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MESENCHYMAL STROMAL/STEM CELLS - EXPANSION, ENGRAFTMENT AND IMMUNE MODULATION

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To my family and friends,
for trust and love.

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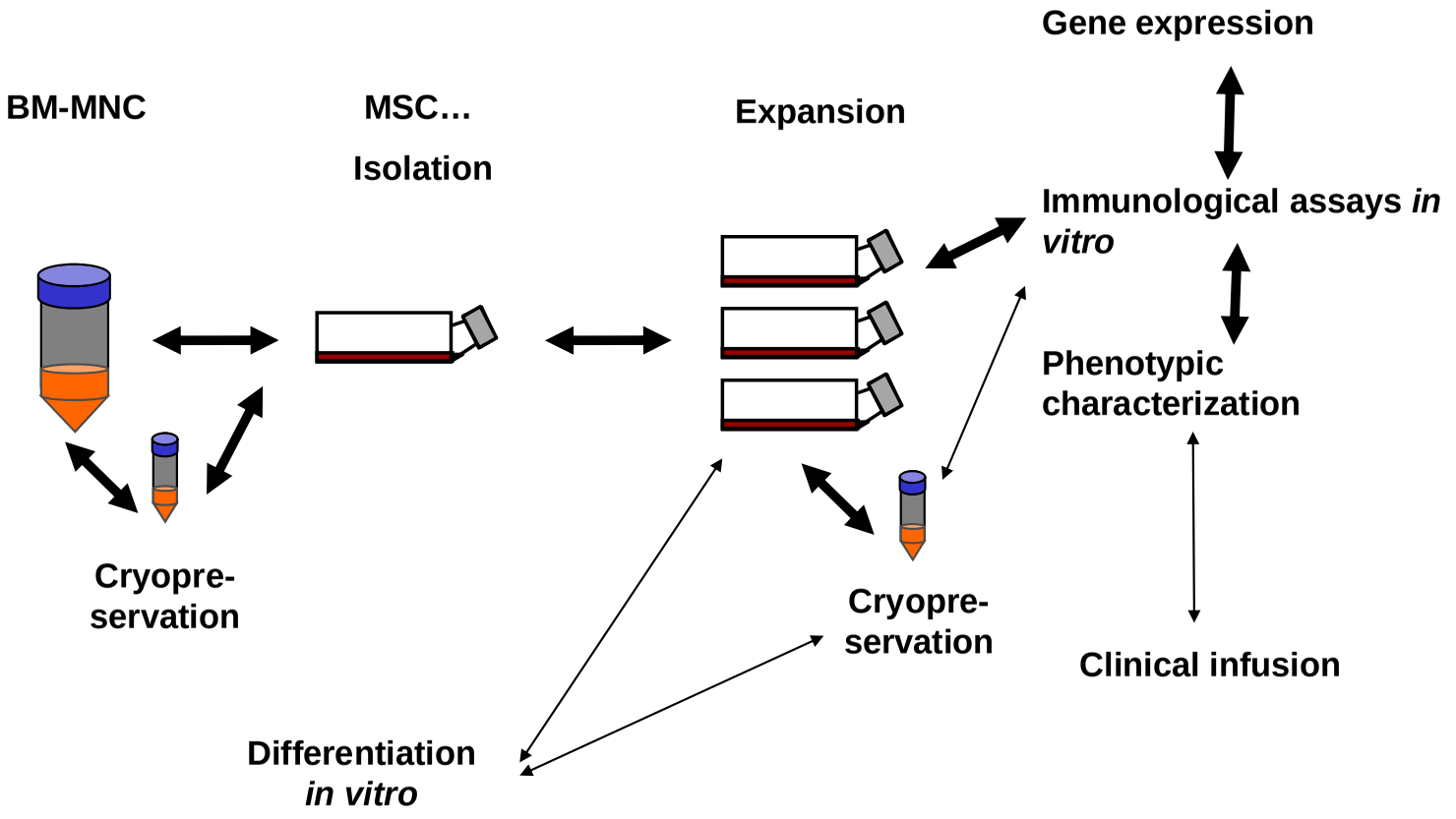

1. SUMMARY

Due to their production of hematopoietic growth factors and their immunomodulatory capacity, bone marrow-derived mesenchymal stromal/stem cells (MSC), are a promising tool in the procedure of hematopoietic stem cell transplantation (HSCT).

Seven patients undergoing allogeneic HSCT were co-infused with MSC. Three of the patients were regrafted after a previous rejection. Either HLA-identical or haploidentical MSC were given at the time of HSC infusion. Engraftment was successful in all patients with no untowards effects.

Large-scale expansion of MSC intended for clinical use, requires standardized and optimized handling of the cells *ex vivo*. For safety and regulatory reasons, we explored if fetal calf serum (FCS) in the culture medium could be replaced by human blood group AB serum. Characteristics and immune suppressive function of MSC were not affected by culturing MSC in the alternative serum, and the cell yield was slighlty improved.

Cryopreservation of bone marrow derived mononuclear cells negatively influenced the final MSC yield. An increased number of MSC was achieved by increasing the FCS levels above 10% and decreasing the replating density. The suppressive effect of MSC was unaffected by the number of passages MSC had been cultured *in vitro*. Cell viability and immunosuppression *in vitro* remained high even after several years' storage in liquid nitrogen. Mixing MSC from several donors improved suppression.

When selecting MSC for immunosuppressive therapy, it would be helpful to have defined markers relating MSC characteristics to their immunosuppressive function. We compared phenotype, growth characteristics and gene arrays of MSC with differing immunosuppressive capacity, derived from two donors. MSC from donor 1 consistently suppressed proliferation of activated lymphocytes. This was in contrast to MSC from donor 2, where MSC had no or little inhibition or a stimulating effect. Furthermore, single cell derived cultures from the suppressive donor generated suppressive and non-suppressive clones, indicating that the suppressive phenotype is not inherent to the MSC donor. However,

phenotypic differences between all cells from the two donors were too diverse to be efficient for prospective isolation of MSC with suppressive capacity. Thus, the only currently reliable means of validating MSC remain the proposed set of standards focusing on the cells adherence to plastic, the phenotype and trilineage potential. Functional testing by *in vitro* assays is required to assess the immunosuppressive function.

2. LIST OF PAPERS

1. Le Blanc K, SAMUELSSON HÅKAN, Gustafsson Britt, Remberger Mats, Sundberg Berit, Arvidson Johan, Ljungman Per, Lönnies Lena, Nava Silvia, Ringdén Olle. *Transplantation of mesenchymal stem cells to enhance engraftment of hematopoietic stem cells.* Leukemia, 2007. 21(8): p. 1733-8.

2. Le Blanc Katarina, SAMUELSSON HÅKAN, Lönnies Lena, Sundin Mikael, Ringdén Olle. *Generation of immunosuppressive mesenchymal stem cells in allogeneic human serum.* Transplantation, 2007. 84(8): p. 1055-9.

3. SAMUELSSON HÅKAN, Ringdén Olle, Lönnies Lena, Le Blanc Katarina. *Optimizing in vitro conditions for immunomodulation and expansion of mesenchymal stromal cells.* Cytotherapy, 2009: p. 1-8.

4. SAMUELSSON HÅKAN, Ringdén Olle, Kumagai-Braesch Makiko, Rosendahl Kerstin, Le Blanc Katarina. *Characterization of clonal and polyclonal mesenchymal stromal cells with different immunosuppressive capacity*- Manuscript

3. ABBREVATIONS

AB serum - Human blood group AB serum
APC - Antigen-presenting cells/function
BM - Bone marrow
CIITA – Class II transactivator
CD - Cluster of differentiation
CPM - Counts per minute
CTLA - Cytotoxic T lymphocyte associated
CMV - Cytomegalovirus
DC - Dendritic cells
DC1 - Dendritic cells type 1
DC2 - Dendritic cells type 2
FCS - Fetal calf serum
FSC – Forward scatter
GM-CSF – Granulocyte macrophage – colony stimulating factor
GVHD - Graft-versus-host-disease
GVL - Graft-versus-leukemia
HSC - Hematopoietic stem cells
HSCT - Hematopoitec stem cell transplantation
HLA – Human leukocyte antigen
IDO – Indoleamine 2,3-dioxygenase
Ig - Immuneglobulin
KIR - Immune globulin - like receptor
INFγ – Interferon gamma
IL - Interleukin
MFI – Mean fluoroscense intensity
MHC – Major histocompatibility complex
MLC – Mixed lymphocyte culture
MNC – Mononuclear cells
MSC - Mesenchymal stromal/stem cells
MUD - Matched unrelated donor
NK cell - Natural killer cell
NO – Nitric oxygen
OI – Osteogenesis imperfecta
PBL – Peripheral blood lymphocytes
PGE2 – Prostaglandin E2

PHA – Phytohemaglutinin
RS-1 - Recycling stem cell type 1
RS-2 - Recycling stem cell type 2
RT-PCR - Real time - polychain reaction
RIC – Reduced intensity conditioning
SCID - Severe combined immunedificiency
SSC – Side scatter
TBI - Total body irradiation
T-cell – Thymocyte cell
TCR – T-cell receptor
TGF - Transforming growth factor
TH1 – T helper 1 (cell or mediated response)
TH2 – T helper 2 (cell or mediated response)
Tregs - T regulatory cells
TRM - Transplant-related mortality

4. MESENCHYMAL STROMAL/STEM CELLS

4.1 *Origin and natural function of MSC*

MSC reside in the stroma, a network of supporting tissue in bone marrow and other tissues. MSC give rise to stromal cells [1, 2] and produce stromal components such as collagen, fibronectin, laminin and proteoglycans [3-5].

MSC, for *in vitro* and *in vivo* studies, can be derived from several tissues of the human and animal body. The most common site for isolation is adult bone marrow, but also from adipose tissue, cord blood as well as fetal liver, blood, bone-marrow and lung can also be used [6-9].

4.2 *Charaterization of MSC*

In the late 1960s Friedenstein and his colleagues were the first to characterize MSC as adherent fibroblast-like cells in guinea pig bone marrow that could regenerate bone and cartilage in syngeneic animals [10-13]. Since then, the morphological, phenotypical and functional characterization was further defined [14, 15]. In 2006 the Non Hematopoietic Mesenchymal Stem Cell Committee of the International Society for Cellular Therapy proposed some minimal criteria to define human MSC. MSC must be plastic-adherent when maintained in standard culture conditions. MSC should also express CD73, CD90 and CD105, and lack expression of CD14, CD34 and CD45 or CD11b and CD79alpha or CD19 and HLA-DR [16].

Human MSC are positive for the surface markers CD29, CD44, CD106 and HLA class I [17] and can be induced to express HLAII, iCAM-1 and other markers [18]. As an MSC-specific marker has not been established, and due to the cells' rarity *in vivo* [19], existing knowledge is based on studies performed on cells expanded *in vitro.* The neural ganglioside 2, GD2, was reported to be a single surface marker that uniquely distinguishes MSC from other bone-marrow elements and could be used to isolate MSC from other cells of the bone marrow. GD2 is mainly found in the nervous system and why MSC express it is not fully understood, as well as why MSC express other neural epitopes [20-22]. Thus,

MSC are reported to inherit surface epitopes typical of cells derived from different germ layers.

In vitro and *in vivo*, MSC give rise to osteoblasts, chondrocytes, adipocytes, myocytes, neuronal [23] and stromal cells [1] and may be able to self-renew [24-26].

By induction MSC differentiate into osteocytes, adipocytes and chondrocytes, *in vitro* [12, 19, 27]. The ability of multilineage *in vitro* differentiation of clonal populations of MSC was reported in 1999 in standard conditions at low passage [19]. However, after extensive passaging in culture, MSC are more prone to osteogenic than adipogenic differentiation [28]. The immunosuppressive action of MSC is retained after differentiation into osteocytes, adipocytes and chondrocytes [29]. After *in utero* transplantation, Flake et al found multilinage differentiation and long-term persistence of human MSC in fetal sheep [30, 31].

Mesenchymal stem cells have recently often been termed mesenchymal stromal cells as the majority of plastic adherent bone marrow cells do not meet the criteria for stem cells [32]. However, CD146 (melanoma cell adhesion molecule, MCAM) positive bone-marrow cells, with a differentiation potential similar to that of MSC [2], can also self-renew [33]. Further CD146 is assumed to be a possible isolation marker for MSC [2, 34]. CD146 might be involved in the recruitment of activated T-cells to inflammatory sites [35]. Recycling stem cells type 1 and 2 (RS-1, RS-2) [26, 36, 37] are proposed to be less mature MSC that self-renew. The new suggested nomenclature for MSC, Mesenchymal stromal cells, is not fully established according to the nomenclature in articles published since then.

4.3 *Expansion of MSC*

MSC are normally resident in the bone marrow in numbers as small as 0.001–0.01% of the nucleated cells. The number of MSC in the bone marrow decreases during the human life [19]. The ability to expand MSC in culture, made MSC research possible [38-40]. MSC can be purified from bone-marrow mononuclear cells by plastic adherence in culture, unlike murine cells [41]. MSC cultures become free of hematopoietic precursors, that not are

adherent, after one or two passages [42]. According to the standard protocol MSC are plated as 4000-5000 cells per cm^2 with various media-types supplemented with 10% fetal bovine serum (FBS) or fetal calf serum (FCS) for *in vitro* [15, 17] or *in vivo* studies [43, 44]. In MSC culture, Dulbecco's Modified Eagle's Medium (DMEM) or alpha minimal essential medium (αMEM) [28, 44-46], are most often used.

Maintenance of multipotentiality was better using very low plating density [42, 47]. MSC cultures are reported to be heterogeneous [28, 48] and to grow in cycles from immature to mature MSC when they are cultured *in vitro*. It was as well showed that plating a lower number of MSC could be of advantage as the cell-yield increased with an optimal yield at 1-3 cells per cm^2 [26]. Later Prockop and colleagues also reported that more immature, primitive and potent MSC were obtained with the lowest plating condition in the range of 50 to 1000 cells per cm^2 when the culture media αMEM was supplemented with 20% FCS [42]. Prockop et al further suggest that MSC plated at low density grow with an extended lag phase, where a higher number of cell-cycles can occur, as dickkopf-1 (Dkk-1) synthesis early in culture is low but increases to levels higher than under standard conditions. Dkk-1 is an inhibitor of wnt-signalling. Wnt-signalling promotes proliferation [49, 50] and differentiation [51]. Dkk-1 decreases the cell-to-cell contacts that are required for differentiation of the cells, keeping a higher number of undifferentiated MSC with the capacity to form new colonies [28, 42, 51].

Additional essential compounds in medium are the animal/human serum, plasma or growth factors. MSC cultured in the presence of FCS, were reported to retain FCS proteins in their cytoplasm [52]. Whether FCS contained in MSC gives rise to immunoreactions in MSC treated patients is not clear. However, anti-FCS antibodies have been detected in treated patients. These antibodies are also found in the normal population. Whether such antibodies are important for interactions with MSC *in vivo* remains to be proven [53].

Addition of fibroblast growth factor 2 (FGF2) in media with 10% FCS promotes MSC proliferation and maintains the cells in a more immature state [54]. Growth factors such as epidermal growth

factor (EGF), transforming growth factor β (TGFβ), insulin-like growth factor (IGF), and FGF2 are mandatory in culture of MSC in serum-free media [24, 55]. Human plasma enriched by activated platelet factors is also an effective alternative to FCS [56]. Further MSC obtained in fresh frozen plasma and platelets (FFPP) showed similar morphologic and functional properties to MSC grown in the presence of FCS. FCS can be replaced safely by FFPP in cultures of MSC for clinical purposes according to the report [57].

Standardized procedures are necessary for cultures of clinical-grade MSC but may vary slightly between different institutions. In Europe, handling of the cells according to good manufacturing practices (GMP) is required to insure a delivery of cells that is safe, reproducible and efficient. All parts of the GMP process must be defined and the cells have to be cultured in an as closed system as possible. Quality control of cells must at least include phenotype, functional potential, microbiological safety, and ensure that the cultured cells remain untransformed [58].

Microbiological testing should optimally be done both on MSC cultures as close as possible to transplantation [43, 59, 60]. Likewise, karyotyping of MSC should be performed towards the end of the culture. If MSC not are cryopreserved, karyotyping and microbiological testing may be performed in early passage of the MSC. No MSC derived malignancies have been described in humans after transplantation, but karyotypic changes have been observed in human MSC expanded extensively *in vitro* [61, 62]. Therefore, culture periods are limited to a few weeks before MSC transfusion [57].

4.4 *MSC interactions with immune-cells, in vitro*

MSC may have both autocrine and paracrine effects and secrete factors that modulate cells of the immune system. These include the secretion of growth factors and cytokines such as vascular endothelial growth factor (VEGF), stem cell factor (SCF-1), leukemia inhibitory factor (LIP), granulocyte colony stimulatory factor (G-CSF), macrophage colony stimulating factor (M-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukins (IL-1, -6,-7, -8, -11, -14, and -15), stromal cell-derived

factor (SDF-1), transforming growth factor beta 1 (TGFβ1), prostaglandin E2 (PGE2), nitric oxide and others [19, 63-69].

The expression and secretion of these factors may be modulated through interactions with other cell types [67, 69, 70]. For example monocytes secreted IL-1β that stimulated MSC to secrete TGFβ1 that in turn inhibited T-cell activation and proliferation [67]. Explanations of the suppressive effect of MSC are complex and likely to be multifactorial. Most research about the immune-regulatory effects of MSC is focused on T-cells, but it also includes interactions of many other immune-cells as monocytes, Dendritic cells (DC), Natural killer cells (NK) and B-cells. *In vitro*, Aggrawal and Pittenger examined the immune modulatory functions of MSC by co-culturing them with purified subpopulations of immune cells. They reported alterations in the cytokine secretion profile of various lymphocytes and anti-inflammatory or tolerant phenotypes to be induced [69]. Allo-stimulated T-cells were dose-dependently suppressed by MSC [71-74].

Soluble factors of particular interest in for the clinical use of MSC in HSCT, are IL-10 [75, 76], PGE2 [69], TGFβ1 [67] and nitric oxide [68] as well as the enzyme indoleamine 2,3-dioxygenase (IDO) [77] all reported to propagate the suppressive action of MSC on PBL or purified T-cells. MSC contain the enzyme IDO which catalyzes tryptophan to its metabolite kynurenine [77]. IDO is important in lymphocyte suppression but is also thought to have key roles in health and disease. For fetal survival, IDO activity suppresses proliferation of maternal T-cells avoiding rejection [78] and cancer cells are reported to use intrinsic IDO to avoid attacks from T-cells of the host [79].

Membrane-bound HLA-G was detected on fetal MSC, but not on adult MSC [80]. Nasef et al. [81] later showed that both membrane-bound and soluble forms of HLA-G were constitutively expressed by adult MSC and that HLA-G mediates inhibitory effects on T-lymphocyte proliferation. Another group confirmed that observation and further suggested a contribution in expansion of Tregs and inhibition of NK cell–mediated cytolysis of HLA-G [82].

MSC were reported to not affect early T-cell activation, but to promote division arrest of already activated T-cells [83]. While

survival of inactivated T-cells is sustained, proliferation of activated cells is reduced [84]. This is in line with earlier studies on the role of MSC in T-cell apoptosis, in which MSC were shown to apoptosis activated T-cells [85].

Some groups explain the decreased proliferation in allo-stimulation or in stimulation of T-cells with IL-2, to depend on formation of a higher number of T regulatory cells (Tregs) in presence of MSC [69, 86] while others demonstrated no increase of FoxP3 mRNA when CD4 positive T-cells were co-cultured with MSC [87].

Studies of murine and human MSC and B-cells suggest that MSC reduce the proliferation, differentiation, and chemotaxis of B-cells [88, 89]. These immune modulatory effects appear to be mediated partly by soluble factors [88]. High numbers of MSC are also suggested to suppress immune responses by regulation of antibody production by B-cells [90]. The effects of MSC on antibody production by B-cells are various and depend on the level of B-cell activation. In weak activation of B-cells by antigen, MSC stimulates the antibody production. A higher number of activated B-cells, on the other hand, were inhibited in antibody production [91].

In vitro, studies of interactions between MSC and NK-cells generated variable results. Aggrawals and Pittengers data examined the immune modulatory functions of MSC by co-culture with purified subpopulations of immune cells and reported alterations in the cytokine secretion profile of NK cells to induce a more anti-inflammatory or tolerant phenotype [69]. Later, these data were partly confirmed as MSC, at low NK-to-MSC ratios, altered the phenotype of NK cells and suppress proliferation [92]. Other reports indicate that MSC were killed by IL-2 activated NK cells [93, 94]. The killing was suggested to be performed via LFA-1 on NK cells binding iCAM-1 on MSC [95]. MSC inhibited proliferation [93] by IDO and cyclooxygenase 2-enzyme (COX-2) activity in synergy but not by one of the enzymes alone. The cytotoxity and INFγ production of the NK-cells were also inhibited by MSC [94]. Another group suggested contribution of HLA-G in inhibition of NK cell–mediated cytolysis [82].

DC can be differentiated to DC type 1 (DC1) that direct GVHD typical TH1 responses or to DC type-2 (DC2) that direct less GVHD typical TH2 responses. Fully mature DC express CD80 (co-stimulation), CD83 (maturation marker), CD86 (co-stimulation), CD1a (APC), HLA class II (APC) and CD11C (DC1) or CD123 (DC2) [86, 96].

DC derivation from CD14 or CD34 positive cells is suppressed by MSC [97]. CD14 derived DC secreted less IL-12 (TH1 typical) [98-100] and more IL-10 (TH2 typical) [101] when the DC progenitors were co-cultured with MSC. MSC were reported to impair monocyte differentiation into DC by interfering with the cell cycle by down regulating Cyclin D2, accumulating cells in the G0 phase [102]. The reversible [100] inhibition of DC maturation and function by MSC on monocyte derived DC, was shown to be propagated by specific soluble factors like IL-6, macrophage-colony stimulating factor (M-CSF) [97] PGE2 and IL-10 [69, 75, 100]. The anti-DC effects by MSC, may be propagated by PGE2 [69]. The DC1 population was increased in the responding PBL in allostimulation with irradiated PBL. DC1 expansion was blocked if autologous or allogenic MSC were present during the 5 days of the MLC. The level of DC2 was stable with or without MSC [86, 96]. The result suggests that DC1 maturation was blocked but that DC1 cells not were transformed into DC2 cells in MLC including MSC.

4.5 *Animal studies including MSC*

In animal models infused MSC differentiate into several cell-types [103-109]. Human and rat MSC engrafted after *in utero* injection, in sheep and chicken, respectively [110, 111]. MSC mainly home to sites of injury [38, 112-114]. In animal models, MSC not only repair mesenchymal tissues as chondrocytes [103] osteocytes [104, 105] cardiac myocytes [106, 115-117], as they also were found to differentiate into neurones [107], and epithelia of multiple organs beside the mesenenchymal linage [108, 109, 113].

MSC secrete cytokines important for haematopoiesis and they also support engraftment of HSC [63, 64, 74, 118-121]. Optimal ratio of HSC and MSC for co transplantation, to support engraftment, might be around 1:10 [122, 123]; whereas, an

excessive dose of MSC might be less efficient [98]. By a shift from the production of pro-inflammatory to anti-inflammatory cytokines at the site of injury and by paracrine effects MSC improves the outcome of renal, neural and lung injury in animal models [109, 124, 125]. MSC improved experimental autoimmune encephalitis (EAE) and decreased the relapse-rate [125, 126]. However, in another mouse model of arthritis, no benefit from MSC treatment was observed [127].

Murine donor dendritic cells [128], but even recipient DC were shown to be strongly involved in GVHD [129], after total body irradiation (TBI). Donor DC were not involved in *Graft-versus-leukemia* (GVL) [128]. That makes them ideal therapeutic target to decrease GVHD without affecting GVL, but DC could perhaps not totally be depleted as they have key roles both in initiation of both up- and down regulation of immune-responses *in vitro* and *in vivo* [130, 131]. *In vitro* results indicate that MSC may direct maturing DC toward a suppressor or inhibitory phenotype, which in mice has been shown to be protective against the development of GVHD [132].

4.6. *MSC therapy against tissue disorders, enzymatic disorders and autoimmunity*

Five children with osteogenesis imperfecta (OI), were treated with a bone marrow transplant from an HLA-identical sibling [133] and later infused with MSC derived from the same donor [134]. A low level of engraftment was observed, particularly when the MSC had been minimally expanded *in vitro*.

Limited data is available on transplantation of MSC across HLA-barriers in the absence of immunosuppression. Mismatched allogeneic fetal liver-derived MSC were transplanted into an immunocompetent fetus in the third trimester of gestation [135]. Engraftment in bone was 0.3-7.4% found by fluorescent *in situ* hybridisation. Immunocompetence of the fetus was confirmed by MLC prior to transplantation. However, alloreactivity against donor MSC was not detected *in vitro* either before or after transplant.

MSC have also been used to correct metachromatic leukodystrophy and Hurler's disease. Four out of five patients showed improvement in nerve conduction velocity [136].

Systemic sclerosis is an autoimmune connective tissue disorder characterized by activation of the immune system. A first report described the feasibility and safety of transplanting bone marrow-derived human MSC from an allogeneic haploidentical-related donor~~s~~ after *in vitro* expansion into a patient suffering from systemic sclerosis [137]. A 41-year-old female with refractory disease including painful ulcerations and rapidly progressive skin sclerosis improved three month post MSC infusion. After six month, almost all ulcerations had healed and the blood circulation improved in the hands and fingers. Both the Rodnan skin score and the visual analogue scale for pain were reduced.

MSC suppressed Collagen II-stimulated T-cell proliferation and activation in a dose-dependent manner without inducing T-cell apoptosis in rheumatoid arthritis patients [138]. In contrast, favourable effects of MSC were not observed in a human multiple sclerosis trial [139].

4.7 *HSCT*

One of 4 siblings inherits identical HLA isotypes, and matched siblings may be optimal donors in HSCT. These donors can be found in around one third of the patients and unrelated donors or partly donors are often used.

Engraftment and initial hematopoiesis monitored by platelet and neutrophil counts frequently during the first month post transplantation. Another way to estimate engraftment is by analyzing the origin of the developing blood cells after HSCT. Donor origin, donor chimerism, of blood cells after HSCT, suggests successful engraftment in contrast to mixed chimerism with mixed origin of the blood cells. Mixed chimerism has been more frequently found after T-cell depletion of the donor graft and after nonmyeloablative conditioning [140].

Conditioning, prior to HSCT, with chemotherapy or/and irradiation is performed for more efficient engraftment. Depending on the cause of leukaemia or other disorders the conditioning is regulated from total elimination of patient blood cells, myeloblative

regimens, to milder reduced regimes. Conditioning agents include fludarabine, busulphan, cyclophosphamide and melphalan. Total body irradiation (TBI) is sometimes used [141-144]. Thymoglobulin (ATG) can be added to eliminate T-cells and decrease the risk of graft-versus-host disease (GVHD).

Even if engraftment is secured, other complication can occur such as GVHD, a complication where the donor cells attack the tissues of the patient. Epithelial cells of the skin and/or the gut are often the main targets. The liver is another target organ. T-cells of the graft, whose receptors have specificity for the allogeneic MHC class I or class II molecules in combination with peptide, attack the host. These alloreactive T-cells are stimulated by recipient and/or APC. Recipient T-cells and APC vice versa can attack the allogeneic donor cells in host versus graft reactions, which may result in rejection and graft failure. Recipient cells normally are sparse in number after myoablative regimes. T-cell depletions of the graft decreases GVHD but may increase the risk of rejection and leukemic relapse [145, 146].

An attack by the graft can be partly favourable, since leukemic cells are attacked, an effect termed graft-versus-leukaemia. Detection of minimal amounts of leukemic cells by real time polychain reaction (RT-PCR) may initiate immunotherapy by donor lymphocyte infusion with the aim to prevent relapse [147]. GVHD after HSCT decreases leukaemia relapse, but can be mortal for the patients if severe and uncontrolled [148]. Immunosuppressive drugs are used to reduce allo-responses. Treatment and prophylaxis of GVHD include immunosuppressive drugs such as cortisone, methotrexate, cyclosporine A, tacrolimus and sirolimus [142, 143, 149, 150].

Infections by bacteria, fungi and virus are common complication after HSCT. Infections are due to pancytopenia, immune suppression and HLA in-compatibility between the donor and the host. Treatment to promote engraftment and in particular to control GVHD, further dampen immune functions. After reduced intensity conditioning (RIC) cytomegalovirus (CMV) infection was more frequently found with unrelated compared to related matched donors [151].

4.8 *Co-transplantation of MSC in HSCT*

Co-transplantation of expanded MSC together with HSC might promote hematologic reconstitution [8, 152]. MSC and HSC derived from an HLA-identical donor were transplanted in 46 patients to promote hematopoietic engraftment and to limit GVHD [153]. MSC engraftment in bone was observed in two out of 19 examined patients at 6 and 18 months post transplant. The incidence of acute GVHD and chronic was not statistically different from controls not receiving MSC. No acute or long-term MSC-associated adverse events were observed.

Parental MSC and HSC were transplanted to 14 children. Compared to HLA-identical transplantation, the risk of graft rejection is higher with haploidentical HSC graft, but co-infusion with MSC improved engraftment, which was well tolerated. No adverse reactions or increased number of infections were observed [154].

Haploidentical MSC were infused into a patient with treatment-resistant severe acute GVHD of the gut and liver, with the aim of harnessing the tissue-reparative effect observed in animal models and the immune modulatory effects seen *in vitro* [46]. Recovery in terms of improved liver values and intestinal function was prompt. Upon discontinuation of cyclosporine, the patient's acute GVHD recurred but was still responsive to a second MSC infusion.

MSC infusion in eight patients, with similar steroid-refractory grades III to IV acute GVHD, was successful [155]. In one patient, DNA from both MSC donors, one haplo-identical and one mismatched unrelated, was detected in colon and lymph nodes of the gastrointestinal tract one month after infusion. In a collaborative effort of five academic centers generating MSC according to a similar culture protocol, 55 patients with steroid-refractory GVHD were treated with MSC infusion [156]. 30 patients showed a complete response to the treatment. Survival two years post transplant was significantly higher in complete responder patients compared to partial and non-responders. Similarly, treatment related mortality one year post transplant was significantly lower in responders.

Third party mismatched MSC, with some exceptions, were used to treat therapy-induced tissue toxicity after HSCT. In 5 of 7

patients, the severe hemorrhagic cystitis cleared after MSC infusion. Two other patients with pneumomediastinum and one patient with perforated diverticulitis and peritonitis improved [157].

5.AIMS OF THE STUDY

- To improve expansion and handling of MSC.

- To characterize surface expression of suppressive or non-suppressive MSC, in different levels of activation.

- To improve engraftment after HSCT, to high-risk patients, with the use of MSC.

6.MATERIALS AND METHODS

The material and methods used in this thesis are described in detail in each article or manuscript.

6.1 *Patients and donors (all papers)*

The studies in this thesis were approved by the Regional Ethics Review Board and complied with the declaration of Helsinki on medical research involving human subjects. All patients and donors gave informed consent to participate in the studies, without financial compensation.

The patients underwent HSCT according to the institutional guidelines and internationally accepted protocols. MSC infusions were performed on the day of HSCT to promote engraftment of hematopoietic cells. Three of the patients underwent a second or third transplant after previous rejection. Two of the other four patients suffered from a severe combined immunodeficiency disorder, as their transplant indication.

6.2 *MSC expansion (all papers) and infusion (papers I)*

MSC were isolated and expanded from bone marrow aspirates taken from the iliac crest. Mononuclear cells were isolated by a gradient centrifugation and plated at a density of 160,000 cells/cm^2 and cultured in medium with 10% FCS. When near confluence, the cells were passaged and replated at 4,000 cells/cm^2 .

For clinical transplantation, when a sufficient number of MSC was achieved, the cells were detached, washed and resuspended in NaCl with 10% AB plasma and given as an intravenous infusion. MSC classification was based on morphology, flow cytometric analysis and the cells' ability to differentiate into bone, fat and cartilage.

6.3 *Expansion improvement protocols (paper II, III)*

To study the effect of freezing mononuclear cells, bone marrow MNC from each donor were divided into two aliquots. One aliquot was directly seeded in culture flasks while the other was frozen in 10% DMSO for one week, thawed and subsequently cultured as described above.

To examine how cell density and FCS concentration affect MSC expansion, MSC derived from three different individuals were cultured under standard conditions until confluence, passaged and replated at 500, 1,000 or 4,000 cells/ cm^2 with either 10% or 20% FSC.

To replace FSC, we expanded MSC in the presence of AB-serum from the blood bank. MNC were isolated from bone marrow aspirates as described above and seeded at 160,000/cm^2 in culture medium with the addition of either 10% FCS or 10%, 5% or 2,5% AB-serum and cultured until passage 6.

6.4 *Cell freezing procedure (all papers)*

MSC and MNC were first stored in +8C^o for 30 minutes without DMSO and then rapidly mixed with freezing media. MNC were frozen at a concentration of 5-40 x 10^6 cells/mL in RPM-1640 with 10% human AB serum and 10% DMSO (final concentration). MSC were frozen at a concentration of 1-5 x 10^6/mL in in cell culture media with 10% FCS and 10% DMSO (final concentration).

6.5 *Mixed lymphocyte cultures (paper II, III, IV)*

Alloreactivity was measured in one-way MLC. Purified PBL were cultured in RPMI-1640 medium with 10% heat inactivated pooled human AB serum. PBL were stimulated with an equal number of irradiated (20 Gy) lymphocytes from one or several donors. To evaluate the immunosuppressive effect of MSC *in vitro*, third-party MSC from one or more donors were added to the wells in concentrations ranging from 0.1-100% of the total number of responder cells.

6.6 *DC1-stimulated lymphocyte experiments (paper IV)*

To generate DC1 stimulators, CD14+ cells were obtained from PBL by negative selection with magnetic beads. Monocytes were cultured for one week in the presence of GM-CSF and IL-4. TNFα was added for the last 24 hours. DC were identified as cells positive for HLA class II, CD83 and CD86 and negative for CD3, CD16, CD19, CD56 and CD14. DC1 phenotype was distinguished by positivity for CD11c. DC1 were added to responder PBL at a 1:10 stimulator-to-responder ratio. To evaluate the suppressive capacity of clonally derived MSC, where cell numbers were limited, MSC were plated in round bottom 96 well plates and cultured for two days. DC1 and responder lymphocytes were then added and lymphocyte proliferation evaluated seven days later by incorporation of radioactive thymidine.

6.7 *Phenotypic characterisation of MSC (all papers)*

Due to the lack of a specific marker, MSC are typically characterised as cells strongly positive for CD73, CD105 (endoglin receptor) and CD90, which are negative for hematopoietic markers. For flow cytometric analysis, MSC were resuspended in PBS and incubated on ice with the respective antibodies. The cells were analysed in a flow cytometer (FACSort) and the data analysed with Cell Quest software.

The advantages of using cell ELISA to study the MSC phenotype, is that minimal manipulation is required (such as treatment with trypsin) and that the cells exist in their natural adherent form when they are analysed. MSC were seeded in flat bottom 96-well plates in MSC media and cultured to confluence. After fixation in formaldehyde and incubation with blocking solution, monoclonal antibodies were added. Mouse IgG served as isotype control. Horseradish peroxidase-conjugated goat anti-mouse IgG was added and absorbance read in a microplate reader.

6.8 *Microarray analysis (paper IV)*

We used the Affymetrix setting to study expression of mRNA in MSC cultured in MLC supernatants. It is a method that does not provide absolute values but indicates differences in gene expression

between compared cell populations. Responder PBL were stimulated with allogeneic DC1. MSC were added alone in transwell inserts and subsequently removed for RNA isolation. Gene expression profile in suppressive and non-suppressive MSC were compared by pair wise comparisons.

7. RESULTS AND DISCUSSION

7.1 *Co-transplantion of HSC and MSC to promote HSC engraftment (paper I)*

Seven transplant recipients were co-infused with MSC. Three of the patients were retransplanted for graft failure and two patients suffered from severe combined immunodeficiency. MSC were derived from HLA-identical or haploidentical related donors. MSC infusion was safe with no side effects. Co-transplantation of MSC resulted in fast engraftment of neutrophils and platelets. All patients achieved 100% donor chimerism by three months. One patient, diagnosed with aplastic anaemia and graft failure after her first transplantation, also suffered from Henoch-Schönlein purpura. After co-transplantation, she recovered from both the Henoch-Schönlein purpura and the aplasia.

A beneficial effect from co-infusion of HSC and MSC remains to be investigated in future prospective trials. Human MSC promote engraftment of human HSC in xenotransplant models [74, 118, 121]. A beneficial effect of MSC infusion in allogeneic transplant is less clear [153] and may be most evident in high risk transplants. Haploidentical HSCT is associated with an increased risk of graft failure. Compared to historic controls, rejection was lower in 14 children co-infused with parent-derived HSC and MSC, corroborating our findings of successful engraftment by co-transplantation even after previous rejection.

7.2 *Effect of freezing and storage on the cell product (paper III)*

Due to the cells rarity *in vivo*, *ex vivo* expansion is necessary to generate the numbers of cells required for a clinical effect. We studied conditions required for optimal function, expansion and reproducibility of MSC products, particularly when indented for immunosuppressive treatments.

Freezing of BM MNC decreased the potential to generate MSC. MSC yield was significantly higher when MNC were plated fresh, rather than frozen thawed marrow. In addition, the immunosuppressive effects and the bone and fat differentiation potentials decreased by freezing MNC in 10% DMSO. In contrast,

freezing already expanded MSC, for as long as several years, had little effect on both cell viability and suppression of MLC and PHA-stimulated lymphocytes. Instead, storage less than 6 months and more than 12 months, respectively, slightly increased the suppressive effect.

Proliferation, cellular characteristics and the final endothelial differentiation were similar in cultivated MSC derived from freshly isolated MNC or cells that had been frozen in 5% DMSO [158]. The composition of cells in the MNC fraction, possibly necessary for a by-stander effect in the initiation of MSC cultures could be altered by MNC freezing, particularly in high concentrations of DMSO. CD34+ cells have superior viability if cryopreserved in 5% rather than 10% DMSO [159].

Minimal *ex vivo* manipulation is preferred for cells indented for clinical use. However, passage number did not affect the degree of MLC suppression.

The degree of suppression induced by MSC in MLC is variable and may depend on the degree of mismatch between responder and stimulator lymphocytes but, likely, also on currently unknown mechanisms inherent to the MSC. Pooling of MSC from two or three donors generated higher and more stable suppression both in MLC and in PHA-stimulated cultures. Higher and more stable suppression was observed even when using combinations of weakly suppressive MSC.

7.3 *Expansion protocol improvements (paper III)*

We examined how replating density and FCS concentration affected MSC expansion. At first passage, MSC derived from three different individuals were re-plated at different densities in the presence of 10% or 20% FCS. Plating MSC at low density or increasing FCS to 20%, during passage 1 and 2, only improved cell yield to a low degree. Low re-plating density in combination with higher FCS increased cell-yield, though. Plating 1,000 MSC/cm2 in passage 1 and then 500 MSC/cm2 in passage 2, reached the highest cell-yield. Low re-plating densities may result in reduced contact inhibition (regulated by wnt-signalling), promoting expansion. Further, several cell divisions can occur without interruption by trypsin treatment and re-plating and culture-promoting substances

may accumulate in the culture media. However, initial plating at very low density (500/cm^2) resulted in cell death.

Intra-venous infusion of MSC generated in the presence FCS has so far been safe, without toxic side effects, but it is still unclear whether unfavourable immune responses toward FCS can occur [45, 53]. Other possible risks with the use of FCS include and bacterial infections and prions [160-162]. For these reasons reasons, alternative culture protocols replacing FCS with platelet lysate, serum free media or autologous serum have been suggested [56, 163-165].

We expanded MSC in the presence of AB-serum and cultured the cells according to our standard protocol. Proliferation of MSC in early passages was increased in the presence of 10% AB-serum compared to FCS, while lower concentrations of AB-serum (5% or less) were not sufficient to support expansion over several passages.

Both AB-serum and FCS generated MSC differentiated *in vitro* into fat and bone and exhibited similar morphology. Phenotypic expression of typical MSC markers and HLA class I was similar. HLA class II expression was higher in MSC generated in AB-serum. When cultured beyond passage 6, AB-generated MSC senesce to a higher degree compared to MSC cultured in FCS. One possible explanation for the increased HLA class II expression in AB-serum generated MSC could be cell-stress due to a partial lack of nutrients required for stable expansion. In later studies, adding platelet lysate to promote expansion, a similar increase in HLA class II has not been observed in our hands. However, AB-serum remains a reliable and safe additive for the generation of large numbers of cells in low passage for clinical use.

7.4 *Characterisation of MSC with different immunosuppressive capacity (paper IV)*

The mechanisms underlying the MSC suppressive effect are not well defined. When selecting MSC for immunosuppressive therapy, it would be helpful to have well defined markers relating MSC characteristics to their immunosuppressive function.

We compared phenotype, growth characteristics and gene arrays

of MSC with differing immunosuppressive capacity *in vitro*, derived from two donors. MSC from one donor showed consistent suppression in MLC and the other little or no suppression and sometimes stimulation. Under resting conditions, the expression of HLA class I and II was slightly higher on adherent MSC derived from the less suppressive donor. When exposed to DC1-stimulated lymphocytes, adherent MSC increased their cell surface expression of vCAM1, iCAM-1 and PDL-2 but no differences in expression were found between cells from the two donors. Only the increase in expression of HLA class II was distinctly higher in the non-suppressive than in the suppressive MSC. A fold change more than 2 was found for 734 probe sets with increased and 444 with decreased expression in MSC from the suppressive donor. We decided to focus on the most significant expression of immune response genes and in particular on surface markers.

We found genes involved in Toll-like receptor (TLR) signalling [166-168] higher expressed in the suppressing MSC of donor 1 origin. TLR signalling induces maturation of DC for enhanced efficiency in antigen presentation. The mediators then play a crucial role in the organization of acquired immunity and, together with matured DC, activate lymphocytes [169]. We also found genes involved in antigen presentation to be distinctly expressed on the poor suppressor polyclonal MSC from donor 2. HLA DR and class II transactivator (CIITA) [170], and genes of the complement cascade [171-178] were clearly higher expressed in MSC from the non-suppressive donor 2 the be expression of the suitable alpha or beta subunits were expressed below our criteria. The expression of HLA class II (DP, DQ, DR), were the only epitopes that were further analysed on the protein level as the number of cells was limited.

A role in APC by HLA class II on MSC is assumed to be limited as APC is so complex [179]. Our results uniformly show that MSC from donor 2, expressing more HLA class II, are less suppressive. DC1 and PBL were different for each experiment, suggesting a limited role of HLA class II on MSC during alloreaction, as a match to the responders are crucial for an effect.

To investigate if the degree of suppression is intrinsic to the MSC donor of origin, single cell derived colonies were cultured

from the suppressive donor. While one clone suppressed lymphocyte proliferation to a degree similar to that of polyclonal MSC from the same donor, the other clone were stimulatory. Again, differences, although small, between the two clones became most apparent when the cells were exposed to activated lymphocytes. Compared to resting condition the stimulating clone distinctly increase its expression of iCAM-1 and the suppressive clone its expression of CD90 and CD105. Expression of iCAM-1, CD90 and CD105 on single cell clones exposed to activated lymphocyte, remained clearly different.

8.CONCLUSIONS

- MSC improved engraftment in high risk patients after HSCT, even after regraftment.

- Human blood group AB serum, was not affecting characteristics and immune suppressive functions of mesenchymal stem cells, but slightly improved expansion compared to FCS.

- Number of passages or length of storage had minor effects on MSC, in our set-ups.

- Mixing of 2-3 MSC improved suppression.

- Cryopreservation of bone-marrow mononuclear cells, our MSC source, decreased the final MSC yield.

- A lower replating density and higher FCS level improved cell-yield of MSC, after 2 passages.

- The immune modulatory actions of MSC probably include alternative mechanisms – monitored by surface expression, in MLC.

9. PERSPECTIVES

The observation that infusion of MSC may have immunomodulatory effects *in vivo* has led to excitement over the potential use of MSCs in the clinic, such as in the treatment of acute GvHD, organ allograft rejection and autoimmune disorders.

MSC populations are heterogenic, even after clonal expansion (Paper IV), but future research may find functionally distinct sub-populations of cells with defined *in vitro* characteristics that can selected for specific therapies. A correlation between such characteristics in laboratory testing, and a clinical effect from MSC infusion also needs to be established.

Development of MSC as a clinical therapy requires product standardization, access to GMP facilities and possibilities to upscale to multicenter studies. Optimal handling of the cells must be defined to assure that cell products of reproducible quality are generated. Future improvements in the handling of the cells may include some of our here presented methods.

10. POPULÄRVETENSKAPLIG SAMMANFATTNING

Mitt forskningsområde handlar om så kallade mesenkymala stam eller stromal celler, som jag här förkortar till MSC-celler och bara till MSC i avhandlingen i övrigt (se ”overview of the subjects of my thesis”, före sidan 1). Dessa MSC-celler kallas även bindvävsstamceller. MSC-celler finns dock mer än i bindväv och vi använder MSC-celler från benmärg. I benmärg ingår MSC-celler i en vävnad som stimulerar och skyddar blodcellsbildningen. MSC-celler är så kallat multipotenta därför att de kan utvecklas till mer specialiserade celler som ben-, fett- och broskceller, i detta fall. MSC-celler har i olika syften transplanterats till patienter. Detta har rapporterats ge goda effekter i flertalet sjukdomar. MSC-celler har visats ha oanade förmågor. Som exempel har patienter efter MSC-cellstransplantation, fått starkare benstruktur i svåra genetiska bensjukdomar. De som lider av autoimmuna sjukdomar har i vissa fall också förbättrats, med MSC-cellstransplantation. MSC-celler vandrar till skadade områden i kroppen och anpassar sig till omgivningen, utan att själv stötas bort av immunsystemet. MSC-celler antas ha en förmåga att slå på en utsöndring av faktorer som kan bidra till att förbättra en del sjukdoms- och bristtillstånd. Patienter med vissa typer av enzymbrister, har till exempel visats bli kompenserade med en transplantation av MSC-celler.

Vår specifika forskning berör den immundämpande förmågan, som MSC-celler dessutom visats sig ha. Cellerna är ibland mer effektiva än många immundämpande mediciner som patienter har fått efter benmärgstransplantation. Benmärgstransplantation ger man till exempel för att bota mycket allvarliga blodsjukdomar, som leukemi. Benmärgs- eller rättare sagt blodstamcellstransplantation, ges då för att byta ut felaktiga blodceller i patienten. Blodstamcellerna måste sedan etablera sig i patienten och börja omvandla sig till alla olika immunceller, som behövs i kroppen. Eftersom blodstamceller är stamceller, självförnyar de sig samtidigt i denna process. Immuncellerna ska kunna ge ett försvar mot infektioner av främmande partiklar och organismer (immunitet) och samtidigt tolerera patienten (tolerans).

Ett fokus i mina MSC-cellsstudier har varit att öka förutsättningarna för blodstamcellerna att etablera sig i patienten

och bilda immunceller. Patienter fick MSC-celler tillsammans med blodstamcellerna, i svårbehandlad leukemi, och resultatet blev väldigt lyckat. De transplanterade blodstamcellerna hade i tidigare försök, utan MSC-celler, ej lyckats etablera sig i flera av patienterna. I denna forskning anses MSC-celler ha en dos-beroende effekt, så att fler MSC-celler ger en bättre effekt. Vi har därför även utvecklat odlingen, så att vi kan odla MSC-celler 3 gånger snabbare än tidigare. Vi har också funnit att frysning och odlingslängd inte förändrar MSC-cellernas funktion negativt.

För övrigt har vi preliminärt kommit en liten bit på vägen till att i framtiden kunna välja ut, de allra mest immunhämmande MSC-cellerna. Vi har vidareutvecklat en immunologisk experiment modell i laboratoriet, som på sätt och vis mer liknar vad som sker i patienten vid en blodstamcellstransplantation, genom att inkludera strålade MSC-celler. Sedan har vi först studerat två mycket olikt immundämpande MSC-celler i denna modell. Därefter har vi sått ut celler från den mest immunhämmande MSC-cellen, en och en, och låtit dessa mångfaldiga sig i antal i laboratoriet. Dessa så kallat klonalt expanderade MSC-celler, har sedan inkluderats i vår experiment modell. Resultaten av våra studier visar på tendenser att MSC-celler är olika, och troligtvis reglerar immunresponser på olika sätt, även om de kommer från samma människa. Många pusselbitar fattas ännu, men vi hoppas att vi i framtiden kan göra behandlingen med hjälp av MSC-celler, ännu mer användbar och effektiv.

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