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IMMUNE MODULATION BY MESENCHYMAL STEM CELLS

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Var inte rädd för mörkret
ty ljuset vilar där.
Vi ser ju inga stjärnor
där intet mörker är.

Erik Blomberg, 1920

CONTENTS

1 SUMMARY

Mesenchymal stem cells (MSCs) were discovered as adherent cells in the bone marrow stroma that could form bone and stimulate hematopoiesis. Later, MSCs were shown to be hypoimmunogenic and to suppress proliferation of activated T cells. Cytotoxic T lymphocytes (CTLs) constitute important effector cells of the immune system, but can also cause severe tissue destruction. The first article in this thesis shows that MSCs suppressed activation of CTLs against allogeneic peripheral blood mononuclear cells (PBMCs). MSCs did not affect the lysis performed by already activated CTLs. PBMCs but not MSCs were lysed by CTLs although both target cells were derived from the same individual. Additionally, MSCs potentiated NK-cell mediated lysis of K562, and were resistant to lysis by NK cells, despite a KIR-ligand mismatch.

Cytokines are important mediators in immune signaling. MSCs increased the levels of interleukin-2 (IL-2), IL-2 Receptor, and IL-10 in mixed lymphocyte cultures (MLCs), while the levels decreased in mitogen-stimulated cultures, as presented in paper II. Inhibition of prostaglandin E_2 synthesis partially restored proliferation in mitogen-stimulated cultures inhibited by MSCs, but not in MLCs. These results indicate possible different mechanisms of inhibition by MSCs after mitogenic and allogeneic stimulation of PBMCs. MSCs also suppressed phorbol myristate acetate activation of PBMCs, indicating that MSCs exert the suppressive function downstream of the receptor level.

MSCs stimulated the production of immunoglobulin G (IgG) by splenic mononuclear cells (MNCs) and enriched B cells. This stimulation by MSCs was mediated by soluble factors when MNCs were used as responder cells. In contrast, when enriched B cells were cocultured with MSCs, cell-cell contact was required for increased IgG production. MSCs did not induce proliferation of splenic MNCs. When MSCs were added to stimulated MNCs, they both stimulated and inhibited IgG production induced by lipopolysaccharide, cytomegalovirus or varicella zoster virus, depending on the degree of stimulation.

The resistance to lysis of MSCs reported in paper I, was explored further using alloreactive and peptide-specific CTL clones in paper IV. MSCs were resistant to lysis compared to other cells with similar expression of HLA class I. MSCs as target cells generated only weak tyrosine phosphorylation in CTLs. Furthermore, CD25 upregulation and CD3 and CD8 downregulation were minimal. We also showed that MSCs failed to induce TNF-α and IFN-γ production by the CTLs.

Based on the *in vitro* results on MSC-induced inhibition of T cells and preliminary clinical studies, we transplanted haploidentical MSCs to a patient with severe treatment-resistant grade IV acute graft-versus-host disease (GVHD) of the gut and liver. Clinical response was striking, with rapidly decreased liver enzymes and reduced diarrhea. Biopsies indicated possible engraftment of MSCs in the intestine. *In vitro* data showed no sign of immunization by the MSCs and a second infusion was given with similar positive results. This case encourages prospective, controlled studies with MSCs for prophylaxis and treatment of GVHD.

2 LIST OF PUBLICATIONS

I **Mesenchymal stem cells inhibit the formation of cytotoxic T lymphocytes, but not activated cytotoxic T lymphocytes or natural killer cells**
Rasmusson I, Ringdén O, Sundberg B and Le Blanc K
Transplantation 2003;76(8):1208-13

II **Mesenchymal stem cells inhibit lymphocyte activation by mitogens and allogens by different mechanisms**
Rasmusson I, Ringdén O, Sundberg B and Le Blanc K
Experimental cell research 2005; 305: 33– 41

III **Mesenchymal stem cells induce IgG production by human B cells**
Rasmusson I, Le Blanc K, Sundberg B and Ringdén O
Manuscript 2005

IV **Mesenchymal stem cells fail to trigger effector functions of cytotoxic T lymphocytes**
Rasmusson I, Uhlin M, Le Blanc K and Levitsky V
Manuscript 2005

V **Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells**
Le Blanc K, Rasmusson I, Sundberg B, Götherström C, Hassan M, Uzunel M and Ringdén O.
Lancet 2004; 363 (9419): 1439-41

3 LIST OF ABBREVIATIONS

APC	Antigen Presenting Cell
BM	Bone Marrow
CD	Cluster of Differentiation
CFU-F	Colony-Forming Unit-Fibroblast
CML	Cell-Mediated Lympholysis
CMV	Cytomegalovirus
CNS	Central Nervous System
ConA	Concanavalin A
COX	Cyclooxygenase
CSF	Colony-Stimulating Factor
CTLA	Cytotoxic T Lymphocyte-Associated antigen
DC	Dendritic Cell
EBV	Epstein Barr Virus
ELISA	Enzyme-Linked Immunosorbent Assay
ELIspot	Enzyme-Linked Immunospot
ESC	Embryonic Stem Cell
FBS	Fetal Bovine serum
FISH	Fluorescence In Situ Hybridization
GFP	Green Fluorescent Protein
GVHD	Graft-Versus-Host Disease
GVL	Graft-Versus-Leukemia
HLA	Human Leukocyte Antigen
HSC	Hematopoietic Stem Cell
HSCT	Hematopoietic Stem Cell Transplantation
IDO	Indoleamine 2,3-Dioxygenase
IFN	Interferon
IL	Interleukin
IgG	Immunoglobulin G

KGF	Keratinocyte Growth Factor
KIR	Killer Immunoglobulin-like Receptor
LCL	Lymphoblastoid Cell Line
LFA	Leukocyte Functional Antigen
LPS	Lipopolysaccharide
MAPC	Mesodermal Adult Progenitor Cell
MLC	Mixed Lymphocyte Culture
MNC	Mononuclear Cell
MSC	Mesenchymal Stem Cell
NK cell	Natural Killer Cell
NOD/SCID	Non-Obese Diabetic / Severe Combined Immunodeficient
OI	Osteogenesis Imperfecta
OPG	Osteoprotegerin
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase Chain Reaction
PD	Programmed Death
PD-L	Programmed Death Ligand
PGE_2	Prostaglandin E_2
PHA	Phytohemagglutinin
PKC	Protein Kinase C
PMA	Phorbol Myristate Acetate
RANK-L	Receptor Activator for NFκB-Ligand
SCF	Stem Cell Factor
SDF	Stromal cell-Derived Factor
TCR	T-Cell Receptor
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor
VZV	Varicella Zoster Virus

4 INTRODUCTION

MESENCHYMAL STEM CELLS

Stem cells are defined as unspecialized cells that have the capacity to differentiate into other cell types as well as to continuously self renew. The totipotent fertilized oocyte gives rise to pluripotent embryonic stem cells (ESCs), which in turn form multipotent cells. Multipotent stem cells have been isolated from most postnatal tissues, where the hematopoietic stem cells (HSCs) are the most studied. HSCs reside in the bone marrow (BM) and provide a continuous source of progenitors for erythrocytes, platelets, monocytes, granulocytes, and lymphocytes. BM also contains non-hematopoietic cells referred to as mesenchymal stem cells (MSCs) or marrow stromal cells. MSCs are multipotent, adherent cells that reside in the stroma and can produce stromal components, such as collagen, fibronectin, laminin and proteoglycans.[1-3] Because of the current inability to isolate MSCs prospectively, due to their rarity *in vivo*[2] and lack of characteristic markers, existing data are based on studies performed on cells expanded *in vitro*.

MSCs were first recognized in the late 1960s by Friedenstein et al., who identified an adherent, nonphagocytic, fibroblast-like population that could regenerate rudiments of normal bone *in vitro* and *in vivo*.[4-7] They cultured whole BM and analyzed the adherent cell populations. He reported that the small number of adherent cells was heterogeneous in appearance. After several passages in culture, the adherent cells became more homogenously spindle-shaped. MSCs had the ability to differentiate into colonies that resembled small deposits of bone or cartilage. This isolation method based on the adherence of fibroblast-like cells to plastic, and a lack of adherence of hematopoietic cells, is still the standard protocol to isolate BM-derived MSCs. The precursors of MSCs were initially referred to as colony-forming-unit fibroblasts (CFU-F), because they readily adhered to culture dishes and formed fibroblast-like colonies.[8]

Characterization of MSCs

MSCs have been isolated from most non-human species such as mouse,[9,10] rat,[11] cat,[12] dog,[13] guinea pig,[14] rabbit,[15] pig,[16] cow,[17] horse,[18] and baboon.[19] Apart from BM, MSCs have also been found in other postnatal human tissues such as adipose,[20] placenta,[21] and scalp tissue,[22] as well as in various fetal tissues.[23-28] MSCs are stable cells, that can be expanded *in vitro* for many cell doublings without loss of phenotype and without showing signs of karyotype changes.[2] Alterations in phenotype have so far been discovered first after approximately 40 cell doublings, at which time the cells finally became broader and flattened before degenerating.[3,29,30]

Whether MSCs are true stem cells remains a matter of debate. Human MSCs *in vitro* are tri-differential, with a capacity to form bone, cartilage and fat.[31,32] *In vitro* studies have also shown that MSCs can form myocytes,[33-36] hepatocytes,[37] endothelium[38] and cells of the central nervous system.[39-41] The cell plasticity of MSCs is controversial. Plasticity refers to the proposed ability of adult stem cells to cross over lineage barriers

and to adopt the expression profiles and functionality of cells unique to other tissues. Most studies base the conclusions on gene profiling, protein expression and phenotype. Proofs of functional effects are often absent.

Progenitor cells for MSCs

Is there a progenitor cell for MSCs? Verfaillie et al. described the isolation and *ex vivo* expansion of cells from human BM that differentiated into bone, cartilage and fat, as well as endothelium, myocytes, hematopoietic-supportive stroma[42] and hepatocyte-like cells.[43] These mesodermal adult progenitor cells (MAPCs) were isolated using magnetic beads to deplete white ($CD45^+$) and red (glycophorin-A^+) hematopoietic cells. The $CD45^-$ $GlyA^-$ cells constituted 0.1% to 0.5% of BM mononuclear cells (MNCs), and when cultured on fibronectin with epidermal growth factor, platelet-derived growth factor BB, and 2% or less fetal bovine serum (FBS), 0.02% to 0.08% of those cells gave rise to adherent clusters.[42] MAPCs differ in surface expression from MSCs, predominantly lacking human leukocyte antigen (HLA) class I and only showing a low expression of CD44. The expression of these antigens increased with increased serum concentration, or when the MAPCs were cultured on type IV collagen or laminin instead of fibronectin. Differentiation to skeletal muscle and endothelium required that the cell population was CD44 and HLA class I negative.[42] In addition, the pluripotency of MAPCs was confirmed *in vivo*, where single murine MAPCs transplanted into mouse blastocysts contributed to most tissues and organs, including cell types in the central nervous system.[44]

Conget et al. found a small population of quiescent cells within their mesenchymal progenitor population.[45] MNCs were isolated from human BM and cultured in 20% FBS, that after one passage was decreased to 10%. After elimination of proliferative cells, a discrete population (5-20%) of quiescent uncommitted and undifferentiated MSCs remained. These cells needed to be activated by FBS for proliferation and differentiation. Infusions in mouse showed engraftment of quiescent MSCs in BM, spleen, bone and skeletal muscle, whereas expanded MSCs were only detected in BM and spleen.

Marrow-isolated adult multilineage inducible cells (MIAMI cells) were characterized by D'lppolito et al. in Miami, USA. [46] These cells were small adherent cells when cultured on fibronectin in low oxygen tension (3% compared to around 20%) and 2% FBS. The cells expressed numerous markers found among ESCs as well as mesodermal-, endodermal- and ectodermal-derived lineages. However, these cells have not been further characterized.

Engraftment of MSCs

A harsher definition of stem cells is the *in vivo* capacity to regenerate or maintain a tissue compartment at a single-cell level as well as to be transplanted, isolated, and re-transplanted into multiple generations of recipients. Friedenstein et al. showed *in vivo* differentiation of clones of MSCs.[47] Liechty et al. have shown engraftment and

differentiation of human adult MSCs after *in utero* transplantation in sheep.[48] Baboon BM-derived MSCs transduced with green fluorescent protein (GFP) were infused into baboons following lethal total body irradiation and hematopoietic support.[49] Engraftment was analyzed up to 21 months after infusion using polymerase chain reaction (PCR), with the highest engraftment in gastrointestinal tissue. Kidney, lung, liver, thymus and skin also showed MSC engraftment. MSCs derived from other non-human species have demonstrated engraftment in various tissues, such as bone, BM and heart.[19,50,51] Most studies have only been able to find a small fraction of the implanted cells after days or weeks. The presence of small numbers of cells, usually detected by fluorescence in situ hybridization (FISH), PCR, or labeling of implanted cells, has been stated as evidence of specific homing of MSCs. Whether the engraftment is due to specific homing or lodgment of MSCs is unknown.[52,53]

It is doubtful if all MSCs are truly multipotent stem cells. Clonal analyses of MSCs revealed a heterogeneous population of cells, with varying differentiation potential and expansion capacity.[2,7,30] Cell fusion has been suggested as an explanation for *in vivo* differentiation. Spees et al. showed that a subset of human MSCs that was cocultured with damaged epithelial cells rapidly differentiated into epithelium-like cells. [54] The MSCs acquired a morphology similar to that of the epithelial cells and began to express keratin and other epithelial markers. However, up to 1% of the MSCs were recovered as bi-nucleated cells expressing an epithelial surface epitope. Another study demonstrated that differentiation of human MSCs into hepatocytes in rat liver was not due to cell fusion, since both human and rat chromosomes were independently identified by chromosomal analysis.[55]

In vivo potential of MSCs

The *in vivo* differentiation capacity of MSCs was shown in animal studies. Osteogenic differentiation to create new bone in bone defects was demonstrated in the athymic rat implanted with human MSCs on a ceramic carrier, leading to significantly stronger bone.[56] Positive effects were also seen in a canine model using autologous MSCs.[56,57] Autologous culture-expanded MSCs were demonstrated to regenerate cartilage defects and repair Achilles tendon ruptures in rabbit models.[15,58] Human marrow-derived fibroblasts only differentiated into osteogenic cells in association with a carrier material containing calcium phosphate when transplanted into immuno-compromised mice, in contrast to mouse-derived cells that could form bone *in vivo* with several carrier materials.[59] This indicates possible species-specific differences concerning *in vivo* bone formation. Due to possible xenoreactivity[60] and a lack of human tissues, most *in vivo* studies use MSCs from the same species as the model animal.

MSCs are believed to be of therapeutic value in cardiac regenerative medicine. Human MSCs *in vitro* differentiated into cardiomyocyte-like cells.[34] Animal-derived MSCs have demonstrated cardiomyocyte specific features *in vivo* and improved heart function.[61-64] Zhao et al. showed that human MSCs were grafted into the cortex surrounding the area of infarction one week after experimental stroke in rats

concomitant with significantly improved functional performance.[65] In a clinical trial, 69 patients who underwent primary percutaneous coronary intervention within 12 hours after onset of acute myocardial infarction, were randomized to receive intracoronary injection of autologous culture-expanded MSCs or saline. Imagining techniques demonstrated that MSCs significantly improved left ventricular function.[66] A small clinical study also showed positive effects of cell transplantation. Patients who underwent transcoronary transplantation of both MSCs and endothelial progenitors experienced significantly better wall motion, contractility and scar healing.[67] Tang et al. reported that infusion of MSCs lead to increased levels of angiogenic factors in the heart, decreased proapoptotic protein expression in ischemic myocardium and increased capillary density, in a model of myocardial infarction.[68] A similar study ruled out transdifferentiation of MSCs into cardiomyocytes and increased vascularization,[69] hence the mechanism for improvement of cardiac function remains elusive.

MSCs may have potential in healing of injured tissue. Using a human-sheep *in utero* xenotransplantation model, Mackenzie et al. demonstrated increased localization of human MSCs at the site of wound healing.[70] This tendency of MSCs to migrate towards injured areas and a possible role in enhanced healing was demonstrated in other studies as well; in chronic rejection of heart allo-grafts (rat MSCs),[71] spinal cord injuries (rat MSCs),[72] lung-injury after bleomycin exposure (mouse MSCs),[73] neurodegenerative lysosomal storage disorders (mouse MSCs),[74] traumatic brain injury (rat MSCs)[75] and experimental liver cirrhosis (rat MSCs).[76]

Transplantation of MSCs demonstrated promising results in clinical studies. A human study with three patients suffering from large bone defects showed improved healing after autologous MSCs were placed in macroporous hydroxyapatite scaffolds, and implanted in the wound site along with external fixation.[77] MSCs placed in a calcium hydroxyapatite ceramic scaffold also resulted in improved motor function when transplanted into the knee of a patient with a large osteochondral defect. Biopsies of the repaired tissue revealed cartilage-like regeneration and bone formation.[78] Horwitz et al. have performed transplantation of allogeneic BM in children with the genetic disorder osteogenesis imperfecta (OI).[79,80] Donor osteoblast engraftment was detected and histological changes indicated new dense bone formation. All patients had increases in total body bone mineral content. These improvements were associated with increases in growth velocity and reduced frequencies of bone fracture. The same group also used gene-marked MSCs to treat six children who had undergone standard BM transplantation as treatment for severe OI. The MSCs were derived from the same donor as the BM. All patients received two infusions, five of six patients showed engraftment in one or more sites, including bone, skin, and marrow stroma, and showed an acceleration of growth velocity.[81] Le Blanc et al. recently transplanted human male fetal liver-derived MSCs *in utero* to improve the condition of a female fetus with multiple intrauterine fractures, diagnosed as severe OI. After birth, engraftment of donor cells was observed in bone and the patient has had fewer fractures than anticipated.[82]

Several studies have shown positive effects, without detection of engrafted MSCs. Zhao et al. proposed that MSCs exert a positive effect by soluble factors, after transplantation of human MSCs into a stroke rat-model, and saw functional recovery but no neural phenotype of the transplanted cells.[65] MSCs may secrete important factors in healing, rather than reconstituting the repaired tissue. For example, bone damage yield signaling substances including bone morphogenic proteins (BMP), transforming growth factor-β (TGF-β), insulin-like growth factor-1 (IGF-1) and basic fibroblast growth factor (bFGF), which can recruit cells that participate in tissue repair.[83-87]

MSCs and hematopoiesis

MSCs express surface molecules that can interact with cells of the hematopoietic lineage, including intercellular adhesion molecule (ICAM-1, CD54), ICAM-2 (CD102), vascular cell adhesion molecule 1 (VCAM-1, CD106), lymphocyte function-associated antigen 3 (LFA-3, CD58), activated leukocyte cellular adhesion molecule (ALCAM, CD166), hyaluronate receptor (HCAM, CD44) and integrins, such as very late antigen (VLA, CD49).[2,3,88] Stimulation of CD44 were shown to increase the colony formation of $CD34^+$ stem cells.[89] Simmons et al. demonstrated that adhesion of $CD34^+$ cells to cultured allogeneic MSCs was largely inhibited by both monoclonal antibodies to VLA-4 and to its ligand VCAM-1.[90] These studies indicate the importance of interactions between hematopoietic cells and stroma.

At the same time as MSCs provide physical support for HSCs, they constitutively secrete cytokines important for HSC differentiation, including Interleukin-1 (IL-1), IL-6, IL-7, IL-8, IL-11, IL-12, IL-14, IL-15, IL-27, leukemia inhibitory factor (LIF), FMS-like tyrosine kinase-3 (Flt-3) ligand, stem cell factor (SCF), macrophage colony stimulatory factor (M-CSF), granulocyte-CSF (G-CSF) and GM-CSF.[91-95] When cocultured with hematopoietic progenitors *in vitro*, MSCs have the capacity to maintain and expand lineage-specific colony-forming units from $CD34^+$ marrow cells in long-term BM cultures.[88] Whole cell-binding assays with MSCs and hematopoietic cells showed that T cells bound MSCs with higher affinity than did B cells or myeloid cells. In coculture experiments, MSCs provided key signals to stimulate megakaryocyte and platelet production from $CD34^+$ hematopoietic cells.[96]

In addition to providing critical cell–cell contact and producing growth factors for hematopoiesis, MSCs may also attract infused HSCs to the marrow. Peled et al. assayed the influence of stromal cell-derived factor-1 (SDF-1) in recruiting $CD34^+$ cells to the marrow in a NOD/SCID model of human hematopoiesis. SCF and IL-6 induced expression of the SDF-1 receptor CXCR4 on $CD34^+$ cells, which potentiated migration towards SDF-1.[97] Activation of $CD34^+$ cells by SDF-1 led to adhesion and transendothelial migration by activation of various adhesion molecules.[98] The importance of SDF-1 and homing was also strengthened by a report of decreased SDF-1 levels in BM after treatment with granulocyte-colony stimulation factor (G-CSF).[99]

Stromal cells are also essential for lymphopoiesis. Early B cells adhered to stromal cells, and differentiation did not occur when lymphocytes and stromal cells were separated in a diffusion chamber system.[100,101] A murine study showed that stromal cells prolonged the survival of plasma cells and potentiated antibody secretion by IL-6 and VLA-4 interactions.[102] Murine thymocytes plated onto a BM stromal culture displayed differential sensitivity for adherence. The highest affinity was seen for the double-negative T cells ($CD4^-CD8^-$), and to a lower extent the double-positive cells. Single positive $CD4^+$ or $CD8^+$ populations did not show significant binding to stromal cells.[103] Prolonged culturing resulted in production of replicating immature T cells, suggesting that BM stroma may function as an extrathymic site of T-cell maturation. A murine study showed evidence of stromal-cell migration from bone grafts to the thymus. Donor-type BM stromal cells existed in the thymus of mice that received BM and bone grafts but not in the mice that received BM cells alone. The T cells of such mice showed donor-type HLA restriction.[104] There are conflicting results exist on whether stromal components acquire donor-genotype after allogeneic hematopoietic stem cell transplantation (HSCT). A pioneering study showed donor-derived stroma after HSCT,[105] corroborated by a report of low mixed chimerism in the stroma after extensively T-cell-depleted HSCT.[106] High donor engraftment in stroma was shown by transplantation of bone fragments intraperitoneally and directly into bone on day 0 of the non-myeloablative BM transplantation, in a clinical report of three patients.[107] Contradictory to this, others only detected host-derived stromal cells after HSCT.[108-110] Selective analysis of the MSC fraction of BM stroma, demonstrated that MSCs remain host-derived after HSCT.[111]

Co-transplantation of MSCs with HSCs was reported to enhance HSC-engraftment. Human *ex vivo* expanded fetal lung-derived MSCs co-transplanted with human $CD34^+$ cells isolated from cord blood injected into NOD/SCID mice demonstrated a 10 to 20 fold increase in engraftment as determined by human $CD45^+$ cell expression when compared to transplantation of the isolated $CD34^+$ cells alone.[25] Co-transplantation of human MSCs with $CD34^+$-selected HSCs enhanced myelopoiesis and megakaryocytopoiesis in NOD/SCID mice, when a limited dose of $CD34^+$ cells was administered.[112,113] Koc et al. infused autologous MSCs after myeloablative therapy of breast cancer patients receiving autologous peripheral-blood progenitor-cells.[114] There were no adverse effects after delivery of MSCs. The hematopoietic engraftment was rapid, although a control group of patients not receiving MSCs was not included.

IMMUNE SUPPRESSION BY MSCs

BM-derived adult MSCs have been shown both *in vivo* and *in vitro* to suppress activation of T cells. The *in vivo* role of this will remain purely speculative until a method to selectively knock out MSCs is developed. It may possibly be a way for the body to maintain homeostasis and inhibit immune activation in distinct compartments, such as the BM or the fetal/maternal interface. MSCs modulate the immune function of the major cell populations involved in alloantigen recognition and elimination, including antigen presenting cells (APCs), T cells, and natural killer (NK) cells. The

molecular mechanism mediating this immunosuppressive effect of MSCs is not completely understood.

MSCs and allogeneic recognition

An emerging body of data indicates that MSCs escape recognition of alloreactive cells, or at least possess a hypo-immunogenic character,[32,94,113,115,116] even when co-stimulatory CD28 signals were delivered.[117] Human and rat MSCs did not elicit interferon-gamma (IFN-γ) production by human PBMCs, whereas human and murine fibroblasts did.[113] This allogeneic escape mechanism may be of therapeutic value, since transplantation of allogeneic MSCs in stock would be readily available, compared to culture of autologous MSCs or MSCs from related donors for each patient. An *in vitro* study suggested a stronger immunosuppressive effect of allogeneic MSCs compared to autologous cells.[118] Klyushnenkova et al. saw a significant proliferation in response to allogeneic MSCs that peaked on day 8, compared to allo-reactions against PBMCs that peaked on day 6. Still, the response was never greater than 40% of the response against PBMCs.[119] No proliferative response was left after removal of cells expressing HLA class II, CD14 and CD19. HLA expression show variations in different studies, but MSCs are generally believed to express HLA class I and can be induced by IFN-γ to up-regulate HLA class II. Nevertheless, Krampera et al. used murine MSCs devoid of both HLA class I and II,[120] while Potian et al. used human MSCs that expressed both class I and II.[94] Neither population showed immunogenic potential and both could inhibit immune responses. This questions the importance of HLA expression on MSCs for immune suppression. Furthermore, up-regulation of HLA class II by IFN-γ, still did not elicit a proliferative response.[32,94,117,119]

In vitro suppression by MSCs

MSCs have shown to suppress lymphocyte proliferation induced by allo-antigens in mixed lymphocyte cultures (MLCs),[94,115-117] mitogens, such as phytohemagglutinin (PHA),[116,121,122] concanavalin A,[116,123] and tuberculin,[113] as well as activation of T cells by CD3 and CD28 antibody stimulation[95,117,120] in a dose-dependent mode. Interestingly, a low concentration of MSCs or MSC-culture supernatants have shown to stimulate rather than inhibit MLCs.[94,113,116] The suppression by MSCs was greatest when added at the beginning of the MLC, but the MSCs also showed effect when added later.[119,121] Murine MSCs have been reported to inhibit the activation of T cells by a profound inhibition of cyclin D2 as well as induced upregulation of the cyclin dependent kinase inhibitor p27kip1.[124] Without activation of cyclin D, the T cells remained in G_0 phase of the cell cycle.

Suppression of immune responses by MSCs is most likely mediated by soluble factors, since separation of MSCs and the activated PBMCs by a semi-permeable membrane (Transwell) that allows exchange of soluble factors but not cell contact, still inhibit proliferation.[117,119] Contradictory to this, supernatants from MSC-cultures show no suppressive capacity,[94,113,122,125] unless the MSCs have been cocultured with lymphocytes.[113,123] Groh et al. cultured human MSCs with different enriched immune

cell populations and found $CD14^+$ cells to induce the immunosuppressive feature of MSCs.[126] Fibroblasts have not shown a suppressive capacity in MLCs when run in parallel with suppressive MSCs,[94,113] thereby excluding crowding of cells as the suppressive mechanism. The possibility of a bulk effect has also been evaluated by the addition of irradiated T cells autologous with the responder cells in MLCs. This did not alter proliferation.[121]

The effect of MSCs on lymphocytic subpopulations has been evaluated in several studies. Mitogen- and alloantigen-activated $CD3^+$, $CD4^+$, and $CD8^+$ T cells were all inhibited by MSCs.[118,121,122] Murine BM-derived MSCs were shown to inhibit mitogenic stimulation of murine B cells and T cells.[125] In these experiments, MSC-culture supernatant showed no effect on T-cell activation, while a significant suppression was observed on stimulated B cells. The inhibitory effect of MSCs on B cells was confirmed after stimulation of murine splenic B cells with anti-CD40 and IL-4 in the presence or absence of murine MSCs.[124]

Several studies have shown similar suppressive effects both when using MSCs autologous or allogeneic to the responder cells, indicating a genetically unrestricted suppression.[115,116,118,119] Djouad et al. showed that both human and mouse-derived MSCs could suppress xenogeneic MLCs.[123] Similar xenogeneic suppression was reported for minipig-derived MSCs, which inhibited proliferative responses of human PBMCs to mismatched allogeneic and xenogeneic PBMCs.[127] Combined, these results indicate general inhibitory mechanisms that may cross species barriers.

MSCs and antigen presenting cells

MSCs modulate dendritic cell (DC) and T-cell function and promote the induction of suppressor or regulatory cells. MSCs inhibited up-regulation of APC-related molecules, such as CD1a, CD40, CD80 (B7-1), CD86 (B7-2), and HLA-DR during DC maturation.[118,128,129] Jiang et al. showed that MSCs inhibited the *in vitro* generation of DCs from monocytes, both in contact and when the MSCs were present in transwell inserts.[130] This inhibition was abrogated by removal of MSCs and continuous culturing of the monocytes in DC-promoting medium (GM-CSF+IL-4+lipopolysaccharide). The cells isolated from cultures that had been cocultured with MSCs showed a reduced potential to activate $CD4^+$ T cells to proliferation, measured in MLCs (DCs+$CD4^+$ cells), as well as by pulsing DCs with Keyhole-Limpet hemocyanin and culture them with $CD4^+$ cells.[130] Maccario et al. also confirmed a reduction of DC-formation in the presence of MSCs.[118] Reduced pro-inflammatory cytokines, such as IFN-γ, IL-12, and tumor necrosis factor-alpha (TNF-α) in MSC/monocyte cocultures have also been reported, together with increased production of suppressive cytokines e.g. IL-10.[95,129,130] Taken together, these results suggest that a key mechanism of allogeneic inhibition of lymphocyte proliferation is mediated by MSCs directing maturing APCs toward a suppressor or regulatory phenotype that results in an attenuated or regulatory T-cell response. Nevertheless, MSCs inhibited T-cell proliferation by mechanisms that did not require

APCs, using direct stimulation with CD3 and CD28 antibodies, enriched T-cell populations or clones.[120]

MSCs and anergy

Naive T cells circulate in the blood and lymphatic system in the quiescent G_0 phase of the cell cycle. Encounter with the appropriate antigen, they proceed through the cell cycle and form effector or memory cells. Interactions are formed between the T-cell Receptor (TCR)/CD3 complex and HLA/peptide, as well as costimulatory signals provided by CD28 (T cell) and B7 (APC). CD28 is expressed on both resting and activated T cells, but activated T cells also express cytotoxic T-lymphocyte antigen-4 (CTLA-4), an inhibitory ligand to B7, that down-regulates the activation of the cell. Without costimulation, the naive cell becomes anergic and unresponsive. An anergic cell does not proliferate or secrete IL-2 in response to appropriate antigenic stimulation. It does however express the IL-2R, and the anergy can be abrogated by exogenous IL-2.[131]

MSCs lack surface expression of the T cell costimulatory molecules B7-1, B7-2, and CD40.[88,117,119] Therefore, MSCs as APCs could render cells anergic. Several studies have shown that proliferation of suppressed T cells to allogeneic cells, mitogens or IL-2 was restored after removal of MSCs.[119-121] Klyushnenkova et al. showed that the lack of response against MSCs was not due to a deficiency in costimulation, since retroviral transduction of MSCs with B7 did not result in T-cell proliferation.[119]

Recently, Augello et al. published a report on programmed death-1 (PD-1) and its ligands PD-L1 and PD-L2 in murine MSCs.[125] PD-1 is a coinhibitory molecule of the B7-CD28 family expressed on lymphocytes upon activation.[132] PD-1 signaling were shown to both stimulate and inhibit lymphocyte activation and cytokine production after interacting with the ligands PD-L1 and PD-L2.[133-135] Coculture of MSCs and allogeneic splenocytes in the presence of PHA induced a sharp decrease of PD-1 expressed by MSCs and an increase of PD-L1 and PD-L2, compared to MSCs cultured alone.[125] Proliferation of mitogen-stimulated T- or B-cell cultures was partially restored by neutralizing antibodies against these factors. PD-L1 mediated inhibition of $CD4^+$ and $CD8^+$ murine cells could be overcome by the addition of exogenous IL-2, indicating that the cells maintain IL-2 responsiveness.[136] The importance of proper PD-1 signaling for lymphocyte homeostasis and immune tolerance was further emphasized by the observation that mice deficient in PD-1 expression developed spontaneous autoimmune diseases.[137,138] Comparable to the abrogated MSC-suppression, increased proliferation was also seen when neutralizing antibodies against PD ligands were added to cultures of human $CD4^+$ T cells and allogeneic DCs.[139]

MSCs have been demonstrated to induce a split anergy phenotype in T cells. Glennie et al. showed that removal of MSCs from inhibited cultures only restored IFN-γ production, and not proliferation of murine PBMCs, despite addition of exogenous IL-2.[124] Whereas others have shown a resumed proliferative capacity upon secondary

stimulation,[119-121] Maccario et al. demonstrated resumed proliferation of $CD4^+$ and not $CD8^+$ cells.[118]

MSCs and regulatory T cells

Regulatory T cells are thought to have a critical role in the suppression of immune responses. This naturally occurring subset of $CD4^+$ cells that express CD25 (the α-chain of the IL-2 Receptor) was first described by Sakaguchi et al. in mice.[140] Regulatory T cells have showed to be important in protection against autoimmune diseases.[140,141] This subgroup of naturally occurring suppressor cells were also described in humans and constitutes about 5-10% of peripheral $CD4^+$ T cells.[142]

MSCs increased the proportion of the regulatory subsets $CD4^+CD25^{bright}$, $CD4^+CTLA\text{-}4^+$ and $CD4^+CD25^+CTLA\text{-}4^+$cells in MLCs.[118] When PBMCs were cultured with MSCs in the absence of stimulatory PBMCs, less than 10% of $CD4^+$ T cells expressed CD25 and/or CTLA-4 molecules, indicating that lymphocyte stimulation other than the presence of MSCs was needed to increase the number of regulatory T cells. Aggarwal and Pittenger also demonstrated an increase in the proportion of $CD4^+CD25^+$ in IL-2 stimulated PBMCs cocultured with MSCs.[95] In contrast, Beyth et al. showed that depleting $CD25^+$ cells from the $CD4^+$ subpopulation before stimulation with monocytes, had no effect on inhibition by MSCs.[129] This may indicate that MSCs potentiate the expansion of regulatory T cells, but do not stimulate to activation of new regulatory cells from naive T cells. Other studies have evaluated CD25 as an activation molecule, where MSC-induced inhibition of mitogen-stimulated T cells reduced the expression of CD25, as well as CD69 and CD38.[122,126] The hypoimmunogenic state of MSCs was confirmed by reduced expression of CD25 on T cells cocultured with MSCs compared to PBMCs as stimulator cells.[119]

MSCs and cell-mediated cytotoxic responses

NK cells and cytotoxic T cells (CTLs) are important cytotoxic effector cells for elimination of transformed or infected cells. CTLs are generated from CTL-precursors (CTL-p) that are incapable of killing. CTLp require an antigenic signal and a costimulatory signal from APCs to upregulate the IL-2R. IL-2 from activated $CD4^+$ T cells further drive the cells to active CTLs. $CD4^+$ cells are important effector cells in that they produce cytokines that mould the immune response, e.g. IL-2. $CD4^+$ T cells are divided into T_{H1} cells that produce inflammatory cytokines, and suppressive T_{H2} cells. CTLs are reactive against peptides expressed on HLA class I. In contrast to CTLs, NK cells are constitutively cytotoxic cells that mainly target cells that lack HLA class I expression.[143] NK cells express several different inhibitory and activating receptors, where the inhibitory Killer Immunoglobulin-like Receptors (KIR) recognize HLA class I alleles. Therefore HLA class I expressing cells can be lysed by NK cells if the targets are KIR-ligand mismatched and the target cells don't express inhibitory HLA class I alleles.[144] Interactions between cytotoxic cells and target cells induce either release of preformed cytotoxic mediators, or expression of ligands on target

cells that transduce apoptotic signals, e.g. Fas Ligand (FasL). Besides direct interaction with target cells, NK cells also express CD16, a receptor for the Fc fragment of immunoglobulin G (IgG). NK cells can thereby eliminate cells not normally recognized by NK cells if they have been bound by IgG, though antibody-dependent cell-mediated cytotoxicity (ADCC).

Murine MSCs inhibited cell proliferation, cytotoxicity and IFN-γ production by $CD8^+$ cells in a dose-dependent fashion when present in murine splenocyte cultures

in vitro with their cognate antigen, the murine male antigen HY.[120] Human MSCs were also reported to inhibit IFN-γ production by IL-2 stimulated NK cells.[95] MSCs themselves did not induce IFN-γ production by NK cells, indicating that they do not activate NK cells.

Several studies have shown a suppressive effect of MSCs on cytotoxicity. Maccario et al. showed that MSCs were able to display a dose-dependent inhibitory effect on alloantigen-specific cytotoxic activity, when present during the priming of cytotoxic cells in MLCs.[118] Cytotoxic activity against human MSCs was not detected and addition of MSCs in the lysis assay showed no effect on lysis. Addition of non-labeled (cold) NK-sensitive target cells decreased cytotoxic activity, thus confirming a contribution of NK effector cells, beside alloantigen-specific CTLs in MLCs. Analysis of the cultures particularly revealed a down-regulation of the expansion of $CD8^+$ T cells and NK cells, with increased numbers of $CD4^+$ T cells. Angoulvant et al. showed that human MSCs suppressed the induction of cytotoxic responses to alloantigens.[145] PBMCs and MSCs derived from the same donor were used as stimulators to trigger CTLs. PBMCs induced the formation of active CTLs that could lyse various targets, including MSCs, whereas MSCs did not stimulate to lysis of any target cells. Addition of MSCs to cultures stimulated with PBMCs, dose-dependently led to a lower frequency of active CTLs. There was a partial recovery of target cell lysis by addition of IL-2. Potian et al. proposed that MSCs could blunt the cytotoxic effects of alloreactive CTLs to stimulator target PBMCs, whereas fibroblasts derived from the same donor as the MSCs had no effect.[94]

It has been proposed that MSCs can function as "veto cells". Veto-mediated suppression is based on infusion of a low dose of cells that transiently delete $CD8^+$ T cells reactive against the infused cells, thereby inducing a transient state of tolerance in the host.[146] Potian et al. reported that MSCs could inhibit lysis when added to the lysis assay, and suggested that this was a "veto-effect".[94] Djouad et al. reported that murine MSCs induced formation of $CD8^+$ regulatory cells that were responsible for the inhibition of allogeneic lymphocyte proliferation.[123] After depletion of $CD8^+$ cells from the responder population, MSCs showed no effect on proliferation. Splenocytes that were depleted of $CD8^+$ cells after primary MLCs, showed no inhibitory effect in secondary MLCs, whereas the portion that contained $CD8^+$ cells transferred suppression to the culture.[123]

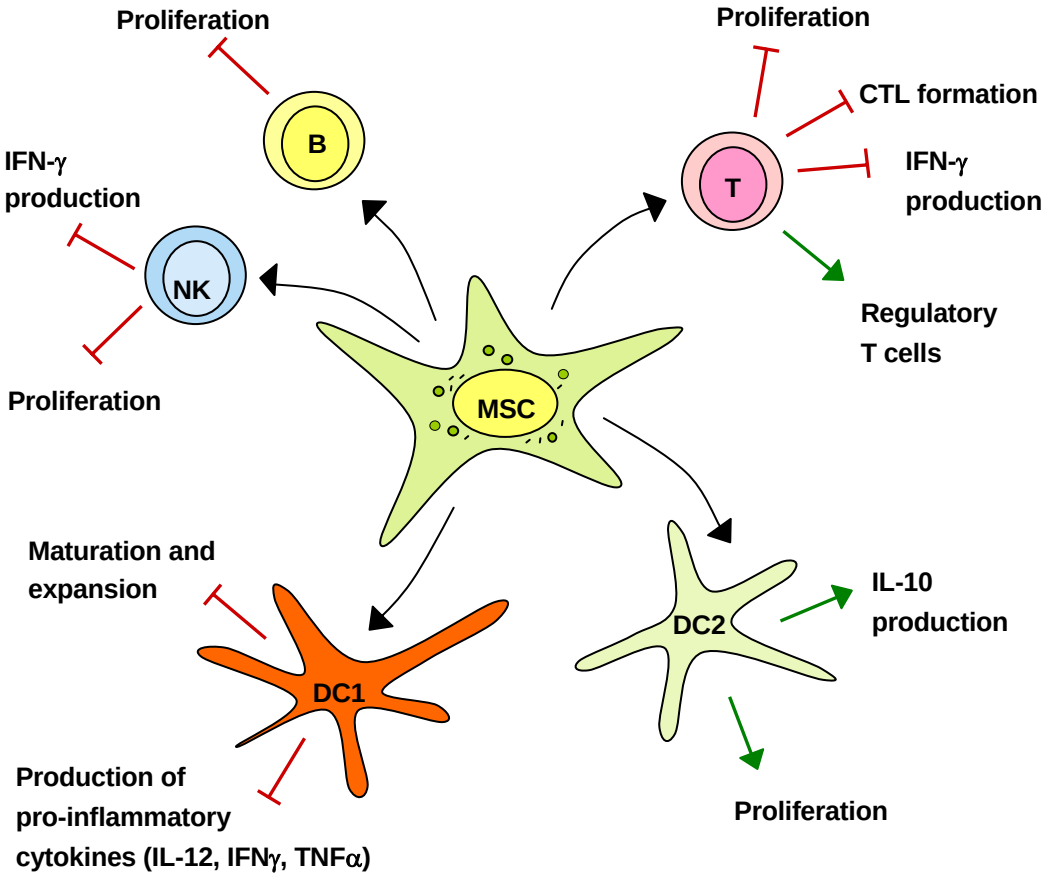

Figure 1 Schematic illustration of the effects of MSCs on the immune system. NK, B and T refer to NK, B and T cells. DC1 refers to mature monocytic dendritic cells (DC1) and DC2 mature plasmacytoid dendritic cells (DC2)

Inhibitory effect ⊣ Stimulatory effect →

Soluble versus contact dependent inhibition by MSCs

Several studies have shown that the inhibition elicited by MSC is mediated by soluble factors. TGF-β has been the most studied potential candidate. Di Nicola et al. showed that the MSC-induced suppression of responder T cells against stimulator PBMCs could be abrogated by high concentrations of neutralizing antibodies against TGF-β1 and hepatocyte growth factor (HGF).[121] Blocking each factor separately resulted in a minimal effect on inhibition, whereas neutralizing the cytokines simultaneously restored all proliferation of T cells. Simultaneous addition of recombinant TGF-β1 and HGF to MLCs induced a similar suppression as when using MSCs. Le Blanc et al. failed to reproduce this.[122] These reports might not be comparable, since Di Nicola et al. used enriched T cells and allogeneic stimulation, whereas Le Blanc et al. analyzed proliferation in unseparated mitogen-stimulated PBMCs. In another study, neutralizing these factors partially restored CTL-formation after suppression by MSCs.[145] Enriched T cells were stimulated against allogeneic PBMCs to yield active CTLs, strengthening

the evidence that TGF-β1 and HGF could mediate the suppression by MSCs in alloantigen-stimulated enriched T cells. Similar to Di Nicola, several studies have excluded a single role for TGF-β in MSC induced suppression.[117,120,129]

The characterization of cytokines produced by MSCs is still rudimentary and is hampered by the diversity of cells and culture systems used. MSCs do not constitutively produce IL-2, IL-4 and IL-10.[119,147] However, IL-1, IL-6, IL-7, IL-8, IL-11, IL-12, IL-14, IL-15, IL-27, GM-, G-, and M-CSF can be detected.[91,93-95] Di Nicola et al. tried to restore proliferation by neutralizing IL-6 and IL-11, but neither of these factors appeared to be of importance in MSC-mediated suppression of alloresponses.[121] Even though IL-10 was not constitutively secreted by MSCs, increased IL-10 levels have been reported in MLCs when MSCs were present.[119,129] The inhibitory effect of MSCs on cytokine release and proliferation of T cells has been partially reverted by blocking IL-10 signaling.[129] Enriched $CD4^+$ cells were cocultured with monocytes and staphylococcal enterotoxin B (SEB) in the presence of MSCs. The addition of neutralizing antibodies against IL-10R partially restored proliferation as well as IFN-γ and TNF-α production.

Several possible mechanisms concerning MSC-mediated suppression have been evaluated. SDF-1 was analyzed as a potential candidate. SDF-1 exerts chemotactic activities at low doses, whereas high concentrations can repel T cells.[148] Moreover, BM and thymic stroma that produce abundant SDF-1 lack extensive infiltration of mature T cells.[149] SDF-1 was not detected on the cell surface of MSCs even after treatment with inflammatory cytokines.[122] However, a low level of soluble SDF-1 was detected in culture supernatants, but the addition of neutralizing antibodies against SDF-1 to MLCs cocultured with MSCs showed no effect on inhibition.

Another investigated mechanism was the possible role of RANK-L and osteoprotegerin (OPG) interactions. RANK-L is expressed by activated lymphocytes and promotes DC survival and function, T-cell activation and T cell-DC communication *in vitro*.[150] RANK-L signaling is blocked by OPG, a soluble decoy receptor produced by stromal cells.[151] Human MSCs express OPG mRNA[152] and undifferentiated MSCs secreted low levels of OPG. However, neutralization of OPG had no effect on the inhibition of PHA-stimulated lymphocytes.[122]

PGE_2 is at present one of the more intriguing candidates of MSC induced immune suppression. PGE_2 influences numerous immune functions, including B-cell activation[153] and induction of regulatory T cells.[154] Cyclooxygenase (COX) enzymes are involved in the synthesis of PGE_2. COX-1 isoform is constitutively expressed and COX-2 is upregulated upon inflammation. MSCs constitutively express both COX-1 and COX-2, resulting in the constitutive production of PGE_2.[95,155] Both COX-2 and PGE_2 production were increased upon coculture of MSCs with PBMCs.[95,117] However, the role of PGE_2 in MSC-mediated suppression is contradictory. Inhibition of COX activity and subsequently PGE_2 synthesis by indomethacin decreased PGE_2 in cocultures of MSCs and PBMCs stimulated by CD3/CD28 antibodies, but without

restored proliferation.[117] Aggarwal and Pittenger showed that inhibition of PGE_2 synthesis by indomethacin or NS-398 both could restore the majority of proliferation of mitogen-activated PBMCs cocultured with MSCs.[95] Even the obstructed TNF-α and IFN-γ secretion by activated DCs and T cells was restored when PGE_2 synthesis was blocked.

Indoleamine 2,3-dioxygenase (IDO) expression is induced by IFN-γ and catalyzes the conversion of tryptophan to kynurenine. Active IDO depletes tryptophan essential for T-cell proliferation, resulting in reduced lymphocyte proliferation.[156-158] Regulatory suppressor T cells were induced by depletion of tryptophan by IDO-expressing plasmacytoid DCs.[159] Human MSCs do not constitutively express IDO, but IDO protein and functional IDO activity were seen upon stimulation of MSCs with IFN-γ.[160] IDO activity was also detected in MLCs suppressed by MSCs with significantly reduced tryptophan levels. Addition of tryptophan to MLCs significantly restored allogeneic T-cell proliferation.[160] Besides tryptophan depletion, the conversion of tryptophan to kynurenine can result in kynurenine breakdown products that also mediate inhibition of T-cell proliferation.[161] Tse et al. excluded a role of IDO in MSC-induced suppression, since the addition of tryptophan or an IDO-inhibitor (1-methyltryptophan) showed no effect on suppression.[117] IDO-mediated suppression of T cells was reported to induce apoptosis of thymocytes and T_{H1} cells, but not T_{H2} cells.[162] Several studies demonstrated that MSCs do not increase apoptosis in the suppressed cultures.[117,121,163] However, a recent report proposes that MSC inhibit proliferation by inducing apoptosis of activated T cells.[164] This apoptosis was related to the conversion of tryptophan into kynurenine by IDO.

The results concerning MSC-induced suppression of cells of the immune system are contradictory and may include different inhibitory mechanisms. The mechanism(s) might be dependent on the use of model systems, enriched cell populations or un-fractionated PBMCs, the species and source of MSCs, the isolation protocol and the variable timings for measurement. The variation in these parameters could possibly lead to different results. The lack of a clear-cut definition of what constitute MSCs also makes the analysis and comparisons difficult. Most studies on immune regulation by MSCs use human or mouse BM-derived MSCs. A recent report showed that minipig-derived MSCs did not induce proliferation of human PBMCs while minipig-derived PBMCs did.[127] Minipig MSCs inhibited mitogenic stimulation as well as allo- and xenogeneic proliferation of human PBMCs. Neutralizing antibodies against FasL (CD95L) and TGF-β1 could separately restore all proliferation of ConA-stimulated cultures. Neutralizing IL-10 slightly increased inhibition. This study partly corroborates studies using human or murine MSCs. However, a significant role of TGF- β1 has been excluded in several reports, and it remains to be seen if this could be a porcine-specific feature. This report can exemplify the complexity of the various systems used.

Responder	Stimulator	MSC	Neutralizing factor	Reduced inhibition	Response	Ref
TGF-β and/or HGF						
PBMCs	Anti CD3+Anti CD28	+	Anti TGF-β1	NO	Proliferation	[117]
PBMCs	Superantigen SEB	+	Anti TGF- β1, 2	NO	IFN-γ production	[129]
$CD2^+$ T cells	PBMCs	+	Anti TGF- β1	NO	Proliferation	[121]
$CD2^+$ T cells	PBMCs	+	Anti HGF	NO	Proliferation	[121]
$CD2^+$ T cells	PBMCs	+	Anti TGF- β1+Anti HGF	YES	Proliferation	[121]
PBMCs	Mitogen PHA	+	Anti TGF- β1+Anti HGF	NO	Proliferation	[122]
T cells	PBMCs	+	Anti TGF- β1+Anti HGF	YES	CTL formation	[145]
PGE_2						
PBMCs	Anti CD3+Anti CD28	+	Indomethacin	NO	Proliferation	[117]
PBMCs	Mitogen PHA	+	Indomethacin	YES	Proliferation	[95]
DC1*	LPS	+	NS-398	YES	TNF-α production	[95]
T_{H1}**	Mitogen PHA	+	NS-398	YES	IFN-γ production	[95]
IDO						
T cells	PBMCs	+	Tryptophan	YES	Proliferation	[160]
PBMCs	Anti CD3+Anti CD28	+	Tryptophan	NO	Proliferation	[117]
PBMCs	Anti CD3+Anti CD28	+	IDO-inhibitor	NO	Proliferation	[117]
Various factors						
$CD2^+$ T cells	PBMCs	+	Anti IL-6	NO	Proliferation	[121]
$CD2^+$ T cells	PBMCs	+	Anti IL-11	NO	Proliferation	[121]
PBMCs	PBMCs	+	Anti SDF-1	NO	Proliferation	[122]
PBMCs	Mitogen PHA	+	Anti OPG	NO	Proliferation	[122]
$CD4^+$ T cells + monocytes	Superantigen SEB	+	Anti IL-10R	YES	IFN-γ production	[129]
				YES	TNF-α production	
				YES	Proliferation	

Table 1. Potential candidates responsible for MSC-induced immune suppression. Addition of MSCs to stimulated cells inhibited the various responses. This table illustrates the different factors neutralized in the various assays, and if the inhibition was effected. Where the inhibition was reduced (indicated by YES), only a partial restoration of the effector response was seen.

* DC1 cells refer to $CD1a^+$ cells of the myeloid lineage, cultured in GM-CSF and IL-4
** T_{H1} cells refer to culture of $CD45RA^+$ T cells with IL-2 + IL-12 + antiIL-4

In vivo immune suppression by MSCs

The immunosuppressive capacity of MSCs has also been evaluated *in vivo*. Bartholomew et al. demonstrated that intravenous administration of MSCs derived from BM of baboons prolonged the survival of allogeneic skin grafts.[115] The magnitude of suppression obtained by a single dose of MSCs injected intravenously was similar to that of potent immunosuppressives currently used in the clinic.[165,166] A second infusion of MSCs did not extend skin graft survival, and neutrophils eventually infiltrated the graft and rejection occurred. Grinnemo et al. studied if human MSCs could survive and engraft in experimentally induced ischemic rat myocardium. Rat PBMCs were

analyzed for xenogeneic responses against human MSCs *in vitro* one week after injection of MSCs. MSCs induced a significant lymphocyte proliferation in PBMC cultures of immunized rats, but no proliferation was seen in PBMCs from rats not injected with MSCs. There was a significant infiltration of primarily macrophages in the area of injection in immunocompetent rats. Although MSCs have been transplantable across allogeneic barriers, this study suggests that xenogeneic transplant rejection may occur.[60]

Djouad et al. demonstrated two aspects of *in vivo* suppression of MSCs.[123] Allogeneic murine MSCs could engraft and form bone in immunocompetent mice. However, lymphocytic infiltrates were seen in the periphery of the newly formed bone, possibly indicating that MSCs awoke an immune response. Still, the allogeneic bone was not rejected. Suppression of the immune system is a vital therapeutic tool, but Djouad et al. also showed a negative side of this. MSCs facilitated tumor development, when MSCs were infused systemically or adjacent to subcutaneously placed melanoma cells in allogeneic immunocompetent mice. When injected subcutaneously, the MSCs were seen in the stroma surrounding the tumor, whereas systemically infused MSCs could not be detected. Melanoma cells or MSCs injected alone did not give rise to tumors.[123]

Murine MSCs prevented experimental autoimmune encephalomyelitis (EAE) in mice. EAE is a mouse inflammatory disease model of human multiple sclerosis.[163] Intravenous administration of MSCs before disease onset ameliorated EAE. The therapeutic scheme was effective when MSCs were administered at disease onset and at the peak of disease, but not after disease stabilization. CNS pathology showed decreased inflammatory infiltrates and decreased demyelination in mice transplanted with MSCs. MSCs transfected with GFP were detected in the lymphoid organs of treated mice.[163]

MSCs and graft-versus-host disease

Acute graft-versus-host disease (GVHD) is a complication after allogeneic HSCT where the immunocompetent cells in the graft react against host-derived antigens.[167-169] The HSC graft contains a mixture of cells, including mature T cells. At the HSCT, cells are infused into a host that has been profoundly damaged by underlying disease and by conditioning, which result in activation of host cells with secretion of proinflammatory cytokines, such as TNF-α and IL-1.[169-171] A mild form of GVHD is beneficial to avoid relapse of the underlying disease, especially leukemia, but can in its more severe forms be lethal.[172-174] Acute GVHD occurs when donor T cells react to host APCs with sequential activation of donor T cells.[175,176] MSCs suppress formation of CTLs as well as alter the cytokine profile and maturation of APCs. MSCs may therefore be a potential cellular therapy for GVHD. Murine MSCs significantly increased the survival rate after HLA-mismatched murine allogeneic HSCT. Co-transplantation of MSCs with hematopoietic cells resulted in a lower GVHD score as well as reduced serum levels of IFN-γ.[177] Lazarus et al. have performed two phase I studies to evaluate the feasibility of transplanting MSCs to improve engraftment of HSCs as well as to reduce GVHD. In

1995, they isolated and culture-expanded BM-derived MSCs from 23 patients with hematological malignancies in complete remission. Autologous MSCs were infused intravenously and no adverse reactions were observed.[178] In 2005, a second phase I trial was reported, where culture-expanded MSCs were coinfused with HLA-identical HSCs in 46 hematological malignancy patients. MSCs were administered 4 hours before infusion of HSCs without any infusion-related adverse events, ectopic tissue formation or increased GVHD as a response against the allogeneic MSCs.[179] These studies focused on evaluating the safety of MSC-infusions, but a clinical benefit of MSC infusions still remains to be established. A case report presented a 20-year-old woman with acute myeloid leukemia treated with HSCT combined with MSCs from her haplo-identical father.[180] The patient engrafted rapidly without acute or chronic GvHD with a reported follow-up of 31 months.

The ability of MSCs to inhibit the development or reverse acute GVHD may be due to soluble factors secreted by the MSCs. There are two possibilities of this; 1) the inhibition of alloreactive T cells by some immunosuppressive factor, and 2) the release of factors that could increase the healing rate of wounded tissues. Keratinocyte growth factor (KGF) is a mitogen for epithelial cells[181] that reversed severe GVHD in murine models.[182-184] KGF decreased expression of costimulatory molecules on infiltrating cells and increased expression of anti-inflammatory cytokines.[185] KGF also decreased damage induced by conditioning.[186,187] KGF is expressed by a variety of mesenchymal cells such as fibroblasts[188] and vascular smooth muscle cells.[189] However, so far there are no studies on the secretion of KGF by MSCs, so this remains purely speculative.

Maternal-fetal immune suppression

A recent review by Barry et al. focused on the striking similarity between immune suppression by MSCs and the maternal acceptance of the fetal allograft.[190] Careful immune regulation is needed to keep the fetus alive and well in the womb, since the mother is not immunologically ignorant and can delete circulating fetal cells without rejection of the fetus.[191] These immunomodulatory changes are linked to suppression of inflammatory cytokines and to the induction of T cells with regulatory or suppressive phenotypes. Foremost among the mediators of such effects are IL-10,[192,193] TGF-β,[194], HGF,[195] PGE_2[196] and IDO[197]. Tryptophan concentration in maternal circulation falls steadily during pregnancy.[198,199] The fact that MSCs can be isolated from several fetal tissues, as well as placenta, amniotic fluid,[200] fetal blood[24] and term umbilical cord blood[201] strengthen the theory that MSCs may have a role in fetal acceptance. Fetal MSCs show similar expression of surface markers as adult BM-derived MSCs.[24,26,28] Fetal MSCs express HLA class I but not HLA class II.[202] Mitogen-stimulated proliferation of PBMCs was inhibited by fetal MSC. However, unlike adult MSCs, fetal MSCs did not inhibit MLCs.[26] Additional studies showed that fetal MSCs could suppress proliferation in MLCs after treatment of the MSCs with IFN-γ.[202] Further comparisons between adult and fetal MSCs may provide key clues to understanding the mechanisms of inhibition.

5 AIMS OF THE PRESENT STUDY

- To investigate the effect of human BM-derived MSCs on the formation of CTLs and on CTL and NK-cell mediated lysis.

- To analyze if MSCs are lysed by alloreactive CTLs and KIR-ligand mismatched NK cells.

- To examine potential differences between alloantigen- and mitogen-stimulated PBMCs suppressed by MSCs.

- To study the effects of MSCs on IgG secretion by human spleen-derived MNCs and enriched B cells.

- To further explore the reduced immunogenicity of MSCs using HLA-restricted CTL clones.

- To explore possible *in vivo* effects by infusion of haplo-identical culture-expanded MSCs in a patient with grade IV therapy-resistant acute GVHD.

6 METHODS

The materials and methods are described in detail in each article or manuscript.

CULTURE OF MSCs (PAPER I-V)

MSCs were isolated and expanded from BM aspirates taken from the iliac crest of adult volunteers, approved by the Ethics Committee at Karolinska University Hospital. MNCs were isolated by Percoll-gradient centrifugation and plated at a density of 160,000 cells/cm^2 in culture flasks. The nonadherent cells were removed after 48-72 hours and the adherent cells were further cultured until colonies had formed. Cells were detached by trypsin and EDTA and replated at a density of 4000 cells/cm^2. The cells were classified as MSCs based on morphology, flow cytometric analysis and ability to differentiate into bone, fat, and cartilage.

One major setback in MSC-research is the lack of specific markers. It is generally accepted that MSCs are devoid of hematopoietic and endothelial markers, including, CD11b, CD31, CD34 and CD45. The antibodies SH-2, SH-3 and SH-4 were designed in 1992 by Haynesworth et al. by immunizing mice with human BM-derived MSCs. The antibodies secreted *in vitro* by hybridoma cell lines produced from these mice, showed specificities for MSCs.[203] Today, it is known that SH-2 recognizes CD105 (endoglin)[204] and SH-3 and SH-4 recognize CD73 (5'-nucleotidase).[205] We use a panel of CD29, CD44, CD73, CD105, CD166 and HLA class I as positive detection of MSCs, and CD14, CD34 and CD45 as negative detection. The antigenic phenotype of MSC is not unique, sharing features of mesenchymal, endothelial, and epithelial cells. A recent study reports on antibodies produced against human MSCs that react with other epitopes than the SH antibodies,[206] but the specificities remains to be confirmed.

IN VITRO STIMULATION OF PBMCs (PAPER I-V)

The experimental work in this thesis is mainly based on different *in vitro* methods. *In vitro* models have potential for the study of specific, isolated events that are integrated in a more complex biological process. However, *in vitro* models have to be interpreted with caution since the *in vivo* situation is more complicated and the results may not be directly applicable to the clinical situation. To evaluate the effect of MSCs on activated PBMCs, we have used allogeneic stimulator cells in MLCs, the mitogen PHA as a potent polyclonal T-cell stimulator, IL-2 and phorbol myristate acetate (PMA) to active protein kinase C (PKC) and thereby induce proliferation. Proliferation was evaluated by tritium-labeled thymidine that incorporated into newly synthesized DNA. Cells were harvested on filters and the radiation emitted from the labeled thymidine was measured in a beta-counter.

Alloreactivity was measured in one-way MLCs, where responder PBMCs were cultured with unrelated stimulator PBMCs. Reactivity in MLCs has been correlated with risk for rejection after transplantation.[207] Proliferation in MLCs increased when a pool of stimulator cells derived from several donors was used, increasing the histo-

incompatibility. MLCs were also used in paper V to evaluate the risk of *in vivo* immunization of infused MSCs.

MNCs and enriched B cells derived from human spleens were used to assess the effect of MSCs on IgG secretion induced by stimulation by lipopolysaccaride (LPS), cytomegalovirus (CMV) and varicella zoster virus (VZV). We also measured the potential alloresponse against allogeneic MSCs regarding proliferation, cytokine release and IgG secretion. LPS is a potent B-cell stimulator and induced high antibody production in most responder cells, whereas CMV and VZV only occasionally generated a humoral response. These stimulators where chosen based on their importance in the clinic, where especially CMV infection and disease constitute a major problem after allogeneic HSCT.[208,209]

CYTOTOXIC T-LYMPHOCYTES (PAPER I AND IV)

The effect of MSCs on CTLs was analyzed in paper I and IV. In paper I, cytotoxic cell precursors in PBMCs were activated in MLCs. The cells were harvested on day 5 and analyzed in a chromium-release assay. We used unfractionated PBMCs as effector cells and a more purified system was required to analyze the interactions between CTLs and MSCs. In paper IV, we used already primed CTL clones that were sustained in special media supplemented with cytokines and growth factors. The activated CTLs originated from PBMCs stimulated with allogeneic PBMCs or autologous lymphoblastoid cell lines (LCLs). LCLs are EBV-infected PBMCs, that presents EBV peptides to the PBMCs and activate EBV-reactive CTLs. This triggering was repeated 3-4 times to ensure enrichment of the proper CTLs and during that time, cells with other specificities died. The cells were thereafter cultured in the presence of IL-2 and growth factors. To obtain pure cultures it was vital that other cells had died before IL-2 was added to avoid IL-2 induced activation of all T cells regardless of specificity. Limiting dilution was performed, to obtain CTL clones. Specificity of the effector cells was controlled by lysis of a panel of targets, including various LCLs, allogeneic and autologous PHA blasts and K562.

CELL-MEDIATED LYMPHOLYSIS (PAPER I AND IV)

Cell-mediated lympholysis (CML) was evaluated using the standard chromium-release assay. CTLs activated in MLCs, freshly isolated NK cells or CTL clones were used as effectors. Target cells were labeled by radioactive chromium (^{51}Cr). After extensive washing of target cells, target and effector cells were mixed and incubated for 4 hours. Half of the supernatants were harvested and the radiation emitted from the released chromium from lysed cells was measured in a gamma counter.

The chromium-release assay is the most commonly used method to detect lysis. Other non-radioactive assays are being developed, such as the lactase dehydrogenase (LDH) assay. This assay is based on enzyme release from lysed cells. In paper I, MSCs were added as third party to see possible effects on the lysis of PBMCs. The chromium-

release assay has the advantage of only measuring the lysis of one labeled target cell population. Using the LDH assay it may be difficult to truly assess the lysis, because the released LDH could come from the PBMC blasts as well as the MSCs.

ENZYME-LINKED IMMUNOSPOT ASSAY (PAPER III)

Enzyme-linked immunospot (ELIspot) assay was used to analyze IgG production. ELIspot assay has the advantage of detecting even very few IgG-producing cells amongst several thousands. MNCs or enriched B cells were cocultured for 48 hours with MSCs, or with LPS, CMV or VZV and MSCs. Cultured cells were washed several times to remove as much as possible of the serum, that otherwise could interfere with the assay. Cell-counts were adjusted and the cells were plated in 96-well plates pre-coated with rabbit antibodies against human IgG. Cells were incubated for additional 18 hours and secreted IgG bound to antibodies. The cells were washed away, before addition of an alkaline phosphatase (ALP)-labeled anti-human antibody. IgG-specific spots were developed by the addition of ALP substrate and spots were manually counted in a light microscope.

CYTOKINE ANALYSIS (PAPER II-IV)

Cytokine levels were analyzed in paper II-IV. Cytokine concentration in culture supernatants were measured by enzyme-linked immunosorbent assays (ELISA) and an automated chemoluminescence immunoassay (IMMULITE®) (paper II-III). In paper IV, intracellular flow cytometry (or fluorescence-activated cell sorting analysis) was used to analyze cytokine production. Flow cytometry has the advantage of rapid and simultaneous quantification of several parameters on or in one cell. CTLs were treated with Golgistop (containing monensin), a substance that sequester produced proteins in the Golgi complex. After stimulation, the CTLs were permeabilized and stained with antibodies against IFN-γ and TNF-α. Staining was performed on ice. All flow cytometry analyses were performed with a negative isotype control.

We wished to confirm the alterations of protein levels of IL-2 and soluble IL-2R (sIL-2R) at the mRNA level (paper II). Complementary DNA was synthesized from mRNA in the samples. Standard RT-PCR measures an end-product, while the use of real-time RT-PCR permits accurate quantification during the exponential phase of the reaction without post-PCR processing such as gel analysis. The detection system is based on fluorescent signals generated during the PCR process and is measured after each cycle. The TaqMan probes used contain a reporter (FAM) and a quencher (TAMRA) flourochrome. As long as the two flourochromes are in close vicinity of each other, the quencher silences the reporter flourochrome. During the polymerization phase of the PCR cycle, the TaqMan probe binds to the DNA. Upon amplification by the Taq polymerase, the endonuclease activity of the enzyme breaks the probe and separates the reporter from the quencher, and the reporter signal is detected. The computer software calculates the threshold cycle (C_t), defined as cycle number at which the fluorescence passes a fixed threshold, where samples with high copy numbers of

target reaches the threshold value at earlier cycles than samples with fewer copies. We compared the C_t value of IL-2 and IL-2R with the value for the housekeeping gene glucose 6-phosphate dehydrogenase (G6PD) to measure fold increase compared to the background.

CULTURE-EXPANDED MSCs FOR CLINICAL USE (PAPER V)

Paper V is a case study report of a boy suffering from life-threatening acute GVHD who received culture-expanded MSCs isolated from his mother, to ameliorate the severe immune reaction. After Ethics Committee approval and informed consent from the donor, MSCs were isolated from the iliac crest under local anaesthetics. The cells were cultured in a sterile room exclusively used for MSC cultures with a separate incubator for cells for clinical use. The culture conditions had been approved of by the Swedish Medical Products Agency. A carefully tested FBS derived from Swedish animals were used, to limit the risk of transferring infectious agents. After 3 weeks of cell culture, $90x10^6$ MSCs were harvested. These cells expressed CD29, CD44, CD105 and CD166, but not CD34 or CD45. $2x10^6$ cells/kg bodyweight were given intravenously on day 73 after HSCT. A second infusion was later given when the GVHD reoccurred after discontinuing the immunosuppressive treatment. MSCs stored in liquid nitrogen were thawed, washed several times and $1x10^6$ cells/kg were infused on day 170 post HSCT.

FLUORESCENCE IN SITU HYBRIDIZATION (PAPER V)

Fluorescence in situ hybridization (FISH) can be used to detect single copies of genes in a biopsy or selected cell material, by constructing a probe that will specifically bind to the DNA and emit signals that can be detected in a fluorescence microscope. Multiple fluorochromes can be used to examine several sequences within the same cell. Such was the case in paper V, when we wanted to examine the presence of male and female sex chromosomes in colon epithelium. By simultaneous staining for X and Y chromosomes, we could detect mainly cells expressing XY since the patient was male, but also occasional XX cells. Besides the risk of false negative results due to different alignment of the chromosomes, there was also a risk of false positive results by superimposed cells, especially in this setting where we analyzed XX cells in a XY individual. Using FISH analysis, we could also rule out fusion of MSCs and epithelial cells, since the XX cells were diploid.

7 RESULTS AND DISCUSSION

MSCs INHIBIT THE FORMATION OF CTLs BUT NOT LYSIS BY CTLs OR NK CELLS (PAPER I)

CTLs primed against allogeneic PBMCs in MLCs showed a high cytotoxic response in chromium-release assays. MSCs added to the MLC inhibited the CTL-mediated lysis in a time- and dose-dependent manner. MSC-mediated inhibition was most pronounced when the MSCs were added at the beginning of the MLCs and at a 10:1 effector:MSC ratio. The magnitude of suppression was not reduced when the MSCs were separated from the lymphocytes in transwell inserts, indicating that cell-cell contact was not required. However, when MSCs were added in the lysis assay, no significant inhibition of CTL-mediated cytotoxicity was seen. MSCs inhibited T cells in the early activating phase of the allograft reaction, presumably by preventing the formation of active CTLs.

Both alloantigen-specific CTLs and alloreactive NK or NK-like cells can mediate alloantigen-induced cell-mediated cytotoxic activity with alloreactive NK cells contributing to cytolytic function when effector and target cells are KIR-ligand incompatible. A study by Maccario et al. showed that MSCs inhibit the cytotoxic activity in MLCs. The addition of nonlabeled NK-sensitive target cells decreased cytotoxic activity, thereby confirming a contribution of NK effectors to the cytolytic function elicited in MLCs.[118] We evaluated the effect of nonlabeled MSCs on negatively selected NK cells in paper I. MSCs did not suppress NK-cell lysis, but rather dose-dependently increased lysis of labeled K562 (to 123±6% in 10:1 effector:MSC ratio ($p<0.05$) and to 107±4 in 1000:1, when the control without MSCs was set to 100%, n=6; data not shown in the article).

It will be interesting to examine this phenomenon further. Studies of the effect of MSCs on CTL-mediated lysis of malignant cells may be an *in vitro* measure of MSCs' ability to decrease the graft-versus-leukemia (GVL) effect. It is possible that when MSCs suppress the T-cell response, both GVHD and GVL are inhibited. Patients with acute and/or chronic GVHD experience leukemic relapses less frequently than patients with no GVHD.[172-174,210,211] Therefore, it might not be desirable to abolish GVHD completely. Djouad et al. showed, using a murine melanoma tumor model, that the subcutaneous injection of melanoma cells led to tumor growth in allogeneic recipients only when MSCs were coinjected.[212] So far, these findings have not been corroborated by other groups. It remains to be seen if MSCs increase the risk of leukemic relapse after allogeneic HSCT. Prospective clinical studies are needed and ongoing. Since the effect of MSCs was transient in paper V, the effect of MSC infusion may show optimal results if given shortly after transplantation to modify the T cells in the graft when antigen presentation occurs. If used to treat severe acute GVHD, MSCs may show the greatest potential when combined with antibody treatment to eliminate already existing CTLs. NK cells have been thought to be more important for GVL than GVHD.[213-215] Lysis of NK cells was slightly potentiated by MSCs in paper I. However, others

showed a reduced expansion and IFN-γ release when NK cells were cocultured with MSCs.[95,118]

MSCs ARE NOT LYSED BY CTLs OR NK CELLS (PAPER I)

The immunogenicity of allogeneic MSCs was evaluated by activating CTLs against allogeneic PBMCs. Both stimulator PBMCs and MSCs from the same donor were used as targets in chromium-release assays. Even though the two target cell populations were HLA identical, MSCs were only weakly lysed by the CTLs, whereas the PBMC blasts were strongly lysed. The finding that MSCs escape recognition by CTLs is surprising since MSCs express HLA class I antigens. We chose to trigger the CTLs against PBMCs, since previous reports indicated that MSCs do not induce a proliferative response. Our data is different from a later report by Angoulvant et al.,[145] who showed that when allogeneic PBMCs were used to stimulate T cells, a high CTLp frequency was detected toward MSC targets. However, when MSC were used as stimulators, CTLp frequency was markedly altered whatever the targets used. The method used differed from ours, mainly by the longer (10-day instead of 6-day) MLCs, and by the addition of exogenous IL-2 on day 3 and 6.

Similar hypoimmunogenicity was observed when NK cells only weakly lysed KIR-ligand mismatched MSCs. It remains to be shown whether MSCs express inhibitory molecules and therefore are not lysed by NK cells. Götherström et al. showed that MSCs do not express HLA–E or –G on the surface,[216] but several other inhibitory molecules could be involved. Taken together, our *in vitro* data would suggest that MSCs can be transplanted between HLA incompatible persons since they are not destroyed by CTLs or NK cells and do not induce an immune response.

MSCs ALTER IL-2 AND IL-10 LEVELS IN ACTIVATED PBMCs (PAPER II)

Cocultures of PBMCs and MSCs showed a weak increase in IL-2 and sIL-2R. When MSCs suppressed proliferation of MLCs, the addition of MSCs significantly increased IL-2 and sIL-2R, as measured by ELISA and real-time RT-PCR. MSC-culture supernatants did not contain measurable levels of IL-2 or sIL-2R. In contrast to the increase seen in MLCs, MSCs decreased IL-2 levels in PHA-stimulated cultures, while sIL-2R levels were not significantly decreased. The PCR analyses demonstrated strong downregulation of both IL-2 and sIL-2R. Analyses of the adherent cells were negative for both IL-2 and sIL-2R.

We have previously shown that MSCs decreased surface bound IL-2R on PHA-activated T cells, consistent with a reduced activation.[122] It is possible that when we analyzed the mitogenic setting, MSCs inhibited activation of the T cells, and the up-regulation of IL-2R that is necessary for a continued activation. Instead, in the allogeneic setting the cells continue to produce IL-2, maybe to overcome the inhibition of proliferation. Alternatively, the MSCs may exert an effect downstream of the

activation of the IL-2 pathway, suggesting that the PBMCs recognize and respond to the stimulator cells, but that the intracellular response to IL-2 is extinguished.

Interleukin-10 levels increased in MLCs cocultured with MSCs, consistent with recent findings by others.[119] However, the increase in IL-10 levels did not correlate with the inhibition of proliferation by MSCs. IL-10 production in PBMC cultures stimulated by PHA were not affected by the addition of MSCs. Addition of antibodies to neutralize IL-10 further increased the inhibition of proliferation in MLCs, particularly when MSCs were present, but had no effect in cultures stimulated by PHA. Increased IL-10 levels have been reported in reactions against allogeneic tissue *in vivo*. Cytokine studies in patients with kidney transplant rejection demonstrated that IL-10 increased with acute but not in chronic rejection.[217,218] Increased IL-10 levels have also been associated with acute GVHD.[219] Thus, while discrepancies in published data regarding positive and negative mechanistic findings may reflect different MSC culture techniques, they more likely reflect the different stimuli used and the different lymphocyte populations tested.

MSCs INHIBIT INTRACELLULAR ACTIVATION OF PBMCs (PAPER II)

Several studies show that MSCs suppress lymphocyte proliferation induced by alloantigens and mitogens *in vitro*.[113,115-117,119,121,122] We reported in paper II that MSCs also suppress proliferation induced by IL-2. However, all tested stimuli depend on binding to an extracellular receptor. No previous study has analyzed the effects of MSCs downstream of the receptor level in T cells. When we stimulated PBMCs with PMA, MSCs inhibited the proliferative response both when the MSCs and the lymphocytes were in contact and when the cell populations were separated by transwell inserts. This would suggest that lymphocyte proliferation is inhibited downstream of PKC.

DIFFERENT ROLES OF PGE_2 DEPENDING ON THE STIMULATION OF PBMCs (PAPER II)

PGE_2 has been suggested as the factor mediating MSC-induced suppression of T cells.[95] To evaluate a possible role of prostaglandins, we inhibited synthesis of PGE_2 by indomethacin. The proliferation in MSC-suppressed PHA-stimulated cultures was partially restored by the addition of indomethacin, suggesting that inhibitory prostaglandins were involved in the MSC-induced suppression. However, indomethacin added to MLCs did not restore proliferation. These results may indicate a different role for PGE_2 in the two systems. It is possible that the MSCs affect lymphocytes differently depending on the type of lymphocyte activation. Fetal MSCs, in contrast to adult MSCs, only inhibited mitogen-stimulated cultures and not MLCs.[202] The ability of MSCs to inhibit alloreactions may only be acquired beyond the first trimester.

Inhibition of PGE_2 synthesis by indomethacin decreased PGE_2 levels in cocultures of MSCs and PBMCs stimulated by CD3/CD28 antibodies, but did not restore proliferation.[117] Activation by CD3/CD28 stimulation resembles lymphocyte activation in alloreactions. Aggarwal and Pittenger showed that inhibition of PGE_2 restored most of the proliferation when mitogen-activated PBMCs were cocultured with MSCs. Even the obstructed TNF-α and IFN-γ secretion from activated DCs and T cells was restored when PGE_2 synthesis was blocked.[95] Our data may explain the divergent findings in these two reports, confirming a role for PGE_2 in the suppression of mitogenic but not allogeneic stimulations.

MSCs STIMULATE IgG PRODUCTION BY B CELLS (PAPER III)

MSCs stimulated IgG production when cocultured with splenic MNCs or enriched B cells measured in an ELIspot assay. In cultures of unfractionated spleen cells the stimulation appeared to be mediated by a soluble factor or factors, whereas cell-cell contact was required after B-cell enrichment. Cocultures of MSCs and B cells increased IFN-γ responses, despite an absent proliferation. IgG secretion increased when PBMCs were cocultured with MSCs, but to a lower extent than in splenic MNCs cultures. This may indicate fewer MSC-reactive clones in the peripheral blood compared to the spleen. This kind of compartmentalization has been shown previously. Lymphocytes from different lymphoid organs responded differently to various bacterial stimuli.[220] It is also possible that MSCs induce maturation of B cells into plasma cells, resulting in an increased IgG secretion. MSCs secrete IL-6, originally discovered as a B-cell differentiation factor.[221] Plasma cells purified from BM or lymph nodes of mice died rapidly when plated in media, unless stromal cells were present. Stromal cells also induced a high secretion of antibodies, that was not seen when stromal cells were derived from IL-6 knock-out mice.[102]

We wished to analyze a specific B-cell response, and antibody secretion is a true B-cell specific feature. However, we cannot clearly say from our observations that the effect of MSCs is a direct effect on B cells, rather than interactions between MSCs and other cells, such as T helper cells or monocytes that in turn stimulate B cells to IgG production. Even though we used highly enriched B cells, since we used a negative selection procedure, we may have had a small proportion of contaminating cells. With positive selection there is a risk that antibodies bound to the B cells interfere with the ELIspot assay. Still, we believe that the stimulatory effect of MSCs was foremost a direct effect on B cells. Stimulation was more pronounced in unfractionated cell populations, even though the absolute number of B cells in these samples was lower than in enriched samples (>50% B cells in MNCs, versus >90% in enriched cultures). There may be two mechanisms regarding IgG stimulation by MSCs. One mechanism between B cells and MSCs that is contact dependent and one dependent on soluble factors, where MSCs interact with other immune cells than B cells that in turn induce IgG production.

MSCs both stimulated and inhibited IgG responses induced by LPS, CMV and VZV in splenic MNCs, depending on the level of stimulation. A strong stimulation by LPS combined with stimulation by MSCs may have resulted in paralysis of the B cells. Antigens and mitogens that stimulate overlapping populations can together potentiate responses when added in low concentrations, but when added together in high concentrations result in paralysis.[222]

MSCs DO NOT TRIGGER ACTIVATED CTL CLONES (PAPER IV)

In paper IV we used alloreactive as well as EBV peptide-specific HLA class I restricted CTL clones. MSCs remained resistant to CTL lysis even after pulsing the MSCs with the EBV peptide at high concentrations. This was seen in spite of a relatively high surface expression of the relevant HLA class I allele. MSCs were also much less sensitive to lysis by allo-specific CTL clones than HLA-matched LCLs were. EBV peptide-pulsed MSCs could not stimulate tyrosine phosphorylation of intracellular signaling molecules in CTLs. MSCs induced only a weak CD25 upregulation, and no CD3 or CD8 downregulation on the surface of CTLs. Furthermore, MSCs failed to induce IFN-γ and TNF-α production by CTLs. We propose that MSCs induce only an abortive activation program in fully differentiated effector CTLs, which does not involve activation of major CTL effector functions.

MSCs were less lysed, despite an intermediate to high expression of HLA class I, consistent with previous findings that PBMCs do not proliferate against MSCs even after IFN-γ induced HLA class I upregulation.[32] This may increase the ability of MSCs to survive even in an inflamed tissue where the production of IFN-γ by infiltrating cells is high. This study is consistent with previous reports where MSCs decreased the production of inflammatory cytokines, such as IL-12, IFN-γ and TNF-α from APCs, NK cells and $CD4^+$ cells.[95,129,130] Our results further strengthen the current evidence that MSCs can be used in the clinic without awaking an adverse immune reaction.

MSCs MITIGATE ONGOING SEVERE GVHD (PAPER V)

A 9-year-old boy with acute lymphoblastic leukemia (ALL) in third complete remission received allogeneic HSCT from an HLA A, B and DRB1 identical, unrelated, female donor. On day 11 after HSCT, the patient developed grade I GVHD of the skin that progressed despite cyclosporin and steroid treatment. By day 22, the patient developed severe diarrhea and abdominal pain requiring morphine, he stopped eating and required total parenteral nutrition. Increased bilirubin and alanine aminotransferase levels indicated liver damage. Despite continuous immunosuppressive treatment, by day 70, the patient fulfilled the criteria for grade IV acute GVHD of the intestine and liver. By then, BM had already been harvested from his mother, and was expanding in culture. The hope of reducing the severity of GVHD with the use of MSCs originated from our *in vitro* results,[32,116,223] phase I clinical trials showing no infusion-related toxicities with MSCs[114,178] and a preliminary report of co-transplantation of MSCs and HSCs from HLA-identical

siblings indicating a reduction in acute and chronic GVHD.[224] By day 73 the patient received an infusion of $2x10^6$ MSCs/kg. There was no infusion-related toxicity, and 4 days later, the frequency of diarrhea promptly decreased. A decline in total bilirubin was noted 5 days after the transplantation, and 2 weeks later the patient resumed oral food intake.

By day 143, DNA analysis revealed the presence of minimal residual disease (MRD) in the BM.[225] Cyclosporin treatment was discontinued to allow maximum GVL effect. By day 150, the patient again had diarrhea, but no abdominal pain, and biopsies showed only a mild GVHD in the intestine. Bilirubin again rose to an alarming level. He received a second MSC infusion of $1x10^6$ MSCs/kg on day 170. After 1 week, his stools were normal, the bilirubin level declined and he started to eat again. He was discharged on day 220, with no sign of MRD or acute GVHD.

In vitro studies indicated no alloreactivity between the patient's PBMCs and MSCs from the mother, whereas a response was seen against the mother's PBMCs. This was tested on several occasions before and after the first and second MSC infusion. The patient was highly immunosuppressed at the time of transplant, as indicated by absent lymphocyte responsiveness to mitogens and in MLCs. After MSC infusions immunologic recovery was prompt. MSCs from the mother continued to inhibit *in vitro* PBMC proliferation before and after the MSC infusions.

Biopsy specimens showed 4% female epithelium in the intestine by FISH analysis. The double-positive XX cells also stained positive for cytokeratin, a marker for epithelial cells. Local engraftment of donor MSCs and their differentiation could not be unequivocally demonstrated because the female hematopoietic stem cell transplant could not be excluded as a possible source of female epithelial cells. The cells were CD68 negative thereby excluding macrophage contamination of the specimen. All XX cells appeared to be diploid. The findings accord with those of a study in baboons where mismatched MSCs engrafted in gastrointestinal tissue after intravenous infusion.[49]

Findings published in paper I suggest that treatment of acute GVHD with MSCs may be optimal if combined with a treatment to eliminate already existing CTLs. This patient received most treatments available to reduce the activity of alloreactive T cells. This included psoralen and ultraviolet-A light (PUVA),[226] infliximab (anti-TNFα), daclizumab (antiIL-2R) and mycophenolate mofetil (MMF), an inhibitor of purine nucleotide synthesis leading to impaired proliferation of activated lymphocytes.

GVHD is not only severely damaging due to the destruction of tissues, but also because the immune system is dysfunctional. The patient was treated for repeated bacterial, viral, and invasive fungal infections. Despite the ability of MSCs to master the GVHD, the patient later succumbed to a viral-induced pneumonia and died, more than two years after HSCT. This makes the research on immune modulation by MSCs even more important. MSCs can suppress proliferation of PBMCs reactive against bacterial, viral and fungal antigens, showing an *in vitro* risk of decreased T-cell

responses against infections (Sundin et al., manuscript 2005). Nevertheless, there have been no reports suggesting an increased risk of infections after infusions of MSCs. IDO activity resulting in tryptophan depleting has been suggested as one mechanism mediating MSC suppression.[160] Tryptophan depletion has also been reported to limit microbial proliferation,[227,228] but whether IDO activation by MSCs is important in suppression of GVHD is unknown.

The inhibitory effect of MSCs was transient, and reduced the GVHD as long as no other challenges were presented. When the immunosuppressive drugs were discontinued to increase the GVL effect, the GVHD recurred and a second infusion of MSCs was needed. This shows that MSCs did not induce tolerance in the host, but more likely a transient immune suppression, possibly together with rapid healing of wounded tissues. *In vitro* data by Klyushnenkova et al. suggested that MSCs do not induce tolerance.[119] The authors cultured T cells with allogeneic PBMCs or MSCs derived from the same donor. T cells proliferated vigorously to PBMCs whereas they did not respond to MSCs. When the responder cells were rechallenged by the stimulator PBMCs, the responses were nearly identical, independent of if the first stimulator were PBMCs or MSCs.

8 CONCLUSION

- Human BM-derived MSCs inhibited the formation of CTLs reactive against allogeneic APCs. MSCs did not affect already activated CTLs, or lysis by NK cells.

- MSCs were not lysed by CTLs activated against PBMCs derived from the same donor as the MSCs, nor were KIR-ligand mismatched MSCs lysed by NK cells.

- MSCs increased IL-2, sIL-2R and IL-10 levels in MLCs, while the levels decreased or remained unchanged in mitogen-stimulated PBMC cultures. Inhibition of PGE_2 synthesis by indomethacin restored proliferation in mitogen-stimulated cultures suppressed by MSCs, but showed no effect in MLCs. These differences indicate the possibility of different mechanisms of inhibition depending on the T-cell stimuli.

- MSCs suppressed proliferation by IL-2 and PMA-activated PBMCs.

- MSCs increased the number of IgG-secreting cells, but not proliferation in splenic MNCs or enriched B cells. MSCs induced IgG secretion via soluble factors in MNCs while cell-cell contact was required for stimulation of enriched B cells. MSCs inhibited IgG production in splenic MNCs if B-cell stimulation by LPS, CMV or VZV was high. Suboptimal stimulation was potentiated by MSCs.

- MSCs did not activate allo- or EBV-reactive CTL clones despite presentation of their cognate antigen, when measured as tyrosine phosphorylation, cytokine release, surface activation markers and lysis.

- Infusion of haploidentical MSCs was safe, without infusion-related toxicity. MSCs reduced the severity of treatment-resistant grade IV acute GVHD in the intestine and liver. MSCs did not induce tolerance. MSCs did not immunize the patient, and a second infusion was given with similar good results.

MSCs possess immune modulatory functions *in vitro* and *in vivo* and the results presented in this thesis strengthen the evidence that MSCs may be used as cellular therapy for immune modulation and tissue repair.

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9 POPULÄRVETENSKAPLIG SAMMANFATTNING

Alla kroppens olika celler härstammar från det befruktade ägget och de embryonala stamceller som bildas från det. Även efter det att alla kroppens celler och vävnader har bildats, finns stamceller kvar. De behövs för att nya celler ska kunna bildas, eftersom celler hela tiden dör och behöver ersättas. Stamcellerna i den vuxna individen är begränsade och kan bara ge upphov till vissa typer av specialiserade celler. Exempelvis blodstamceller som ger upphov till blodkroppar. **Mesenkymala stamceller** (MSC) kan isoleras från bland annat benmärg. Benmärgen tas ut med en spruta som suger ut benmärg ur höftbenet medan givaren är lokalbedövad. MSC kan bilda ben-, brosk- och fettceller, vilka är mesenkymal vävnad. MSC producerar även många tillväxtfaktorer som stimulerar blodcellsbildningen i märghålan och interagerar med blodstamcellerna på ett sätt som främjar bildningen av nya blodceller.

Den här avhandlingen fokuserar på det faktum att MSC kan påverka immunförsvaret. T- och B-celler är viktiga komponenter i vårt specialiserade immunförsvar. T-cellen interagerar med till exempel virusinfekterade celler eller cancerceller och dödar dem. B-celler producerar antikroppar som är lösliga molekyler som cirkulerar i kroppen. Antikroppar till binda till exempelvis bakterier som därmed känns igen av immunförsvaret och dödas. Ett fungerande immunförsvar är väsentligt för att vi ska må bra. Dock finns det inflammatoriska sjukdomar där immuncellerna inte fungerar önskvärt. Då kan det vara positivt att kunna hämma immunförsvaret.

Vi har studerat hur MSC påverkar T- och B-celler. MSC kunde hämma T-celler, dels den snabba celldelning som sker efter aktivering, dels bildandet av så kallade mördar T-celler, vilka är de som dödar andra celler och skadar vävnader. Det kan vara önskvärt att hämma denna effekt om den skadar kroppen, men samtidigt vill vi inte öka risken för infektioner och tumörer. MSC stimulerade bildningen av antikroppar från B-celler, vilket kanske kan ge ett ökat skydd vid vissa infektioner. Idag räknas cellterapi med MSC som mycket experimentellt, därför används MSC bara när ingen annan behandling hjälper. I den här avhandlingen rapporteras om en patient som behandlats med MSC odlade från hans mamma. Pojken som tidigare hade fått transplantation av blodstamceller för att bota leukemi (blodcancer), drabbades av en livshotande transplantat-kontra-värd reaktion (graft-versus-host disease, GVHD). GVHD medför skador som orsakas av givarens T-celler efter blodcellstransplantationen. Förutom att olika vävnader angrips så drabbas patienten även lättare av allvarliga infektioner till följd av att patienten får starka immunhämmande mediciner för att minska GVHD reaktionen. GVHD orsakas främst av mördar T-celler som vi har visat i provrör kan hämmas av MSC. Därför valde vi att behandla patienten med MSC som sista utväg. Pojken hade skadad lever och tarm, konstant diarré och kunde inte äta. Knappt en vecka efter transplantationen av MSC, upphörde diarrén och han kunde börja äta vanlig mat igen. Han är den första patienten på Karolinska Universitetssjukhuset som har överlevt en så allvarlig GVHD. Den här fallstudien gör oss hoppfulla att MSC kommer att kunna användas i framtiden för att läka vävnad i kroppen som skadats av mördar T-celler.

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