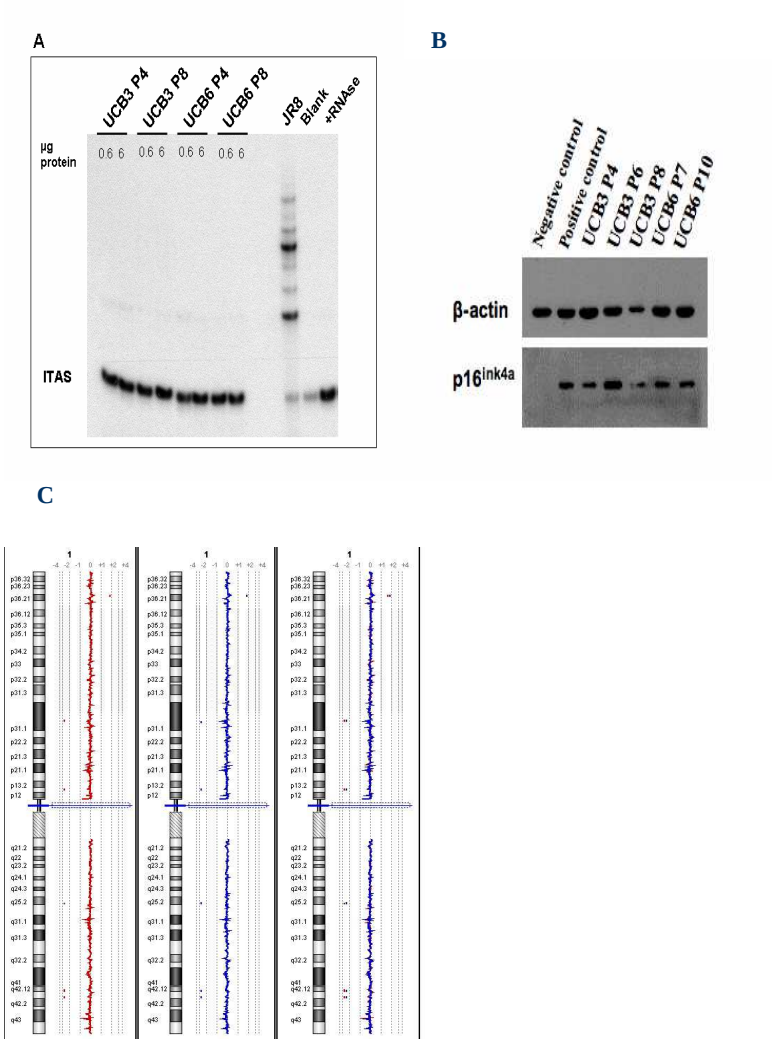
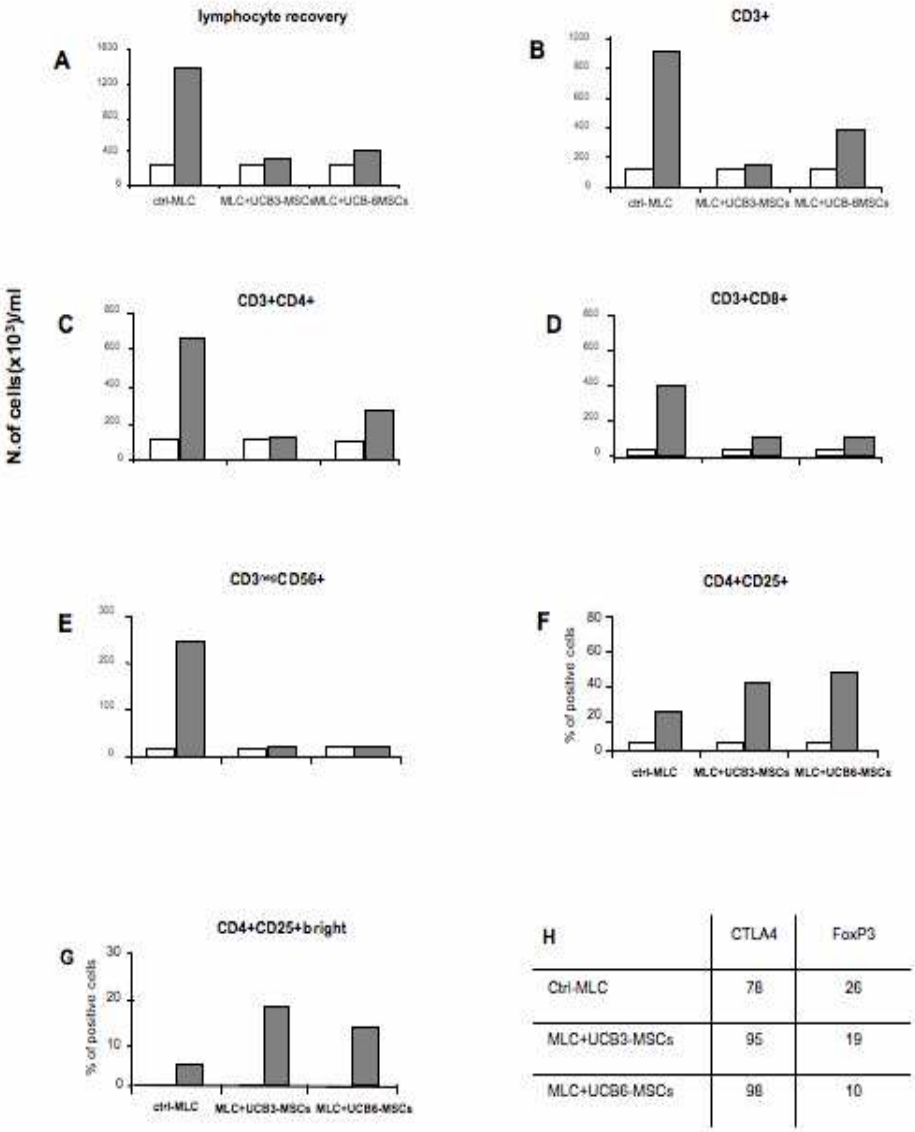


Figure 2. (A) Telomerase activity of UCB3- and UCB6-MSC cultures at P4 and P8. Telomerase activity was detected by the TRAP assay using two protein concentrations. The telomerase-positive cell line JR8 was used as a positive control. The blank represents a negative control to which no protein extract was added. The lane labelled +RNAse represents an additional negative control containing 0.6 µg cell extract of JR8 pretreated with RNAse. The location of the internal amplification standard (ITAS) is reported. (B) $P16^{ink4a}$ expression in UCB3- and UCB6-MSC cultures at different *in vitro* passages as evaluated by Western immunoblotting. The $p16^{ink4a}$-positive DU145 cell line and the $p16^{ink4a}$-negative U2OS cell line were used as positive and negative controls, respectively. (C) Representative array-CGH profiles of chromosomes 1 of UCB6-MSCs at P3 (left, red profile) and P9 (middle, blue profile). The array-CGH profiles are linear and perfectly overlappeing (right), thus demonstrating that *in vitro* expanded UCB- MSCs do not show unbalanced chromosomal rearrangements.

Immune regulatory properties of UCB-MSCs

In a first set of experiments, the immune regulatory capacity of UCB-MSCs was evaluated by assessing UCB-MSC interaction with alloantigen-specific immune response, elicited *in vitro* in primary MLC. In agreement with previously reported studies,[4] we observed that UCB-MSCs were able to strongly inhibit alloantigen-induced lymphocyte proliferation (Figure 3A). A strong inhibitory effect was evident on whole T lymphocytes and their subsets ($CD3^+$, Figure 3B; $CD3^+CD4^+$, Figure 3C; $CD3^+CD8^+$, Figure 3D), as well as on NK lymphocytes ($CD3^{neg}CD56^+$, Figure 3E). The percentage of $CD4^+CD25^+$ T cells considerably increased, as compared with day 0, after 10-day primary MLC, both in the presence and absence of UCB-MSCs, even though the percentage of this subset was higher in UCB-MSC/MLC as compared with ctrl-MLC (Figure 3F). In an attempt to discriminate $CD4^+CD25^+$ Tregs from conventional, recently activated $CD4^+CD25^+$ T lymphocytes, the degree of expression of CD25 ($CD4^+CD25^{bright}$ T cells, Figure 3G), as well as of CTLA4 and FoxP3 molecules was evaluated within the $CD4^+CD25^+$T cell subset (Figure 3H). We found a higher percentage of $CD4^+CD25^{bright}$ and $CTLA4^+$ cells in UCB-MSC/MLCs as compared with ctrl-MLCs, while the percentage of FoxP3+ was lower in the presence than in the absence of UCB-MSCs (Figure 3H).



H	CTLA4	FoxP3
Ctrl-MLC	78	26
MLC+UCB3-MSCs	95	19
MLC+UCB6-MSCs	98	10

Figure 3. Immune modulatory effect of UCB-MSCs on the expansion of T and NK-lymphocyte subsets, induced by allogeneic stimulus. Recovery of total number of lymphocytes (A), $CD3^+$ (B), $CD3^+CD4^+$ (C), $CD3^+CD8^+$ (D), $CD3^{neg}CD56+$ NK cells (E), $CD4^+CD25^+$ (F), $CD4^+CD25^{bright}$ (G) T-lymphocytes subsets and with respect to the initial number (white columns), was assessed after 10-days primary culture (gray columns). Percentages of $CTLA4^+$ and $Foxp3^+$ cells were calculated on gated $CD4^+CD25^+$ T cells (H). MLC was performed in the absence (Ctrl-MLC) or presence of third-party MSCs derived from UCB3 (MLC+UCB3-MSCs) or UCB6 (MLC+UCB6-MSCs). Results are expressed as the number of cells/ml in panels A-E and as percent of positive cells in panels F-H.

The cytokine production kinetics induced *in vitro* by allogeneic stimulus documented that addition of UCB-MSCs: (*i*) inhibits IFNγ secretion; (*ii*) strongly increases IL-6 secretion in MLC supernatants (see Supplementary data, Table 1s). Differently from what shown in a previous study where the addition of BM-MSCs grown in 5%PL to the MLC substantially increased IL-10 secretion,[27] UCB-MSCs only slightly enhanced IL-10 secretion in 24-hour MLC supernatants; (*iii*) IL-2, IL-7, IL-12, IL-15 were undetectable in all experimental conditions.

In order to assess the effect of UCB-MSCs on alloantigen-induced cytotoxic activity, effector cells recovered after 10-day MLC were tested in a cytotoxicity assay, employing as targets either MLC-stimulator PHA–blasts (Figure 4A) or third-party UCB-MSCs from the same lots added to the MLC at day 0 (Figure 4B, Figure 4C). Results obtained in all experiments showed a striking inhibitory effect mediated by both lots of UCB-MSCs (UCB3- and UCB6-MSCs) on alloantigen-induced cytotoxic activity.

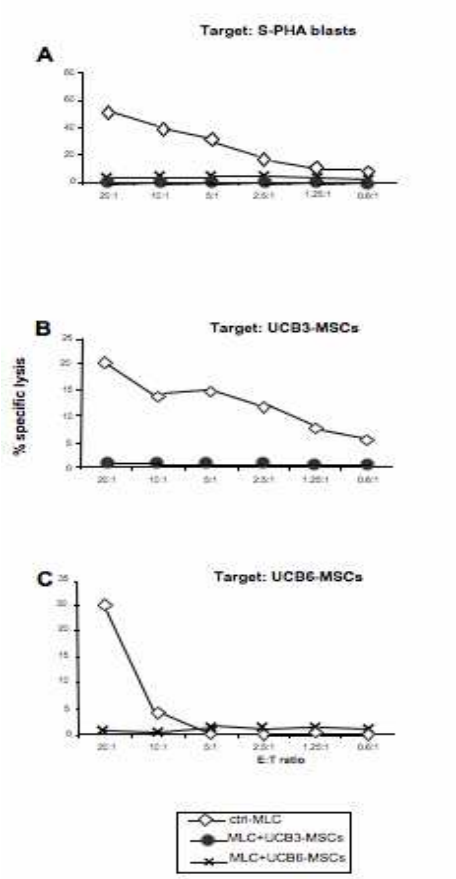


Figure 4. Immune modulatory effect of third-party UCB-MSCs on cell-mediated cytotoxic activity induced by an allogeneic stimulus (MLC). ^{51}Cr-labeled target cells included PHA-activated S-PBMCs (S-PHA) (A) and the same UCB3-MSCs (B) and UCB6-MSCs (C) added to MLCs. Effector to target (E:T) ratios ranged between 20:1 and 0.6:1. Results are expressed as percent specific lysis of target cells.

With the aim of better understanding the biological mechanisms responsible for the immunosuppressive effect exerted by UCB-MSCs on alloantigen-induced immune response, in a second set of experiments, MLCs were carried out in the presence of IDO- or PGE2-specific inhibitors. As control, the same experimental conditions were also tested either in the presence or in the absence of BM-MSCs cultured in PL-

supplemented medium.[27] Moreover, the constitutive expression of intracellular, membrane and soluble HLA-G (iHLA-G, mHLA-G, sHLA-G, respectively) was evaluated in UCB-MSCs, as well as in BM-MSCs harvested at P3.

In terms of alloantigen-induced lymphocyte proliferation, neither IDO-specific inhibitor, nor PGE2-specific inhibitor were able to reverse the MSC-induced suppressive effect (data not shown). By contrast, when alloantigen-induced cytotoxic activity was evaluated, a clear-cut effect of the PGE2-specific inhibitor was observed; indeed, addition of PGE2-specific inhibitor to MLC was able to reverse the suppressive effect exerted by both UCB-MSCs and BM-MSCs on alloantigen-specific cytotoxic activity, even though this reagent was apparently more effective when BM-MSCs were employed (see Figure 5). On the contrary, IDO-specific inhibitor was not able to reverse the suppressive effect exerted by both UCB-MSCs and BM-MSCs on alloantigen-specific cytotoxic activity (Figure 5).

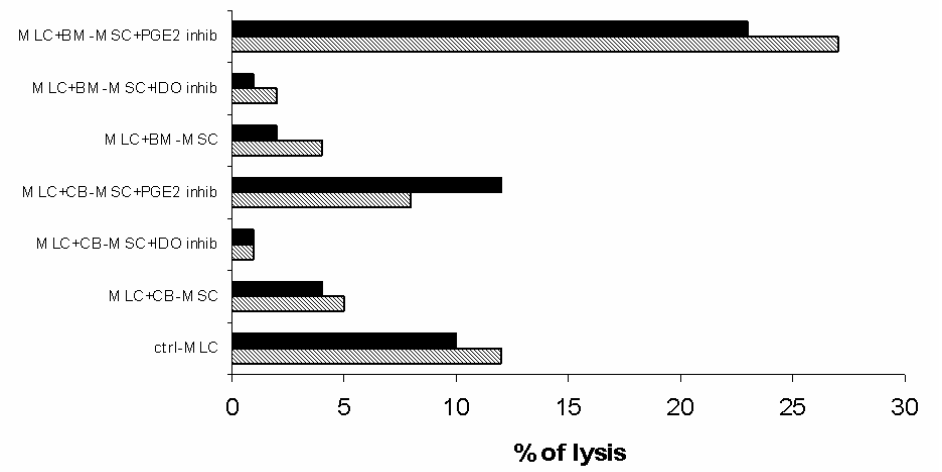


Figure 5. Involvement of IDO and PGE2 in UCB- and BM-MSC mediated suppression of cell-mediated cytotoxic activity induced by an allogeneic stimulus (MLC). ^{51}Cr-labeled target cells were PHA-activated S-PBMCs (S-PHA). Effector to target (E:T) ratio ranged between 10:1 and 1:1; results are expressed as %

specific lysis of target cells. BM-derived MSCs (BM1-MSCs and BM2-MSCs) expanded in PL-supplemented medium were employed as a control.[27] IDO or PGE inhibitors (inhib) were added to the MLC as described in the "Design and Methods" section. Results obtained at the E:T of 1:1 for Exp. 1 (broken bar) and 10:1 for Exp. 2 (full bar) are shown, UCB3-MSCs and BM1-MSCs were employed in Exp. 1 whereas UCB6-MSCs and BM2-MSCs were tested in Exp 2.

As shown in Table 1, PGE2 concentrations were considerably higher in supernatants of MLCs carried out in the presence of BM-MSCs than in the presence of UCB-MSCs. These data might explain the striking effect of a PGE2-specific inhibitor observed in MLCs performed using BM-MSCs (Figure 5). The presence of PGE2-specific inhibitor considerably decreased PGE2 secretion in MLC experiments carried out with either BM-MSCs or UCB-MSCs (Table 1). Interestingly, a striking increase in PGE2 secretion was observed in the presence of the IDO-specific inhibitor (Table 1).

This observation may explain why IDO-specific inhibitor was unable to reverse the suppressive effect exerted *in vitro* by MSCs on alloantigen-specific cytotoxic activity (see Figure 5). In fact, the striking increase in PGE2 secretion, induced by the presence of IDO-specific inhibitor, could exert an effective suppressor function on alloantigen-specific cytotoxic activity, thus masking the effect of the IDO-specific inhibitor on IDO-mediated suppressive activity.

	Exp 1			Exp 2		
	PGE2	***Tryp***	***kyn***	***PGE2***	***tryp***	***Kyn***
Control-MLC	117	232±15	≤0.01	45	193±11	≤0.01
MLC+BM-MSCs	1440	164±10	1.54±0.4	700	95±4	1.6±0.21
MLC+BM-MSCs+IDO inhib	7500	215±15	≤0.01	1500	220±20	≤0.01
MLC+BM-MSCs+PGE2 inhib	234	*NA	≤0.01	35	*NA	≤0.01
MLC+UCB-MSCs	180	54±1	4.53±0.1	60	184±12	3.06±0.76
MLC+UCB-MSCs+IDO inhib	270	200±14	≤0.01	156	240±21	≤0.01
MLC+UCB-MSCs+PGE2 inhib	60	*NA	≤0.01	20	*NA	≤0.01

Table 1. Concentration of PGE2, Tryptophan (tryp) and Kynurenine (kyn) in MLC-supernatants Concentrations of PGE2, tryptophan and kynurenine were quantified in MLC-supernatants collected after 72-hours culture in the absence (ctrl-MLC) or presence of BM-MSCs (MLC+BM-MSCs) or UCB-MSCs (MLC+UCB-MSCs) and in the absence or presence of IDO inhibitor (MLC+MSCs+IDO inhib) or PGE2 inhibitor (MLC+MSCs+PGE2 inhib). PGE2 levels are reported as pg/ml. Tryptophan and kynurenine levels are reported in µM as mean ±SD of three repeated runs for the same samples. Two independent experiments are presented, in which UCB3-MSCs and BM1-MSCs from donor 2^{27} (Exp 1) and UCB6 MSCs and BM2-MSCs from donor 5^{27} (Exp 2) were tested.*NA = not assessable. The tryptophan value could not be evaluated in the experimental condition as its fluorescence signal was superimposed by the fluorescence signal of the PGE2 inhibitor.

IDO activity was evaluated in MLCs supernatants, as indirect evidence of IDO-mediated tryp degradation. Results reported in Table 1 demonstrate the presence of detectable levels of kyn only in culture supernatants recovered from MLCs carried-out in the presence of either UCB- or BM-MSCs. This finding indicates that IDO-activity is dependent on the interaction between MSCs and cells active in MLC. Kyn was more abundant in the presence of UCB-MSCs, as compared with BM-MSCs. As expected, kyn was undetectable in culture supernatants collected from MSC/MLCs carried out in the presence of IDO-specific inhibitor; however, kyn was also undetectable in culture supernatants collected from MSC/MLCs carried-out in the presence of PGE2-inibitor. The latter observation is in accordance with recently published data demonstrating that PGE2 is able to up-regulate IDO activity in dendritic cells.[39,40] The inter-relationship among PGE2 secretion and IDO activation could also hypothetically explain why, even though PGE2 levels detected in MLC+BM-MSC+PGE2 inhibitor culture condition of Experiment 1 is comparable to that detected in Ctrl-MLC (see Table 1), alloantigen–induced cytotoxic activity is strikingly higher in the former than in the latter culture condition (see Figure 5).

Evaluation of constitutive HLA-G-expression in MSCs showed that UCB-MSCs displayed a higher percentage of mHLA-G^+ cells as compared with BM-MSCs, while more than 95% of both UCB-MSCs and BM-MSCs were iHLA-G^+. UCB-MSCs and BM-MSCs secreted similar amount of sHLA-G in culture supernatants (see Supplementary data, Table 2s).

Discussion

In this study, we have demonstrated that MSCs generated from full-term UCB in a culture medium containing 5% PL are similar to BM-MSCs in terms of morphology, differentiation potential, immunophenotype and

proliferative ability. On the contrary, they differ for clonogenic efficiency. In spite of their high proliferative potential, UCB-MSCs do not show any signs of *in vitro* transformation. Moreover, in view of our findings, although obtained in a limited number of UCB-MSC samples, UCB-MSCs are apparently as effficient as BM-MSCs when tested in terms of capacity of suppressing an alloantigen-specific immune response (see Figure 3s and Figure 4s).

Bieback *et al.* have demonstrated that MSCs can be isolated from full-term UCB with a success rate greater than 60%, when critical parameters for the selection of 'good quality' units are employed.[22] These critical parameters included: a storage time shorter than 15 hours, a net UCB volume greater than 33 ml; a MNC count greater than 1 x 10^8 and absence of clots or signs of hemolysis. The quality criteria adopted in our study were less stringent in terms of storage time and UCB cellularity, but included a viability test on the manipulated cells. Despite this, the 5% PL-supplemented culture medium, which we had documented to be superior to 10% FCS in terms of clonogenic efficiency and proliferative capacity when employed for BM-MSCs,[27] did not provide higher efficiency of isolation of UCB-MSCs, as compared to a FCS-based medium.[22] In fact, only 20% of UCB units we tested gave rise to MSCs, whereas in the remaining UCB units MSCs could not be obtained, despite the extended culture time. We cannot exclude that either the relatively long storage time of the UCB units employed or their inferior cellularity might have influenced our inferior isolation rate, despite the use of a PL-supplemented medium.

Once obtained, UCB-MSCs expanded in the presence of 5% PL display the typical MSC morphology, immune phenotype and differentiation capacity (see Figure 1s and Figure 1A and 1B). UCB-MSCs possess high proliferative potential, yielding numbers comparable to BM-MSCs cultured

with the same medium[27] at P5, although starting from lower cell counts at P0 (Figure 1C).

Given the high proliferative capacity and the published reports on *in vitro* transformation of MSCs,[41,42] we monitored UCB-MSCs during their whole culture period and, particularly, during their senescence phase, which occurred after 9 and 10 passages for UCB3- and UCB6-MSCs, respectively. Neither phenotypical, nor functional alterations of the cells were observed; the favorable bio-safety profile of UCB-MSCs was further demonstrated by the absence of telomerase activity and hTERT expression, the expression of $p16^{ink4a}$ protein, the absence of anchorage-independent cell growth and by a normal molecular karyotype as proved by array-CGH analysis (Figure 2).

These data suggest that UCB-MSCs, expanded in our culture system in the presence of 5% PL, do not display a tendency for spontaneous transformation, in accordance with data published evaluating BM-MSCs cultured with both PL- and FCS-supplemented media.[27,33] This is, to our knowledge, the first report to include a thorough investigation of the genetic stability of MSCs derived from UCB. This type of evaluation, also in view of the high proliferative potential of UCB-MSCs, is mandatory, in our opinion, for any clinical application of these cells.

When tested for their capacity to influence the alloantigen-specific immune response, in comparison to BM-MSCs grown in 5% PL (see also Figure 3s and 4s for details on BM-MSCs),[27] UCB-MSCs have similar suppressive effect on T- and NK-lymphocyte subset proliferation and on alloantigen-induced cytotoxic activity, while only slightly increase IL-10 in MLC supernatants. The results obtained in our study suggest that UCB-MSCs are able to exert an immunosuppressive effect on alloantigen-specific immune response by means of several mechanisms, including IDO activation and production of kyn, PGE2 secretion and HLA-G expression.[4,11,36,43-49] All

these biological mechanisms have been previously described to be active in MSCs derived from other sources, such as BM-MSCs.[4,11,35,43-49] In particular, it is noteworthy that our data confirm and extend previously reported results, underlying the inter-relationship among PGE2 secretion and IDO activation, two well-known mechanisms involved in anti-inflammatory immune response.[39,40] Indeed, the presence of PGE2-specific inhibitor, besides reducing PGE2 levels in culture supernatants, was also able to inhibit IDO activity. Moreover, in the presence of IDO-specific inhibitor, we observed a striking increase in PGE2 secretion.

A distinctive feature of UCB-MSCs seems to be the constitutive surface expression of HLA-G on the majority of cells, while it has been reported that BM-MSCs mainly express only the soluble isoform of HLA-G.[45] However, it is worth considering that MEM-G/9 monoclonal antibody, which is specific for both membrane-bound (HLA-G1) and soluble (HLA-G5) HLA-G isoforms was employed to evaluate HLA-G expression in flow cytometry. Therefore, we were unable to formally prove that soluble HLA-G5 isoform is the only one expressed by UCB-MSCs, as documented by Selmani et al.[45] for BM-MSCs. Further experiments are warranted to clarify this point.

Membrane HLA-G expression, as well as sHLA-G isoforms have been demonstrated to exert a strong suppressive effect on proliferation and activation of effector functions of both T and NK lymphocytes. For instance, it is well known that HLA-G expression at the feto-maternal interface is one of the most potent mechanisms protecting the fetus from maternal immune attack.[48] Moreover, surface HLA-G expression is one of the systems employed by tumor cells to evade the cytotoxic activity of both tumor-specific T lymphocytes and NK cells, and it has been recently suggested that transfer of membrane patches containing HLA-G molecules from mHLA-G^{pos} cells to activated T and NK lymphocytes (“trogocytosis”)

might be a mechanism of immune suppression protecting HLA-G^{neg} tumor cells.[49] It may, thus, be speculated that UCB-MSCs expressing mHLA-G may be more protected than BM-MSCs from attack mediated by the host immune system. However, it has been recently demonstrated in a murine model that while local implantation of MSCs results in ectopic bone formation in syngeneic recipients, it leads to transplant rejection in allogeneic mice.[50] This is in line with previously published data that MSCs can be lysed by cytotoxic T-lymphocytes, when infused into MHC-mismatched mice, resulting in their rejection.[51] These observations supports the use of MSCs, in hard tissue repair strategies, preferably in an autologous or tolerant host.[50] Further studies specifically addressing this issue are underway.

In conclusion, while the ability of BM-MNCs to generate MSCs reaches 100% under appropriate culture conditions, the success rate of isolating MSCs from UCB ranges, according to different reports, from 20 to 63%.[21-24,52-54] In particular, Reinisch *et al.* have recently shown that MSCs can be obtained from full-term UCB in the presence of human PL, yielding cell numbers suitable for clinical application. The same authors report an isolation efficiency of 46%, considering both FCS-expanded and PL-expanded MSCs. However, their PL preparation procedure, percentage of PL employed in the culture medium (10%), as well as MSC plating density, differ from our approach. These differences might explain the different results obtained, in particular in terms of isolation efficiency, as compared with our data. Despite this, also in our experience, UCB-MSCs display a high proliferative capacity, which allow the expansion of sufficient cell numbers for clinical application, in a reasonable time-frame.

Given their high proliferative capacity, immunosuppressive properties and potential for avoiding attack by immune cells, UCB-MSCs, also in view of their easy collection, could be considered to be used in clinical practice for

prevention and treatment of alloreactive-related immune complications, namely severe GvHD and graft rejection, following HSCT. However, as note of caution, recent data, obtained in a xenogenic model of NOD/SCID mice, showed that human UCB-MSCs, when administered in multiple doses, are effective in the prevention, but not in the treatment of GvHD.[55] This discrepancy with the clinical efficacy displayed by MSCs on acute GvHD[5] in human HSCT might be explained by the animal model employed and also by the unfavorable ratio between the number of UCB-MSCs and the huge number of effector cells mediating the tissue damage at time of acute GVHD onset.

UCB-MSCs could also serve as a tool in strategies of reparative/regenerative medicine, where the combination of the immunosuppressive and tissue repair properties could ameliorate the management of autoimmune and chronic inflammatory diseases.[9-11]

Our results, although obtained in a limited number of MSC samples tested, suggest that the differences between BM- and UCB-derived MSCs and between cells expanded in the presence of PL and FCS may be relevant for the clinical application of MSCs.

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CHAPTER 9

GENERAL DISCUSSION

9.1 Introduction

To completely exploit the potential of MSCs as new treatment modality, more *in vitro* and *in vivo* work is required. These studies will aim to increase our knowledge on how MSCs mediate their suppressive effect and reduce inflammatory responses.

Once more precisely defined, the *in vivo* biological activities of MSCs could be applied in novel therapeutic strategies to stimulate tissue repair, and to modulate immune response against allo- and autoantigens.

9.2 Characterization of MSCs

9.2.1 The lack of markers and functional assays

In most laboratories, MSC isolation still relies on their adherence to plastic, resulting in a heterogeneous population of cells, referred to as MSCs. Immunephenotyping by flow cytometry is applied to characterize *ex vivo* expanded MSCs and to define their purity. However, at present, no specific marker or combination of markers has been identified that specifically defines true MSCs and *ex vivo* expanded cells are currently stained with a number of positive markers (CD105, CD73, CD90, CD166, CD44, CD29) in combination with negative markers (CD14, CD31, CD34, CD45). Although recently many research groups have reported the identification of new MSC markers,[1-4] none of the available has demonstrated to be singularly capable to identify the true mesenchymal progenitors. Different cell subsets might be responsible for specific functions and might be characterized by different cell surface markers. Therefore, research should include the identification of MSC-specific markers. This will allow to dissect the developmental hierarchy of MSCs and will facilitate the generation of homogenous cell populations.

Novel techniques, such as proteomic approaches, might be useful to define new MSC surface antigens that can be used to identify substes. To this aim, in

collaboration with Fondazione Istiuto Nazionale di Genetica Molecolare INGM, Milano and PRIMM s.r.l. Milano, we have designed an approach to identify cell subsets based on the use of a library of polyclonal antisera specific for human membrane proteins with unknown function. We have selected, cloned and expressed in bacteria the open reading frames coding for all proteins that are predicted to be either transmembrane or secreted. With the recombinant proteins we have generated a library of 1,700 mouse antisera that, in principle, can be used to discover new subset defining proteins on every cell population of interest. We are currently screening the antisera library by flow cytometry on *ex-vivo* expanded BM-MSCs, with the aim of possibly identifying and functionally characterize new proteins expressed by subsets of MSCs.

Microarray analysis is another useful instrument to characterize MSCs. Microarray analyses have being employed to compare gene expression profiles of MSCs and fibroblasts[5,6] or MSCs and differentiated cells,[7,8] but no definitive conclusions have been drawn. MSCs derived from different donors using the same culture conditions might yield consistent and reproducible gene expression profiles, whereas several genes might be differentially expressed in MSCs derived from different sources or culture conditions.[9] In order to develop novel markers able to identify MSC subsets with specific functions, microarray analysis should focus on: i) comparison of gene expression profiles in different MSC populations (*i.e.* MSCs from different tissue sources, cultured in different conditions, at different *in vitro* passages; ii) changes induced in the course of *in vitro* differentiation.

9.2.2 The effect of tissue source

Altough similar MSCs can be cultured from various fetal and adult tissues,[10-14] clinical experience has been mainly gained with *ex vivo* expanded BM-derived

cells; only few studies have employed different sources, such as adipose tissue.[15] The frequency of mesenchymal progenitors, the proliferative capacities and differentiation potential, as well as phenotypical and immunomodulatory properties have been shown to vary in different sources.[11,12,16] Whether one source might be more useful in a defined clinical setting needs to be investigated.

Recently, Sacchetti *et al.* have shown that stromal progenitors expressing MCAM/CD146 in human BM are capable of transferring, the hematopoietic microenvironment to heterotopic sites, giving rise to identical bone and stroma.[17] These authors believe that the functional properties of 'MSC-like' cells isolated from tissues other than BM and expressing CD146+CD34-CD45- phenotype, are different and not linked with the ability to establish the hematopoietic microenvironment *in vivo* (*Bianco P., personal communication*).

To gain more insights into tissue-dependent functional differences, we have compared the chondrogenic differentiation potential of culture-expanded MSCs derived from fetal and adult tissues (chapter 2). We demonstrated that fetal BM- and adult BM-derived MSCs exhibit a superior capacity to differentiate into chondrocytes, than fetal lung and placenta-derived MSCs. We speculate that the cell source, rather than their fetal origin, could account for this higher differentiation capacity, since no differences could be found in chondrogenic potential between fetal and adult BM-MSCs. Intrinsic diversities of MSCs residing in the tissue, as well as their physiological role in a specific tissue, might explain the properties of a specific tissue source, as compared to other sources. Alternatively, the frequency of cells with lineage-specific differentiation capacity may differ between tissue sources and therefore chondrogenic MSCs might be present in higher frequency in BM rather than in fetal lung or placenta. Also the culture conditions employed for

the expansion of the cells might influence their differentiation ability, leading to the committment of the cells towards a specific lineage.

In chapter 8 we focused on UCB as a potential novel source of MSCs for clinical application. To this aim, the biological and functional properties of *ex vivo* expanded UCB-derived MSCs were investigated, in comparison with BM-MSCs. We found that differences exist *in vitro* in terms of clonogenic efficiency, proliferative capacity and immunomodulatory properties between UCB- and BM-MSCs. These differences should be taken into account when considering the clinical application of MSCs in the various clinical settings. For example, given their high proliferative capacity, immunosuppressive properties and potential for avoiding attack of immune cells, UCB-MSCs might be employed in the clinics for the prevention and treatment of alloreactive-related immune responses after HSCT, namely severe GvHD and graft rejection. UCB-MSCs might also be useful in regenerative medicine, where the combination of immunosuppressive and tissue repair properties could ameliorate symptoms of autoimmune and chronic inflammatory diseases. These *in vitro* findings need to be confirmed in the clinical setting; altough UCB-MSCs might be as suppressive as BM-MSCs upon interaction with alloantigen-specific immune response *in vitro*, this might not happen *in vivo*. Moreover, while it is possible to isolate MSCs from BM with a success rate of 100%, the isolation efficiency of MSCs from UCB varies from 20 (in our hands) to 63%.[10,18-21] This could represent a limitation for the clinical application of UCB-MSCs, despite the fact that this source offers the advantage of easy collection.

9.3 In vivo use of MSCs in experimental animal models

9.3.1 MSC homing and survival

Data on the fate of transplanted MSCs *in vivo* are scarce. Whether they home to specific sites and engraft or they dye soon after releasing the mediators

responsible for their effect is still largely unclear. It might be possible that therapeutic benefit is obtained by local paracrine growth factors produced by the cells and/or by the local microenvironment and that survival it is not necessary for their clinical effect. In rats, radiolabeling experiments showed localization of MSCs after intraarterial and intravenous infusion mostly in the lungs and secondarily in the liver and other organs.[22] Studies in baboons using a green fluorescent retroviral construct suggest engraftment in the gastro-intestinal tract and in various tissues in the range of 0.1-2.7 %, with comparable results for autologous and allogeneic cells.[23] Other authors have shown that active homing of MSCs to BM depends on stromal-derived factor-1 (SDF-1) which interacts with CXCR4 on the MSC surface.[24] Similar mechanisms have been shown for migration to pancreatic islets[25] and ischemic tissues.[26] MSC mobilization and homing might depend on cytokines, chemokines and growth factors released during systemic and/or local inflammatory conditions and might be mediated by the interaction with integrins and selectins expressed on the surface of MSCs. Homing of MSCs to inflamed and ischemic tissues would increase the feasibility of cellular therapy in the setting of autoimmune diseases (AID) and tissue repair.

A possible strategy to facilitate homing of MSCs, involves the modification of surface structures that play a role in migration to specific tissues, as suggested by Sackstein *at al*.[27] These authors converted the native CD44 glycoform expressed on MSCs into E-selectin/L-selectin ligand (HCELL) (expressed on hematopoietic cells) using fucosyltransferase. Intravital microscopy in NOD/SCID mice showed BM infiltration by HCELL(+) MSCs within several hours after intravenous infusion.

In vivo labelling of MSCs will allow to investigate *in vivo* 'trafficking' and biodistribution of MSCs both in animal models and in humans. Supermagnetic iron-oxide nanoparticles can be employed to label MSCs and to trace them *in vivo* by magnetic resonance (MR) imaging, hopefully without

interfering with MSC biological functions and without inducing toxic effects in the recipients.[28,29]

One potential limitation of this technique is ingestion of iron particles by macrophages that prevents specific labelling of MSCs. Further efforts should focus on the development of new tracers; the acquisition of information on survival of MSCs *in vivo*, on their ability to engraft in host tissues and on the mechanisms that regulate their interaction with damaged tissues.

9.3.2 Disease models in experimental animals

The implementation of experimental disease models is essential for a better understanding of MSC biology and for producing pre-clinical data that could be useful for therapeutic in humans. Several animal models of tissue protection and autoimmunity have been recently developed and tested. Similar results have been obtained by the infusion of MSCs in two different murine models of acute lung injury and hepatic fibrosis.[30,31] In both models a protective effect of MSCs was noted despite limited engraftment in the target organs. In a rat model, MSC-derived conditioned medium proved effective in reversing fulminant hepatic failure.[32] In addition, MSCs displayed tissue-protective effects in animal models of kidney, retinal and central nervous system injury.[33-36] These effects do not seem to be mediated by MSC transdifferentiation; bystander mechanisms including inhibition of pro-inflammatory cytokines and anti-apoptotic effects on target cells seem to be involved.

9.4 In vivo use of MSCs in patients

The role of MSCs in the clinical setting has been exploited mainly in allogeneic stem cell transplantation, where MSCs have been infused either to facilitate engraftment (chapter 5) or to treat steroid-resistant acute GvHD (chapter 6). Many potential clinical application are being discussed, in

particular those relating to the repair of damaged tissues and/or requiring an anti-inflammatory effect. An international registry of patients treated with MSCs has been recently launched under the auspices of the EBMT Developmental Committee.[37] This allows to collect data on patients treated with MSCs for any disorder, as well as to analyze their clinical characteristics and outcome. Besides retrospective data analyses, the registry represents the basis for future multicenter clinical trials.

9.4.1 Hematopoietic stem cell transplantation

In chapter 5 the role of MSCs in sustaining hematopoietic engraftment and reducing the risk of graft failure after haploidentical T-cell depleted HSCT from a HLA-partially matched family donor was explored in a phase I/II study. Feasibility of expansion of comparable BM-MSC products in 2 different sites (Pavia and Leiden) was demonstrated, as well as safety of MSC clinical use. The data obtained also suggest that co-infusion of *ex vivo* expanded BM-MSCs might help to overcome graft rejection, since none of the 25 study patients experienced graft failure as compared to 20% (11 out of 52 children) graft failure rate in the historical controls. One possible explanation for this finding is that MSCs might display an immunosuppressive/anti-proliferative effect on alloreactive host T lymphocytes escaping the preparative regimen, resulting in an engraftment promoting effect. Alternatively, MSCs might favor the engraftment of donor HSCs through non-immunological mechanisms; for instance, by contributing to the hematopoietic stem cell *niche* or by stimulating the functional recovery of the BM-microenvironment through the secretion of paracrine mediators. Chimerism analysis of *ex vivo* expanded MSCs derived from recipient BM after HSCT did not show evidence of donor cells in the majority of patients. This finding suggests that sustained engraftment of MSCs might not be necessary to induce therapeutic benefit.

The use of MSCs for the treatment of steroid-resistant, severe acute GvHD was evaluated in a phase II multicenter clinical trial conducted within the EBMT Developmental Committee (chapter 6). The 5 participating centers adopted a common MSC expansion protocol that allowed the generation of similar products at the different sites.

The safety of the infusion of HLA-identical or disparate, *in vitro* expanded, BM-MSCs was demonstrated. A complete response rate was observed in 55% of the patients and the overall response rate was 69%. The 2-year probability of survival of complete responders was significantly better that that of patients with partial or no response; whereas transplantation-related mortality (TRM) was significantly lower in complete responders. There was a non-statistically significant trend for a better response rate in children, as compared with adult patients. We concluded that the infusion of BM-MSCs might be a safe and effective treatment for patients with severe, acute GvHD who do not respond to steroids and/or other immunosuppressive therapies.

Based on the experience of the co-transplantation of MSCs and CD34+ peripheral blood stem cells (chapter 5), we have designed and conducted a similar phase I/II study on the co-transplantation of MSCs and UCB-derived HSCs in 3 centers (Pavia, Leiden and Stockholm). Thirteen pediatric patients with haematological malignancies received co-infusion of UCB cells and parental-derived BM-MSCs, and were compared with 39 historical controls. The feasibility and safety of the approach was confirmed also in this setting, since no MSC-related toxicities were registered. In contrast with pre-clinical results[38] and our own experience in the haploidentical transplants (chapter 5), there was no difference in haematological recovery between the 2 groups, although less G-CSF was administered in the study patients as compared to the controls ($p<0.05$). This difference may reflect that graft disfunction in UCB transplantation is more pronounced also in relation to the low numbers

of HSCs infused. Moreover, the relatively variable number of CD34+ cells administered and the use of G-CSF in historical controls might have masked any effect of MSCs in this setting. The overall rate of acute GvHD did not significantly differ between MSC patients and controls, but severe grade III-IV acute GvHD did (0% vs 26%). Although overall survival was not significantly improved by the addition of MSCs, early TRM showed a reduction, related to the decrease in death due to severe GvHD. This suggests that co-infusion of MSCs at the time of transplantation might allow to sufficiently reduce donor T cell alloreactivity to abrogate the most severe manifestations of acute GvHD, thus reducing TRM in UCB transplantation (*manuscript in preparation*).

9.4.2 Crohn's Disease

MSCs are currently investigated as a novel cellular therapy for patients with refractory CD.[39,40] In chapter 7, we investigated the potential role of autologous BM-derived MSCs as immunomodulatory/anti-inflammatory treatment to stimulate tissue repair in CD and demonstrated that both isolation and *ex vivo* expansion of BM-MSCs from these patients are feasible. Moreover, CD-MSCs proved effective in inhibiting *in vitro* polyclonally-induced proliferation of both autologous and allogeneic peripheral blood lymphocytes.These findings, although limited to an *in vitro* observation, might suggest that patient-derived MSCs, rather than third party cells, could be employed for the treatment of refractory CD patients. The use of autologous MSCs might offer significant advantages over allogeneic cells, in light of the observations that MSCs can be lysed by both allogeneic T cells[41] and NK cells.[42] In non-profoundly immunodepressed subjects, such as most CD patients, allogeneic MSCs might be rejected after infusion without having the chance to display their beneficial tissue-healing effect.

9.4.3 The concept for treating autoimmune diseases

On the basis of their immunomodulatory properties, anti-inflammatory and tissue-protective effects, MSCs may be used in the treatment of refractory AID. Several reports on experimental models of autoimmunity have shown a beneficial effect of MSCs on various AID[43-46] Despite this, few clinical data on the use of MSCs in human AID are available. The few published reports include a feasibility study of 10 patients[47,48] with multiple sclerosis (MS) and phase I/II trials in CD patients refractory to conventional treatment.[15,49,50] Discussion is underway also concerning other AID such as type 1 diabetes mellitus, systemic sclerosis (SS) and Systemic lupus erythematosus (SLE).

Contradictory results have been published on the properties of *ex vivo* expanded MSCs from AID patients.[51,52] Whether these "diseased" cells are functionally impaired or whether they display similar characteristics as those of healthy donors needs further investigation. Although most studies, including our own experience (chapter 7), support the use of autologous cells for transplantation purposes. Whereas in many acute clinical situations the time necessary for MSC expansion (3-4 weeks) precludes the use of autologous cells, in AID it is feasible to isolate and culture the cells from patient tissues. Moreover, the immune privilege observed in heavily immune suppressed patients, such as patients with steroid-refractory acute GvHD, might not be guaranteed in immunocompetent hosts and, therefore, allogeneic MSCs might be rejected. On the other hand, this might not be important if MSCs are capable to home to target organs and to survive long enough to exert a therapeutic effect.

9.4.4 Efficacy of MSC treatment and future clinical prospects

Thus far, MSCs have been employed in phase I/II clinical trials, addressing the issues of feasibility and safety of infusion. To date no adverse effects have been registered after MSC administration, although a longer follow-up is

necessary to draw definitive conclusions on potential late adverse events. No demonstration of efficacy of MSC therapy has been obtained; this requires the execution of large multicenter randomized clinical trials specifically addressing response to MSC therapy, in comparison with more conventional treatment modalities.

In the setting of haploidentical T-cell depleted HSCT in children, preliminary data of the phase I/II study suggest that MSCs might help to overcome graft rejection. This finding should be confirmed in adults undergoing transplantation from disparate donors; in this group of patients, where a lower number of CD34+ cells is infused per kilogram of recipient body wieght, co-infusion of MSCs might provide useful data on the engraftment promoting effect of these cells. The execution of a randomized clinical trial in this context is difficult to implement. Calculations revealed that at least 100 patients per arm should be enrolled in the trial to statistically prove efficacy of this approach. The use of haploidentical transplantation is relatively limited by the number of patients undergoing this procedure and by the center experience in this type of transplant.

Regarding GVHD treatment, recently the first phase III double blind, placebo controlled, randomized clinical trial has been launched within the EBMT Developmental Committee. The primary objective of this study is to establish efficacy of infusions of allogeneic MSCs on steroid-resistant grade II–IV acute GvHD, as compared to second line treatment of GVHD. Patients are randomized to receive either MSCs (2 intravenous infusions at a dosage of 2 x 10^6/kg recipient weight) or equal volume of saline infusions, in combination with second line treatment. Besides efficacy, a follow-up of 2 years will also document any long-term side effect of MSC infusion, such as increased TRM, relapse and infection.

In the setting of refractory AID and tissue repair, phase I/II studies of MSC therapy are underway.[15,49,50] Although promising, preliminary results need to be confirmed in larger cohorts of patients and in clinical studies aimed at evaluating efficacy. Open issues also include patient selection, disease stage and activity, MSC source and expansion conditions. The possibility of obtaining functional MSCs and in sufficient number for clinical applications from patient's material needs to be confirmed in the different diseases.

We are currently conducting, in collaboration with the Department of Internal Medicine and Gastroenterology of Fondazione IRCCS Policlinico San Matteo, a phase I/II study aimed at assessing the feasibility and safety of local intrafistolous infusion of autologous BM-MSCs in patients with refractory CD and perianal fistulas. Preliminary results in 8 patients demonstrate the feasibility/safety of this approach and suggest a potent reparative effect of MSCs on the damaged intestinal mucosa, characterized by complete healing of the fistulas in the majority of the patients and a critical decrease in their disease activity indices (*manuscript in preparation*).

9.5 Safety issues

The utilization of *ex vivo* expanded MSCs for clinical application is associated with potential risks i.e. the immunogenicity of the cells or the medium components, *in vitro* transformation of the cells during expansion, and ectopic tissue formation.

9.5.1 Culture conditions

At present, MSCs are extensively expanded *ex vivo* before used in the clinical setting. The adoption of different isolation methods and culture conditions may lead to multiple MSC populations with slightly different biological and functional characteristics. For instance, differences in culture medium or supplements (FCS, human serum, PL, addition of GFs), plating density, level

of confluency at cell detachment may influence their proliferative capacity, expression of surface markers or differentiation capacity, leading to the committment towards a specific phenotype or tissue lineage. This supports the need for the definition and validation of common isolation and expansion protocols for the preparation of MSCs both for experimental and clinical purposes. The use of a uniform expansion method facilitates the comparisons between cell-products generated at different sites and allows to perform large multicenter collaborative studies.

To avoid the potential risks associated with the use of FCS as culture supplement for MSCs (transmission of infections, formation of antibodies against bovine proteins), alternative expansion methods have been investigated. The possibility of using autologous or allogeneic human serum for *in vitro* expansion of MSCs has been tested;[53] the reduction of bovine antigens by a final 48-hour incubation with medium supplemented with 20% human serum has also been proposed.[54] Platelet-rich plasma (PRP) or PL, containing high levels of PLT-derived GFs, have been tested in the clinical setting.[55-58,21] In chapter 3 we have employed PL as alternative culture supplement for *in vitro* expansion of human BM-MSCs and compared PL-expanded MSCs with those cultured in the presence of FCS. We were able to demonstrate the superiority of a culture medium supplemented with 5% PL, as compared with 10% FCS, in terms of clonogenic efficiency and proliferative capacity. Moreover, we showed that PL-expanded MSCs maintain their immunoregulatory properties *in vitro*. Despite the fact that expansion procedures using PL have demonstrated their interest and have been implemented in different laboratories, definitive standards to produce clinical-grade PL-MSCs are lacking. It remains to be studied whether the clinical safety and efficacy profile of PL-expanded MSCs is similar to FCS-expanded MSCs.

9.5.2 Genetic stability and risk of malignant transformation

Given the reports of potential transformation of adult human MSCs after *ex vivo* culture,[59-62] genetic stability of MSCs should be routinely assessed prior to infusion In chapter 4 we investigated the potential susceptibility of human BM-MSCs, expanded in the presence of FCS, to undergo transformation after *in vitro* culture. We found that these cells can be cultured for long term without loosing their phenotypical and functional characteristics. Using genetic studies, performed through conventional and molecular karyotyping, the absence of chromosomal abnormalities was observed. Similar findings have been obtained in 18 BM-MSC cultures for clinical application prepared in the last 2 years in our Center, that showed no signs of cell transformation. These latter cells were prepared following the common expansion protocol developed within the EBMT Developmental Committee and their expansions were interrupted at passage 3 in order to minimize the risk of transformation. The genetic profile of MSCs expanded in the presence of PL and isolated both from BM (chapter 3) and UCB (chapter 8) was also tested and these cultures revealed no signs of transformation. In particular, both MSC sources displayed a normal molecular karyotpype by array-Comparative Genomic Hybridization (array-CGH); moreover, in case of UCB-MSCs, $p16^{ink4a}$ was normally expressed and anchorage growth independence in soft agar was never observed.

Recently, French researchers have reported the presence of aneuploidy in a number of MSC preparations for clinical application, after cultivation both in the presence of FCS+ Fibroblast Growth factor-2 (FGF-2) and PL.[63] To further characterize the genetic abnormalities, quantitative analysis of genes related to transformation and senescence was performed. Normal and stable expression of c-myc, p53 and p21 was demonstrated, whereas human telomerase reverse transcriptase (hTERT) was never expressed. Moreover, MSCs normally expressed $p16^{ink4a}$ and anchorage growth independence in soft

agar was never obtained. These data suggest that, although aneuploidy can occur during MSC expansion, it does not reflect cell transformation, but rather senescence of the cells. Based on these results, the French MSC clinical trials which were temporary interrupted due to the potential risk of transformation of the cells, have been recently re-opened.

In light of these observations, phenotypic, functional and genetic assays, although of limited sensitivity, should be routinely performed on MSCs before *in vivo* use to demonstrate whether their biological properties, after *ex vivo* expansion, remain suitable for clinical application.

9.6.1 Risks associated with MSC-mediated immunemodulation and ectopic tissue formation

Whether MSC treatment can further aggravate immune incompetence and increase the risk of developing infections in patients with severe acute GvHD, as well as favor relapse in patients with malignant disorders, needs to be further investigated. Karlsson *et al*. performed specific analysis of subsequent Epstein-Barr virus and Cytomegalovirus reactivity in 2 patients included in the phase II GvHD study and demonstrated that effector functions of virus-specific T-cells were retained after MSC infusion.[64] Recent data in 10 patients suggest that co-transplantation of MSCs and HSCs may result in increased risk of relapse in patients with hematologic malignancies, as compared to patients receiving standard HSCT.[65] Data obtained so far in the phase I/II trials performed within the Developmental Committee do not show an incerased risk of developing both infection and relapse, as compared with historical controls. Large collaborative randomized studies, encountering long-term follow-up, are necessary to define whether MSCs might induce suppression of the host antitumor immune response, abrogate or weaken graft-versus-leukemia (GvL) activity and reduce the ability to respond to infectious agents in various groups of patients.

Recently, calcifications were observed in the infarcted hearts of mice that received local infusion of MSCs.[66] This study reveals the potential risk of ectopic tissue formation in patients treated with MSCs for myocardial infarction and other diseases. So far clinical data have shown the safety of MSC infusion without any occurrence of ectopic tissue or tumor formation *in vivo;* however, factors governing post-infusion fate of MSCs and the influence of the local environment on MSC behaviour are largely unknown and need further investigation. Given the paucity of available clinical data and the rather short follow-up, it is reasonable to advise strict and long-term follow-up for patients treated with MSCs.

9.6 Open clinical and experimental issues

9.6.1 Clinical issues

- Autologous versus allogeneic MSCs –

Whether autologous or allogeneic MSCs should be preferred will depend on the clinical setting in which the cells are employed and on the desidered therapeutic effect. In clinical situations of 'urgent' MSC treatment, such as in patients suffering from severe acute GvHD, allogeneic third-party, 'ready off the shelf' MSCs should be preferred. In disorders in which sufficient time for MSC harvest and *ex vivo* expansion is available, such as in refarctory AID patients, autologous cells can be employed, provided that they are functionally active. The potential rejection of infused MSCs should be carefully considered in the different clinical contexts. This might be either unfavourable or profitable, when only a temporary effect of MSCs is needed. In some cases a 'hit and run' effect of MSCs might be sufficient to induce a clinical response and might be useful to protect the patient from the risks of MSC-mediated immunosuppression. The rejection of MSCs would also avoid the risk of ectopic tissue formation and of the potential engraftment of a transformed MSC population in the host. The repetitive infusion of MSCs might cause

sensitization in the patient, characterized by the formation of alloantibodies directed against MSCs and responsible for their rejection. Alternatively, the manipulation *in vitro* of MSCs might be sufficient to alter their biological properties and to cause the development of antibodies against components of the culture medium incorporated in the cells during expansion, finally leading to cell rejection. In contrast to this hypotesis, we have recently reported the outcome of 2 children undergoing unrelated UCBT with parental BM-MSCs co-transplantation who initially rejected the graft. They were later succesfully re-transplanted using the father as the donor of both haploidentical PBSCs and MSCs. Despite previous exposure to paternal MSCs, they did not subsequently reject the paternal stem cell graft. This observation suggests that, in heavily immunodepressed patients, such as those undergoing myeloablative HSCT, multiple infusions of allogeneic MSCs might not be immunogenic and might not induce rejection (*manuscript submitted*).

- Dose and schedule -

Optimal timimg of MSC administration, cell dose and additional immunosuppressive therapy need also to be defined. Dose and schedule of administration will probably depend on the clinical context. For facilitating engraftment one MSC infusion might be sufficient to prevent graft failure, in case of severe acute GvHD several MSC administration might be necessary to control the disease. In chronic inflammatory and autoimmune diseases, MSCs might not be a ‘once-in-a-life treatment’, but could represent a helpful tool during the active and severe phases of the disease. Whether the simultaneous administration of other immunosuppressive treatments could potentiate or abolish MSC therapeutic benefit needs also to be addressed in future experimental and clinical studies.

9.6.2 Experimental issues

Most of the available data on MSC immunomodulatory properties have been obtained *in vitro* on *ex vivo* expanded cells. The mechanisms by which MSCs display their effects *in vivo* are still largely unknown and need to be further investigated. Several mechanisms of action, including cell-cell contact as well as the release of soluble mediators by MSCs or upon interaction with immune cells,[41,67,68] have been proposed, but no definitive conclusions can be drawn. The high variability of the reported results might be partly explained by employing different culture conditions, different MSC and lymphocyte populations, as well as different MSC:lymphocyte ratios. Defined animal models, *in vitro* and *in vivo* studies are necessary to more precisely unravel the mechanisms underlying the anti-proliferative/anti-inflamatory effect of MSCs. In light of the observations made in chapter 8 on UCB- and BM-MSCs, we plan to more precisely investigate the role of the different biological mechanisms employed by MSCs. For instance, PGE2 secretion and IDO activation, two well known mechanisms involved in anti-inflammatory responses *in vitro*,[69,70] might be crucial in attenuating the inflammatory state in AID. We therefore aim to investigate the relevance of these 2 mechanisms in the context of refractory CD to test whether they are functionally active in patient-derived cells. Expression of HLA-G, is known to be one of the most potent mechanisms protecting from immune attack at the feto-maternal interface.[71] This will be investigated in UCB-derived MSCs where it is likely to be functionally relevant, as well as in the setting of HSCT and organ transplantion where MSCs are infused with the aim to prevent rejection. The ability of MSCs to favor *in vitro* the differentiation of $CD4^{+}CD25^{+}FoxP3^{+}$Tregs,[72] will be tested *in vivo* by analyzing the percentage of this lymphocyte population in peripheral blood after MSC treatment in both severe acute GvHD and AID patients. We also plan to investigate the interaction between MSCs and B lymphocytes *in vitro* and *in vivo*, in light of

the recent experimental observation that MSCs may promote proliferation and differentiation of transitional and naive B cells isolated from healthy donors and patients with SLE.[73] If confirmed *in vivo*, MSC therapy in the context of AID might be detrimental and could lead to worsening of signs and symphtoms of autoimmunity.

9.7 Conclusions

Over the past years MSCs have been broadly applied in a variety of clinical settings. Areas of clinical application include modulation of alloimmune responses in the setting of allogeneic stem cell and organ transplantation and AID, as well as direct promotion of tissue repair (bone, cartilage and heart repair). MSC therapy appears to be relatively safe and encouraging therapeutic results have been obtained in several pilot studies. However, MSC therapy is still experimental and no standard treatment has emerged. In the coming years randomized studies will be completed to establish therapeutic efficacy. At the same time *in vitro* and *in vivo* studies will help to understand mechanisms underlying efficacy and will identify specific subsets that mediate repair.

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LIST OF ABBREVIATIONS

AID	autoimmune diseases
Array-CGH	array-Comparative Genomic Hybridization
AT	adipose tissue
BM	bone marrow
CD	Crohn's Disease
CIA	collagen-induced arthritis
DC	dendritic cell
DLI	donor lymphocytes
EAE	experimental autoimmune encephalomyelitis
EBMT	European Group for Blood and Marrow Transplantation
FCS	fetal calf serum
G-CSF	granulocyte colony stimulating factor
GFs	growth factors
GvHD	graft-versus-host disease
GvL	graft-versus-leukemia
HGF	hepatocyte growth factor
HSCs	hematopoietic stem cells
HSCT	hematopoietic stem cell transplantation
IDO	indoleamine 2,3-dioxygenase
MAPCs	multipotent adult progenitor cells
MLD	metachromatic leukodystrophy
MR	magnetic resonance
MS	multiple sclerosis
MSCs	mesenchymal stem cells
NK	Natural Killer
O.I.	*Osteogenesis Imperfecta*
PBSCT	peripheral blood stem cell transplantation
PGE2	prostaglandin E2
PL	platelet-lysate
PRP	Platelet-rich plasma
SDF-1	stromal-derived factor-1
SLE	Systemic lupus erythematosus
SS	systemic sclerosis
SSEA-1	stage-specific embryonic antigen-1
TGF-β3	Transforming growth Factor-β3
Treg	regulatory T cells
TRM	transplantation-related mortality
UCB	umbical cord blood
USSCs	Unrestricted Somatic Stem Cells

Nederlandse samenvatting

Dit proefschrift richt zich op de karakterisering van de biologische en functionele eigenschappen van humane Mesenchymale Stromale Cellen (MSC's). Hierbij is een vergelijking gemaakt tussen MSC's afkomstig uit foetale weefsels, waaronder placenta en navelstrengbloed welke zijn vergeleken met MSC's afkomstig uit het beenmerg van volwassen individuen. Navelstrengbloed (Umbilical Cord Blood, UCB) is onderzocht als een potentieel nieuwe bron van MSC's voor klinische toepassing. Om deze reden zijn de immunomodulatoire eigenschappen van MSC's afkomstig van navelstrengbloed vergeleken met die van MSC's afkomstig uit beenmerg. Daarnaast zijn de experimentele condities waaronder MSC's in het laboratorium kunnen worden geëxpandeerd onderzocht en verder geoptimaliseerd. Hierbij zijn ook alternatieve kweekmethoden onderzocht, waarbij geen gebruik gemaakt wordt van dierlijke eiwitten die aanwezig zijn in "fetal calf serum". Als alternatief hiervoor werd een expansiemethode onderzocht gebaseerd op het gebruik van trombocyten lysaat waarin zich groeistimulerende factoren bevinden.

Eén van de belangrijkste potentiële risico's van MSC expansie is de mogelijkheid van maligne transformatie. Dit risico treedt met name op bij het langdurig ex-vivo kweken van MSC's. Daarnaast zou het risico verder kunnen worden vergroot door de toepassing van deze cellen bij immuun gecompromitteerde patiënten. Er werden geen structurele of numerieke chromosomale afwijkingen gevonden bij MSC's die gekweekt werden onder serumbevattende dan wel onder serumvrije kweekcondities. Andere potentiële risico's van MSC therapie omvatte immunogeniciteit bij het toedienen van allogene cellen en het risico van ectopische weefselformatie na systemische of lokale toediening.

Eén van de problemen bij het karakteriseren van MSC's is het ontbreken van specifieke oppervlaktemarkers waarmee MSC's uit weefsels kunnen worden geisoleerd. Als gevolgd hiervan zijn de klinische MSC producten heterogeen wat betreft hun immuun fenotype en ook wat betreft hun functionele eigenschappen. Nieuwe technieken waaronder gen-expressieprofielen en eiwitprofielen worden voorgesteld om MSC's nauwkeuriger te karakteriseren.

De in-vivo biodistributie en "trafficking" van ex-vivo geëxpandeerde MSC's is nog weinig onderzocht. Het belang van de ontwikkeling van geschikte labelings technieken om MSC's in-vivo te kunnen volgen na inspuiting wordt besproken.

De belangrijkste toepassing van MSC therapie ligt in de context van hematopoietische stamceltransplantatie. Dit betreft ondermeer de co-transplantatie van MSC's en Hematopoietische Stamcellen (HSC's) om het aanslaan van het transplantaat ("engraftment") te bevorderen. Daarnaast zijn MSC's vooral toegepast ter behandeling van steroïd resistente ernstige acute graft-versus-host ziekte. Een relatief nieuwe indicatie voor MSC therapie omvat de behandeling van "Inflammatory Bowel Disorders", waaronder de ziekte van Crohn. Bij een kleine groep patiënten werd de rol van autologe MSC's afkomstig uit beenmerg onderzocht met betrekking tot hun immunomodulatoire en ontstekingsremmende eigenschappen. Behandeling met MSC's vindt nu reeds plaats bij een groot aantal aandoeningen die gekenmerkt worden door inflammatie en weefselschade. Het is te verwachten dat in de nabije toekomst MSC therapie als een nieuwe therapeutische modaliteit kan worden toegepast bij een groot aantal verschillende immunologische-, en ontstekingsgemedieerde ziekten, met het doel om afweerresponsen te moduleren en weefselschade te herstellen.

Summary in English

This thesis focuses on the characterization of the biological and functional properties of human mesenchymal stromal cells (MSCs), isolated from different tissue sources. The differentiation capacity of MSCs from fetal and adult tissues has been tested and compared. Umbilical cord blood (UCB) has been investigated as a potential novel source of MSCs for clinical application and the immunomodulatory properties of UCB-derived MSCs have been characterized in comparison with those of MSCs of bone marrow (BM) origin. Moreover, it has been attempted to optimize the experimental conditions for MSC *ex vivo* expansion. Alternative culture methods, devoid of animal proteins, have been applied by introducing expansion procedures based on platelet-derived growth factors. The potential susceptibility of MSCs to undergo malignant transformation after long-term *in vitro* culture has been investigated and discussed in view of their clinical application, especially in immunocompromised hosts. The immunogenicity of the cells and/or their medium components, the risk of ectopic tissue formation and that of MSC-mediated immunosuppression have been also discussed as potential risks associated with the clinical use of *ex vivo* expanded MSCs.

The lack of surface markers and functional assays to specifically identify MSCs and to facilitate the generation of homogenous cell products has been analyzed and efforts for future MSC development have been discussed. Novel techniques, such as proteomic approaches and microarray analysis, have been proposed with the aim to improve the knowledge on MSC biological and functional properties; imaging studies, based on the use of suitable labeling techniques and able to investigate *in vivo* MSC 'trafficking' and biodistribution, have been discussed.

The role of MSC therapy in the context of hematopoietic stem cell transplantation (HSCT) has been explored, focusing in particular on the co-

transplantation of MSCs to promote engraftment of hematopoietic progenitors, and on the administration of MSCs for the treatment of steroid-resistant, severe acute graft-versus-host disease (GvHD). The role of MSCs in the treatment of inflammatory bowel diseases refractory to conventional therapies, as well as the concept for treating refractory autoimmune diseases has been discussed. In particular, the potential role of autologous BM-derived MSCs as immunomodulatory/anti-inflammatory treatment to stimulate tissue repair in Crohn's Disease (CD) patients has been investigated. Based on these experimental and clinical findings and by broadening the knowledge on MSC biological activities, these cells could be employed in the near future as a novel therapeutic strategy to stimulate tissue repair and modulate immune responses in a variety of immune-mediated and inflammatory diseases.

Riassunto in Italiano

Questa tesi verte sulla caratterizzazione delle proprietà biologiche e funzionali delle cellule stromali mesenchimali (MSC) umane isolate da differenti tessuti. In questo lavoro sono state testate e confrontate le capacità differenziative delle MSC umane derivate da tessuti fetali e di tipo adulto. Quale potenziale sorgente di MSC per l'applicazione clinica è stato studiato il sangue placentare; le proprietà immunomodulatorie delle MSC da sangue placentare sono state confrontate con quelle delle MSC di derivazione midollare. Sono state, poi, ottimizzate le condizioni di coltura per l'espansione *ex vivo* delle MSC. Un terreno basato sull'impiego di fattori di crescita derivati dalle piastrine è stato preparato ed impiegato quale sistema di coltura alternativo in quanto privo di proteine di origine animale. É stata, inoltre, valutata la potenziale suscettibilità delle MSC alla trasformazione in senso neoplastico dopo coltura *in vitro* a lungo termine; ciò risulta particolarmente interessante in funzione del loro impiego clinico, in particolare in soggetti immunocompromessi. Sono stati valutati quali potenziali rischi associati all'uso clinico delle MSC i seguenti fattori: l'immunogenicità delle cellule e/o dei componenti del terreno di coltura, il rischio di formazione *in vivo* di tessuto ectopico e l'eventuale immunosoppressione favorita dalle MSC stesse *in vivo*.

In questo lavoro sono stati altresì discussi ed approfonditi eventuali sviluppi futuri riguardanti l'identificazione di marcatori di superficie e di test funzionali atti tanto all'identificazione specifica delle MSC, quanto alla generazione di prodotti per terapia cellulare omogenei. Inoltre, al fine di migliorare la conoscenza delle proprietà biologiche e funzionali delle MSC, sono state esplorate tecnologie innovative, quali tecniche di proteomica e analisi con *microarray*. Sono stati, quindi, proposti studi di immagine basati

sull’impiego di appropriati sistemi di marcatura e capaci di studiare il *trafficking* e la biodistribuzione delle MSC *in vivo*.

E’ stato, inoltre, testato il ruolo delle terapie cellulari con MSC nel contesto del trapianto allogenico di cellule staminali emopoietiche. In particolare, sono stati condotti studi clinici di fase I/II basati sia sul co-trapianto di MSC allo scopo di facilitare l’attecchimento dei progenitori emopoietici sia sulla somministrazione di MSC per il trattamento della malattia del trapianto contro l’ospite steroido-resistente di grado severo. Sono stati discussi tanto il ruolo della terapia con MSC nel trattamento delle malattie infiammatorie croniche intestinali non responsive alle terapie convenzionali, quanto il potenziale utilizzo delle stesse nel trattamento delle malattie autoimmuni refrattarie. In particolare, è stato quindi valutato l’impiego di MSC autologhe di origine midollare quale approccio immunomodulante/anti-infiammatorio con lo scopo di stimolare la riparazione tessutale in pazienti con malattia di Crohn refrattaria.

Sulla base di quanto emerso da questo studio e attraverso l’ampliamento della conoscenza biologica delle MSC, queste cellule potranno, in un prossimo futuro, essere impiegate, quali strumenti innovativi, per facilitare la rigenerazione dei tessuti e modulare la risposta immune in numerose affezioni a patogenesi immuno-mediata ed infiammatoria.

Curriculum Vitae

Maria Ester Bernardo was born in Voghera (Pavia), Italy on September 14, 1975. In 1994 she took her high-school diploma at Liceo Classico "U. Foscolo" in Pavia. In July 2000 she took her Medical Degree in Medicine and Surgery, University of Pavia with a thesis entitled "Immunological Reconstitution after placental stem cell transplantation" (*cum laude*). In April 2001 she was licensed to practice medicine and surgery.

From June 2004 to June 2005 she served an internship at the Department of Haematology, Leiden University Medical Centre (head Prof. Dr. R.Willemze), under the supervision of Prof. Dr. W.E. Fibbe, focusing on research projects in hematopoietic stem cell transplantation utilizing mesenchymal stromal cells as therapeutic support.

In October 2005 she specialized in Pediatrics, University of Pavia, with a thesis entitle "Study of the chondrogenic potential of mesenchymal stem cells isolated from different tissue sources"(*cum laude*). From November 2005 to March 2007 she served as a fellow at the Department of Paediatric Oncohematology, Foundation IRCCS Policlinico San Matteo, Pavia. From April 2007 to present she is a Staff Paediatrician, specialized in Paediatric Oncohaematology, Department of Paediatric Oncohematology, Foundation IRCCS Policlinico San Matteo, Pavia.

Maria Ester Bernardo is a member of the Italian Association of Paediatric Haematology and Oncology (AIEOP) and of the American Society of Haematology (ASH). She is a member of the *Editorial Review Board* of the journal *Bone Marrow Transplantation.*

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