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Multipotent mesenchymal stromal cell transplantation: possible viral complications and alloimmunity

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To my late grandparents Sonia and Åke

Contents

Summary

In the last few years, human multipotent mesenchymal stromal cells (MSC) have been increasingly used in novel therapeutic strategies due to their intrinsic immunosuppressive, anti-inflammatory and regenerative properties. In hematopoietic stem cell transplantation (HSCT), a curable treatment of hematological malignancies and several non-malignant conditions, MSC have been used as a therapy for graft-versus host-disease (GvHD) and other complications. The aim of MSC infusions in HSCT is to use the cells' immunomodulatory effects to reduce the immunological reactions giving rise to GvHD and to achieve tissue regeneration. This thesis evaluates the clinical safety from a virological point of view and the immunogenicity of MSC in HSCT recipients.

MSC were PCR screened for human herpesviruses and parvovirus B19 (B19), pathogens associated with severe infections in HSCT. The cells did not harbor herpesviruses, but presence of B19 DNA was detected in one MSC out of 20 screened. The presence of B19 is surprising since B19 is known for its extreme tropism for erythroid bone marrow cells. Upon exposure to the viruses, MSC supported infection of cytomegalovirus, herpes simplex virus and B19, but not Epstein-Barr virus as visualized by immunofluorescence. These infections could be passed to other uninfected cells implying that the infections of MSC were productive.

Even though MSC are typically regarded as lowly immunogenic, data on rejection exist. MSC immunogenicity was evaluated at both the humoral and cellular levels. No alloantibodies could be detected by flow cytometric cross matches. However, MSC bound antibodies directed to FCS – a component of the MSC culture medium. These antibodies are of uncertain clinical significance as they are constitutively expressed in humans. When evaluating MSC recipient lymphocytes in lymphocyte proliferation assays, there was no sign of allosensitization against the MSC donor, i.e. no immunological memory, 1 week to 6 months post-MSC infusion. In all instances, donor and third-party MSC failed to mount proliferative responses. *In vitro* studies revealed that MSC failed to prime responder cells to rechallenge with lymphocytes from the MSC donor and MSC rechallenge after PBL priming only gave weak responses. MSC failed to induce activated and effector CD4+ and CD8+ T lymphocyte subsets regardless of priming.

To conclude, MSC occasionally carry viruses and may constitute a viral reservoir of persistent viruses associated with considerably disease in HSCT recipients. The MSC do not seem to induce humoral or cellular immune responses after infusions of HLA disparate MSC as therapy of complications to HSCT.

Sammanfattning

Celler är kroppens byggstenar. Alla organ och vävnader består av celler med olika egenskaper; t ex leverns celler som kan avgifta blodet från ämnen som är skadliga, bukspottskörtelns celler som sänker blodsockret och gör vätskor som bryter ned maten vi ätit, de vita blodkropparna som försvarar kroppen mot infektioner. Stamceller är cellernas ursprung. De kan dela sig och bli till nya stamceller, men de kan också bli till flera olika sorters celler med olika egenskaper. Kroppens allra första stamcell är det befruktade ägget som gett upphov till den kropp vi idag har. Stamceller från det befruktade ägget kallas "embryonala stamceller". I de flesta vävnaderna i kroppen finns det också "adulta stamceller", vilka är celler som ser till att kroppens vävnader och organ hela tiden får nya celler och reparerar skador som organ och vävnader fått av olika anledningar.

Under de senaste åren har det forskats intensivt kring stamceller. Forskarna vill förstå hur de fungerar för att kunna använda stamcellerna som behandlingar vid olika sjukdomar: t ex Parkinsons sjukdom, diabetes, hjärtinfarkt, tandlossning, benbrott. Den enda stamcellen som idag används på sjukhus världen över är blodstamcellen. När man har blodcancer (leukemi) är blodstamcellen sjuk och gör defekta blodceller. Blodcancer botas med cellgifter, men ibland behövs en transplantation av nya friska blodstamceller (benmärgstransplantation) från vävnadstyplika släktingar eller frivilliga donatorer.

Benmärgstransplantationer är ingen lätt behandling, utan det finns flera livshotande komplikationer. Den vanligaste, som också kan bli mycket farlig, är transplantat-kontra-värd-reaktion. Då ger sig de nya transplanterade cellerna på patienten som fått dem. Det kan leda till svår diarré, elaka hudutslag, leverpåverkan och i värsta fall döden. För att undvika detta ger man mediciner som dämpar reaktionen, t ex kortison. Om dessa mediciner inte fungerar, har det inte funnits något att göra. Fram till nu…

Det finns i kroppen också ett slags adulta stamceller som kallas mesenkymala stamceller, eller sk multipotenta mesenkymala stromaceller. Dessa celler är bra på att hämma reaktioner som avstötning och transplantat-kontra-värd-reaktion. Varför det är så vet man inte. I en stor europeisk studie, där flera läkare och forskare runt om i Europa samarbetat, har det visats att mesenkymala stamceller verkar ha en positiv effekt på svår transplantat-kontra-värd-reaktion där ingen medicin fungerat. Mesenkymala stamceller kan inte bara lindra ovälkomna reaktioner vid transplantation, utan kan också bli till olika celler som ben-, brosk-, fett- och muskelceller.

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För några år sedan, vid ett rutinmässigt ultraljud på en gravid kvinna fann man ett foster med sjukdomen "osteogenesis imperfecta". Det är en sjukdom där det är fel på skelettbildningen. I lindriga fall föds en bebis med benbrott och i värsta fall dör bebisen på vägen ut. Fostret man sett hade flera benbrott redan i mammans mage och skulle sannolikt inte överleva en förlossning. Någon botande behandling finns inte. Fostret fick en injektion mesenkymala stamceller via navelsträngen och relativt komplikationsfritt föddes det en liten flicka som idag närmar sig skolåldern. Hon mår bra, växer och har bara haft något enstaka benbrott.

Den här avhandlingen handlar om risken att överföra virus med mesenkymala stamceller och hur mesenkymala stamceller inte känns igen av immunförsvaret:

- Precis som blodet på blodcentralen kontrolleras innan det ges till den som behöver blod, så måste stamceller och organ som ska transplanteras kontrolleras. I mesenkymala stamceller finns det sällan virus. Herpesvirusfamiljens virus hittades inte, men det gjorde parvovirus B19 – som ger upphov till "femte sjukan" hos friska barn och svår brist av blodceller hos transplanterade patienter. Det är möjligt att mesenkymala stamceller i benmärgen utgör en virus-reservoar, vilket måste beaktas inom transplantationsmedicinen.

- Mesenkymala stamceller kan stänga av reaktioner som transplantat-kontra-värd-reaktion, men de verkar dessutom själva inte kännas igen av immunförsvaret. Vid transplantationer bildas det anti-kroppar och immunceller som kan göra sig av med det trans-planterade (avstötning), om vävnadstypen inte överensstämmer mellan donator och mottagare. Studierna i den här avhandlingen visar att det inte riktigt är så för mesenkymala stamceller. Det verkar som att vem som helst skulle kunna ge vem som helst sina mesenkymala stamceller utan att de avstöts.

Sammanfattningsvis, mesenkymala stamceller är celler som kan bilda flera olika vävnader och hämma oönskade reaktioner vid transplanta-tioner. De bär sällan på virus, men kan göra det. Mesenkymala stamceller behöver inte vara vävnadstyplika mellan donator och mottagare, eftersom antikroppar och immunceller inte verkar avstöta dem. I framtiden kan cellerna kanske användas till att bota andra sjukdomar, som t ex benbrott, tandlossning, hjärtinfarkter, artros och ledgångsreumatism.

List of publications

This thesis is based on the four papers below, referred to by their Roman numerals:

I   Mesenchymal stem cells are susceptible to human herpesviruses, but viral DNA cannot be detected in the healthy seropositive individual. **Sundin M**, Örvell C, Rasmusson I, Sundberg B, Ringdén O, Le Blanc K. Bone Marrow Transplantation, 2006, 37: 1051-1059.

II Persistence of human parvovirus B19 in multipotent mesenchymal stromal cells expressing the erythrocyte P antigen: implications for transplantation. **Sundin M**, Lindblom A, Örvell C, Barrett AJ, Sundberg B, Watz E, Wikman A, Broliden K, Le Blanc K. Biology of Blood and Marrow Transplantation, *accepted*.

III No alloantibodies against mesenchymal stromal cells, but presence of anti-fetal calf serum antibodies, after transplantation in allogeneic hematopoietic stem cell recipients. **Sundin M**, Ringdén O, Sundberg B, Nava S, Götherström C, Le Blanc K. Haematologica, 2007, 92:1208-1215.

IV HLA mismatched MSC suppress T lymphocyte alloresponses *in vitro* and do not induce immunological memory in recipients of MSC infusion. **Sundin M**, Barrett AJ, Ringdén O, Uzunel M, Lönnies H, Dackland Å-L, Christensson B, Le Blanc K. *Submitted*.

List of abbreviations

ALS	amyotrophic lateral sclerosis
B19	parvovirus B19
CD	cluster of differentiation
CMV	cytomegalovirus
EAE	experimental autoimmune encephalitis
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
EMBT	European Group for Blood and Marrow Transplantation
FCS	fetal calf serum
FCXM	flow cytometric cross match
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte macrophage-colony stimulating factor
GvHD	graft-versus-host disease
GvT	graft-versus-tumor effect
HGF	hepatocyte growth factor
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HO-1	heme oxygenase-1
HSCT	hematopoietic stem cell transplantation
HSV	herpes simplex virus
IDO	indoleamine 2,3-dioxygenase
IFN-γ	interferon-γ
Ig	immunoglobulin
IL	interleukin
ISCT	International Society for Cellular Therapy
LIF	leukemia inhibitory factor
mHAg	minor histocompatibility antigens
MHC	major histocompatibility complex
MI	myocardial infarction
MLR	mixed lymphocyte reaction
MRD	minimal residual disease
MS	multiple sclerosis
MSC	multipotent mesenchymal stromal cells
MUD	HLA-matched unrelated donor
NO	nitric oxide
OI	osteogenesis imperfecta
PBL	peripheral blood lymphocytes
PGE2	prostaglandin E2
PTLD	post-transplant lymphoproliferative disorder

RIC	reduced intensity conditioning
RT	reverse transcription
SCF	stem cell factor
SCID	severe combined immunodeficiency disorder
SOS	sinusoidal obstruction syndrome
TAM	transplant-associated microangiopathy
TGF-β	transforming growth factor-β
Treg	regulatory T lymphocytes
VZV	varicella zoster virus

Stem cell transplantation

A stem cell is defined as a cell with self renewal potential which can continuously produce unaltered daughter progeny or produce daughter cells which display different, but more restricted properties. The two broad types of human stem cells are embryonic stem cells that give rise to all specialized fetal tissues and adult stem cells, which act as a repair system for the body, replenishing specialized cells, but also maintain the normal cell turnover in regenerative organs, e.g. liver, blood, skin and gastrointestinal epithelia.

Stem cell potency specifies the ability to differentiate into various specialized cell types: *totipotent* stem cells (i.e. cells derived from the first few divisions of a fertilized oocyte) have the ability to differentiate into all cell types giving rise to a human organism, *pleuripotent* stem cells are descendants of the totipotent cells and can differentiate into specialized cells from any of three germ layers, *multipotent* stem cells can only produce cells of a closely related family of cells (e.g. hematopoietic stem cells giving rise to the different types of blood cells), *unipotent* stem cells can only differentiate into one specialized cell type, but has the property of self-renewal that distinguishes them from non-stem cells.

To date the hematopoietic stem cells, which give rise to the lympho-hematopoietic system are the most studied human stem cells. Their clinical uses are dating back to the 1960's, when they were first transplanted between humans to cure diseases arising from defect or malignant hematopoietic stem cells. The hematopoietic stem cell transplantation (HSCT) has evolved from an experimental treatment into routine clinical practice. The remarkable success of HSCT has promoted interest for the use of other stem cell types as therapeutic options in the field of regenerative medicine. However, to date only hematopoietic stem cells are currently used in clinical transplantation.

Hematopoietic stem cell transplantation

The aims of HSCT are to replace abnormal hematopoiesis and to achieve an immunological strike against malignant cells. Nowadays, HSCT is used for the treatment of non-malignant hematological disorders, e.g. aplastic anemia, severe combined immunodeficiency disorder (SCID), thalassemia and as an enzyme replacement for some inborn errors of metabolism, and malignancies, e.g. leukemia and lymphoma. In oncology, HSCT is experimentally used as a treatment regime against solid tumors.

The HSCT can be divided into three groups: autologous, syngeneic and allogeneic. *Autologous* HSCT is when the patient receives the own stem cells. If the stem cell donor is genetically identical, i.e. a monozygotic twin, the transplantation is *syngeneic*. *Allogeneic* HSCT is performed between different individuals, mostly human leukocyte antigen (HLA) identical siblings or HLA-matched unrelated donors. The autologous HSCT results in neither a risk of graft-versus-host disease (GvHD), nor the potential advantageous immunological strike (graft-versus-tumor effect, GvT) on malignant cells. Furthermore, there is also a risk of relapse of malignancy, since the autologous graft may contain malignant cells[1]. The counterpart of all HSCT is allogeneic, and this thesis will focus on allogeneic stem cell transplantation.

History of hematopoietic stem cell transplantation

Hematopoietic stem cells, i.e. bone marrow, were identified prior to the knowledge of blood groups and HLA, and introduced as a part of treatment against anemia and leukemia. Quine reviewed the fumbling attempts to use bone marrow as a "remedy" during the 1890's. In only a few instances the treatment was successful, but the positive effects seen were interpreted as a possible clinical application in the future[2]. In the 1930's, Josefsson tried to cure pernicious anemia and other anemia by injecting preparations of liver into the myeloid cavity of the sternum. The treatment was successful in several cases and once again the replacement of a defect hematopoiesis was proposed as a cure of hematological disorders[3]. In 1939, Osgood and colleagues transplanted bone marrow from a blood group matched sibling to a patient with aplastic anemia. Although the patient deceased one month post-transplantation, regenerating islets of bone marrow were seen in the postmortem bone marrow examination[4].

During the post-world war period, bone marrow failure became associated to exposition to ionizing radiation. Experimental animals were lethally irradiated and developed subsequently an acute irradiation syndrome – severe anemia, leucopenia, and thrombocytopenia – which was revoked by injections of bone marrow cells[5, 6]. Later it was determined that the recovery was due to donor cells engrafting the bone marrow cavity[7, 8].

In 1956, Barnes and colleagues suggested the usage of bone marrow to treat humans[9] and the first successful bone marrow transplantation was performed in 1968 by Robert A. Good at University of Minnesota Medical School[10]. The first systematic transplantations were during the same time period performed by E. Donnall Thomas, first at Mary Imogene Basset Hospital and thereafter at Fred Hutchinson Cancer Research Center.

Despite poor results, it was shown that bone marrow grafts could be administered intravenously without severe adverse events[11, 12]. The first 200 transplants were reviewed by Mortimer M. Bortin, who described a generally poor outcome. Most patients were terminally ill in advanced therapy-resistant malignancies and succumbed before it was possible to evaluate the effect of the transplantations. Other displayed hematological recovery, but died from immunological reactions[13]. Today, this immunological reaction characterized by skin lesions, weight loss and diarrhea is recognized as GvHD[14-17].

In the end of the 1960's and early 1970's, the results improved and several successful bone marrow transplants were reported for both benign and malignant hematological disorders[18-20]. However, the major breakthrough was the discovery of the HLA system[21]. In the mid-1970's, it was evident that successful outcome very much depended on the matching of donor and recipient HLA[22]. HSCT as a clinical application has improved gradually due to the advancement of medical knowledge and the development of modern pharmaceuticals.

Transplantation immunology

Histocompatibility is principally determined by the HLA system, which is the human version of the major histocompatibility complex (MHC). Over 200 genes on the short arm of chromosome 6 constitute the HLA complex. More than 40 genes encode leukocyte antigens and the remaining is an assortment of genes without evolutionary relationship to the HLA. Although, some of these genes are involved with HLA genes functionally, several genes within the complex have no defined role in immunity. The HLA genes involved in immune responses are a multigeneic system encoding structurally homologous cell surface molecules. These glycolproteins are characterized by a high degree of allelic polymorphism[23].

The HLA are divided into three classes. Class I and II present antigens in contrast to class III, which are involved in immunity by expression complement proteins and cytokines. Most somatic cells express HLA class I, presenting intracellular antigens to CD8+ T lymphocytes. HLA class II present endo- and phagocytosed extracellular antigens on "antigen presenting cells", i.e. B lymphocytes, activated T lymphocytes, macrophages, dendritic cells and thymic epithelial cells, to CD4+ T lymphocytes[23].

A heterozygous individual may, due to co-dominant expression, have up to 12 different HLA molecules of HLA class I and II. There are three class I molecules: A, B and C, encoded by one gene respectively for the

heavy chain. β-microglobulin is non-covalently associated with those heavy chains to form the HLA class I molecules. The HLA class II molecules; DP, DQ, DR are heterodimers encoded by an α-chain and a β-chain gene that colocalize. To allow recognition of a wide range of foreign proteins, HLA diversity has probably been preserved throughout the evolution[23]. The HLA genes are the most polymorphic in the human genome, as illustrated in **figure 1**.
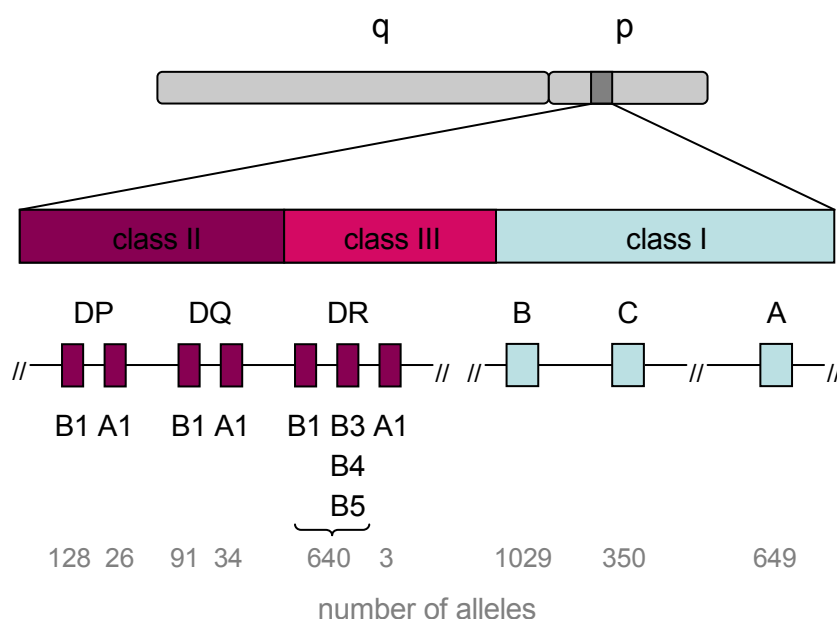
Figure 1 – The histocompatibility is mainly determined by the HLA system. On the short arm of chromosome 6, the HLA genes are clustered. Multiple genes with high polymorphism constitute the HLA system of each individual. The number of alleles known in June, 2008, is 3187 (http://www.anthonynolan.org.uk/research/hlainformaticsgroup).

The HLA diversity, which is beneficial to achieve immune responses against almost all possible antigens, may be a problem in the transplantation setting. Minor differences between donor and recipient HLA can provoke alloreactivity, i.e. unwanted immune responses, jeopardizing a successful outcome of the transplantation. Thus, matching of HLA has significantly improved engraftment and decreased presence of GvHD. Matching is also essential for the HSCT recipient to recover a working immune system. Donor derived T lymphocytes need to recognize antigens presented by both donor and recipient HLA to act powerfully[24-26].

HLA are the central means in selecting donors, but polymorphism in other genes may also have impact on the HSCT. Polymorphism in genes of killer immunoglobulin-like receptors expressed by NK cells, in the tumor necrosis factor-α gene (located in the HLA class III region) and other

cytokine genes have been correlated to occurrence of GvHD and transplantation outcome[27-32]. Even if the donor and recipient are HLA-matched, but not monozygotic twins, there are differences in numerous other endogenous antigens presented by HLA. Such differences arise from polymorphism in non-HLA genes and genomic differences between the two sexes, and these antigens are designated *minor histocompatibility antigens* (mHAg)[33-35]. The *AB0-histo blood group antigens* may also have an impact on the HSCT outcome. As all individuals, HSCT recipients produce anti-A/B antibodies against the missing blood group antigens. More than one third of all transplants are performed across the AB0-barrier, which may result in different complications – e.g. hemolysis, delayed engraftment and GvHD[36].

The procedure

The ideal HSCT donor is an HLA matched sibling. Due to the closely linked HLA genes that constitute a single genetic locus, any pair of siblings has a 25% chance of being HLA identical. Clinically, sibling donors are available in one third of the HSCT[37]. In 1972, the first attempts of using matched unrelated donors (MUD) were performed[38] and since 1974 MUD have been collected in registries[39]. The probability of finding a suitable donor is about 80% depending on the ethnic background[40].

Previously, bone marrow was the source of hematopoietic stem cells used in HSCT. The cells were collected by aspirations from the donor iliac crest under anesthesia[41]. Today, hematopoietic stem cells for trans-plantation are commonly collected from peripheral blood after mobil-ization by granulocyte and granulocyte/macrophage colony stimulating factor (G-CSF and GM-CSF)[42-46]. It is also possible to use hematopoietic stem cells from umbilical cord blood[47-49].

For the recipient, the HSCT starts with a conditioning regimen. There are three main objectives; creating space, immunosuppression and eradication of disease. However, the ultimate role of conditioning is long-term disease control[12, 41, 50]. The cytostatic drug cyclophosphamide in combination with irradiation or other cytostatics are used to achieve a myeloablative conditioning[12, 51-54]. This type of conditioning is associated with toxicity and tissue damage. Nowadays, many conditioning regimens are reduced in intensity and instead focused on immunosuppression. In transplantation after reduced intensity conditioning (RIC), the trans-planted cells will gradually eradicate the lymphohematopoietic system, including the remaining malignant cells (i.e. GvT). This implies a lower toxicity and that fragile, older and co-morbid patients can have a HSCT[55].

After the conditioning, the HSCT recipient is administered the hematopoietic stem cells intravenously through a central venous line. Surface molecules on the stem cells direct them to the bone marrow cavity, i.e. homing, and gradually healthy blood cells are produced[56].

Immunosuppression is an important factor that has improved the HSCT outcome. Methotrexate and cyclosporine A are the most common drugs in preventing GvHD, but newer agents such as tacrolimus, sirolimus and mycophenolate mofetil have been introduced. The common mechanism of these drugs is to inhibit T lymphocyte proliferation[57-60]. Depletion of T lymphocytes in the graft can be used, but is associated with complications such as rejection and relapse[61-64]. Furthermore, supportive care, i.e. transfusion policies, pain relief, parenteral nutrition and pro-phylactic antimicrobial therapy are crucial to succeed with the HSCT[65-67].

Monitoring the outcome

Although the HSCT is a successful treatment, the outcome needs to be surveyed, i.e. relapse of malignancy and possible rejection has to be monitored. This is mainly performed trough analyses of *chimerism* and *minimal residual disease* (MRD). In Greek mythology the "Chimera", as described by Homer, was a fire-breathing creature with the head of a lion, the body of a goat and the tail of a snake. Thousands of years later, Anderson and colleagues introduced the term chimerism in medicine to describe the phenomenon of organisms with cells derived from more than one zygote lineage[68]. In HSCT, chimerism refers to the number of donor-derived lymphohematopoietic cells post-transplant. Nowadays, most chimerism analyses are PCR-based. The methodology exploits that some core DNA sequences are tandemly repeated in the genome and that the number of those Mendelian concomitant inherited repeats varies among individuals. Chimerism is described as percentage recipient-derived B lymphocytes, T lymphocytes and myeloid cells over time[69, 70]. Deviation in chimerism may indicate relapse or rejection, which have to be intervened[70-72]. The MRD is defined as presence of a small number of cells expressing molecular markers of disease detected at a threshold far below what can be detected by standard methodology. By monitoring MRD, with immunophenotyping and PCR, potential relapses can be identified for immediate treatment[73-75].

Complications

The *early complications* of HSCT arise several days after the conditioning and up to weeks after the infusion of the hematopoietic stem cells. One significant early complication is the "hemorrhagic cystitis" that present as symptoms ranging from painless microscopic hematuria to severe hemorrhage along the entire urinary tract. The incidence post-HSCT is reported to vary from as low as 10 to as high as 70%[76-78]. Early onset is related to the conditioning regimen[52, 76, 79]. Later onset seems associated to viruses such as BK virus[80, 81], adenovirus[82, 83], cytomegalovirus[84, 85] and to GvHD[76, 77, 86]. Prevention and treatment are based on hyper-hydration. Irrigation of drugs, embolization and cystectomy has been used as treatment[87]. Another entity of complications arises from injured vascular endothelium and comprises a heterogeneous and overlapping group of clinical syndromes. The two most threatening conditions in this group are: "sinusoidal obstruction syndrome" (SOS) and "transplant-associated microangiopathy" (TAM). SOS, previously called venoocclusive disease of the liver, is characterized by a painful hepatomegaly, jaundice and fluid retention. Anticoagulants and thrombolysis along with symptomatic treatment have been used as therapy[88, 89]. TAM, previously referred to as thrombotic microangiopathy, is characterized by anemia, presence of schiztocytes, elevated lactate dehydrogenase, thrombo-cytopenia, fever and renal insufficiency. Discontinuation of cyclosporine A or tracrolimus and usage of other immunosuppressants may resolve the syndrome. Plasmapheresis and thrombolytics have been used with varying results[90, 91].

One of the most important causes of morbidity and mortality after HSCT is *infections.* The breakdown of mucosal integrity, by toxic effects of the conditioning, and the immunodeficient state (due to the GvHD, the conditioning and the immunosuppressants) generate an opportunity for invasive infections. Bacteria are one major concern and in one third of the patients positive blood cultures are found[67, 92, 93]. Also fungal infections and reactivation of latent viruses constitute to the spectrum of infectious diseases seen in HSCT[94-96]. In **figure 2**, the infectious complications of HSCT are illustrated. Almost all patients reactivate cytomegalovirus (CMV) post-transplant and up to one third develop symptomatic infection. Pre-emptive treatment strategies, based on PCR-surveillance of CMV, have greatly reduced the risk of fatal disease[94, 97-100]. Reactivation of Epstein-Barr virus (EBV) is also seen after HSCT, but it seldom causes direct viral end-organ disease. Nevertheless, the important complication of EBV is a lymphoma-like condition called post-transplant lympho-proliferative disease (PTLD)[94, 101]. This complication seems to be markedly increased in cord blood transplantation with usage of antithymocyte globulin[102]. After development of strategies to improve early diagnosis,

prevention and treatment of opportunistic infections the early post-transplant outcome has been improved. Due to loss of specific immunity, active immunizations to tetanus, poliovirus and diphtheria is generally performed in all transplant populations[92, 94]. Live vaccines are no longer prohibited, since they have been safely used in children after HSCT[94, 103, 104].
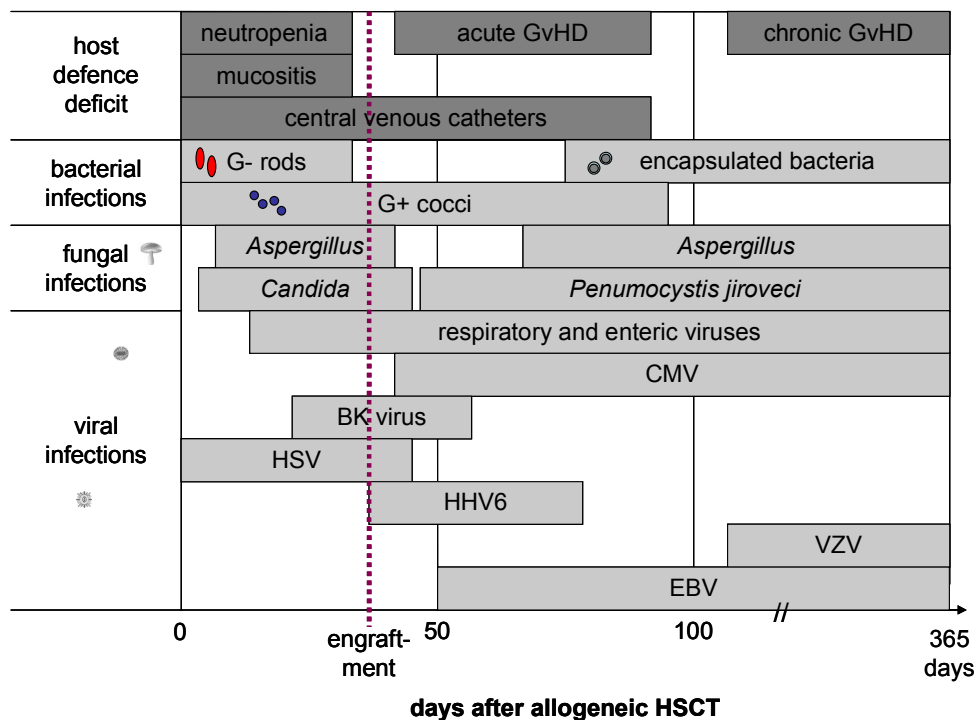
Figure 2 – Infectious complications after HSCT. The graph represents a general overview of post-HSCT infections. However, it should be noted that infectious complications vary between different transplant centers. G-, Gram negative; G+, Gram positive; HHV6, human herpesvirus 6. Figure adopted, but modified, from Marty and Rubin, 2006[92].

GvHD is the most important complication, associated to the greater number of morbidity and mortality, in HSCT. The clinical manifestations depend on the degree of HLA mismatch between donor and recipient and the alloreactivity of the graft to host antigens. A high degree of HLA matching is associated to early engraftment and reduced severity of GvHD[25]. Despite this, patients still develop GvHD due to reactivity to mHAg[105]. The pathogenesis behind GvHD is complex and divided in three phases. Firstly, the conditioning induces tissue damage that activates host cells to produce pro-inflammatory cytokines leading to enhanced allo-recognition by transplanted cells. Secondly, alloreactive donor-derived cells expand in response to presented host alloantigens. Thirdly, the immune effector cells exert their functions along with further secretion of cytokines.

All these cellular and inflammatory effector events result in tissue destruction[106]. The acute GvHD classically develops within the first 100 days post-HSCT. Main targets are the skin, liver and gastrointestinal tract. Cell death leads to exanthema, mucosal denudation with subsequent diarrhea, and biliary stasis[14, 107]. Acute GvHD is staged within each organ system and weighed together to a functional grade. Grade I has a favorable prognosis, grade II is a moderate disease and grade III-IV represents severe multiorgan to life threatening disease[108, 109]. The incidence of GvHD varies from 10 to 80% depending on the degree of HLA matching, number of lymphocytes in the graft, patient age and GvHD prophylaxis[26, 110]. High-dose corticosteroids, cyclosporine A, anti-thymoglobulin[111, 112] and psoralen with subsequent ultraviolet light[113, 114] can be used as treatment. Recently, multipotent mesenchymal stromal cells (MSC) have been tried in therapy-resistant GvHD with prosperous results[115, 116] (see »clinical applications« in next chapter). The chronic GvHD is poorly understood, however donor-derived T lymphocytes seem to play an important role[117]. Almost all organs can be affected by the disease, which is associated to reduced quality of life and mortality. The incidence varies from 40 to 60% in long-term survivors. The treatment is overall the same as for acute GvHD[118-123].

Graft failure and *rejection* are two major HSCT complications associated with poor outcome. The principal reason of graft rejection is an insufficient conditioning regimen. Immunocompetent recipient cells survive and mediate rejection of donor cells (see »transplantation immunology« above). With improved treatment protocols and better HLA matching, rejections can be reduced[124-126]. Occasionally, it can be difficult to distinguish graft rejection from graft failure – a rejection subsequently gives rise to graft failure. The failure can be early, i.e. lack of hematopoietic recovery, or late, associated to recurrence of disease or reappearance of recipient cells after an initial engraftment[127, 128]. The treatment of these complications is the administration of a secondary conditioning with subsequent infusion of a new graft, however re-transplantations are riskful[129].

The *late complications* of HSCT have become more evident over the past years since there are a large number of patients now surviving long-term. Late adverse events can be divided into malignant and non-malignant conditions. The non-malignant complications, impairing quality of life, represent a heterogeneous spectrum of disorders[130], e.g. ocular side effects[130-132], liver complications[130, 133-135], pulmonary dysfunction[130, 136, 137], endocrine complications[130, 138-140], bone-joint-dental damages[130, 141, 142] and neuropsychological impairments[130, 143]. It was first in the early 1990's that the risk of secondary malignant diseases post-HSCT was described. The improved survival resulted in a need to assess secondary malignancies,

which are divided into three groups: lymphoma (including PTLD)[144-147], leukemia[148-152] and solid tumors[150, 153-156].

Immune reconstitution

After HSCT, function is variably impaired in all branches of the immune system. The immune reconstitution, both tempo and diversity of the repertoire, is influenced by several factors – patient age, stem cell source, graft manipulation and conditioning. Recovery of innate immunity appears to occur rapidly, at least in terms of quantitative evaluation, whereas recovery of adaptive immunity is typically delayed and often incomplete.

Innate immunity includes complement, NK cells, granulocytes and antigen presenting cells. The complement is not generally deficient post-HSCT[157]. NK cells normalize within the first month and are important for the antiviral immunity, GvT and graft rejection[158-160]. Granulocytes, especially neutrophils, are important to prevent infections. Using mobilized stem cells and non-myeloablative conditioning, time to neutrophil recovery has been reduced[161]. Conversely, cord blood is associated to a prolonged neutropenic period[162, 163]. GvHD further impairs the commonly seen early neutrophil dysfunction[164, 165]. Antigen presenting cells recover from 1 month to 1 year post-HSCT and a defective antigen presentation may result in both B and T lymphocyte dysfunctions[166].

Adaptive immunity, the cell mediated immunity, is orchestrated by functioning T lymphocytes. The T lymphocytes seen early post-transplant are mainly donor-derived, even though residual recipient cells are seen occasionally. Homeostatic peripheral expansions provide an early pool of reconstituting T lymphocytes, explaining the predominating memory phenotype seen[167]. Antigen driven expansions may occur, which results in a skewed reconstitution[168]. CD8+ T lymphocytes recover rapidly, by 3 months post-HSCT, and normalize within 1 year. The reconstitution of CD4+ T lymphocytes is slower, up to 20 months, and dependant on the thymic activity. Children recover CD4+ T lymphocytes faster than adults, probably due to a larger thymic mass[166, 168]. Faster T lymphocyte recovery is associated to the number of T lymphocyte inoculum, i.e. mobilized stem cells contain more T lymphocytes than bone marrow, which is superior to cord blood in this regard[166, 168]. B lymphocyte counts are low in the 2 first months post-HSCT, but they subsequently rise and are normalized by 1-2 years post-transplant[169]. Plasma cells are resistant to both irradiation and chemotherapy, which is why the antibodies become primarily of recipient in origin several months post-HSCT[170]. The serum immunoglobulin (Ig) levels decreases gradually and are followed by a donor-derived normal-

ization. Serum isotypes develop in the order they develop in childhood: IgM levels recover within 2-6 months followed by isotype switching (dependent of CD4+ lymphocytes), with subsequent increase of IgG (in the order IgG1, IgG3, IgG2 and IgG4) in 3-18 months and finally IgA levels within 6-36 months[171, 172].

Future stem cell transplantation

In the last years, the field of stem cell research has grown greatly and interest in stem cells for reparative and regenerative medicine has increased extensively. HSCT is the only stem cell based therapy that is implemented in the clinical practice. In ongoing studies, the HSCT is optimized and new indications are tried. The next chapter will describe transplantation of MSC, which seem to be the second most transplanted stem cell in humans. Still, the usage of MSC in clinical medicine is under evaluation. Currently, there are several studies exploring other, embryonic and adult, stem cells for clinical applications. Time will show whether stem cell transplantation will end up as the universal remedy of human diseases…

Hematopoietic stem cells

It is over a decade since the RIC was introduced as a new direction in the field of HSCT. The major limitations have been to separate GvT from GvHD and disease relapse[173]. Preparative regimens with usage of low-toxicity targeted therapeutics such as imatinib, bortezomib, rituximab to partner with RIC HSCT to combine GvT and targeted treatment are under development[173-176]. The RIC HSCT may also be used as a platform for controlled immune reconstitution, where the beneficial GvT is promoted without risking GvHD and profound immunoincompetence leading to severe infections. Several investigators are trying different approaches such as antibodies against antigen presenting cells, suicide-gene transfected T lymphocytes and anti-CD25 immunotoxins[173]. Selective depletion may be another mean in reducing GvHD. Alloreactive cells are removed or inactivated by antibodies (against CD25, CD69 or CD95) or photo-depletion. The removal of such cells seems to reduce the GvHD and improve the outcome[177].

The best results with allogeneic HSCT have been obtained in patients receiving allograft from HLA matched siblings. As the chance of finding such donors is only 25%, much attention has been given overcoming the HLA barrier, i.e. using mismatched or haploidentical donors instead. In general, the investigators have utilized T lymphocyte depletion and

selection of CD34+ cells in myeloablative or RIC settings with varying results[178]. Mismatched and haploidentical HSCT provides an opportunity when fully matched siblings are lacking. Some studies of haploidentical HSCT display a beneficial GvT by alloreactive NK cells, which is expected to encourage a greater use for leukemia patients[179-181]. Still, better selection of recipients and donors, development of safer conditioning regimens promoting engraftment and reduction of GvHD are prerequisites for the use of mismatched and haploidentical HSCT as a routine[178].

The indications for HSCT are widening. Regeneration of a healthy immune system is of interest in the treatment of multiple sclerosis (MS). More than 300 MS patients have received HSCT as treatment with varying results[182]. Most were autologous, but a few allogeneic transplants have been performed[183]. Currently, HSCT for MS is regarded highly experimental. HSCT may also be used as treatment of inflammatory bowel diseases. Positive clinical effects have been shown for both allogeneic and autologous HSCT. Autologous HSCT is preferred, due to the higher transplantation related mortality in allogeneic HSCT. However, allogeneic HSCT might be a upcoming treatment option in gastrointestinal diseases, as the allogeneic HSCT gradually improves[184]. The experience with HSCT as a therapy for metabolic diseases has demonstrated unquestionable success as well as limitations. Improved HSCT in combination with enzyme replacement, chaperone therapy and substrate inhibition are believed to be the way to go for archival of optimal outcome in these patient categories[185].

Other stem cells

The evidence to date suggests that embryonic stem cells are not ready to be used in the clinic. However, their enormous potential in regenerative and reparative medicine cannot be ignored. Theoretically, embryonic stem cells can be used for therapy of all organs. When it comes to transplantation of these stem cells, their immunogenicity is poorly understood and still rudimentarily described[186].

Endothelial progenitor/stem cells might be used in treatment of ischemic heart disease and for various applications of tissue engineering[187]. In cardiology, unfractionated bone marrow cells (assumed to contain different progenitors) have been used in clinical trials with conflicting results and embryonic stem cells are under investigation in different animal models[188, 189]. Almost all fields of medicine have some confidence in stem cells, so even hepatology. Recent preclinical studies propose *ex vivo* differentiation of stem cells into hepatic cells for transplantation, rather than transplantation of undifferentiated stem cells. Embryonic as well as

amniotic and fetal liver cells might be used for therapeutic regeneration of the liver[190]. Both adult and embryonic stem cells are of interest as possible stem cell therapy in the endocrine pancreas, but also in this field there are a lack of conclusive results and only preclinical studies have been performed[191, 192]. In regenerative nephrology, adult bone marrow derived stem cells, embryonic stem cells and stem cells derived from specific kidney niches have been proposed as therapy of ischemic, toxic and chronic kidney diseases[193]. There are also several attempts to cure neurological disorders, such as Parkinson's disease, using stem cells[194].

As described in this section, both embryonic and adult stem cells have a theoretical potential in clinical medicine. At present, there is a lack of convincing clinical results for the usage of stem cells other than the hematopoietic. However, it is worth bearing in mind that it took several decades for the HSCT to develop into the present unquestionable and successful treatment as it is nowadays, thus future advances may allow for use of non-hematopoietic stem cells in clinical settings.

Multipotent mesenchymal stromal cells

The plastic-adherent cells isolated from human bone marrow and other tissues have come to be widely known as mesenchymal stem cells. However, generally accepted stem cell criteria do not seem to be fulfilled by the biological properties of the unfractionated cell population. The name "mesenchymal stem cells" is therefore inaccurate and potentially misleading to the inexperienced person. Nonetheless, it is believed that *bona fide* mesenchymal stem cells exist, although this has yet to be proven. To address the discrepancy between nomenclature and biologic properties, the International Society for Cellular Therapy (ISCT) has proposed that plastic-adherent fibroblast-like cells, regardless of source, should be termed "multipotent mesenchymal stromal cells" (MSC). Hence, the term mesenchymal stem cell is reserved for cells fulfilling the criteria of a "true" stem cell[195]. During the last decade, the scientific achievement in the field of MSC has increased exponentially. Unfortunately, investigators have used different approaches in characterizing their MSC. To stimulate the development of the field and to facilitate exchange of results between investigators, the ISCT proposed minimal criteria for defining MSC in 2006. The human MSC are defined according to three criteria: (1) plastic-adherence in standard culture conditions, (2) surface marker expression: CD14- or CD11b-, CD19- or CD79α-, CD34-, CD45-, HLA-DR-, CD73+, CD90+, CD105+, and (3) tri-lineage differentiation *in vitro*: chondrocyte, osteoblast and adipocyte[196].

The discovery

Tissue regeneration has interested researchers for a long time. The hematopoietic stem cells were identified after an extensive search for cells that could allow survival after radiation exposure. At the same time, several studies displayed formation of bone when bone marrow was transplanted to an ectopic site[197]. In the late 1960's, Friedenstein and colleagues were the first to isolate the cells, from bone marrow, that could form ectopic bone[198, 199]. In parallel to the hematopoietic stem cell and its lineages, the idea of a mesenchymal stem cell developed. This stem cell, a single cell, was believed to be capable of forming bone, cartilage and other mesenchymal tissues. The notion of a stromal stem cell was proposed, largely based on Friedenstein's work, in the 1980's by Owen and colleagues[200]. In the 1990's, Caplan and colleagues popularized the term mesenchymal stem cell[201]. Haynesworth and colleagues thereafter developed a reliable *in vivo* bone-

forming assay and were able to isolate and *ex vivo* expand human MSC in therapeutic quantities[202].

Sources, multipotency and phenotype

Human MSC were first identified in the bone marrow, which still seems to be the most common source in the field. MSC constitute a minor fraction, i.e. 0.001 to 0.01% of all marrow cells[203, 204]. The prevalence of MSC decreases over time, illustrated by the fact that a neonate has 200 times more MSC than an 80 year old[203]. MSC have also been isolated from prenatal[205] and various postnatal tissues, e.g. fat, synovium, cartilage, periosteum, placenta, and cord blood[206].

An extensive proliferative capacity is displayed by the MSC. The cells can be passaged more than 25 times *in vitro*, i.e. over 50 cell doublings, without signs of differentiation. MSC are easily *ex vivo*-expanded, since a small volume of bone marrow can render billions of MSC. As for the majority of adult stem cells, cell division is accompanied by telomere shortening which reaches, with age/multiple divisions, a critical size beyond anomalies of cell division occur. Hence, signs of senescence and apoptosis appear[207-209]. The ability of MSC to differentiate into bone, cartilage and fat has been thoroughly examined, as reviewed[206]. Although the MSC are defined by the tri-lineage differentiation, they display a broader potential to differentiate. Differentiation into other mesenchymal lineages, such as myocytes, tendinocytes, ligamentocytes[210], cardio-myocytes[211] have been described. Reports also indicate a possible non-mesenchymal differentiation, as the MSC have been described to become neural[212, 213], endothelial[214] and hepatic cells[215]. However, most of the studies have been performed in different cell populations, which make it impossible to draw any firm conclusions regarding the multipotency of MSC. Using MSC clones, it has been shown that not all cells are able of supporting a tri-lineage differentiation. Nevertheless, all cells differentiated into bone, implying a default pathway[216]. The MSC multipotency is illustrated in **figure 3**.

MSC are stromal cells that play a role of hematopoiesis support by their interaction with the hematopoietic stem cells and the secretion of cytokines and growth factors crucial for production of blood cells[217]. Stromal cells are also important in the development of functional B lymphocytes[218]. Membrane molecules belonging to the integrin family ($\alpha1\beta1$, $\alpha5\beta1$), the Ig superfamily (ICAM-1, VCAM-1, HCA), and CD44 are expressed by MSC[204, 209, 219]. Among the innumerable cytokines and growth factors, the MSC produce: interleukin (IL)-6, IL-7, IL-8, IL-11, IL-12, IL-14, IL-15, IL-27, leukemia inhibitory factor (LIF), GM-CSF, G-CSF,

M-CSF and stem cell factor (SCF)[220-224]. In co-cultures, MSC are able to maintain and expand lineage-specific colony-forming units from CD34+ cells in long-term cultures[219].
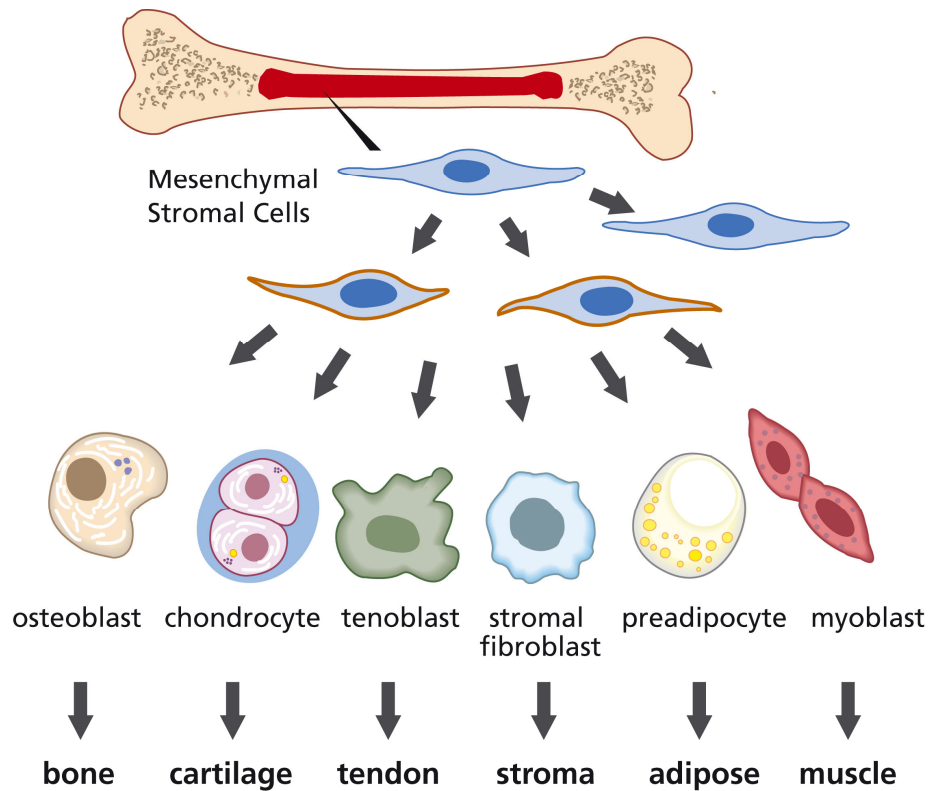

Figure 3 – The MSC multipotency. MSC are able to proliferate and divide without loss of phenotype. However, the cells can also differentiate, preferentially into the mesenchymal lineages displayed above.

The MSC are characterized by the criteria proposed by the ISCT. Currently, the *ex vivo*-expanded MSC should be negative for hematopoietic and endothelial markers, but positive for others[196]. However, the search for an MSC specific marker has resulted in a long list of alternative positively expressed surface markers: ALCAM (CD166), ICAM-1 (CD54), ICAM-2 (CD102), ICAM-3 (CD50), NCAM (CD56), HCAM (CD44), VCAM (CD106), ITG-α1 (CD49a), ITG-α2 (CD49b), ITG-α3 (CD49c), ITG-α4 (CD49d), ITG-α5 (CD49e), ITG-α6 (CD49f), Tetraspan (CD9), MUSC18 (CD146), BST-1 (CD157), NGFR (CD271) and STRO-1[225]. No MSC specific marker has so far been identified and the *in vivo* phenotype of MSC remains to be defined.

Interplay with the immune system

Evidence has emerged that MSC deploy an array of mechanisms allowing escape from allogeneic immune responses and that the cells are able to suppress various immune reactions. The exact mechanism of action has still to be elucidated. However, interesting results partly explaining the interaction with the immune system on several levels will be presented in this section. The immunology of transplantation is briefly introduced in »transplantation immunology« of the previous chapter.

Immunogenicity

Most studies have described human MSC as HLA class I positive and HLA class II negative[219, 226, 227]. The expression of HLA class I is important, since expression protects the cells from NK cell effector mechanisms. For instance, a major function of NK cells is to kill tumor cells that downregulate HLA class I[228]. In co-culture experiments, human MSC fail to elicit proliferative responses of allogeneic lymphocytes[223, 226]. Because HLA class II antigens are potent alloantigens, the lack of/low expression on MSC is another crucial factor for the reduced immunogenicity of MSC. Even if MSC were induced by interferon (IFN)-γ stimulation to express HLA class II they seem to escape alloreactive lymphocytes[223, 226, 227]. Additionally, MSC do not express the co-stimulatory molecules CD40, CD40L, CD80 or CD86 required for initiation of effector T lymphocyte function[219, 227]. After provision of a co-stimulatory signal by anti-CD28 antibodies or MSC transfection of CD80 and CD86, immune responses against the MSC were absent[227, 229]. MSC have been reported to escape both lympholysis and NK cell mediated lysis[230]. This is corroborated by more recent *in vitro* studies, suggesting that MSC are able to present tumor or viral antigens with limited efficiency and avoid lympholysis by specific cytotoxic lymphocytes[231].

In humans it has been demonstrated that MSC seem to avoid normal alloresponses when transplanted in HSCT recipients[232]. Additionally, an immunocompetent fetus with osteogenesis imperfecta (OI) transplanted with fetal MSC did not mount immune responses against the allogeneic cells, tested by *in vitro* assays, and engraftment was observed[233]. Multiple infusions of high-dose allogeneic MSC in baboons revealed host T lymphocyte hyporesponsiveness to donor alloantigens. However, production of antibodies reactive to donor cells was seen[234]. Further *in vivo* evidence for tolerance comes from the observation that mismatched MSC engraft in rodents, dogs, pigs and non-human primates[235-237]. Furthermore, human MSC infused *in utero* persist in fetal sheep, although at low

numbers[238, 239]. Data on the relationship between survival of infused MSC and HLA disparity is limited to anecdotal reports suggesting absence of rejection, as MSC engraft at low levels, of both HLA matched and mismatched MSC[232, 233, 240-243]. However, in two murine models MSC have been suggested to be immunogenic. MSC induced alloimmunization[244] and memory T lymphocyte responses resulting in rejection[245]. Species-specific differences might explain the discrepancies between the studies on MSC immunogenicity.

Inhibition of immune responses in vitro

Upon addition of animal or human MSC to mixed lymphocyte cultures (MLR, i.e. allostimulation)[223, 246, 247] and cultures of lymphocytes stimulated to proliferation by mitogens[247-249] or antibodies[221, 227, 247], T lymphocyte proliferation is suppressed in a dose-dependant fashion. At very low concentrations MSC display stimulatory effects on T lymphocyte proliferation[223, 247, 249]. Human MSC exert their immunosuppressive effects even if separated from the target cells (e.g. in transwell systems), why the mediator appears to be soluble[248, 250-252].

MSC seem to modify the functions of several cellular components in the immune system. Neither apoptosis nor an anergic state, induced by MSC, of responder T lymphocytes can explain the failure of lymphocyte proliferation in the presence of MSC, as responses are seen after MSC removal[221, 229, 230, 246, 249, 253]. In contrast, lymphocytes recovered from an MLR with murine MSC present displayed cell cycle arrest and failed to proliferate[254]. Recently, it was proposed that immunosuppression mediated by human MSC is a consequence of an anti-proliferative effect on the lymphocytes, rather than an inhibition of lymphocyte activation and effector function[255]. This contrasts to other reports indicating that MSC suppress both lymphocyte activation[256, 257] and cytotoxic activity[230, 253]. However, the exact mechanisms of lymphocyte inhibition are not established and might involve several components. There is evidence that MSC can direct lymphocytes to a suppressive phenotype, i.e. formation of regulatory T lymphocytes (Treg) and when MSC are present in MLR, the Treg population significantly increases[221, 253]. On the contrary, depletion of Treg do not seem to affect MSC mediated immunosuppression[250]. MSC have been demonstrated to have both stimulatory and inhibitory effects on B lymphocytes and the effect was dependant on the dose of MSC[258, 259]. Moreover, recent studies displayed MSC suppression of allospecific antibody production by decreased IL-5 levels[260]. It has been suggested that MSC inhibit IL-2 and IL-15 driven NK cell proliferation and IFN-γ production[221, 230, 253, 261]. Lysis by freshly isolated NK cells are not inhibited by

MSC[230], whereas NK cells cultured for some days with IL-2 and MSC have a reduced cytotoxic potential[262]. Dendritic cells, i.e. a type of antigen presenting cell, are inhibited in their maturation and differentiation by MSC. One mechanism in the MSC mediated immunosuppression might be a direction of dendritic cells towards a suppressor phenotype, resulting in an attenuation of T lymphocyte responses[250, 253, 263, 264]. To summarize, MSC have the potential to modulate or inhibit almost all aspects of cellular immunity.

Several soluble factors ascribe to be responsible for the immuno-suppressive effects of MSC. However, the effect of the soluble factors is a source of controversy. Despite theses caveats, certain MSC derived products are able to create an immunosuppressive milieu. MSC may secrete hepatocyte growth factor (HGF)[256, 265], IL-10[250, 266] and transforming growth factor (TGF)-β[250, 267], which all are known to act immunoinhibitory. Nevertheless, blocking of these molecules does not fully restore proliferation in MLR[248, 267]. MSC express cyclooxygenases and are therefore able to produce prostaglandin E2 (PGE2) that play a role in many immune functions. Indomethacin inhibition of PGE2 only partially restores lymphocyte proliferation in MLR[221, 227, 266, 268]. The indoleamine 2,3-dioxygenase (IDO) catalyses the conversion of tryptophan into kynurenine, resulting in tryptophan depletion that inhibits lymphocyte proliferation and kynurenine accumulation that is cytotoxic. Upon IFN-γ stimulation MSC may produce IDO, but this enzyme cannot solely explain the immunosuppressive features of MSC[225, 269]. IFN-γ secreted by ongoing immune responses seem important for MSC immunomodulation, since blocking of IFN-γ has been reported to markedly abrogate MSC suppressive activity[262]. MSC also express inducible nitric oxide synthase, thus the cells are able to produce nitric oxide (NO), which is known to inhibit T lymphocyte proliferation[270]. In addition, MSC produce soluble HLA-G that correlates to the immunosuppressive effect[271]. The enzyme heme oxygenase-1 (HO-1), which function is to degrade heme, is also reported to act anti-inflammatory or immunosuppressive and can mediate effects of IL-10 and NO. Human and murine MSC express HO-1 and it might be a key in the MSC mediated immunosuppression. In animal experiments, MSC protected murine heart transplants from rejection, but after blockage of HO-1 in MSC the transplants became rejected[272]. Recently, LIF was proposed as another candidate. This cytokine is known to coordinate humoral and cellular immune responses. It is constitutively expressed by MSC and expression increases when MSC are present in MLR. Blocking of LIF resulted in a reduced suppression of T lymphocyte proliferation and decreased levels of Treg[273]. To conclude, none of these

soluble factors can exclusively explain the phenomenon of MSC induced immunosuppression.

Inhibition of immune responses in vivo

MSC seem immunosuppressive *in vivo*. In a baboon allogeneic skin graft model, donor MSC prolonged graft survival of donor and third-party skin transplants without immunosuppressive drugs[246]. Myeloablative conditioning and HSCT combined with MSC infusion from the same mismatched donor resulted in skin graft tolerance of 100 days versus 47 days after HSCT alone in cynomologus monkeys (A. Bartholomew, personal communication). One of the most impressive *in vivo* effects of MSC therapy is the control of lethal GvHD in mice[274]. Therapeutic efficacy of MSC in a murine model of multiple sclerosis, the experimental autoimmune encephalitis (EAE), has been reported. MSC decreased symptoms of demyelinisation[275]. In another murine model, MSC infusions reduced damage and fibrotic effects after bleomycin exposure in lungs[276]. In rat models, MSC protected kidneys from acute renal failure due to ischemia[277] and accelerated glomerular healing in a model of glomerulo-nephritis[278].

Studies suggest a physiological role of MSC. Patients with severe aplastic anemia display MSC deficient in suppressing lymphocyte proliferation and production of cytokines[279]. This MSC defect might be a part in the pathogenesis of the disease. The MSC may also contribute to the maternal tolerance of a fetus, as MSC can be derived from the placenta and produces several of the cytokines known to contribute to the state of fetal "immune privilege"[280].

Engraftment

MSC were detected primarily in the lungs and secondly in the liver after intravenous and intra-arterial infusions in rats. The MSC were also detected in kidney, spleen and long bones. If the rats were pretreated with vasodilator, MSC cleared the lungs resulting in increased proportions in liver and long bones[281]. After intravenous infusion in non-human primates, MSC were distributed to a wide range of tissues. Gastrointestinal tissues harboured the highest concentrations. Engraftment detected several months post-transplant was estimated to 0.1-2.7%[237] . In another non-human primate model, MSC engrafted in numerous tissues after irradiation injury and the level of engraftment was as high as 10% in some tissues[282]. Recently, transplantation of human fetal MSC have been shown

to improve glomerulopathy in collagen type I alpha 2-deficient mice with engraftment around 1%[283]. It has also been shown that intracranially injected MSC were distributed to a wide range of locations in the brain with engraftment at therapeutic levels[284]. These data suggest that MSC initially distribute broadly following systemic infusion, and later may participate in ongoing cellular turnover and replacement in a wide variety of tissues.

In a clinical study of MSC to promote engraftment of hematopoietic stem cells, most patients displayed MSC in the circulation one hour after intravenous infusion[285]. MSC have been tried clinically for treatment of OI. Five of six patients showed engraftment in one or more sites, including bone, skin, and marrow stroma, and had a beneficial clinical effect. Overall, the fraction of donor cells at any biopsy site never exceeded 1%[286]. Human fetal MSC have also been demonstrated to engraft in bone at low levels resulting in positive effects in a patient transplanted *in utero* to treat severe OI[233]. In HSCT patients suffering from toxic damages in various tissues and GvHD (see »complications« in previous chapter), MSC have been used with prosperous results. Engraftment was found in damaged tissues and in regional lymph nodes[240, 241]. Recently, HSCT donor-derived MSC have been isolated from bone marrow of pediatric patients undergoing HSCT. This finding implies that MSC are capable of engraftment in the bone marrow of HSCT recipients[287]. All these data suggest that transplanted MSC may have the ability of homing and that they persist, although at low levels, in tissues after infusions in humans.

Adverse events

MSC have been increasingly used in animal models to explore potential use for clinical applications and phase I/II clinical trials have been conducted with promising results. Nevertheless, as for all new treatment the possible side-effects have to be considered and thoroughly weighed against the benefit.

Immediate adverse events

In the early clinical trials on MSC therapy, it was shown that the cells were safe to administer in patients, i.e. no adverse events were observed upon MSC infusion[232, 288, 289]. In a pediatric population, it was reported that MSC did not induce any clinically significant adverse events, besides one case of urticarial rash after a second MSC infusion[286]. However, in the

recent studies there are no reported side-effects during or immediately after MSC administration[116, 240, 241, 290-292].

Later adverse events

Currently, MSC therapy is a new entity in clinical transplantation which is why long-term follow up still is absent. However, data from some animal studies displayed no long-term side-effects of MSC therapy[246, 284]. Other studies have reported that MSC may support tumor growth. In a murine model, the MSC favoured proliferation of tumor upon co-injection of MSC and melanoma cells[251]. Additionally, MSC supported tumor growth in another model and displayed a molecular signature similar to mesenchymal tumor cells[293]. On the contrary, MSC displayed inhibition of lung carcinoma, melanoma and colon carcinogenesis in other models[294, 295]. MSC-like cells also displayed reduction of brain tumor growth[296] and MSC potently suppressed development of Kaposi sarcomas *in vivo*[297]. In an open-label pilot study of MSC infusion in HSCT patients, MSC treated patients had significantly earlier and increased rate of relapse (i.e. malignant hematological disorders reappearing, see »monitoring the outcome« in previous chapter)[298]. Conversely, in other larger studies the use of MSC has not been correlated to an increased risk of relapse[116, 291, 292]. To properly answer the question of whether MSC increases the relapse risk, further and larger studies are needed.

Since MSC have a high proliferative capacity, one might wonder whether the cells can give rise to new tumors spontaneously. Human MSC have been shown to undergo a two-step transformation when cultured for several months[299, 300] and to form solid tumors in mice[301]. However, other studies of human MSC could not confirm transformation[302, 303]. The issue of spontaneous transformation is a matter of debate, and if it exists it seems to be an extraordinary event.

Possible infectious complications to MSC transplantation are poorly investigated. A recent review proposed that stem cell transplanters need to perform "microbiological risk assessment" of their stem cells products[304]. In clinical trials, MSC treated HSCT patients may have an increased risk for severe infections[116, 240, 298]. However, the survival rate for patients with complete response to MSC treatment was significantly better than for those with partial or no response and those not receiving MSC infusion, suggesting that beneficial effects of MSC are not overridden by a high number of severe infections[116, 240]. Still, the possible infectious side-effects have to be evaluated in large double-blind randomized trials.

Clinical applications

Due to their properties, MSC may be used to relieve and cure diseases, as described in this chapter. Since MSC are easily expanded *ex vivo*, large number of cells derived from a single donor could yield cells for multiple recipients. This expansion potential together with the favorable low immunogenicity implies universal donor features, without need for HLA matching. This section will describe what MSC have been used for and what they might be used for in the future.

MSC in HSCT

In the late 1990's it was demonstrated that the post-chemotherapy bone marrow stroma failed to support hematopoiesis[305]. Autologous MSC were used in trials as "marrow support" in HSCT, demonstrating a rapid engraftment of hematopoietic cells. The feasibility and safety of clinical-scale expansion and infusions of MSC were displayed[232, 285, 288]. Thereafter, allogeneic HLA-identical sibling MSC were tried in HSCT. The infusions were administered safely and the hematopoietic recovery was prompt[289]. Recent studies have established the feasibility and safety of MSC infusions and documented positive results in acceleration of engraftment[291] and graft enhancement[290, 306] in allogeneic HSCT.

The first study of allogeneic MSC in HSCT, by Lazarus and colleagues, also demonstrated reduced incidence rates of both acute and chronic GvHD. Subsequently, other groups have used *ex vivo*-expanded MSC in severe steroid-refractory acute GvHD with success[115, 240, 292, 307]. Recently, the developmental committee of the European Group for Blood and Marrow Transplantation (EBMT) presented their results on 55 GvHD patients and concluded that MSC might be an effective therapy of severe steroid-resistant acute GvHD[116]. This multicenter study has encouraged further, randomized double-blind, EBMT studies of the usage of MSC therapy in HSCT, which might generate important data to answer many of the questions raised concerning safety and efficacy.

Additionally, MSC infusions have been successfully used for repair of tissue injury (hemorrhagic cystitis, colon perforation and pneumo-mediastinum) secondary to allogeneic HSCT[241].

To summarize, MSC seem to have a future in HSCT by acting as an immunosuppressant counteracting rejection and GvHD. The cells may serve as "marrow support" and seem to reduce toxic injuries. Furthermore, MSC might regenerate damaged tissues after homing and can perhaps secrete cytokines and growth factors necessary for rapid healing. The potential of MSC will be determined in the future studies.

*MSC as immunomodulation and in regenerative medicine*

In a murine model, MSC transplanted into the brain promoted proliferation, migration and differentiation of endogenous neural cells[308]. At the same time, in another study, MSC were used for treatment of EAE. Intravenous injection of MSC strikingly ameliorated the disease and it was suggested that MSC effectively interfered with the causative autoimmune attack[275]. Further studies confirmed the MSC ability to interfere with the pathogenic autoimmune response[309]. However, these findings could not be repeated in a small study of adults with MS given intrathecal MSC. Some of the patients showed neurological improvement. Only one patient out of ten showed improvement assessed by magnetic resonance imaging[310]. Autoimmune arthritis in mice has been treated with MSC. As in the EAE studies, the MSC effectively blocked the pathogenic mechanisms[311]. Autologous MSC might be used for rheumatological disorders, since they display the same properties as MSC derived from healthy subjects[312, 313]. Presently, data only exist in preclinical models, but clinical applications seem to be under development. One problem in this field might be that the patients are not immunosuppressed as in the HSCT trials, which is why there may be immune reactions against the MSC that may lead to rejection or other adverse events.

Autologous MSC have been used with promising results in 34 patients with myocardial infarction (MI). The cells were injected in the target coronary artery and resulted in increased heart wall movements with improved left ventricular ejection fraction, i.e. implying a beneficial effect, in the MSC treated group as compared to the placebo[314]. OI is a genetic disorder characterized by production of defective collagen, the principal protein in bone. In a clinical study of OI, six children were transplanted with MSC without preconditioning. Post-MSC infusion, five of six showed engraftment and had an acceleration of growth velocity during the first six months[286]. Amyotrophic lateral sclerosis (ALS) is a progressive disease with poor prognosis. The pathogenic mechanism is loss of motor neurons leading to reduced muscle functionality and current treatment only alleviates symptoms. Autologous MSC were given to ALS patients resulting in a mild trend toward a slowing down of muscle strength decline[315]. Patients with Hurler syndrome and metachromatic leukodystrophy develop significant neurologic and musculoskeletal defects that limit their survival. Both diseases are due to enzyme deficiencies. It was postulated that MSC could participate in correcting these disorders. After a HSCT, the patients were administered MSC from the hematopoietic stem cell donor. Observed improvements in some clinical parameters, i.e. bone mineral density and nerve conducting velocity, warrant further study of MSC as therapeutic tool in these disorders[232].

There are also a number of ongoing clinical trials using MSC; e.g. exploring their use in treatment of peritonitis, chronic myocardial ischemia and distal tibial fractures. MSC are also of interest for biomedical companies. Osiris Therapeutics Inc. (USA) currently has three MSC products in clinical trials; Prochymal™ for GvHD, Provacel™ for MI and Chondrogen™ for orthopedic diseases. Mesoblast (Australia) is involved in pilot clinical trials of MSC for orthopedic and cardiovascular diseases. BrainStorm Therapeutics Inc. (Israel) has a product called NurOwn™ that contain MSC for neurodegenerative diseases[316]. As described in this paragraph, MSC are the focus of intensive research and there are several possible clinical applications.

Epilogue

More than 40 years has passed since the discovery of MSC. The cell has been and remains to be a subject to extensive pre-clinical and clinical research. Each week, new results on MSC biology, i.e. characterization, isolation, *ex vivo*-expansion, properties, and possible clinical applications are published. Still, there are no striking results implying common use of MSC transplantation. However, we should bear in mind the history of HSCT with initial disappointing results that have evolved and still evolve to successful routines in clinical medicine. Thus it is likely that MSC, in the future, will enter the clinic to relieve or correct various diseases.
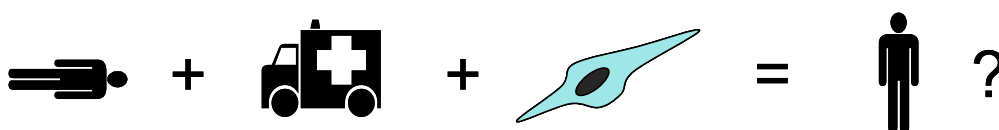
Aims

The general aims of this thesis are to investigate MSC in the context of clinical transplantation. As described in previous chapters, the MSC seem to have a potential as cellular therapy of various conditions. There are several uncertainties that need to be addressed before the cells can be adopted in clinical medicine. This thesis aims to clarify two clinically important concerns:

1. Can the MSC harbor persistent or latent viruses which can be transferred from the donor and give rise to hazardous infections in the recipient undergoing HSCT?

2. Will the HSCT recipients that receive MSC therapy mount humoral and cellular immune responses to HLA discrepant MSC?

Material and methods

All information regarding material and methods applied in this thesis has been described thoroughly in respective paper I-IV. This section describes the methodology used for the different papers in this thesis with some general comments.

Patients and donors (all papers)

The studies included in this thesis were after ethical scrutiny approved by the regional ethics review board and complied with the declaration of Helsinki on medical research involving human subjects. All patients and donors gave informed consent to participate in the studies, without any financial compensation.

Patients were enrolled from the Hematology Centre, the Section for Pediatric Hematology and the Centre for Allogeneic Stem Cell Transplantation at the Karolinska University Hospital in Huddinge, Stockholm, Sweden. All patients were undergoing HSCT according to the institutional guidelines and internationally accepted protocols. MSC therapy was employed for the following indications: to promote engraftment of hematopoietic stem cells, to treat complications of HSCT and to treat acute GvHD.

MSC, peripheral lymphocytes (PBL) and serum donors were all considered healthy after assessment of medical history, physical examination and serological screening of viruses. All of the donors participated voluntarily.

MSC expansion and infusion (all papers)

MSC for clinical use and research were cultured as previously described elsewhere[240, 290]. Briefly, heparinized bone marrow was separated over a density gradient. Mononuclear cells were collected, suspended in human MSC medium containing 10% fetal calf serum (FCS) and plated in plastic culture flasks. The culture procedures were performed according to the guidelines of the MSC consortium of the EBMT and the procedure was approved by the Swedish Medical Products Agency. All expanded MSC fulfilled the MSC criteria according to the ISCT[196] and were culture-negative for bacteria and fungi, and PCR negative for *Mycoplasma pneumoniae*[240, 290].

For infusions, MSC were collected in passage 1-4 and diluted in saline supplemented with 10% human AB plasma. The dosages of MSC were

around $1\text{-}2\times10^6$ cells/kg bodyweight of the recipient. MSC were derived from HLA-matched siblings, HLA-haploidentical and HLA-mismatched (i.e. third-party) donors. All patients received the MSC as intravenous infusions.

Virological methods

A counterpart of clinical virology is the detection of viruses to corroborate clinical diagnoses or explain disease of patients. In this thesis, virological detection techniques were applied on MSC before and after exposure to virus. MSC were either cultured with addition of virus or co-cultured with virus infected cells to study susceptibility of viruses recognized from the clinical course of HSCT recipients.

PCR detection (paper I and II)

PCR based methods are standard techniques for allowing the rapid detection of genetic information by specific amplification of low copy number nucleic acid sequences. The more refined techniques of PCR, also give the opportunity to quantitative information of the magnitude of viral infections, i.e. the number of virus genomic equivalents/copies. In this thesis, both the older "nested" PCR and the modern "real time" quantitative-PCR were used for detection of parvovirus B19 (B19) and herpesviruses; CMV, EBV, herpes simplex virus (HSV) and varicella zoster virus (VZV). PCR is fast and sensitive, but can be hampered by poor primer constructs and contamination of nucleic acids resulting in false positive or negative results.

Immunofluorescence (paper I and II)

To visualize subcellular distribution of biomolecules, immuno-fluorescence can be used. Antibodies labeled with fluorescent dyes are used as probes targeting the desired component. Cells are prepared on glass slides and after labeling, the slides are analyzed by fluorescence microscopy. In virology, antibodies against viral proteins are commonly used to demonstrate infection. In this thesis; B19, CMV, EBV and HSV proteins were visualized. Immunofluorescence is an easy technique that gives information on expression and localization. However, photo-bleaching, unspecific antibodies and difficulties in visualizing small amounts of the target are the major limits to its use.

Titration on susceptible cells (paper I and II)

Viruses can also be detected by inoculating samples on cells susceptible for the test virus. Green monkey kidney and human foreskin fibroblast cell lines are used for displaying HSV and CMV infections, respectively. After the inoculums, the cells are cultured for days to weeks. As the virus infects the cell and start replicating, a cytopathological effect on the cells will be seen. By serial dilution of the sample and inoculating it at least in quadruplicates, the virus titers can be calculated with the Reed-Münch endpoint calculation method. The CMV and HSV infections of MSC were investigated using this method. This type of method is of course limited by problems associated to the usage of living cells.

Serology (paper I and II)

To asses whether donors and recipients had encountered the investigated viruses, serological screening of antibodies was performed. Serology is a routine method used by clinical virology laboratories. Enzyme-linked immunosorbent assay (ELISA) is commonly used (see »immunological methods « in one of the following sections).

Expression studies

In preclinical and clinical research, expression of different markers and genes provide useful information for understanding the complex human biology. New techniques and new markers of normal processes and diseases are introduced frequently.

Immunofluorescence (paper I and III)

As described above, immunofluorescence is a good method of investigating protein and glycoprotein expression. In this thesis, immuno-fluorescence was used to determine expression of CD19 and AB0-histo blood group antigens.

Flow cytometry (all papers)

Flow cytometry is a rapid technique for counting, examining and sorting cells suspended in a stream of fluid. It allows, after staining with fluorescent antibodies or dyes, simultaneous multiparametric analysis of characteristics of single cells flowing through an optical and/or electronic

detection apparatus. The limitations of flow cytometry depend on lack of control cells and the quality of antibodies against the targets. In MSC research another disadvantage is that the cells have to be trypsinized before analysis. This might cleave molecules of interest and change features of the cell.

RT-PCR (paper IV)

To allow analysis of gene transcription, mRNA levels have to be determined. Since regular PCR is based on amplification of DNA and not RNA, the RNA has to be converted to complementary DNA. This process is called "reverse transcription" (RT) and is based on usage of the enzyme reverse transcriptase, which produces a strand complementary DNA with mRNA as template. In this thesis, RT-PCR was used to investigate gene expression levels of proteins important for regulation of immune responses. RT-PCR has the same drawbacks as PCR in general. By correlating mRNA levels for each gene to itself at different time points, instead of using absolute transcript numbers, possible alterations in effectiveness in RT were diminished.

Immunological methods

Numerous methods in modern research are based on immunological reactions. However, this section concerns methods to study immunological phenomena, e.g. immune cells and antibodies.

Lymphocyte proliferation assays (paper I, II and IV)

The lymphocyte proliferation assays are based on the MLR, which primarily was designed to detect HLA discrepancy and is correlated to the risk of rejection in transplantation[317]. Lymphocyte proliferation is determined by incorporation of ^{3}H-thymidine and can also be assessed by declining CFSE fluorescence in flow cytometric assays (see »flow cytometry« in previous section). In this thesis both methods have been used. Responder lymphocytes were either stimulated with PBL, MSC or infectious agents. Lymphocyte proliferation in response to external stimuli is a complex process that is regulated on several levels. However, it is a well-used method for estimation of lymphocyte activity in response to different stimuli.

ELISA (paper II)

The ELISA uses antibodies with specificity for a particular antigen. Sample with an unknown amount of antigen is absorbed to a micro-well or captured by another antibody specific to the same antigen. After the antigen is added, the detection antibody is added, forming a complex with the antigen. The detection antibody can be covalently linked to an enzyme. Finally, adding an enzymatic substrate produces a visible signal, which indicates the quantity of antigen in the sample. In this thesis, ELISA was used to determine antibody levels to viruses and FCS.

Flow cytometric cross match and Flow-PRA (paper III)

In flow cytometric cross matches (FCXM) recipients are investigated for antibodies against donor cells. The serum is incubated with the cells and thereafter fluorescinated anti-human Ig antibodies are added. Finally, the cells are assayed in a flow cytometer (**Figure 4**). To determine specificity of the antibodies giving positive FCXM reaction, the sera can be absorbed by different antigens and subsequently the sample will appear negative if the specific antigen was used. Flow-PRA is also based on flow cytometry. Instead of using cells to investigate anti-HLA antibodies, serum is incubated with beads coated with HLA. The limitations of these methods are the same as in flow cytometry. However, FCXM are used at clinical transplantation laboratories.
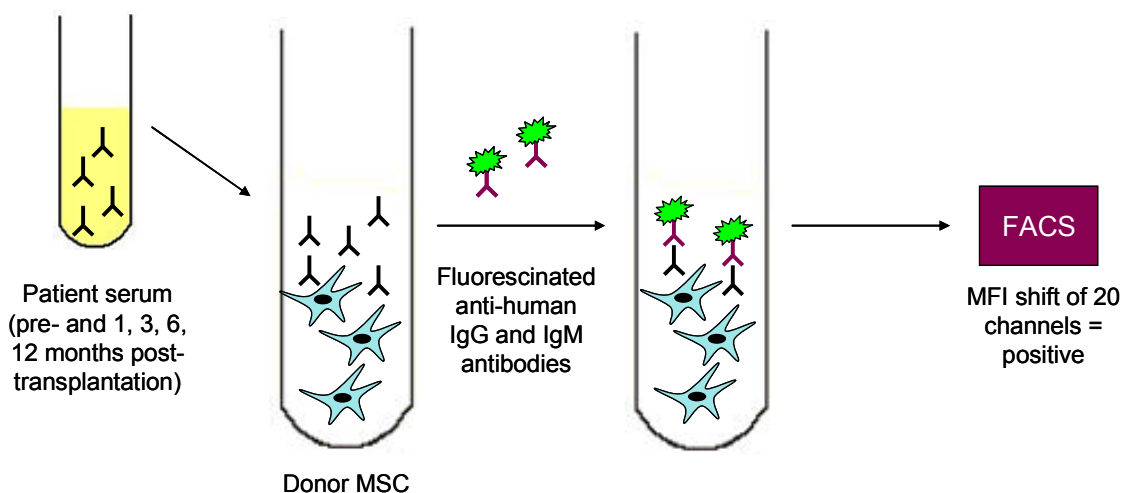

Figure 4 – Flow cytometric cross match. To evaluate possible development of antibodies against donor MSC, patient serum from different time points after MSC therapy was incubated with the cells. Fluorescinated anti-human IgG and IgM antibodies were used to detect, in a flow cytometer (FACS), any antibodies binding the MSC. A shift in mean fluorescent intensity (MFI) more than 20 channels was regarded as a positive reaction.

Results and discussion

All studies included in this thesis aim in general to provide information regarding MSC as cellular therapy. To date, most data on MSC transplantation are derived from animal models. These data are of course important and guiding. However, animal models cannot replace clinical experimental trials. The MSC mediated immunosuppression is established in several model systems and seems to be effective in some pilot clinical trials and a larger multicentre trial within the EBMT. It is now time to evaluate what happens to the MSC when infused in HSCT recipients and if MSC transmit viruses to them. Studies of these two safety aspects of MSC therapy may impart important knowledge of the MSC biology in humans.

MSC as viral reservoirs (papers I and II)

The contemporary cellular therapy constitutes of transfusion of erythrocytes or thrombocytes and HSCT. In transfusion medicine, strategies to avoid transmission of viruses with potential hazard to the recipient have been efficient. Nowadays, blood products are screened for hepatitis virus A and B, human T lymphotropic virus and human immunodeficiency virus (HIV). The same screening, expanded to include the CMV and EBV, is also applied in HSCT[318]. Herpesviruses as well as B19 are known to cause problematic infections in HSCT recipients[94] and are therefore of interest in MSC therapy of complications to HSCT.

Investigated by flow cytometry, MSC express the uptake receptors for CMV (CD13) and B19 (P antigen and Ku80), which make the cells accessible for the viruses. However, MSC did not express the EBV receptor (CD21). MSC were screened for presence of herpesviruses and B19 by sensitive PCR. None of the 12 screened MSC, derived from healthy donors, displayed the presence of herpesviruses. In the search for B19, one of 20 cells screened turned out positive for B19. To further study the MSC susceptibility, MSC were exposed to various viruses. MSC displayed a strikingly cytopathological effect after exposure to HSV and CMV. Conversely, exposure to EBV did not give rise to an established infection of the MSC. By immunofluorescence, HSV and CMV proteins were visualized in the cells. MSC were also exposed to B19 and the cells' infection was determined by means of positive immunofluorescence.

The HSV and CMV infections were further investigated to see if the infections were functional and productive. Supernatants from virus exposed MSC were titrated on green monkey kidney cells and human foreskin fibroblasts, respectively. Infections were found to be productive,

i.e. there was a raise in virus titer over time, and the virus particles produced from infections of MSC were able to infect other cells. In the case of B19, infected MSC could pass B19 to bone marrow cells, but not to other MSC. The discrepancy here might be explained by that infected MSC and uninfected MSC had no cell contact (transwell system). Transmission of B19 from MSC to bone marrow cells could be blocked by adding anti-B19 antibodies to the culture medium. The B19 PCR positive MSC was, without knowledge of this result, used in MSC therapy of GvHD in two HSCT recipients. By simple calculations, the dose in the patients should correspond to around 140 genomic equivalents/mL blood, which is an extremely low dose. None of the patients developed clinical signs of B19 disease, nor were their B19 PCRs positive one and three months post-MSC infusion. Even though both patients were severely immunocompromised, by means of Ig levels and ability to respond in MLR, the usage of B19 infected MSC did not develop into hazardous infection. However, as the virus is potentially dangerous to HSCT recipients these findings suggest the need for the implementation of precautions to limit possible B19 infections after MSC infusions.

PBL were stimulated to proliferative responses to viral, fungal and bacterial agents. When MSC were introduced they acted as immuno-suppressants in a dose-dependant manner, equally to what is observed for MSC inhibition of MLR. This implies that MSC may interfere with immune responses to infectious agents and in the case of HSCT, making already immunosuppressed individuals even more susceptible to dangerous infections. Recently, it was proposed that MSC exerted differential effects on alloresponses and virus-specific immune responses, i.e. MSC would not interfere with immunity against infections[319]. However, in that study MSC were stimulated to virus peptides and not whole particles. This may imply that MSC suppress lymphocyte proliferation in general, but do not interfere with immunity when a specific response is established.

The results of this thesis demonstrate that MSC can be infected with and may carry B19 and herpesviruses. In addition, the MSC might be a reservoir for persistent viruses. This is corroborated by the fact that bone marrow stromal cells are known to be permissively infected by CMV[320] and can transmit disease when transplanted *in utero*[321]. MSC are also known to support persistent infection of human herpesvirus 8/Kaposi sarcoma associated herpesvirus[322]. Furthermore, it has been shown that bone marrow stromal cells are susceptible to HIV and are able to pass the virus to both lymphoid and myeloid cells. MSC were proposed as a reservoir of HIV[323].

It is clear that cell therapy products such as MSC might transmit infectious disease as well as having beneficial effects in HSCT recipients[116]. This result in a controversy that has to be overcome in that MSC products have to be safe for the individual patient's sake, but also to avoid negative publicity that might harm the field of regenerative medicine in general. Protocols for routine viral screening are not established[304], our results suggest a need in the near future. Further studies exploring viruses, not only herpesviruses and B19, in MSC and stem cell products are warranted.

MSC have low immunogenicity (papers III and IV)

In general, MSC are regarded as cells with a low inherent immunogenicity. They do not induce proliferation of allogeneic lymphocytes, in contrast to many other cells derived from HLA disparate individuals. When stimulating lymphocytes with PBL from the MSC donor, the generated effectors cells cannot kill MSC. MSC have been used in clinical trials and seem to have an effect on GvHD following HSCT. As described in a previous chapter, even though HSCT recipients are heavily immunocompromised, they can occasionally mount immune responses giving rise to rejection of the transplants. Rejection can be mediated by antibodies, i.e. humoral, or immune cells, i.e. cellular.

Twelve patients who received MSC therapy for complications of HSCT or to promote hematopoietic engraftment were evaluated whether they displayed signs of humoral sensitization to the MSC. None of the patients had abnormal elevations in Ig levels post-MSC infusion that could be related to the MSC therapy. Two patients had positive FCXM prior to MSC infusions and at the endpoint, 12 months post-MSC infusion, two of five patients were found to display positive FCXM. One patient who had positive FCXM prior to and another one with positive FCXM after MSC infusion were multi-transfused due to their underlying diseases. Using flow cytometry, MSC were also found to be negative for expression of AB0-histo blood group antigens.

In order to determine the specificity of the antibodies giving rise to the positive FCXM, the patient sera were further analyzed (**Figure 5**). Sera were incubated with MSC from another donor, HLA disparate to the donor of the infused cells. Still, the FCXM were positive, implying that the antibodies might be MSC specific and not due to a sensitization to the donor. Anti-HLA antibodies could not be detected by Flow-PRA, confirming lack of sensitization to the donor. Thereafter the sera were absorbed with erythrocytes and FCS-grains, respectively. Erythrocyte absorption still gave positive FCXM, i.e. the antibodies were not directed

against erythrocyte antigens. Using the FCS-grains, the FCXM turned out negative. The antibodies were directed against FCS, a component of the MSC culture medium, which was confirmed by negative FCXM using MSC generated in human AB plasma. Anti-FCS antibodies were mainly of the IgG1 subclass.
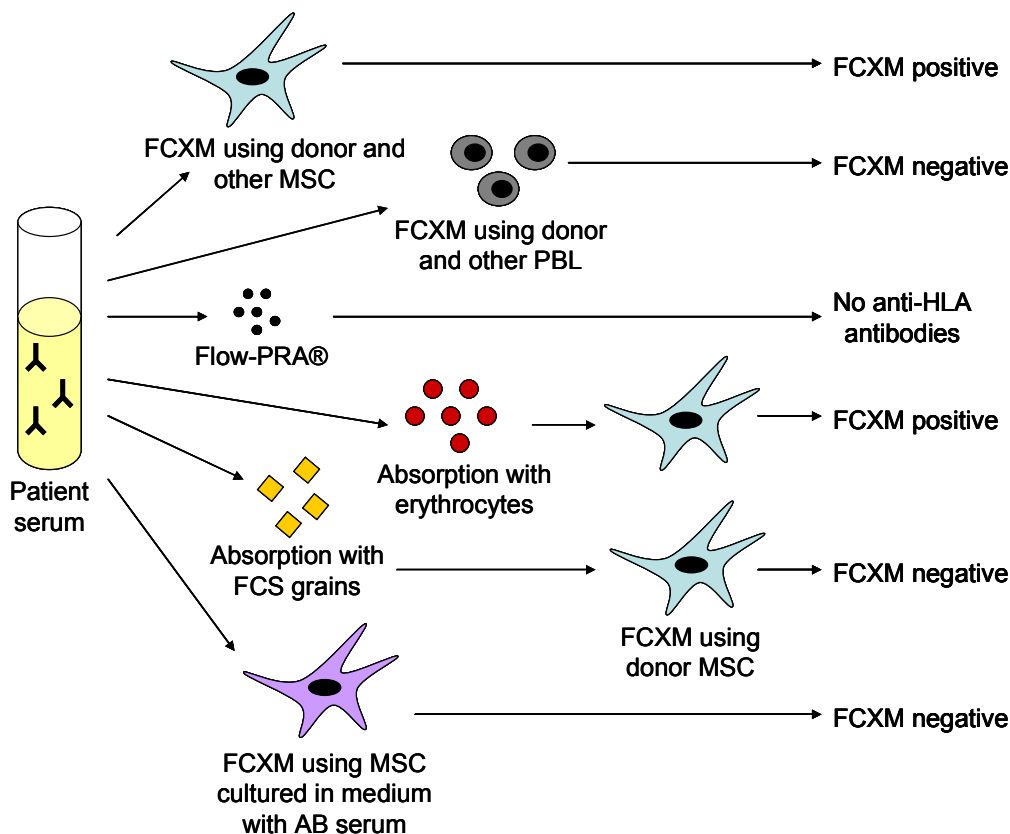

Figure 5 – Specificity determination of antibodies binding MSC. Some patient sera had antibodies that bound the MSC. These antibodies were not reactive to lymphocytes (PBL) and were not specific to HLA, as investigated by Flow-PRA. Nor were the antibodies directed to erythrocytes, since absorption did not change the outcome. However, the specificity of the antibodies were fetal calf serum (FCS), since absorption and using MSC generated in AB plasma turned the flow cytometric cross match (FCXM) negative.

This study points out that HSCT recipient have antibodies that can bind MSC. These antibodies are directed to neither HLA nor AB0-histo blood group antigens, known to contribute to graft rejection[324-327]. No vast clonal expansion of B lymphocytes seems to have occurred since there was no raise in patient Ig levels and no mono- or oligoclonality could be seen. Instead it may have been preformed Ig and newly produced Ig, from engrafted HSCT donor B lymphocytes, against FCS that bound the MSC[169-172]. Patterns of IgG subclasses for these anti-FCS antibodies would give rise to opsonization, antibody-dependant cytotoxicity and complement

activation[328]. However, our own preliminary results indicate that MSC cannot be lysed by complement proteins and that MSC are insensitive to NK cell killing[230]. Are these anti-FCS antibodies functional? Anti-bovine antibodies are regarded as natural occurrance[329-331]. Both in the twelve patients and in the controls antibodies against FCS were common, as determined by ELISA. Most humans have them and all patients with positive FCXM had a clinical effect of the MSC infusion. On the other hand, the absence of MSC engraftment with concomitantly raised anti-FCS antibody titers were shown in a non-immunosuppressed patient[286].

This thesis also investigates cellular immunity against MSC. Eighteen patients received MSC infusions as therapy of life-threatening complications to HSCT. Prior to MSC therapy, PBL from the MSC donor and third-party PBL mounted alloresponses in all patients, whereas donor MSC and third-party MSC did not. During the first 6 months post-MSC infusion, patients continued to display the same response pattern, i.e. no sign of allosensitization to MSC donor PBL or MSC. Since the recipient cells always proliferated in response to MSC donor PBL, there is no evidence for anergy (as reported by others[254, 275]) to the MSC donor after infusions in humans. In primate models, MSC infusion induces T lymphocyte hyporesponsiveness[234]. However, such a phenomenon could not be confirmed in the study of this thesis.

In *in vitro* experiments, lymphocyte proliferation and flow cytometry, did not display accelerated proliferation kinetics after primary stimulation to MSC and secondary stimulation to MSC donor's PBL, implying that MSC do not prime responder PBL. This contrasts to animal experiments, where rats responded with accelerated kinetics to human MSC[332]. MSC were also able to block, but to a lesser extent, rechallenge response to PBL primed with MSC donor's PBL suggesting that MSC are less effective at blocking established alloresponses. When MSC were used as a secondary stimulus after priming to PBL, the responder mounted a dose-dependant alloreaction, albeit smaller than that seen after secondary challenge with PBL. This may have importance in the clinic, since allosensitized individuals (transfused, transplanted and prior pregnant women) might reject infused MSC. In the HSCT setting, therefore it seems more suitable with third-party MSC rather than autologous cells since an immuno-competent hematopoietic stem cell graft could reject the infused cells. Third-party MSC would also be more convenient due to the fact that they could be stored in appropriate doses and do not need to be expanded, as in the case of autologous cells which will require several weeks. Both CD4+ and CD8+ T lymphocytes proliferated poorly in response to primary stimulation and rechallenge with MSC, which supports previous

findings[333]. PBL primed with MSC and rechallenged with PBL displayed a slightly increased proliferation compared to MSC as secondary challenge. Nevertheless, the blocking of alloresponses was not complete, since terminally differentiated CD4+ and CD8+ lymphocytes expanded after MSC priming and PBL rechallenge. Although, *in vitro* results indicate that MSC might be weakly immunogenic, no such observations could be seen *in vivo*. The findings in this thesis contrast with findings by others, displaying immune responses to MSC *in vivo*[244, 245]. Species-specific differences remain the most likely explanation for the discrepancies. However, more sensitive methods such as T lymphocyte precursor frequencies might be useful in further studies. In contrast to others[253, 334], this study could not confirm expansion of Treg. The gene expression studies revealed that MSC induced expression of activation markers as well as molecules associated to immune regulation. MSC also induced earlier and higher lymphocyte secretion of the anti-inflammatory cytokine IL-10.

To summarize, MSC therapy in HSCT recipients does not seem to induce antibody production, but antibodies may bind MSC. Whether these antibodies are functional or not remains to be proven. With regard to cellular immunity, MSC may be transplanted across HLA barriers with low risk for sensitization to HLA. MSC powerfully limit the alloresponses, but there might be responses in already sensitized individuals. Studies exploring MSC engraftment and trafficking may further elucidate whether MSC are rejected or not.

Conclusions

- MSC are susceptible to the human herpesviruses HSV and CMV, but not EBV. These viruses are not regularly carried by MSC. MSC support productive infection of HSV and CMV.

- MSC are susceptible to B19. Occasionally, MSC carries this virus. B19 can be passed from MSC to bone marrow cells *in vitro*. Low doses of B19 in MSC transplant might not develop into clinical infection or viremia in profoundly immunoincompetent individuals.

- MSC in the bone marrow stroma may constitute a reservoir for persistent or latent viruses.

- MSC can inhibit lymphocyte proliferation as a response to viral, fungal and bacterial agents in a dose-dependant manner, similar to what is seen for alloresponses.

- MSC do not express the AB0-histo blood group antigens, known as potent alloantigens.

- HLA-mismatched MSC infusions do not induce production of alloantibodies.

- MSC may bind antibodies directed to FCS, if generated in medium supplemented with that component. These antibodies may be insignificant, as they are found in many individuals.

- Patients receiving MSC infusions do not respond with allo-sensitization to donor antigens.

- MSC are weakly immunogenic *in vitro*, but do neither give rise to secondary lymphocyte proliferation kinetics nor induction of memory cells.

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