Chapter 1.

General introduction and aims of this thesis

1. The bone marrow microenvironment

Red blood cells, cells of the various leukocyte lineages and platelets are continuously replenished by cells originating from hematopoietic stem cells in the bone marrow in a tightly controlled process called hematopoiesis. This process takes place in flat bones such as the pelvic girdle and the sternum and in the medullary cavity of the long bones, such as the femur. Bone-marrow composes of both hematopoietic and supporting cells originating from hematopoietic stem cells and stromal stem cells, respectively. Already in 1867, Conheim, a German pathologist, described non-hematopoietic cells that originated from the bone-marrow cavity and migrated to sites of cell repair.(Cohnheim, 1867) In 1968 Friedenstein identified a type of plastic adherent stroma cells with fibroblast-like appearance in the bone-marrow and they managed to expand these cells *in vitro*.(Friedenstein, 1968) These cells differentiated towards connective tissue including osteoblasts, chondrocytes and adipocytes when transplanted into a mice model.(Owen, 1988) A common precursor for the connective tissue in the bone-marrow microenvironment, bone, fat and cartilage was proposed. (Prockop, 1997)

With increased insight in the different components of the bone-marrow stroma diverse names were used to annotate the expanded cells based on their way of isolation and capacity to differentiate into different mesenchymal tissues. Bone-marrow stroma contains a heterogeneous cell population which is defined by expression of characteristic cell markers and the lack of hematopoietic markers, by adherence to plastic and by differentiation capacity according to criteria proposed by The International Society for Cellular Therapy (ISCT).(Horwitz, 2005) The definition mesenchymal stromal cells is used for cells obtained by expansion of a heterogeneous cell population. This population should not be annotated as stem cells because of the lack of evidence supporting the single cell differentiation capacity of cells defined in this particular way. To properly define mesenchymal stem cells, single cell sorting using amongst others, CD146, has been proposed.(Sacchetti, 2007) Other names used to refer to bone-marrow stromal cells are adventitial reticular cells (ARC) or CXCL12-abundant reticular cells (CAR-cells),(Sugiyama, 2006a) named after their subendothelial position or high ligand production, respectively. Characterization of intracellular expression of *nestin* or *osterix* has also been applied to define particular subsets of stromal cells.(Mendez-Ferrer, 2010; Schepers, 2013) In addition, the term skeletal stem cells, is used in the context of tissue replacement using bone-marrow derived stem cells for transplantation with or without prior *in vitro* expansion. In this thesis, the abbreviation MSC will solely refer to mesenchymal stromal cells as defined by the ISCT criteria (Table 1 and Figure 1).(Horwitz, 2005)

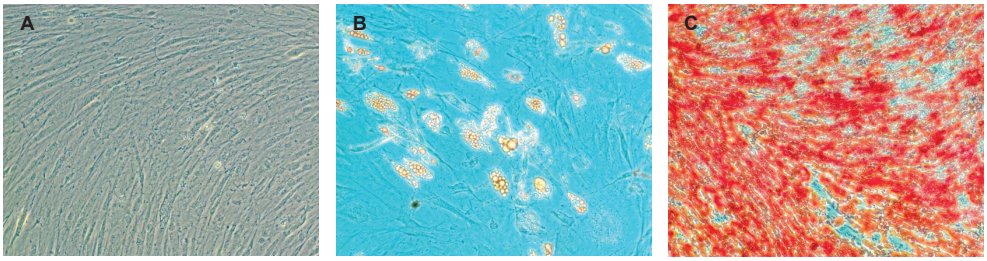

Figure 1. *Differentiation capacity of MSC.* All MSCs described in this study fulfil the ISCT criteria(5) of adherence to plastic (A) and *in vitro* differentiation capacity towards adipocytes (B) and osteoblasts (C).

Table 1. Expression profile of MSCs

Positive	Intermediate	Negative
CD73	HLA class I	CD3
CD90		CD31
CD105		CD34
		CD45
		CD86
		HLA-DR

CD: cluster of differentiation; HLA: human leukocyte antigen.

2. Hematopoietic regulation in the bone-marrow microenvironment

The observation that hematopoietic stem cells (HSC) or progenitor cells (HPC) do not expand and survive *in vitro* without extensive support led to the hypothesis that the stromal cells do not only form the structural network of the bone-marrow, but also contribute in the support and control of hematopoiesis. A balance between hematopoietic stem cells in a quiescent state and stimulation of hematopoietic stem cells to proliferate and differentiate is essential. Exogenous factors as erythropoietin (EPO), thrombopoietin (TPO) and inflammatory cytokines contribute to this process in combination with MSCs derived factors including both cell-cell interaction (*e.g.* via ALCAM(Chitteti, 2013)) as well as soluble factors (*e.g.* stem cell factor (SCF)(Barker, 1997), CXCL12(Dar, 2006) and wnt-signalling(Schreck, 2014)). Beside these factors a critical role for adrenergic stimulation of stromal cells has been suggested. *Nestin* expressing stromal cells were found to be closely situated to adrenergic nerves in the bone-marrow environment.(Mendez-Ferrer, 2010) Adrenergic stimulation leads via increased expression of CXCL12 in stromal cells to increased myeloid proliferation

explaining the circadian rhythm of hematopoiesis and the increased monocyte and neutrophil counts after tissue injury.(Courties, 2015; Mendez-Ferrer, 2008)

The impact of an abnormal bone-marrow microenvironment on hematopoiesis has been clearly demonstrated by Raaijmakers *et al.*(Raaijmakers, 2010) Disturbance of the micro RNA regulation, by knock-down of *Dicer1* specifically in osteoblast progenitors, resulted in an MDS-like hematopoiesis. In the same study, selective knock-down of the *Sbds* gene (Schwachmann-Diamond Blackfan Syndrome) resulted in increased dysplasia and apoptosis in the hematopoietic compartment. Others have used similar murine models to demonstrate disturbed hematopoiesis after selective knock-down of SCF, the 5HT4 serotonin receptor, the retinoblastoma gene or the retinoic acid receptor-γ.(Ding, 2012; Schepers, 2012; Walkley, 2007)

In addition, different groups have looked at the effect of malignant hematopoietic cells on the stromal microenvironment in murine studies. Schepers *et al.* demonstrated in a mice model of chronic myeloid leukemia (CML) that the bone marrow microenvironment is altered in gene-expression and has a decreased capacity to support hematopoiesis.(Schepers, 2013) In a similar murine model Zhang *et al.* showed decreased *CXCL12* gene expression in stromal cells after exposure to CML. This ligand is upregulated in the bone-marrow of patients with CML at diagnosis compared to patients in remission and in healthy controls.(Zhang, 2012a) After the induction of acute myeloid leukemia (AML) in a murine model the number of nestin$^+$ MSCs decreased, which could be rescued by adrenergic agonists.(Arranz, 2014)

The use of MSCs to accelerate hematopoietic recovery has been evaluated in clinical studies based on the capacity of MSCs to support engraftment in sheep and murine models.(Almeida-Porada, 2000; Noort, 2002) Initially, autologous MSCs were expanded from patients with breast cancer for infusion during autologous hematopoietic stem cell transplantation ((HSCT). In this phase I-II trial, MSC infusion was feasible and safe and the rapid hematopoietic reconstitution suggested a supportive role for MSCs.(Koc, 2000) Infusion of allogeneic MSCs at the time of HLA-matched sibling HSCT in adults with hematologic malignancies resulted in rapid reconstitution of absolute neutrophil counts and platelets, but no dose-dependent effect of MSCs was observed.(Lazarus, 2005) Ball *et al.* compared the hematopoietic reconstitution in 13 children receiving co-transplantations of haplo-identical HSC and third party MSCs, with 52 historical controls receiving HSC from a haplo-identical donor only. The reconstitution of platelets and absolute neutrophil counts was comparable, but lymphocyte recovery was faster in the children receiving MSCs.(Ball, 2007) No beneficial effect of MSCs was observed in a pediatric cohort receiving MSCs at the time of umbilical cord blood transplantation.(Bernardo, 2011) MacMillan *et al.* reported similar findings comparing children receiving MSCs plus umbilical cord blood to umbilical cord blood only.(MacMillan, 2009) In these studies no MSC engraftment after

infusion was detected. Interestingly, a case report describing a boy with Wiskott-Aldrich Syndrome demonstrated increased hematopoiesis at the side of intraosseous MSC infusion based on histology.(Resnick, 2010) In a large clinical trial including 55 patients receiving a haplo-identical HSCT in the treatment of leukemia patients were randomized for co-infusion of MSCs. Platelet recovery was significantly faster in the MSC group (22 days) compared with controls (28 days), but no beneficial effect was observed for leukocyte recovery.

2.1 Myelodysplastic syndrome

Pediatric myelodysplastic syndrome (MDS) is characterized by cytopenias, myeloid dysplasia, and a risk of transformation to AML. The spectrum of disease ranges from refractory cytopenia of childhood (RCC) with <5% bone marrow blasts and <2% peripheral blood blasts, via refractory anemia with excess blasts (RAEB), with 5-19% bone marrow blasts or 2-19% peripheral blood blasts, to RAEB in transformation (RAEBt), with 20-29% bone marrow blasts or peripheral blood blasts.(Hasle, 2003; Hasle, 2004; Niemeyer, 2008) In the study described in this thesis we only included patients with primary MDS. Patients with secondary MDS, classified as secondary to (I) inherited or acquired bone marrow failure syndromes or (II) radio- or chemotherapy, were not eligible for inclusion. In addition, patients with Down Syndrome related MDS form a unique entity and these were also excluded from the study.

The treatment for pediatric MDS varies between the different subtypes. RCC-MDS without chromosome 7 abnormalities or complex chromosome abnormalities has a favorable prognosis and watchful waiting can be advocated. However, eventually these patients will need to undergo allogeneic HSCT when becoming transfusion dependent or suffering from neutropenia. In children with RAEB-(t) MDS did not benefit from intense chemotherapy, because of high toxicity and a remission induction <60%.(Woods, 2002) Therefore, HSCT is the therapy of choice in these patients. Recently, immune suppressive therapy has been suggested based on adult data and the role of T cells in the pathogenesis of RCC-MDS.(Aalbers, 2014)

2.2 Juvenile myelomonocytic leukemia

Juvenile myelomonocytic leukemia (JMML) is an aggressive leukemia occurring in young children, at a median age of 2 years.(Loh, 2011)) Children typically present with hepatosplenomegaly, fever and monocytosis. The diagnosis was previously based on clinical criteria. The adapted criteria involve molecular diagnostics as shown in Table 2.(Locatelli, 2015) Hypersensitivity of the JMML cells to granulocyte-macrophage colony-stimulating factor (GM-CSF) and hyperactivity of the RAS-RAF-MAPK signaling pathway is characteristic for the disease and in the majority of patients (90%) a muta-

tion is detected in this pathway.(de Vries, 2010) Monosomy 7 is the most common karyotype abnormality detected in 25% of the patients.

HSCT is the first line treatment. Despite myeloablative conditioning, the one year relapse rate ranges between 30 and 50%.(Locatelli, 2005; Locatelli, 2015) In subsets of patients, particularly those with germline CBL or PTPN11 mutations and in a subset of patients with N-RAS mutations and low HbF, spontaneous resolution is observed and a wait and see approach can be advocated.(Locatelli, 2015)

3. Immunomodulation

The immunomodulatory effects of MSCs were first studied in animal models. In a baboon-model of allogeneic skin transplantation delayed rejection in case of co-injection of MSCs was observed.(Bartholomew, 2002) The number of MSCs present in bone-marrow is relatively low and therefore *in vivo* immunomodulatory effects are difficult to assess and remain largely unrecognized. However, expansion of MSCs from different tissues, *e.g.* bone-marrow, fat, Wharton's jelly and synovial fluid, is well established and the immunomodulatory effects of MSCs are extensively charac-

Table 2. Clinical and laboratory diagnostic criteria of JMML

I. Clinical and hematologic features (all 4 features mandatory)
· Peripheral blood monocyte count $13x10^9$/L
· Blast percentage in peripheral blood and bone marrow, 20%
· Splenomegaly
· Absence of Philadelphia chromosome (BCR/ABL rearrangement)
AND
II. Oncogenetic studies (1 finding is sufficient)
· Somatic mutation in PTPN11 or K-RAS or N-RAS (exclude germline mutations)
· Clinical diagnosis of NF-1 or germline NF1 mutation
· Germline CBL mutation and loss of heterozygosity of CBL‡
III. Only for those patients (10% of the whole number) without any oncogenetic
parameter, beside the clinical and hematologic features listed under I, at
least 2 of the following criteria have to be fulfilled:
· Monosomy 7 or any other chromosomal abnormality
· HbF increased for age
· Myeloid precursors on peripheral blood smear
· Spontaneous growth or GM-CSF hypersensitivity in colony assay
· Hyperphosphorylation of STAT5

terized *in vitro*.(Le, 2012) The increased use of MSCs in clinical studies reveals *in vivo* effects of MSCs after infusion.(Karlsson, 2008; Ringden, 2007) In addition, animal models have given insights in the immunomodulatory effects of MSCs.

3.1 Complex interactions

Mitogen and antigen induced proliferation of T-lymphocytes is suppressed by MSCs in a dose-dependent manner.(Krampera, 2003) Cell proliferation, assessed by flow cytometry or ^{3}H-thymidine incorporation, of CD4+ and CD8+ T-lymphocytes is down regulated. In *in vitro* mixed lymphocyte reaction (MLR) suppression by MSCs is independent of HLA match between responder cells, stimulatory cells and MSCs.(Bocelli-Tyndall, 2007) Further evaluation of T cell subsets using flow cytometry revealed a suppressive effect on both central memory as well as effector cells.(Krampera, 2003; Karlsson, 2008) Whereas the majority of the cell populations is suppressed by MSCs, induction of regulatory T cells has been reported.(Mougiakakos, 2011; Ghannam, 2010)

Both direct contact and soluble factors have been suggested to play a part in the suppressive effect. The potential licensing of MSCs to become suppressive is not fully elucidated. In this concept, MSCs need to be in contact with pro-inflammatory factors such as interferon-γ or with activated monocytes producing interleukin-1 (IL-1).(Groh, 2005) Indirect down-regulation via an increase of regulatory T cells is another potential pathway.(Jitschin, 2013) Presumably more than one system is essential because depletion of various cell populations or the use of antibodies interfering with specific pathways has only resulted in partial blocking of the inhibitory effect.

Preclinical studies exploring the potential for MSCs in auto-immune diseases show that murine and human MSCs suppress proliferation, differentiation and antigen production by B cells.(Corcione, 2006; Che, 2012) The suppressive mechanism is dependent on cell-cell contact and soluble factors with an important role for CCL2. (Schena, 2010; Rafei, 2008; Che, 2014) In contrast, Traggiai *et al*., reported a stimulatory effect of MSCs on proliferation and antibody production using different culture conditions for B cells.(Traggiai, 2008a) This dual effect of MSCs on B cells needs further exploration for tuning MSC therapy in auto-immune diseases.

A controlled response during inflammation is critical to retain a balanced immune system. MSCs contribute to this process via interaction with the innate immune response.(Le, 2012) Interestingly, MSCs have been shown to react differently to toll like receptor 3 (TLR3) and TLR4 activation.(Waterman, 2010) Stimulation of the TLR3 receptor with double stranded RNA leads to pro-inflammatory responses and prolonged survival of neutrophils *in vitro*.(Raffaghello, 2008) Lipopolysaccharide (LPS) induced activation of MSCs via TLR4 results in increased production of CCL2, IL-6 and anti-oxidants. Migration of monocytes to the bloodstream mediated by CCL2 and

skewing of monocyte differentiation towards anti-inflammatory macrophage type II protects the body from overreacting. This has been demonstrated in a murine model resembling sepsis.(Nemeth, 2009) An increased IL-10 production by macrophage type II led to decreased neutrophil infiltration and decreased tissue damage after MSC infusion.

Another example of the precisely balanced immune system is the interaction between MSCs and natural killer (NK) cells. MSCs suppress the *in vitro* activation and cytotoxicity of resting NK cells during stimulation with IL-2 or IL-15 in a dose dependent manner controlling inflammation.(Spaggiari, 2006; Sotiropoulou, 2006) The suppressive effect of MSCs is dependent on HLA-G5, IDO and PGE2.(Spaggiari, 2008; Selmani, 2008) Low expression of HLA class I makes MSCs from a third party donor a potentially suitable tool to establish immunosuppression after allogeneic transplantation because MSCs may escape from recognition by alloreactive T cells responses. Low expression of HLA class I in combination with expression of ligands for the NK cell activating receptors DNAM1, NKG2D and NKp30 renders MSCs susceptible for lysis by activated NK cells. Blocking of these activating receptors decreases the NK cell cytotoxicity. Pre-treatment of MSCs with interferon-gamma (IFN-γ), resembling an inflammatory environment, increases HLA-class I expression causing decreased susceptibility to NK cell lysis but increased susceptibility to T cell mediated lysis.

The *in vivo* effect of MSCs on T-lymphocyte subsets, B cells, monocytes and macrophages will probably depend on the immune modulatory signals produced by the environment and other cells of the immune system.

3.2 MSC treatment in acute inflammatory disease

3.2.1 Hematopoietic stem cell transplantation

Since 1968, allogeneic HSCT has evolved and improved.(Bach, 1968; Gatti, 1968) It is standard of care in multiple protocols for malignant and non-malignant diseases. (Mallhi, 2015) In non-malignant disease the donor derived stem cells repopulate the bone-marrow and constitute a new hematopoietic system without the inborn errors affecting, *e.g.*, red blood cell development or lymphocyte function. The treatment of hematologic malignancies consists of tumor eradication using chemotherapy with or without irradiation, also leading to destruction of the patients own hematopoietic system. HSCT is subsequently performed with donor derived hematopoietic stem cells. An important beneficial impact of the alloreactive donor derived immune system is the so called graft-versus-leukemia (GvL) effect attacking the residual malignant cells. (Horowitz, 1990) However, alloreactivity may also give high morbidity due to evoking acute graft-versus-host disease (aGvHD), which may run a severe course and evolve

into chronic GvHD requiring immunosuppressive treatment for a long period of time. (Ball, 2008c)

Hematopoietic stem cells (HSC) for HSCT in children are derived from bone-marrow, peripheral blood after mobilization and umbilical cord-blood either from related or unrelated donors.(Milano, 2015; Handgretinger, 2008) Whereas HLA-matched related HSC can be transplanted with limited risks of aGvHD, product manipulation should be performed on grafts derived from haplo-identical donors to reduce the aGvHD risk. In addition, *in vivo* depletion of lymphocytes by anti-thymocyte globulin or anti-CD52 (alemtuzumab) is performed to decrease this risk. After HSCT, patients are treated with various types of immunosuppressive regimens to prevent or treat aGvHD.

Immune reconstitution is important to establish the GvL effect, but in the first period after transplantation it is also of the utmost importance for prevention or elimination of viral reactivations. Infections (reactivations) of cytomegalovirus (CMV), Epstein-Barr virus (EBV) and human adenovirus (HAdV) may run a severe course, causing high morbidity and mortality in patients immunocompromised early after HSCT. (Broers, 2000; Boeckh, 2003; Walls, 2003; van Tol, 2005a) Viral reactivations are further complicated in cases of steroid treatment for aGvHD.(Cantoni, 2010)

3.2.2 MSC in treatment of aGvHD

Acute GvHD is a complex disease consisting of multiple steps and involving various intercellular interactions and, thereby, a complication with an unpredictable outcome. It involves triggering of the immune response, amplification of the response and, finally, inflammation and tissue damage.(Ferrara, 2009) Different triggers have been proposed for the initiation of aGvHD. HLA mismatch between patient and donor is the best described factor involved in triggering aGvHD.(Petersdorf, 2013) Genetic polymorphisms, *e.g.*, associated with cytokine production and receptor function, predispose for the strength of the reaction.(Dickinson, 2008) Danger associated microbial peptides (DAMPs) and pathogen associated microbial peptides (PAMPs) have been described to initiate aGvHD. Vossen *et al.* describe a cohort of children in which total gut decontamination prevents aGvHD in HLA matched sibling recipients. (Vossen, 1990; Vossen, 2014) Next to viral and bacterial derived PAMPs and DAMPs endogenous DAMPs such as adenosine triphosphate (ATP) derived from conditioning induced apoptotic cells might be involved in the first steps of aGvHD.(Wilhelm, 2010) After presentation of antigens to T-lymphocytes these cells proliferate, differentiate and home to the site of inflammation amplifying a pro-inflammatory cytokine cascade and neutrophil recruitment resulting in tissue damage of gut, skin and liver. (Holtan, 2014) Table 3 shows the clinical criteria for staging and grading of aGvHD. (Ball, 2008c) Albeit a clinical diagnosis, a tissue biopsy should be taken to exclude other causes of skin, gut or liver involvement.(Ertault-Daneshpouy, 2004)

Acute GvHD grade I did not require treatment. Systemic steroids are the first line of treatment for aGvHD grade II, but patients who fail to reach remission upon steroid treatment, about 50% of the cases, show high morbidity and mortality.(Deeg, 2007; Davies, 2009) MSCs were first used in a 9 years old boy who did not respond to steroids, ciclosporin, Psoralen and ultraviolet-A light (PUVA), infliximab and daclizumab. He responded well to infusion of third party MSCs with a decrease in stool and bilirubin levels.(Le Blanc, 2004) Subsequently, a multi-center compassionate use trial was initiated in Stockholm, Rome and Leiden. In total 55 patients, adults and children, were included at various time-points after onset of severe, steroid refractory, aGvHD.(Le Blanc, 2008) While administration of steroids was continued, single or multiple infusions of MSCs were given. Complete response, defined as no aGvHD at day 28 after (the first) MSC infusion, occurred in 30 out of 55 patients. Patients with complete response had a lower transplant related mortality (37%) compared to patients with partial or no response (72%).This hallmark trial revealed no adverse effects of MSC infusion. A trend towards better survival in the pediatric cohort was observed.

The effectiveness of MSC infusion in steroid refractory aGvHD has not been studied in a placebo controlled trial. However, multiple smaller studies describe the beneficial effects of MSC infusion. Detailed case studies discuss the occurrence of increased viral reactivations and gastro-intestinal stenosis after MSC infusion.(Ball, 2008a; Karlsson, 2008) However, solid conclusions on a causal relationship between MSC infusion and these adverse events could not be drawn.

3.3 MSCs in chronic inflammatory diseases

Inflammatory diseases like systemic juvenile idiopathic arthritis and inflammatory bowel disease have a high morbidity in children caused by disease related events in combination with treatment related toxicity.(Beukelman, 2011; Bandzar, 2013) Although usage of monoclonal antibodies directed to inflammatory cytokines or

Table 3. aGvHD staging and grading

	Skin	Liver	GI tract
Stage	Rash	Bilirubin (µmol/L)	Diarrhea (mL/day)
0	No Rash	<34	<500
1	<25%	34-50	500-1000
2	25-50%	50-102	1000-1500
3	>50%	103-255	>1500
4	Erythroderma (with bullae)	>255	Severe abdominal pain/ileus

Blue: overall grade I; Green: overall grade II; Orange: overall grade III; Red: overall grade IV

their receptors (biologicals) improve response to therapy, patients remain on lifelong therapy.(Nigrovic, 2011; Yokota, 2008) Attempts to treat and cure chronic inflammatory diseases using autologous or allogeneic HSCT showed promising results, but high treatment related mortality hampered further clinical trials.(Brinkman, 2007; Passweg, 2004) Effects of MSCs have been reported in animal models of autoimmunity, such as amelioration of experimental autoimmune encephalitis in a murine model, raising the possibility of MSCs use in the treatment of autoimmune diseases. (Zhang, 2005) In a collagen induced arthritis (CIA) mice model a worsening of disease was seen after administration of an allogeneic MSC cell line(Djouad, 2005), albeit other murine studies using allogeneic MSCs showed significant clinical improvement in CIA.(Augello, 2007) Similarly, animal models of immune colitis/enteropathy and systemic lupus erythematosus (SLE) responded successfully to MSC treatment.(Gonzalez, 2009; Parekkadan, 2008; Sun, 2009; Zappia, 2005) Pilot studies have demonstrated potential beneficial effects in adults with Crohn's disease.(Ciccocioppo, 2011; Duijvestein, 2010) In a prospective study including 78 patients with SLE, a beneficial effect on disease intensity and induction of clinical remission has been reported. (Wang, 2013a) However, for use in further clinical studies safety and better insights in the mechanism of action of MSCs is critical.

Chronic GvHD (cGvHD) occurring after HSCT is a devastating disease with major disabilities in affected children.(Baird, 2010) The immunopathophysiology of cGvHD is poorly understood. Following HSCT in adult patients, alloreactive donor T cells were considered primarily responsible for the development of cGvHD, but randomized studies failed to demonstrate that elimination of donor T cells in the graft reduced the rates of cGvHD.(van Els, 1990) There is no direct correlation between the numbers of minor histocompatibility antigen specific T cells and cGvHD occurrence.(Bueger de, 1993) Recently, B cells have been recognized as being implicated in cGvHD and a coordinated T/B cell response to minor histocompatibility antigens(Zhang, 2006; Schultz, 1995), H-Y antigen antibodies(Miklos, 2004) and the production of specific soluble factors (*e.g.* BAFF) (Baird, 2010; Sarantopoulos, 2009) may all contribute to the development of the clinical syndrome of cGvHD. In total 47 adults and children receiving MSCs for cGvHD were reported in literature.(Ringden, 2006; Zhou, 2010; Weng, 2010; Perez-Simon, 2011; Herrmann, 2012; Muller, 2008; Lucchini, 2010; Fang, 2007) Complete remission was documented in 28% and partial remission in 34% of treated patients, which percentages are substantially lower than the efficacy of MSC treatment observed in aGvHD.(Le Blanc, 2008; Kebriaei, 2009) Due to the variable manifestations of cGvHD, results of the different studies are difficult to compare.

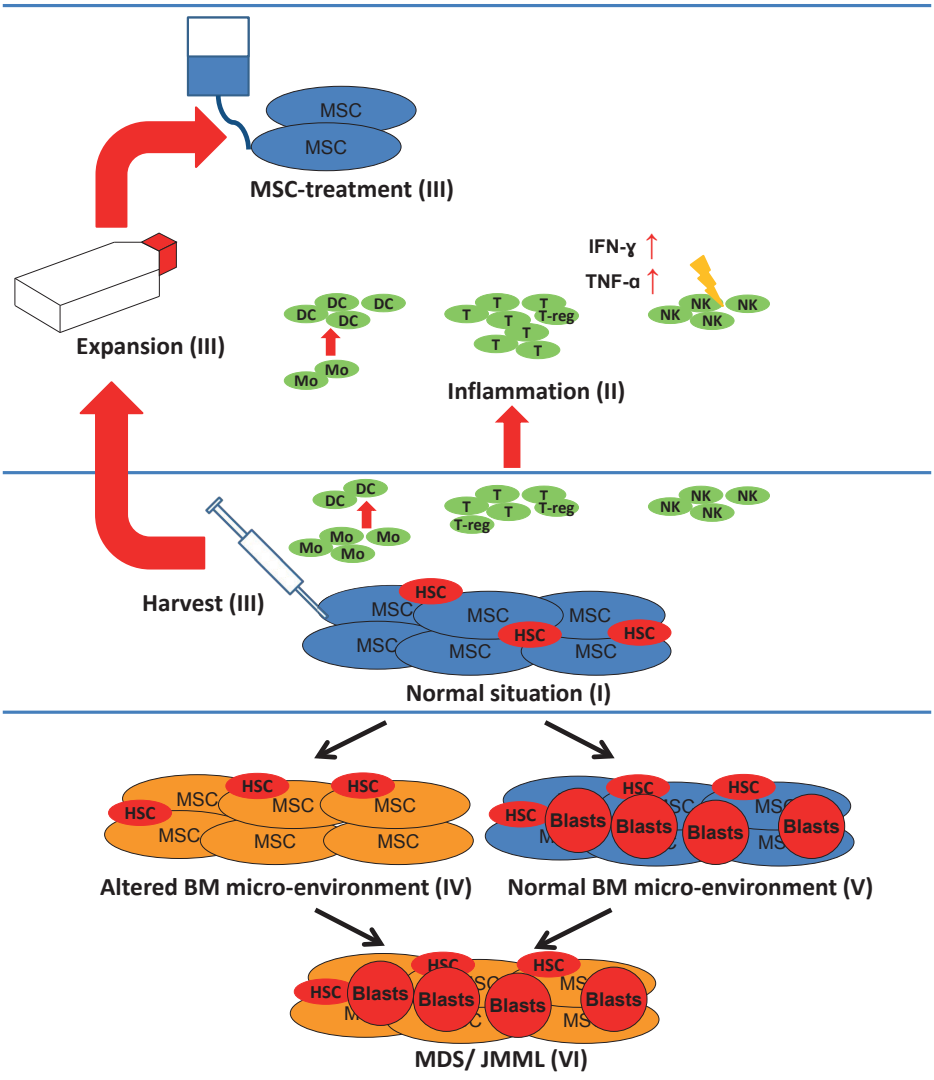

Figure 2. *A simplified overview of the different parts in this thesis* I: MSCs are in close contact with the hematopoietic stem cells (HSC) and contribute to a balanced immunological status. II: During inflammation, e.g. acute Graft-versus-Host disease, antigen presentation by dendritic cells (DC), derived from monocytes (Mo) causes an increase in NK cells (NK) and cytotoxic T cells (T), in combination with relatively low percentages of T-regulatory (Treg) cells resulting in an increase of inflammatory cytokines as TNF-α, IL-1 and IFN-γ. III: After bone-marrow harvest from a healthy donor, MSCs can be expanded *in vitro.* Infusion of MSCs has been reported to suppress T cell proliferation, increase T-regulatory cells, suppress NK cell cytotoxicity and reduce the differentiation of monocytes to dendritic cells. This thesis focusses on the effect and side-effects of MSC treatment in children.
In addition, we hypothesize that MSCs contribute to disease progression in children with MDS or JMML, either by IV: an intrinsically altered micro-environment or V: by alterations induced by affected hematopoietic cells. VI: This altered micro-environment might explain the resistance of these diseases to conventional therapy.

4. Aim of this thesis

Mesenchymal stromal cells (MSCs) support hematopoiesis in the bone marrow, are involved in tissue repair and modulate immune responses. The MSC function may be affected by malignant cells such as in MDS and JMML. The focus of this thesis is to study the impact of MSCs on virus-specific immune recovery and aGvHD after pediatric allogeneic HSCT. In addition, the function of MSC derived from children suffering from a chronic inflammatory disorder, *i.e.,* systemic juvenile idiopathic arthritis, or from a childhood malignant disease, *i.e.,* MDS and JMML, respectively, has been investigated (Figure 2).

The first chapters of this thesis describe the pre-clinical studies on the biological characteristics of patient derived MSCs. We characterized the mesenchymal stromal cells derived from different pediatric diseases. MSCs expanded from bone-marrow of healthy children were used as a control. **Chapter 2** reports our findings on MSCs derived from children with systemic juvenile idiopathic arthritis. MSC treatment has been suggested in chronic inflammatory diseases. An investigation of the immune modulatory capacities of patient derived MSCs was performed to explore the potential of autologous MSC treatment in this disease. We specifically aimed to extensively define the effect of MSCs on different cell populations of the immune system.

In the studies described in **Chapter 3 and 4**, we focused on the role for MSCs in the pathogenesis and sustainment of pediatric hematologic disease. Children with MDS and JMML were included and bone-marrow was collected at diagnosis and after hematopoietic stem cell transplantation. MSCs were extensively characterized by gene-expression analysis. To investigate the possible functional impact of differential gene-expression, assays were performed to investigate the immunomodulatory capacity and hematological support of MSCs.

The next chapters report our findings on the effect of MSCs in a clinical study. To be able to study the effect of MSCs it is essential to properly determine start- and end-points in steroid refractory acute Graft-versus-Host disease (aGvHD). Therefore, gut biopsies taken at diagnosis and, when indicated, after treatment of aGvHD were analyzed. In addition, soluble biomarkers in serum samples at different time-points after HSCT and initiation of MSC treatment were measured to monitor the response to treatment in a less invasive manner. These results are described in **Chapter 5**. Suppression of immune responses to control aGvHD potentially decreases the response to viral reactivation in children after hematopoietic stem cell transplantation. Finally, in **Chapter 6**, we focused at a potential side-effect of MSC treatment. *In vitro* experiments studying the effect of MSCs on virus specific T cell proliferation and activation were performed. In addition, the occurrence of viral reactivations in a cohort of 22 children treated with MSCs for steroid refractory aGvHD was studied. Virus specific

T cells were characterized *ex vivo* from children with a viral reactivation prior to and after MSC infusion.

In **Chapter 7** our main findings and the future role for MSCs in treatment and understanding of pediatric diseases are discussed.