Mesenchymal stromal cells in pediatric disease : pathophysiology and treatment

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Chapter 7.

General discussion
Future perspectives
Samenvatting in het Nederlands

Wij gaan niet opzij.

Mesenchymal stromal cells: Hope or hype?

Are mesenchymal stromal cells a hype, a promising field of research, or the solution for everything? The first papers about mesenchymal stromal cells (MSCs) reporting astonishing results on the efficacy of treatment of steroid refractory acute graft-versus-host disease (aGvHD) and the increase of engraftment after autologous hematopoietic stem cell transplantation (HSCT) led to a widespread renewed interest in these cells. Researchers worldwide explore the possibilities of MSCs as therapeutic agent in a wide range of diseases. Other research groups aim to unravel the pathogenesis of various diseases searching for aberrations in MSC function. Both types of research aim to understand the characteristics of MSCs in order to improve patient care, either by applying animal models, using *in vitro* experimental approaches, or by performing clinical trials. In this chapter I discuss the findings described in this thesis in light of the progress of MSC research over the last decade.

Balance

Homeostasis in haematopoiesis, the constant goal to establish balance in generation of the various cell lineages and their functions, is strived for to enhance survival. Homeostasis has its boundaries in many organ systems in our body. Failing to remain within these limitations causes disease. A clinician will try to treat patients by removing the trigger that disrupts homeostasis (cure) or to restore the balance by adding external factors (care). The immune system, with a continuous supply of new cells and a requirement for adequately regulated functions, is a typical example of homeostasis in which over activity (auto-immune and auto-inflammatory disorders), or inactivity (infection), have an immediate impact on the health status of an individual. In addition, it is of the utmost importance to control the immune reaction to pathogens in order to avoid cause organ damage by the inflammatory response. Studying the different aspects of the immune system has improved the understanding of immunology.

In a similar way we have tried to characterise MSCs *in vitro* from healthy donors and patients with systemic juvenile idiopathic arthritis (sJIA), myelodysplastic syndrome (MDS) and juvenile myelomonocytic leukemia (JMML). In addition, the therapeutic application of MSCs was further evaluated in children with aGvHD after allogeneic HSCT. The side effects of immune modulatory cell therapy on anti-viral T cell mediated immunity were also studied in the latter patient cohort.

MSCs in treatment of inflammatory disease: Restoring balance

Treatment of acute inflammatory disease

MSCs in aGvHD

Prospective studies including patients with steroid refractory aGvHD after allogeneic HSCT reported response rates to MSC treatment ranging from 30% to 80%. (Le Blanc, 2004; Le Blanc, 2008; Kebriaei, 2009; Prasad, 2011; von Bonin, 2009; Lucchini, 2010) The only randomized study, as yet only reported during the 2010 EBMT Meeting, Geneva, could not confirm these observations, with a non-significant difference between complete response rates of 35-40% versus 28-30% in patients treated with MSCs and steroids versus steroids only.(Martin, 2010) This study was performed by Osiris and failed to meet its primary endpoints. Unfortunately, these data have not been published. Although 244 patients were included in this trial, these were spread over a large number of hospitals. However, the beneficial results in pediatric patients amongst this cohort led to the US Food and Drug Administration (FDA) registration of MSCs produced by Osiris for first-line treatment of aGvHD in Canada and New-Zealand. In a sub-group analysis, patients with gastrointestinal or liver aGvHD benefitted from MSCs with higher numbers of patients achieving a complete or partial response. However, all other published studies have compared response rates to those of historical controls. Large variation exists in the time between onset of aGvHD and start of MSC infusion. This seems critical, because one-year overall survival in patients with steroid-refractory aGvHD receiving MSCs as third line therapy was comparable to historical controls (33.3% vs 38.5%), whereas overall survival in patients with MSCs as second line therapy was 73.9%.(Calkoen, 2013b) The need for well-designed, randomized, clinical trials enabling timely MSC infusion is evident. Chapter 5 of this thesis emphasizes the importance of gastrointestinal biopsies at diagnosis and during monitoring after intervention. The results described in chapter 6 report the importance of documentation of side-effects. In addition, feasible techniques to monitor anti-viral T-cell responses are shown.

Difficulties in study design

For inclusion of sufficient patients to assess the effectiveness of MSC infusion a large international collaboration is needed. A laboratory certified for cellular therapy is mandatory for the different centers to join the study and all cellular products should meet similar criteria.(Dominici, 2006) The use of platelet lysate versus fetal bovine serum for expansion of MSCs further complicated this debate and delayed the start of randomized clinical trials.(Schallmoser, 2007) Both supportive agents

are biological products and therefore sensitive to variation in their growth factor concentrations.(Hemeda, 2014) Human platelet lysate has been shown to give higher duplication rates and senescence in higher passages.(Griffiths, 2013; Ben, 2012) Studies reporting differences in immunosuppressive capacity are in favour of MSCs expanded in fetal bovine serum.(Abdelrazik, 2011; Bernardo, 2007a) When considering MSCs for tissue replacement human platelet lysate has been shown to be superior in the formation of oscicles in mice.(Prins, 2009)

Different end-points to document the response to MSC infusion have been used, *e.g.,* best ever response(Le Blanc, 2008) or response on day 28-32 after treatment initiation(Kebriaei, 2009; Prasad, 2011; von Bonin, 2009; Lucchini, 2010), potentially explaining the differences in response to therapy. Criteria for aGvHD are solely based on clinical parameters and so far the definition of response to MSC treatment has been based on severity of skin involvement, amount of diarrhoea and bilirubin levels.(Ball, 2008c) However, diarrhoea is often seen in patients after HSCT caused by either viral reactivation or during recontamination of the gut following decontamination regimens. We observed a patient who had persistent diarrhoea after MSC treatment but had no evidence of active aGvHD as assessed by endoscopy and histology.(Ball, 2008a) Therefore, patients included in a prospective non-randomised phase II trial in our centre, underwent endoscopic evaluation in case of persistent symptoms after MSC infusion in line with studies in Crohn's disease.(Ciccocioppo, 2011; Duijvestein, 2010)

In 18 out of 21 children with steroid refractory aGvHD, persistently profuse diarrhoea was documented despite a first MSC infusion. In 8 out of 12 cases no histological evidence of aGvHD was seen. No complications occurred during endoscopy with biopsies at diagnosis and after MSC infusion. Further clinical management was based upon these findings and patients were successfully tailored of immunosuppression. In contrast, patients with persistent aGvHD received additional MSC infusions with complete response in the majority of the cases. The study underlines the critical importance of histopathological assessment of responses to experimental treatments in GvHD of the gut as clinical symptoms are unreliable as a sole indicator of treatment response. However, many MSC studies to date rely entirely on clinical parameters to document response, calling into question the reliability of the reported response rates. Future studies evaluating the effectivity of MSC therapy for aGvHD should therefore include a standard gastrointestinal biopsy in case of persistent diarrhoea at 28 days after infusion.

Biomarkers

The definition by the National Institute of Health:

Biomarker: A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.(NHI, 2001)

In case of sustained or relapsing diarrhoea after MSC infusion, endoscopic evaluation of the gastro-intestinal tract is the only established method to differentiate between ongoing aGvHD and other causes of diarrhoea. Although safe and well-tolerated in our patient cohort, minimally invasive approaches to measure responses are preferred over endoscopy in critically ill patients. Identification of biomarkers in urine, stool and serum has been proposed. (Rodriguez-Otero, 2012; Landfried, 2011; Paczesny, 2009) A consortium led by Paczesny and Ferrara aimed to define serum biomarkers to 1) estimate the risk of severe aGvHD prior to onset, 2) predict the response of aGvHD to first line or second line treatment at diagnosis, and 3) define the response to steroid treatment after 28 days.(Paczesny, 2009; Paczesny, 2010; Levine, 2012; Harris, 2012; Ferrara, 2011) Pooled serum obtained prior to and during aGvHD was compared using mass-spectrometry to identify candidate proteins. These data were validated in larger cohorts and the predictive value of a selection of these candidate proteins was analyzed. In their cohorts including hundreds of patients, suggested biomarkers can be used to define patients at risk for aGvHD, to stratify patients according to their potential to respond to therapy, and to identify subgroups that have responded to therapy. These results are derived from retrospective analyses and should be confirmed by prospective studies.(Paczesny, 2013) To define the prognostic value of these markers, included patients should receive pre-emptive treatment based on biomarker levels. A control group of patients randomly assigned to receive the selected pre-emptive treatment irrespectively of biomarkers levels, should be included.

Measurement of serum biomarkers in our patient cohort confirmed the increase in biomarker concentration at onset of severe aGvHD for TNFR1, IL-2Rα, HGF and IL-8, and at onset of gastrointestinal aGvHD for cytokeratin 18 (CK18), soluble cytokeratin 18 fragments (sCK18F) and regenerating islet-derived 3α (REG3α). At 28 days after the first MSC infusion, TNFR1 concentrations differentiated between complete (CR) and partial/non-responders (PR/NR).

CK18 and sCK18F serum levels measured at the time of gastrointestinal biopsy were significantly increased when gastrointestinal aGvHD was histologically confirmed (positive biopsies). However, the sensitivity (53.8%; 95% CI 25.1-80.8%) and specificity (86.7%; 95% CI 69.3-96.2%) of the CK18 test needs to be validated in larger cohorts of patients before these markers could be considered replacement for biopsies. Jitschen *et al.* report a decrease of both CK18 and sCK18F in patients after

response to MSC therapy. However, persistent non apoptotic cell-death is associated with lower response to treatment in aGvHD reported by Luft *et al.* demonstrating higher sCK18F to CK18 ratios in steroid refractory versus steroid responsive patients after initiation of steroids. Similarly, in our patient cohort, children with a partial or no response to MSC treatment had lower sCK18F to CK18 ratios already 7 days after MSC infusion. However, the variation amongst the patients did not allow for interventions in individual cases.

Sera of patients included in the randomized controlled studies on MSC therapy in steroid-refractory aGvHD should be collected (1) at onset of aGvHD, (2) at time of randomization, and (3) 7 and (4) 28 days after start of the intervention. Determination of biomarkers in these successive sera should aim to identify patients likely to respond to MSC therapy (1 and 2). In addition, biomarkers at 7 days after randomization may be used as early indication for response of no-response to therapy. Validation of the reported biomarkers on day 28 may be a substitute for gastrointestinal biopsy in the future. Previously reported biomarkers should be determined, but these valuable sera should be used to identify new biomarkers by *e.g.* mass-spectrometry.

Viral reactivations

In surveillance of patients after HSCT, the clinician is constantly outweighing the benefits and the side effects of rapid immune reconstitution. Conditioning, donor selection, graft manipulation, and immune modulatory (suppressive) medication influence the balance between rapid versus delayed recovery. At the cost of increased risk of aGvHD the graft-versus-leukemia effect may be optimized for example by early withdrawal of immune suppression. In addition, delayed recovery of lymphocytes increases the time period during which the patient is at risk for viral reactivations. (Broers, 2000; Boeckh, 2003; Walls, 2003) The interactions between viral reactivations and aGvHD further complicates this challenge. Viral reactivations are associated with an increased incidence of aGvHD, potentially causing the trigger in the first steps of aGvHD.(Akira, 2006) On the other hand, treatment of aGvHD, using immune suppression, decreases the response to viral reactivations, causing high mortality in this subgroup of patients.(Cantoni, 2010)

We addressed this issue comparing our MSC cohort to historical controls in chapter 6. Whereas in adults cytomegalovirus (CMV) reactivations are the most abundant, human adenovirus (HAdV), (Flomenberg, 1994; Howard, 1999; Baldwin, 2000) causes most morbidity and mortality in children, with no established anti-viral drug at hand. (Walls, 2003; Kojaoghlanian, 2003; Leen, 2005; van Tol, 2005b) HAdV infections were particularly associated with decreased survival in MSC treated patients. The one-year overall survival was 100% in children with steroid responsive grade II-IV aGvHD despite occurrence of a viral reactivation. In contrast, patients with a viral reactivation

and steroid refractory grade II-IV aGvHD, treated with MSCs or with other second-line therapy, had a one-year overall survival of 50% or 16%, , respectively. The use of high-dose steroids is a major confounder in this analysis. A decrease of the absolute number of $CD8^+$ T cells after initiation of methylprednisolone has previously been reported. (Aubert, 2001; Cwynarski, 2001) We demonstrated a similar number of IFN-γ producing cells in peripheral blood mononuclear cells (PBMC) prior to and after initiation of aGvHD treatment, whereas the proliferative capacity of T cells is hampered from the start of steroid infusion. No CMV-specific proliferation was detected up to 3 months after MSC infusion in three patients, in whom steroids could not be tapered due to a lack of response to MSC infusion. This could be due to decreased cytokine production induced by methylprednisolone as has been described.(Ozdemir, 2002)

Studying the influence of MSCs on virus specific T cells in *in vitro* experiments demonstrated a suppressive effect of MSCs on PBMC proliferation after stimulation with virus specific peptides derived from HAdV and CMV. In addition, virus specific T cells, cultured for 28 days and mainly consisting of effector memory cells, were suppressed in IFN-γ production and proliferation by MSCs. Although one study reported a diverse effect of MSCs on T cells stimulated with a viral antigen compared to an alloantigen,(Karlsson, 2008) other reports are in line with our data. A recent publication supports our findings by presenting data on suppressed proliferation of and IFN-γ release by specific $CD8^+$ T cells stimulated with a CMV or influenza virus specific peptide or an allo-antigen in co-cultures with MSCs.(Malcherek, 2014) Comparable results were seen in T cell lines. However, *in vitro* experiments do not take into account the number of MSCs at the site of infection and the complex interplay of antigen presentation and lymphocyte proliferation in lymph nodes. This was suggested to be of importance based on a murine study demonstrating that decreased specific T cell proliferation was based on decreased homing of antigen presenting dendritic cells to lymph nodes.(Chiesa, 2011) Interestingly, patients with an onset of viral reactivations prior to MSC infusion had better overall survival compared to patients with viral loads becoming detectable after MSC infusion. Our data do not support the suggestion by Meisel *et al.* that MSCs should not be given to patients with a CMV infection based on the *in vitro* observed decrease of suppression by CMV infected MSCs. The hypothesis that viral antigen triggers MSCs via TLR3 to increase neutrophil activation was also not taken into account in our *in vitro* experiments.(Waterman, 2010)

We aimed to further understand the side-effects of MSC infusion during steroid-refractory aGvHD. The *in vitro* suppressive effect of MSCs on mixed-lymphocyte reactions led to treatment with MSCs for aGvHD, despite a lack of insight in the *in vivo* working mechanism. Interestingly, despite numerous studies reporting a suppressive *in vitro* effect of MSCs on virus specific T cells the *in vivo* effect is disputed based on one *in vitro* study suggesting the contrary.(Karlsson, 2008) As mentioned, all clinical

data are confounded by persistence of aGvHD and the duration of steroids, but there is no reason to believe that MSCs do not negatively affect virus-specific T cells. Still, we propose that the virus specific suppressive effect of MSCs is less compared to that of high dose steroids and, therefore, administration of MSCs in order to rapidly control symptoms and allow steroid tapering is justified in life-threatening aGvHD. However, studies infusing MSCs without concomitant systemic steroids aiming to support engraftment or to prevent aGvHD, should enable investigation of the direct effect of MSCs on viral reactivations.(Ball, 2007)

Treatment of chronic inflammation and auto-immune disorders

The potential of MSCs in the treatment of various chronic diseases has been extensively studied. The *in vitro* observed interactions with cells of the innate and adaptive immune system in combination with the reported prolonged skin graft survival in baboons led to studies in auto-immune diseases and chronic inflammatory diseases.

Response to treatment with MSC infusions in chronic GvHD (cGvHD) varies extensively in literature.(Ringden, 2006; Zhou, 2010; Weng, 2010; Perez-Simon, 2011; Herrmann, 2012; Muller, 2008; Lucchini, 2010; Fang, 2007) The reported cases differ in affected organs, grade of cGvHD and timing of MSC treatment. Although promising results were reported, many challenges remain before MSC infusion will be standard treatment in cGvHD. The recent development of a murine cGvHD model and a humanized cGvHD model, both resembling the human situation, opens possibilities to further understand the pathogenesis of cGvHD and the potential of MSC treatment.(Fujii, 2015; Srinivasan, 2012) Fibrosis of the targeted organs is seen in cGvHD. A potential role of MSC in tissue regeneration has been suggested,(Ringden, 2007) which may be important in the resolution of GvHD. Of note, we reported extensive and persistent gut fibrosis in a pediatric patient following successful MSC treatment of steroid refractory aGvHD.(Ball, 2008a) Weng *et al*. and Herrmann *et al*. also observed that patients with cGvHD and end-stage fibrosis did not benefit from MSC infusions.(Weng, 2010; Herrmann, 2012) These observations cast doubt on the regenerative potential of non-manipulated bone-marrow expanded MSCs when applied in late stage disease. However, beneficial effects were seen on skin lesions in patients with *de novo* systemic sclerosis after systemic MSC infusion suggesting that the sclerodermatous type of chronic skin GvHD may be a candidate disease for MSC treatment.(Scuderi, 2013)

Local administration of MSCs into fistulas in adults with Crohn's disease led to better outcome compared to results normally achieved by surgery. Closure of the fistula was seen in 70% of the patients.(de la Portilla, 2013) In contrast, only 3 out of 10 patients responded to systemic infusion of MSCs.(Duijvestein, 2010) Homing of MSCs to the site of inflammation was not assessed in this study, but an overall

decrease of inflammatory (TNF-α and IL-1β) cytokines in biopsies obtained 6 weeks after infusion was documented.

Murine models of systemic lupus erythematosus (SLE) showed conflicting results after MSC infusion, with one study reporting an increase in disease severity and another amelioration of disease.(Youd, 2010; Sun, 2009) Three human studies, although without inclusion of an appropriate control arm, demonstrated a decline in disease activity in 80-100% of the included patients systemically receiving either autologous bone-marrow derived or allogeneic umbilical cord blood derived MSCs. (Sun, 2010; Sun, 2009; Liang, 2010) An increase in the percentage of regulatory T cells after MSC infusion supports the suggested interaction between MSCs and regulatory T cells. (Sun, 2010) Of note, these three studies were reported by one group.

Both systemic and intrathecal infusion of MSCs has ameliorated disease in multiple sclerosis (MS), a progressive neurodegenerative disease thought to originate from an auto-immune process. This has recently been reviewed by Gharibi *et al.* critically addressing the absence of controls in the published studies.(Gharibi, 2015)

Third party allogeneic MSCs have been used in aGvHD, to enable treatment initiation early after start of symptoms.(Le Blanc, 2008) This approach was considered safe in patients under intensive immunosuppression. The use of allogeneic MSCs in immunocompetent patients without a direct life-threatening disease is controversial. Before autologous MSCs can be used in clinical trials, the characteristics of these cells, with an emphasis on immunomodulation, need to be defined. In our study, the immunomodulatory capacity of the MSCs expanded from the bone-marrow of children with systemic juvenile idiopathic arthritis (sJIA) at time of diagnosis was comparable to healthy pediatric controls.(Calkoen, 2013a)

Currently a total of 120 studies using MSC therapy in children are registered on www.clinicaltrials.gov: *i.e.,* Duchenne Muscular Dystrophy, bronchopulmonary dysplasia, type I Diabetes Mellitus, osteogenesis imperfecta, rheumatic diseases, graft-support, aGvHD, cGvHD, epidermolysis bullosa, medullablastoma (viral transduced MSCs for intrathecal infusion), ischemic heart disease, support of nerve regeneration, autism and liver failure, illustrating the hope (or hype) on MSC in the therapy of a plethora of disorders.

MSCs in disease: Disrupting balance

The function of MSCs in tissue has not been fully elucidated. Promoting a stable micro-environment, supporting other cells and acting as precursor for various cell types are thought to be the most important functions. These functions have been linked to disease. An abnormal MSC function as a cause of disease and abnormal MSC function

as a consequence of disease has been hypothesized. This section deals with these aspects in paediatric disease with a specific emphasis on hematologic malignancies.

The bone marrow niche

The bone marrow microenvironment has been extensively studied since the identification of the hematopoietic and mesenchymal niche.(Friedenstein, 1968) Hematopoietic stem cells (HSC) remain in a quiescent state to maintain differentiation capacity and to escape toxicity.(Cheshier, 1999) *In vitro* expansion of the MSCs and co-culture experiments of MSCs and HSCs demonstrated the importance of MSCs. (Schofield, 1978) However, the mechanisms involved and other potentially important factors remained undefined. Murine studies including conditional knock-out models and *in vivo* imaging of the niche have given new insights.(Lo, 2009) The knowledge about the cell types and molecules involved in the murine bone marrow microenvironment is more extensive compared to the situation in humans. Translation of the concepts derived from animal studies to the human setting involves confirmation by immunohistochemistry,(Kode, 2014; Zhang, 2012b) characterization of expanded cells and, recently, transplanting expanded human MSCs on scaffolds in xenogeneic models.(Groen, 2012)

The main challenges in murine studies unravelling the bone marrow niche focus on identification of (1) the location of the niche, (2) the cell types involved, and (3) associated molecular pathways. These concepts have been extensively reviewed by amongst others Schepers *et al.* and Bianco *et al.*.(Schepers, 2015; Bianco, 2013) Based on the distribution of the HSCs ($CD34^+$ cells) in the bone marrow the main site of quiescent HSC is thought to be situated close to the trabecular bone surrounding the efferent and afferent vasculature.(Kunisaki, 2013) This was confirmed in a human study using immunohistochemistry.(Flores-Figueroa, 2012) Mesenchymal stromal cells have an essential role in the niche concept. However, based on murine studies, MSCs can be subdivided in different subtypes, based on proximity to different vessel types (pericyte-like versus reticular-like), cellular markers (*a.o. Nestin,* leptin receptor and neural/glial antigen 2) and excretion of hematopoietic regulators (CXCL12). In addition, MSCs differentiated to osteoblasts or adipocytes have been shown to, respectively, support and suppress haematopoiesis.(Winkler, 2010; Naveiras, 2009) The supportive function became more recently apparent by selective genetic knockdown of hematopoietic regulators in osteoblasts resulting in myelodysplasia.(Raaijmakers, 2010; Schepers, 2012) The altered gene-expression of *DKK1* and genes in the leptin pathway in JMML patient derived MSCs suggest an altered *in vivo* balance between adipogenesis and osteogenesis. This needs to be confirmed using bone marrow immunohistochemistry.

Endothelial cells lining the arterioles and sinusoids are a major source of hematopoietic regulators contributing to the hematopoietic niche.(Butler, 2010) In addition, differentiated hematopoietic cells, megakaryocytes and macrophages, have also been shown to influence haematopoiesis.

Megakaryocytes directly stimulate the quiescent state or proliferation of HSCs by secreting exocrine molecules (CXCL4, TGF-β1, TPO and FGF1).(Schepers, 2015) Dysfunction of macrophages and osteoclasts leads to disruption of the bone marrow niche by inadequate replacement of bone.(Morrison, 2014) Another mechanism affecting the hematopoiesis is the adrenergic nerve system. *Nestin* expressing MSCs and adrenergic nerves co-localized in the bone-marrow environment.(Mendez-Ferrer, 2010) Stimulation of the adrenergic system is a regulator of the circadian rhythm of hematopoiesis and increased cellular efflux after tissue injury.(Courties, 2015; Mendez-Ferrer, 2008) Communication between MSCs and hematopoietic cells via tunneling nanotubes was added to the complex interaction after the observation that blocking of the tunneling nanotubes leads to decreased induction of cytokine (*e.g.* IP10 and IL-8) secretion by MSCs after *in vitro* exposure to B cell precursor ALL. (Polak, 2015)

The murine knock-out models targeting a wide variety of pathways in the different cell populations resulted in insufficient hematopoiesis with dysplastic characteristics. Disturbance of the micro RNA regulation, by knock-down of *Dicer1* specifically in osteoblastic progenitors, resulted in an MDS like hematopoiesis.(Raaijmakers, 2010) In the same study, selective knock-down of the *Sbds* gene resulted in increased dysplasia and apoptosis in the hematopoietic compartment. Altered gene expression of *Dicer* was confirmed in MSCs derived from adults with MDS. In contrast, the gene-expression of *Dicer* was comparable in MSCs of children with and without MDS in our study. This supports the current understanding that pediatric and adult MDS differ substantially. Others have used similar murine models to demonstrate disturbed hematopoiesis after selective knock-down of stem cell factor (SCF), the 5HT4 serotonin receptor, the retinoblastoma gene or the retinoic acid receptor-γ.(Ding, 2012; Schepers, 2012; Walkley, 2007) Constitutive activation of the *Wnt*-pathway in osteoblasts leads to a hematopoietic system with characteristics of myeloid leukemia. (Kode, 2014)

Malignancies arising from MSCs

MSCs proliferate and differentiate to osteoblasts, adipocytes and chondrocytes. Growth during puberty requires cell replacement and renewal. This is the age at which osteosarcoma typically occurs. Murine MSCs have been shown to transform to osteosarcoma after long term culture.(Mohseny, 2009) Neither in healthy individuals nor in patients with Ewing sarcoma or osteosarcoma, signs of malignant transfor-

mation in culture have been published,.(Buddingh, 2015; Amaral, 2014) This does not exclude MSCs as the progenitor for sarcoma, however, development of sarcoma derived from infused MSCs is unlikely.

Genetic abnormalities in MSCs

Genetic abnormalities specifically in MSCs have been controversial. Raaijmakers *et al.* demonstrated that in a controlled murine setting, specific genetic alterations in the bone-marrow stromal cells of mice lead to malignant transformation in the hematopoietic compartment.(Raaijmakers, 2010) In humans, the isolation procedure of MSCs from the bone-marrow and the *in vitro* expansion of MSCs complicate the analysis of aberrant MSC function because of contamination of isolated cells or culture initiated artefacts. In adults, 16 % of MSCs from patients with AML and MDS showed genetic abnormalities distinct from the genetic abnormalities in the hematopoietic cells.(Blau, 2011) In this cohort, patients without cytogenetic abnormalities in MSCs had a better survival suggesting a survival benefit of the hematologic cells in the presence of affected MSCs. The supportive characteristics of these two groups of MSCS were not evaluated in this study. Other studies in adults report higher percentages up to 68%.(Flores-Figueroa, 2008; Flores-Figueroa, 2005; Blau, 2007; Song, 2012; Oliveira, 2013) Cytogenetic abnormalities were detected in all adult MDS derived MSC using array-CGH.(Lopez-Villar, 2009) However, other studies did not find karyotype abnormalities in the MSCs of adult MDS patients.(Zhao, 2012b; Zhao, 2014; Han, 2007; Soenen-Cornu, 2005) In adults, the acquired abnormalities during lifelong exposure to exogenous factors go concomitant in stromal and hematopoietic cells. In paediatric samples, no genetic abnormalities were detected in ALL.(Conforti, 2013) However, in MSCs derived from one specific subset of ALL, MLL-AF4+ with ALL starting *in utero,* the characteristic fusion gene was detected in MSCs and ALL cells. (Menendez, 2009) We showed that children with MDS or JMML do not have the same structural chromosome abnormality in MSCs as in the affected hematopoietic cells excluding a common genetic mutation as the explanation for altered gene-expression in patient derived MSCs.

MSCs in hematologic disease

As previously described, murine studies have been used to define the different pathways involved in the interaction between hematopoietic cells and MSCs. These murine models potentially resemble rare inherited diseases like Schwachman Blackfan Diamond or osteopetrosis (Mansour, 2012; Raaijmakers, 2010) and their application to investigate the contribution of MSCs to the development or expression of human hematopoietic disorders is controversial.(Raaijmakers, 2012) On the other hand, increasing evidence in adults supports our hypothesis that hematologic malignan-

cies alter the bone marrow microenvironment. This was previously suggested from data obtained in murine models demonstrating altered gene-expression in MSCs after infusion of, *e.g.*, CML and AML.(Arranz, 2014; Zhang, 2012b; Schepers, 2013) Both the homing of HSCs and the inflammatory signals might be altered. Malignant hematopoietic cells are thought to benefit from this altered state by occupying the available spaces and by receiving increased proliferative signals, *e.g.,* IL-6 and IL-1. (Zhang, 2003; Schepers, 2013) In contrast, stromal cell derived IL-6 suppressed the *in vitro* proliferation of chronic lymphocytic leukemia (CLL). (Li, 2015)

Our studies are the first in children addressing the alterations in MSCs induced by hematologic malignancies. JMML and MDS are thought to originate from aberrancies in the hematopoietic system. In our studies we have shown that in both disease entities the MSCs are altered compared to MSCs of age matched controls. This is in line with previously reported data on adult MDS and case-reports in children.

Characteristics of MSC from adults with MDS have been extensively studied focusing on cytogenetic abnormalities(Blau, 2011; Lopez-Villar, 2009; Flores-Figueroa, 2008; Blau, 2007; Flores-Figueroa, 2005; Song, 2012; Oliveira, 2013) and gene and protein expression(Marcondes, 2008; Flores-Figueroa, 2008; Lubkova, 2011; Santamaria, 2012; Flores-Figueroa, 2002; Hirayama, 1993; Aanei, 2012; Aanei, 2011) (Zou, 2015). In addition, abnormal immunomodulation(Wang, 2013b; Zhao, 2012b; Zhao, 2012a; Marcondes, 2008; Han, 2007) as well as decreased hematopoietic support(Zhao, 2012b; Ferrer, 2013; Aanei, 2012; Varga, 2007; Tennant, 2000; Flores-Figueroa, 2012) by MSCs have been reported in adult MDS. However, these data remain conflicting with other studies reporting no abnormalities in stromal function.(Flores-Figueroa, 2008; Coutinho, 1990; Soenen-Cornu, 2005; Klaus, 2010; Alvi, 2001) Different results might be explained by differences in MSC expansion protocols and experimental set-up, but also by the heterogeneity of the disease. (Aizawa, 1999) In our study we describe altered gene expression by pediatric MDS derived MSCs with an emphasis on immunomodulatory genes. The altered immunomodulatory gene-expression in JMML derived MSCs was supported by an altered immunomodulatory function of the MSCs. A decrease in monocyte to dendritic cell differentiation is suggestive for a decrease in immunosurveillance in the bone marrow micro-environment. JMML may benefit from this during progression, which may explain the therapy resistance of the disease. The different subtypes of pediatric MDS differed in their gene expression profile of MSCs. Further evaluation of these alterations may help to explain the differences between these subtypes.

Publications on the role of stroma in the ontogeny and maintenance of pediatric MDS are limited to a case report from a child with MDS,(Narendran, 2004) a study using stroma cells of 7 MDS patients (Borojevic, 2004), and a gene-expression analysis of the stromal compartment by the same research group.(Roela, 2007) Nevertheless

these scarce reports suggest an aberrant support of hematopoiesis associated with an altered gene expression profile of MSCs.

The origin of adult MDS is poorly defined. Based on the inability to engraft HPCs of MDS patients in xenograft models and on murine models showing a dysfunctional bone marrow niche resembling MDS, disturbed MSCs were proposed as the initiator and progressive factor in adult MDS.(Medyouf, 2014) In this study, MDS derived HPC clones better engrafted in mice after injection of MDS derived MSC than without injection of supportive cells and also compared to injection of healthy adult MSC. Interestingly, despite loss of the transplanted MSCs after a few weeks, the MDS clones could be detected long thereafter. This last observation suggests that the murine bone marrow niche was altered by the injected MDS clones. Alterations in the murine micro-environment were not reported in this study, but increased leukemia inhibitory factor (*LIF*) expression was detected in healthy adult MSCs after *in vitro* exposure to MDS derived bone-marrow or MDS cell lines.

The altered gene-expression by MDS and JMML patients derived MSCs is detectable despite multiple passages in culture. In addition, in the above mentioned study, the characteristics were retained after infusion. This suggests an epigenetic regulation of the changes in MSCs. Reversal of the changes after HSCT as described in chapter 3 and chapter 4 and as reported in literature,(Zhang, 2012b) supports this hypothesis. The successful treatment by demethylating agents in adult MDS could potentially be explained by changes in MSC function.(Fenaux, 2009) As discussed previously, the interaction between MSCs and hematopoietic cells is complex and many processes affect these interactions. These specific pathways should be identified and clarified in *in vitro* models. The clinical relevance however, should be studied in murine models or in the future using therapeutic interventions in patients.

Directions for future research

Following the first successful reports of treatment with MSCs, randomized controlled trials in steroid refractory acute Graft-versus-Host Disease (aGvHD), such as that recently initiated at the LUMC hopefully will provide answers about the efficacy of MSCs. Clinical data obtained in these studies in combination with laboratory data on serum biomarkers, lymphocyte subsets in blood and histology of biopsies should be used to define 1) patients likely to respond to therapy, 2) side effects of treatment and, of utmost importance, 3) the working mechanisms of MSC therapy. A better understanding of the different pathways involved in restoration of the balance of the immune system will be essential to proceed with cellular therapy. Experimental studies have identified possible involvement of multiple different pathways, and it

is most likely a combination of these that is responsible for the beneficial effects of MSC infusion in severe aGvHD. Identification of the most important interactions might lead to specific therapies without the need of cellular therapy.

Defining of the response induced by MSCs in aGvHD and the potential therapeutic mechanisms will support a more sophisticated exploration of the use of MSCs in other auto-inflammatory or auto-immune diseases. The use of standardized criteria for the cellular product in combination with proper designed clinical trials including collection of biomaterials will also be relevant to pursuit in these studies. Animal experiments and descriptive clinical trials have predominantly shown beneficial effects in active inflammatory conditions in contrast to ongoing chronic diseases. The suggested mechanism of licensing of the MSCs by *e.g.* double stranded RNA or lipopolysaccharides towards predominantly pro- or anti-inflammatory MSCs might explain these differences. In this way, it is not the question if MSCs are on one side of the equilibrium between anti- or pro-inflammatory responses, they are most likely the balance itself.

As previously discussed, the understanding of the interaction between MSCs and hematopoiesis is increasing. In addition, evidence supporting aberrant interaction during hematologic malignant diseases is more and more available. As demonstrated in this thesis, the disturbed immunomodulatory micro-environment is of importance. Further understanding of the consequences of the alterations will provide therapeutic targets. Support of normal hematopoiesis, resetting of the immune system and disrupting the altered micro-environment should go hand-in-hand.

Clinical trials in pediatric MDS and JMML should aim to 1) target the hematopoietic cell – MSC interaction with *e.g.* anti-IL-6 or CXCR4 antagonist or 2) reverse the aberrant interaction by *e.g.* 5-azacitidine. However, to answer remaining questions three separate, but complementary, approaches should further unravel these interactions:

I. Confirmation of differential RNA expression profiles

The observed differential RNA expression by patient derived MSCs should be studied at the protein level *ex vivo* by staining patient biopsies or measuring serum levels. In addition, the effect on MSC function of altered gene-expression should be characterized using specific up- and down-regulation of the different genes by *e.g.* siRNA.

II. Induction of observed alterations in healthy MSC

In vitro experiments should aim for better understanding of the mechanisms involved in induction of aberrant gene expression and normalization after successful treatment. MSCs should be extensively co-cultured with cells of the affected cell lineage from MDS and JMML patients to verify if differential gene expression

in MSCs and aberrant immunomodulatory capacity and hematopoietic support can be induced. The mechanisms of adaptation, potentially hypermethylation, should be characterized to be able to reverse the induced adaptations *in vitro.*

III. Development of humanized murine models

Patient material is scarce and *in vitro* experiments have its limitations. Humanized murine models using scaffolds for MSC engraftment have been developed for hematologic diseases, *e.g.,* multiple myeloma. These models are not yet available for JMML and pediatric MDS. Experimental data have shown that ossification by JMML and pediatric MDS MSCs is possible on ossicles in mice. However, engraftment of hematopoietic cells has not been performed. In addition, the hematopoietic subset that should be transplanted to resemble JMML or pediatric MDS remains to be defined.

The different approaches are complementary and together should give a complete understanding of the complex alterations in the bone marrow micro-environment in pediatric MDS and JMML. A better insight should lead to therapeutic interventions in these rare diseases. Although new approaches will be developed for comparable adult diseases, our data show that the alterations in the micro-environment cannot be directly translated to pediatric conditions. On the other hand, a complete understanding of the alterations induced during active JMML, characterized by its specific genetic mutations, might be beneficial for other hematologic malignancies.

Samenvatting

In het beenmerg is een continu proces gaande om de verschillende bloedcellen te vervangen, genaamd hematopoëse. De hematopoëse moet strak worden gereguleerd om een tekort of een overmaat aan cellen te voorkomen. Hierbij is het essentieel dat de hematopoëtische stamcellen, de bron voor bloedplaatjes en rode en witte bloedcellen, in stand worden gehouden. Naast hematopoëtische cellen bestaat het beenmerg uit verschillende ondersteunende cellen, zoals vetcellen (adipocyten) en botcellen (osteoblasten). Rond 1970 werd bekend dat mesenchymale stroma cellen (MSCs) de voorloper zijn van deze hematopoëtische cellen en dat deze cellen structuur geven aan het beenmerg. In de afgelopen jaren is bekend geworden dat MSCs van belang zijn voor de regulatie van de hematopoëse en bij het in stand houden van de hematopoëtische stamcellen. De meeste kennis hierover is verworven door onderzoek met muizen. Hierbij kwam naar voren dat een niet goed functionerende beenmergomgeving (MSCs) zorgt voor verstoring van de hematopoëse; daarnaast traden veranderingen op in de MSCs nadat in de muizen bloedkanker was geïnduceerd.

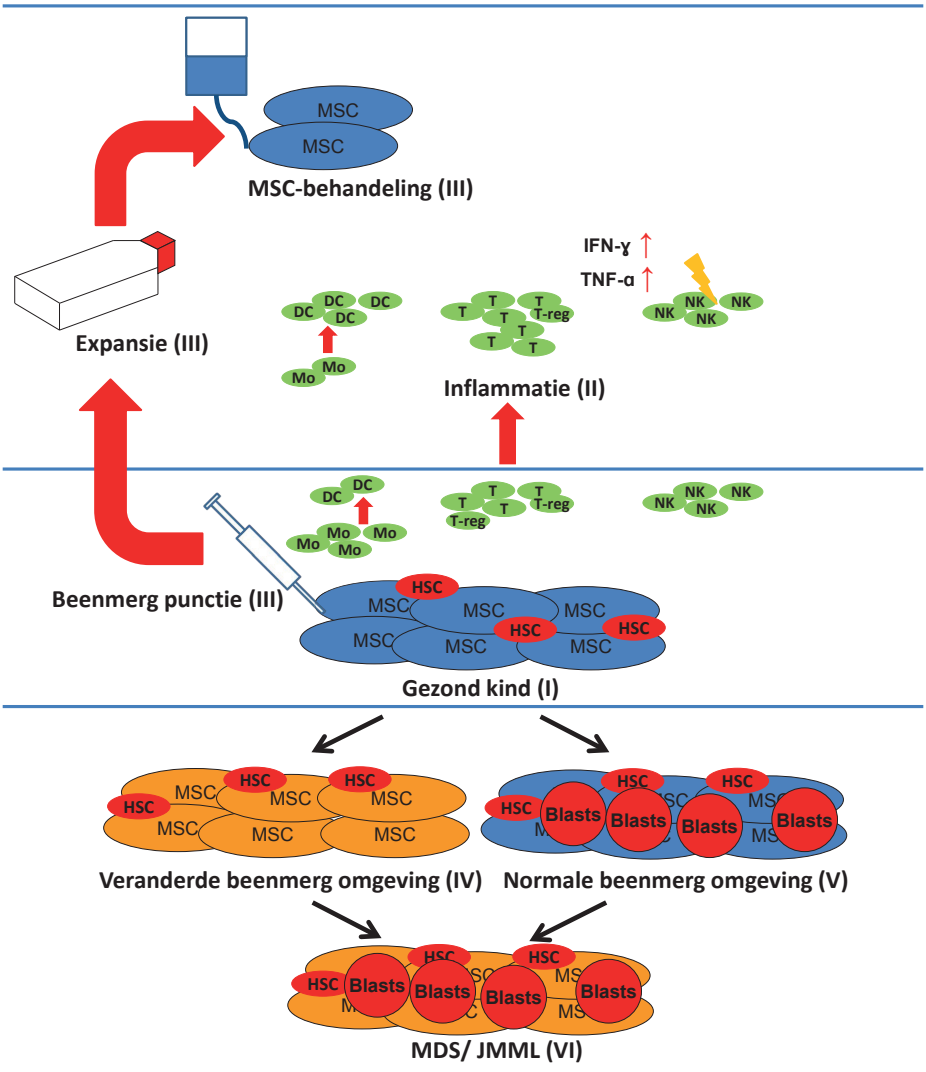
Een andere belangrijke karakteristiek van MSCs is het effect op het immuunsysteem. Nadat uit dierproeven bekend werd dat huidtransplantaten veel minder vaak werden afgestoten als er ook MSCs werden getransplanteerd, werd meer onderzoek gedaan naar de immuunmodulerende effecten van MSCs in *in vitro* experimenten. Dit leidde tot een belangrijke studie waarbij het effect van MSCs op acute *graft-versus-host* ziekte werd bekeken. Deze ziekte kan optreden nadat bij patiënten een hematopoëtische stamceltransplantatie is verricht. Bij acute graft-versus-host ziekte valt het immuunsysteem, afkomstig van de stamceldonor, de huid, lever en darm van de patiënt aan. Bij deze levensbedreigende ziekte werd grote verbetering van de overleving gezien door toediening van MSCs.

De onderzoeken beschreven in dit proefschrift richten zich op het verbeteren van de rol van MSCs bij de pathofysiologie en de behandeling van ziektes bij kinderen. Hierbij hebben wij ons gericht op de karakteristieken van MSCs bij kinderen met systemische juveniele idiopathische artritis, myelodysplastisch syndroom en juveniele myelomonocyten leukemie. Daarnaast hebben wij de effecten van de behandeling van acute *graft-versus-host* ziekte met MSCs in kaart gebracht en onderzocht of MSCs de anti-virale immuunrespons beïnvloeden. Figuur 1 geeft een overzicht van het onderzoek beschreven in dit proefschrift.

Systemische juveniele idiopathische artritis (sJIA) is een chronische auto-inflammatoire ziekte waarbij kinderen koorts, recidiverende huiduitslag, hepatosplenomegalie en artritis krijgen. De behandeling van deze ziekte is gericht op het remmen van de

ontsteking. Hierbij worden zowel aspecifieke middelen zoals NSAIDs en prednison als specifieke monoclonale antilichamen gebruikt. Voornamelijk deze laatste middelen zorgen voor een sterke afname van de klachten. Deze middelen bieden echter geen genezing en moeten dan ook langdurig worden gebruikt met mogelijk ernstige bijwerkingen. Mogelijk kan door toediening van MSCs het eigen immuunsysteem hersteld worden zodat de patiënt kan genezen. Deze hypothese wordt ondersteund door het feit dat patiënten eerder genezen konden worden door middel van hematopoëtische stamceltransplantatie. Hierbij traden echter forse bijwerkingen op die onvoldoende opwogen tegen de ziektewinst. Bij behandeling van patiënten met MSCs heeft het de voorkeur om MSCs te gebruiken van de patiënt zelf om afstotingsreacties te voorkomen. In **hoofdstuk 2** van dit proefschrift hebben wij beschreven dat de MSCs van kinderen met sJIA dezelfde immuunmodulerende eigenschappen hebben als de MSCs van gezonde kinderen. Dit is een eerste stap in het toepassen van de MSCs bij de behandeling van kinderen met sJIA.

Daarnaast hebben wij gekeken naar de eigenschappen van MSCs van kinderen met myelodysplastisch syndroom (MDS) en juveniele myelomonocyten leukemie (JMML). Voor beide bloedziekten is een hematopoëtische stamceltransplantatie noodzakelijk om de ziekte te genezen. De verstoorde hematopoëse bij kinderen met MDS gaat gepaard met een verstoorde immuunmodulatie. Dat is dan ook de reden dat wij hebben gekeken naar de eigenschappen van de MSCs bij kinderen met MDS. De resultaten hiervan staan beschreven in **hoofdstuk 3**. Allereerst is van belang dat de genetische afwijkingen in de hematopoëtische stamcellen niet voorkwamen in de MSCs van die patiënten. Het is dan ook onwaarschijnlijk dat een gemeenschappelijke afwijking verantwoordelijk is voor de veranderingen in het bloedvormende en het ondersteunende compartiment in het beenmerg. De MSCs van kinderen met MDS en de MSCs van gezonde kinderen verschillen niet in hun effecten op immuunreacties bij *in vitro* experimenten. Ook zagen wij geen verschil in de ondersteuning van *in vitro* hematopoëse door de MSCs. Bij analyse van de totale genexpressie van de verschillende MSCs zagen wij wel verschillen. Hiervoor hebben wij gebruik gemaakt van Deep-SAGE, een moderne techniek waarbij de nucleotide volgorde van al het *messenger*-RNA bepaald wordt. Vergelijking van de verkregen profielen toonde clustering van de kinderen met MDS ten opzichte van de gezonde kinderen. De genen met de grootste verschillen waren *interleukine-6*, van belang bij inflammatie; *DKK3*, betrokken bij gereguleerde celdood; *CRLF1* en *DAPK1*, beide geassocieerd met maligne ontaarding. Daarnaast hebben wij specifiek gekeken naar genen waarvan recent is beschreven dat zij differentieel tot expressie komen in MSCs afkomstig van volwassenen met MDS. Wij vonden echter geen veranderde expressie bij de kinderen met MDS. Dit bevestigt het bestaande idee dat MDS bij kinderen een wezenlijk andere ziekte is dan bij volwassenen.



Figuur 1. *Een overzicht van de verschillende onderwerpen in dit proefschrift.* I: MSCs zorgen bij gezonde kinderen voor ondersteuning van hematopoëse en een stabiel immuunsysteem. II: Tijdens momenten van ontsteking, zoals bij acute *graft-versus-host* ziekte, ontstaat er een toename in geactiveerde *natural killer* cellen (NK) en cytoxische T lymfocyten (T) na activeren door van monocyten (Mo) afkomstige dendritische cellen (DC). Gelijktijdig ontstaat een relatieve daling van het aantal regulatoire T lymfocyten (Treg) en toename van inflammatoire cytokinen als TNF-α, IL-1 en IFN-γ. III: MSCs kunnen in het laboratorium worden gekweekt nadat er beenmerg is afgenomen. In dit proefschrift worden de mogelijkheden van MSCs beschreven om de ontregelde immuunrespons weer in balans te brengen.

Daarnaast hebben we de interactie tussen MSCs en de hematopoëtische cellen onderzocht bij kinderen met MDS en JMML. Wij hebben onderzocht of de MSCs mogelijk IV: intrinsiek anders zijn bij deze kinderen of dat er V: door de ziekte veranderingen worden geïnduceerd. VI: de veranderde MSCs dragen mogelijk bij aan de resistentie tegen de huidige behandeling van JMML en MDS.

JMML is een maligne ziekte waarbij verschillende genetische afwijkingen in het hematopoëtische compartiment zijn beschreven. Het is een agressieve vorm van bloedkanker die voorkomt in de eerste vier levensjaren. Bij 30-50% van de kinderen komt de ziekte terug ondanks in eerste instantie succesvolle hematopoëtische stamceltransplantatie. Zoals beschreven in **hoofdstuk 4** hebben wij gekeken naar de MSCs van deze kinderen omdat wij veronderstelden dat de veranderde beenmergomgeving mogelijk een rol zou spelen bij deze agressieve en therapie resistente ziekte. Alhoewel de MSCs niet verschilden op basis van standaardtesten zagen wij bij Deep-SAGE analyse grote verschillen tussen de MSCs van gezonde kinderen en JMML patiënten. Wij hebben vervolgens specifiek gekeken naar de uitrijping van monocyten naar dendritische cellen. Dit proces is van belang voor antigeen presentatie voor cellen van het immuunsysteem. MSCs remmen deze uitrijping. Opvallend was dat MSCs van JMML patiënten een nog sterker effect hadden op de uitrijping dan MSCs van gezonde kinderen. Een verminderde antigeen presentatie speelt mogelijk een rol bij de progressie van JMML. Daarnaast waren de verschillen in expressie van genen betrokken bij de interactie tussen verschillende celtypen (onder andere CXCL12) en genen betrokken bij inflammatie (onder andere de IL-1 familie) opvallend. De verschillen werden niet verklaard door gemeenschappelijke genetische afwijkingen. Na succesvolle hematopoëtische stamceltransplantatie waren de genafwijkingen en de verschillen in remming van monocyt differentiatie niet meer meetbaar. Dit suggereert dat de veranderingen die wij meten in de geëxpandeerde MSCs van kinderen met JMML worden veroorzaakt door de ziekte en niet de oorzaak van de ziekte zijn.

De eerste stap van behandeling van acute *graft-versus-host* ziekte is immuunsuppressie met prednison. Bij onvoldoende of geen respons is er geen verdere standaardbehandeling beschikbaar en is de overlevingskans van kinderen met ernstige acute *graft-versus-host* ziekte die ongevoelig zijn voor prednison ongeveer 30% (historische data). Sinds MSCs worden gebruikt als experimentele behandeling is de overleving echter sterk gestegen. In **hoofdstuk 5** beschrijven wij een groot cohort van kinderen die behandeld zijn in het LUMC. Van deze kinderen zijn uitgebreid gegevensbekend en is frequent serum afgenomen. Bovendien is bij deze kinderen gestart met het afnemen van darmbiopten na behandeling. Een darmbiopt is bij de diagnose van acute *graft-versus-host* ziekte de gouden standaard. Bij de eerste kinderen die waren behandeld met MSCs zagen wij frequent diarree na start van therapie; soms na een in eerste instantie klachtenvrije periode. Bij deze kinderen is een darmbiopt genomen om duidelijkheid te krijgen over de diagnose. Hierbij werd gedacht aan een recidief van de ziekte, een virale infectie of aan diarree geassocieerd met het herstarten van normale voeding. Meerdere keren zagen wij dat bij patiënten geen sprake was van actieve ziekte en dat de immuunsuppressie succesvol kon worden afgebouwd. Vervolgens is bij twaalf kinderen met persisterende of recidiverende

diarree een darmbiopt verricht. In totaal werd bij acht kinderen geen actieve ziekte gezien, maar bij vier kinderen was ondanks MSC behandeling sprake van tekenen van acute *graft-versus-host* ziekte. Bij deze kinderen werden opnieuw MSCs gegeven met een goede respons bij drie van hen. De afname van biopten is bij alle kinderen ongecompliceerd verlopen. Wel is het een ingreep die onder narcose moet gebeuren en een procedure die wordt verricht bij zieke kinderen. Wij hebben dan ook gekeken naar een alternatief door analyse van eerder beschreven biomarkers voor de diagnose acute *graft-versus-host* ziekte in serum monsters. Opvallend is dat bij de start van de behandeling met steroïden de concentratie TNFr1 hoger is bij kinderen die niet reageren op MSC infusie. Dit effect is sterker 28 dagen na de start van MSC behandeling. Daarnaast hebben wij gekeken naar REG3α een biomarker specifiek voor acute *graft-versus-host* ziekte van de darm. Een lage REG3α waarde 28 dagen na start van de behandeling is een goede voorspeller van de eenjaarsoverleving. Alhoewel de concentraties van REG3α, IL2-Rα en cytokeratine 18 (een marker voor cel apoptose) wel significant verhoogd zijn op het moment van biopsie bevestigde ziekte, zijn de specificiteit en sensitiviteit onvoldoende om een biopsie te vervangen. Dit geldt specifiek voor biopten genomen na start van de behandeling. Wij raden dan ook sterk aan om bij toekomstige klinische studies naar acute *graft-versus-host* ziekte in ieder geval darmbiopten te nemen bij het persisteren van gastro-intestinale klachten op het moment van evaluatie van de respons op behandeling.

Het werkingsmechanisme van MSCs bij immuunmodulatie is onvoldoende duidelijk. Ook de bijwerkingen van de behandeling zijn onvoldoende beschreven. In ons cohort hebben we gekeken naar het effect van MSC behandeling op virus infecties. Eerdere studies rapporteren een wisselend effect op T lymfocyten specifiek tegen het cytomegalovirus. Over het effect op de afweer tegen adenovirus, een specifiek probleem bij kinderen na hematopoëtische stamceltransplantatie, was nog niets beschreven. In **hoofdstuk 5** wordt de incidentie van cytomegalovirus, adenovirus en Epstein-Barr-virus infecties vergeleken bij kinderen met acute *graft-versus-host* ziekte van wie 22 patiënten werden behandeld met MSCs; bij 21 kinderen werd alleen prednison gegeven en dertien kinderen kregen een andere tweedelijnsbehandeling dan MSCs. Er werden geen verschillen gezien in de incidentie van de verschillende virale infecties. Opvallend genoeg was de overleving bij kinderen behandeld met MSCs die een adenovirus infectie kregen significant lager. In *in vitro* experimenten werd de specifieke respons van T lymfocyten tegen cytomegalovirus en adenovirus onderdrukt door MSCs. Bij kinderen met een virus infectie rondom de behandeling met MSCs werd geen verandering gezien in het percentage virus-specifieke T lymfocyten. Wel is de proliferatie van de specifieke T lymfocyten onderdrukt bij deze kinderen vanaf het moment dat prednison wordt gestart.

Hoofdstuk 6 geeft een blik in de toekomst met ideeën en adviezen voor toekomstig onderzoek. De gerandomiseerde studies die worden verricht naar de behandeling van acute *graft-versus-host* ziekte kijken primair naar het effect van MSCs, maar daarnaast vormen MSCs een unieke populatie waarbij door kritische en gedetailleerde analyse meer inzicht kan worden verkregen in het werkingsmechanisme en de bijwerkingen van MSCs. Biopten, serum analyse, maar ook lymfocyten subset analyse zijn hierbij essentieel. Verbeterd inzicht kan vervolgens worden gebruikt voor een beter gefundeerde behandeling van chronische auto-immuun- en auto-inflammatoire ziekten met MSCs.

Het onderzoek naar de interactie tussen hematologische maligniteiten en MSCs richt zich op een beter begrip van de pathofysiologie van deze ziekten. Ons onderzoek toont aan dat bij kinderen met myelodysplastisch syndroom (MDS) en juveniele myelomonocyten leukemie (JMML) het gen expressie profiel van de MSCs is veranderd gedurende actieve ziekte. Na succesvolle behandeling herstelt dit profiel zich weer. Voor verder inzicht is bevestiging nodig van onze bevindingen in *ex vivo* botbiopten, *in vitro* inductie van veranderde genexpressie in gezonde MSCs en *in vitro* herstel van veranderde genexpressie in patiënten MSCs. Daarnaast zijn recent beschreven 3D modellen, al dan niet in een gehumaniseerd muismodel, essentieel voor een beter begrip van de complexe interactie tussen MSCs en hematopoëtische cellen. Deze nieuwe inzichten kunnen op termijn consequenties hebben voor de behandeling van kinderen met MDS of JMML. Mogelijke aanknopingspunten hiervoor zijn de immuunmodulatie en de interactie tussen MSC en hematopoëtische cellen.

Het onderzoek naar MSCs ontwikkelt zich snel op vele gebieden. Beter begrip van de complexe mechanismen zal bijdragen aan de zorg voor patiënten. Niet alleen op het gebied van immuunmodulatie en weefselherstel, maar ook bij maligniteiten en wellicht bij talloze andere ziekten.