Cover Page

Mesenchymal stromal cells in pediatric disease

Pathophysiology and treatment

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Friso Calkoen

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Contents

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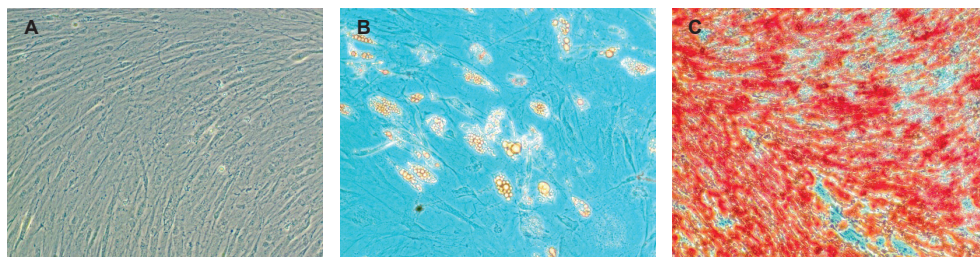
Figure 1. *Differentiation capacity of MSC.* All MSCs described in this study fulfil the ISCT criteria(5) of adherence to plastic (A) and *in vitro* differentiation capacity towards adipocytes (B) and osteoblasts (C).

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 13×10^9/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 de-pendent 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 transplan-tation 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 mac-rophages 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 sys-tem. 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, caus-ing 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, differenti-ate 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.
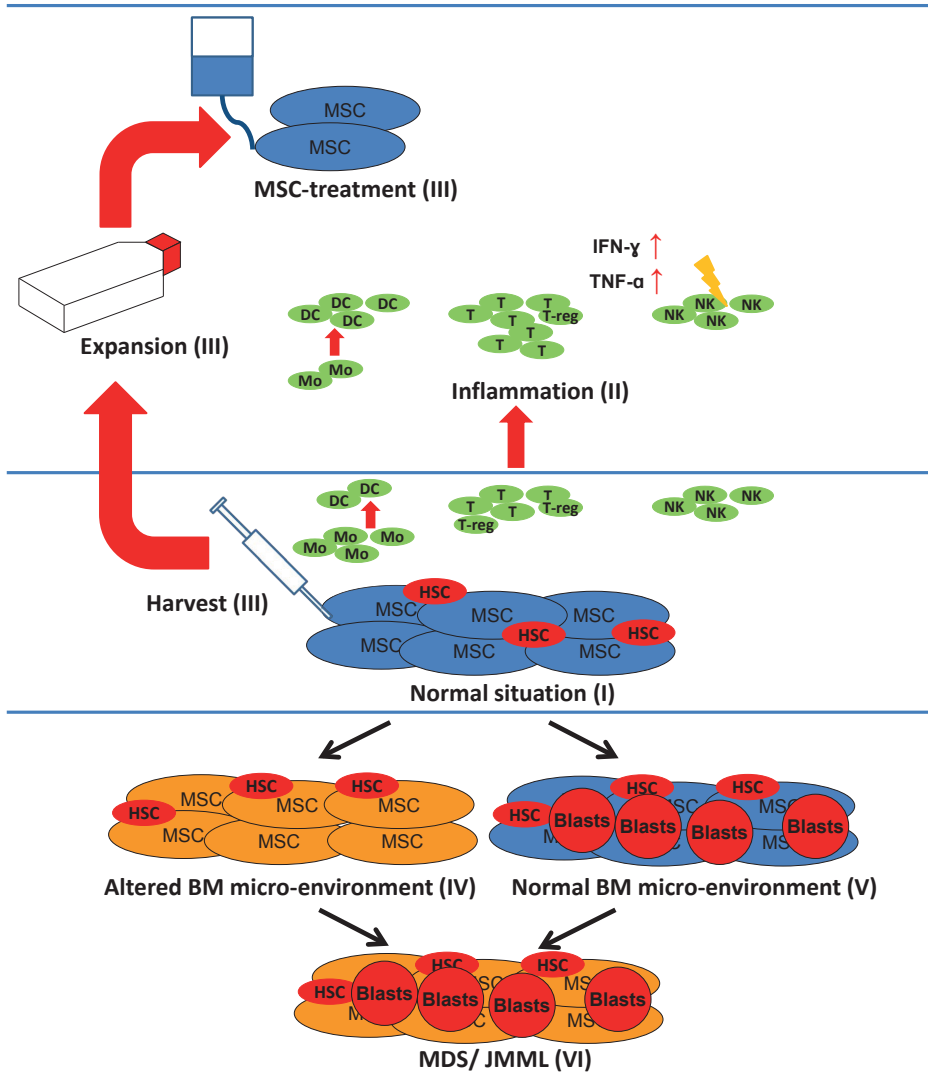
Figure 2. *A simplified overview of the different parts in this thesis* I: MSCs are in close contact with the hematopoietic stem cells (HSC) and contribute to a balanced immunological status. II: During in-flammation, e.g. acute Graft-versus-Host disease, antigen presentation by dendritic cells (DC), 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.

Chapter 2.

Mesenchymal stromal cells isolated from children with systemic juvenile idiopathic arthritis suppress innate and adaptive immune responses.

Calkoen FGJ, Brinkman DM, Vervat C, van Ostaijen-Ten Dam MM, ten Cate R, van Tol MJD, Ball LM.

Abstract

Background aims: Infusion of mesenchymal stromal cells (MSCs) has been reported to be an effective treatment modality for acute Graft-versus-Host disease, and MSCs haven been considered for use in the treatment of patients with autoimmune diseases. Before contemplating clinical studies with MSCs in patients with systemic juvenile idiopathic arthritis (sJIA), the immunomodulatory capacity of MSCs in this setting needs to be explored. A comparative analysis of bone marrow derived MSCs from children with sJIA and healthy paediatric controls was performed.

Methods: MSCs were successfully expanded from 11 patients with sJIA and 10 controls. The phenotype, differentiation and immunomodulatory capacity of these MSCs were compared. The effect of immunosuppressive drugs on MSC function was also investigated.

Results: MSCs from patients with sJIA and controls showed no differences in their suppressive effect using control peripheral blood mononuclear cells. Furthermore, the suppression of the response of peripheral blood mononuclear cells from patients with sJIA autologous sJIA MSCs and allogeneic control MSCs was comparable. The immunosuppressive effect of both groups of MSCs was diminished in the presence of indomethacin ($p<0.05$). MSCs from patients with sJIA and controls suppressed IL-2 induced natural killer cell activation to a similar extent. In addition, MSCs of patients with sJIA and controls inhibited the differentiation of monocytes to dendritic cells.

Discussion: This is the first explorative study in a significant cohort of patients with sJIA to evaluate the effect of MSCs on adaptive and innate immune responses. The comparable immunosuppressive characteristics of MSCs derived from patients with sJIA to age-matched controls support the potential use of patient derived MSC in the treatment of sJIA.

Introduction

Systemic juvenile idiopathic arthritis (sJIA) is an autoinflammatory disease of unknown aetiology that is characterized by spiking fever, exanthema, anaemia, hepatosplenomegaly and arthritis.(Petty, 2004) The outcome of disease and response to therapy is unpredictable. Improvements in treatment outcome with the use of diverse biological therapies, as anti-interleukin (IL)-1 therapy, have been reported.(Lequerre, 2008; De Benedetti F., 2009; Woo, 2008; Martini, 2006; Beukelman, 2011; Quartier, 2011; Nigrovic, 2011; Yokota, 2008) Despite these observations, the disease progresses and/or requires additional therapy in 27% of children treated with etanercept.(Prince, 2011) Moreover, patients treated with these new drugs may experience adverse effects. Recent data relate the use of tumor necrosis factor (TNF)-blocking agents in patients with JIA and other inflammatory diseases to the development of lymphoma and other cancers in children.(Horneff, 2011; Kwon, 2005; Diak, 2010)

Previous studies in children with refractory JIA undergoing autologous hematopoietic stem cell transplantation following chemotherapy and T cell depletion show a progression free survival of 52% after 5 years.(Farge, 2010) However, high morbidity due to macrophage activation syndrome, viral infections and transplant-related mortality occurred.(Brinkman, 2007) Therefore, new treatment modalities need to be explored for therapy-resistant patients.

Mesenchymal stromal cells (MSCs) are multipotent non-hematopoietic stromal cells with anti-inflammatory and anti-proliferative capacities.(Krampera, 2003; Bocelli-Tyndall, 2009) MSC can be expanded easily from different tissues including bone marrow (BM), and remain genetically stable even after numerous passages.(Bernardo, 2007c) No unique marker has been identified for the isolation of MSCs; however, specific criteria to identify MSC have been accepted.(Dominici, 2006; Horwitz, 2005)

T cell and natural killer (NK) cell proliferation, immunoglobulin production by B cells and dendritic cell maturation are suppressed by MSC in *in vitro* experiments.(Bocelli-Tyndall, 2007; Sotiropoulou, 2006; Traggiai, 2008b) The mechanisms underlying these immunomodulatory functions are not fully understood. However, experimental data suggest that cell-cell contact and soluble factors may both be involved in an overall inhibition of the induction of effector functions.(Beyth, 2005; Gieseke, 2010; Krampera, 2003; Meisel, 2004; Rasmusson, 2005; Selmani, 2008; Uccelli, 2008)

The profound immunomodulatory characteristics of MSCs make them potential candidates for use in the treatment of inflammatory diseases. Clinical trials using allogeneic MSCs in the treatment of steroid resistant acute graft versus host disease (aGvHD) have shown promising results.(Le Blanc, 2008) Moreover, the feasibility and safety of autologous MSC infusions have been demonstrated in phase I clinical trials.(Garcia-Olmo, 2005; Duijvestein, 2010; Sun, 2009; Sun, 2010; Karussis, 2010)

However, the efficacy and safety of MSC treatment has yet to be determined in larger cohort studies.(Dazzi, 2011)

Previously, an aberrant suppressive capacity of MSCs from patients with severe aplastic anaemia was previously reported.(Bacigalupo, 2005) In addition, functional aberrations in synovium-derived MSCs from adults with rheumatoid arthritis have been described, suggesting that MSCs from patients with inflammatory diseases may not be comparable to those derived from healthy donors.(Jones, 2010) Therefore, before contemplating clinical studies of autologous MSC infusions in children with treatment-resistant sJIA, the immunomodulatory properties of MSCs of these patients need to be characterized. To our knowledge this is the first study describing comparable characteristics of MSCs from a relatively large cohort of children with sJIA compared to age-matched healthy controls (HC).

Methods

Expansion of MSCs

Bone marrow (BM) of children with sJIA (n=13) was used to initiate MSC cultures according to a protocol approved by a local ethical committee. Fresh BM was harvested from five patients at time of diagnosis. To expand the study population, frozen bone marrow mononuclear cells (BMMC) from patients with sJIA (n=8) were used for MSC cultures. Classification of sJIA was according to the guidelines from the International League of Associations for Rheumatology.(Petty, 2004) Fresh BM of 10 paediatric donors of an allogeneic hematopoietic stem cell graft was used as HC. Parental and patient informed consent forms were signed for each patient and control.

After Ficoll separation BMMC were plated at a density of 0.16×10^6 cells/cm^2 in polystyrene culture flasks. Cells were cultured in Dulbecco's modified Eagle medium with Glutamax (DMEM; Invitrogen, Paisley, UK) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin (P/S; Invitrogen) and 10% fetal bovine serum (FBS; VWR International, Bridgeport, NJ, USA). Medium was refreshed every three to four days. Cultures were harvested at 80% confluency by treatment with trypsin (Invitrogen), replated and maintained for maximally six passages at 37°C and 5% CO_2.

Characterization of MSCs

All MSC cell lines were phenotypically characterized at their second or third passage using antibodies against CD3, CD45, CD86, HLA-DR, CD31, CD34, CD73 and CD90 (all Becton Dickinson Biosciences (BD), San Diego, CA, USA). CD105 was obtained from Ancell Corporation (Bayport, MN, USA). Cells were analyzed on a FACS Calibur flow

Table 1. Clinical characteristics of patients with JIA at the time of BM harvest.

Patient (UPN)	Sex	Age (years)	Disease duration (months)	Active joints (n)	ESR (mm/h)	NSAID[a] (mg/kg/day)	Prednisone (mg/kg/day)	Other immuno-suppressive drugs
1	M	5	7.7	3	19	1.7	-	-
2	F	12	0.3	4	11	2.0	-	-
3	F	7	42.9	2	11	2.9	0.2	Anti-TNF-α
4	F	11	2.4	2	124	2.0	1.0	-
5	M	4	12.9	11	45	2.0	1.0	MTX; Cyclosporin
6	M	11	104.0	11	33	1.7	0.5	-
7	M	7	26.3	32	85	2.0	0.2	Anti-TNF-α
8[b]	M	13	116.7	1	77	1.6	0.3	Azathioprine
9	M	7	74.9	6	46	0.4	0.5	MTX
10[b]	F	5	23.9	8	71	2.0	1.0	MTX; Cyclosporin
11	M	5	0.8	5	91	1.3	-	-
12	F	3	0.5	17	130	1.7	-	-
13	F	1	3.8	10	14	2.5	-	-

ESR, erythrocyte sedimentation rate; NSAID, non-steroidal anti-inflammatory drugs; TNF, tumour necrosis factor; MTX, methotrexate

[a] all patients were treated with indomethacin, except UPN 11 who was treated with diclofenac.

[b] no expansion of MSC was achieved from bone marrow samples of this patient.

cytometer (BD). Mean fluorescence intensity (MFI) was compared with cells stained with isotype-matched negative control antibodies (BD) and with unstained cells.

The osteoblast and adipocyte differentiation potential was evaluated on cells at passage 4-6 as described previously.(Bernardo, 2007b) After 3 weeks, fat vacuoles in adipocytes and calcified depositions in osteoblast were stained with Oil-Red-O (Sigma, St. Louis, MI, USA) or Alizarin Red (MP Biomedicals, Solon, OH, USA), respectively.

Peripheral blood mononuclear cell cultures

To investigate the immunosuppressive effect of MSCs on peripheral blood mononuclear cell (PBMC) proliferation, irradiated (30 Gy) MSCs from patients with sJIA or HC were seeded at 2-fold serial dilutions in flat-bottom 96-well plates. PBMC, isolated by Ficoll separation from buffy coats of healthy blood bank donors (Sanquin, Amsterdam, the Netherlands), were added (100.000 cells/well) after 4 hours. Cells were stimulated with 2 µg/mL phytohemagglutinin (PHA; Murex, Châtillon, France) and cultured at 37°C and 5% CO_2 for 5 days. Fifty microliters of culture supernatant was harvested at day 4 for cytokine analysis. The concentrations of interferon (IFN)-γ and TNF-α in supernatants were measured using standard protocols of enzyme-linked im-

munosorbent assay kits (Sanquin). Cells were pulsed with ^{3}H-thymidine (1 μCi/well; Perkin Elmer, Wellesley, MA, USA) for the final 16 hours and counted with a β-counter (Perkin Elmer). All cultures were performed in triplicate in RPMI 1640 (Invitrogen) medium with 10% pooled human AB serum (Sanquin) and P/S (total volume 200 μL/ well).

To investigate the influence of drugs used in the treatment of sJIA, indomethacin (5 μM; Sigma), dexamethasone (100 ng/mL; Sigma) and the soluble TNF-α receptor adalimumab (10 μg/mL; Abbott Laboratories, Abbott Park, IL, USA) were added at culture initiation.

NK cell cultures

NK cells were isolated from PBMC derived from healthy blood bank donors by negative selection using an NK isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). An NK cell purity greater than 95% was reached. NK cells (1.0×10^6/well) were stimulated with 30 IU/mL IL-2 (Chiron Corporation, Emeryville, CA, USA). NK cells were co-cultured with or without irradiated MSCs in 24 well plates at an MSC:NK ratio of 1:5 or 1:40 in RPMI 1640 supplemented with 10% human AB serum and P/S.

At day 5, NK cells were harvested and counted and cell marker expression and cytotoxicity were assessed. The expression of CD69 (BD) and NKG2D (Beckman Coulter, Brea, CA, USA) was determined by flow cytometry. Cytotoxicity was determined in a standard 4-hour chromium release assay using various target cells (2.500 cells/well) *i.e.*, Daudi cells, which are only sensitive to killing mediated by activated NK cells, and Epstein-Barr virus transformed B cells (EBV-BLCL) coated with antibodies through pre-incubation with anti-thymocyte globulins (ATG; Genzyme, Cambridge, MA; 50 μg/mL for 20 minutes) to measure antibody dependent cell mediated cytotoxicity (ADCC). Target cells were labelled with 100 μCi sodium-51-chromate (Perkin Elmer) for 1 hour. Effector cells were incubated with target cells at ratios ranging from 40:1 to 0.3:1 in triplicate. Spontaneous and maximum release were determined by incubating target cells with medium or Triton X100 (5%; Merck Chemicals, Darmstadt, Germany), respectively. Subsequently, chromium release in the supernatant was assessed in a β-counter. Specific lysis was determined as follows: (experimental release-spontaneous release) / (maximum release-spontaneous release) x 100%.

Dendritic cell cultures

Monocytes were isolated from PBMC derived from healthy blood bank donors with a positive CD14 selection kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Cell purity greater than 90% was reached in all isolations. Monocytes (0.5×10^6) were cultured in 24-well plates for 5 days in RPMI 1640, 10% FCS and 1% P/S supplemented with 800 IU/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) and 40 ng/

mL IL-4 (both from Tebu-Bio, Le Perray en Yvelines, France) to induce dendritic cell (DC) differentiation. At day 5, cells were harvested or similar concentrations of IL-4 and GM-CSF, and CD40-ligand (0.25 ug/mL Beckman-Coulter, Marseille, France) and IFN-γ (500 U/mL, Boehringer, Mannheim, Germany) were added to mature the DC for 2 extra days. All cells harvested on day 5 and 7 were analyzed using flow cytometry for CD14, CD1a, CD80, CD86, and CD163 expression (antibodies from BD). MSC were co-cultured with DC at MSC:DC ratios of 1:5, 1:40 or 1:100.

Statistics

Data were analyzed using GraphPad Prism (LaJolla, CA). For statistic analysis paired and unpaired t-tests were used. *P*-values of <0.05 were considered significant.

Results

Characterization of MSCs

MSCs were successfully expanded from bone marrow (BM) of 11 out of 13 sJIA patients and 10 out of 10 HCs. Age did not significantly differ between the patients and HCs (mean 7.1 years, range 1-12 versus 8.9 years, range 1-19, respectively); 45% of the patients with sJIA and 40% of the controls were female. The characteristics of the patients included in the study are summarized in Table I. Samples from two patients, both frozen BMMC, failed to expand. The cryopreservation time of these samples was 10.2 and 12.3 years, respectively, versus a mean storage time of 7.5 years for the successfully expanded samples (range 2.4-11.1 years). In addition, cultures of these two samples were initiated with relatively low numbers of BMMC ($0.26x10^6$ and $2.8x10^6$). For MSC cultures from frozen BMMC significantly less cells were available compared with cultures from freshly isolated BMMC (mean $3.9x10^6$, range $0.26-7.56x10^6$ versus mean $33.1x10^6$, range $16-60x10^6$ BMMC, respectively).

MSCs were characterized by adhesion to plastic and fibroblast-like appearance. In addition, all MSC fulfilled the phenotypic criteria, (i.e., expression of CD73, CD90 and CD105 and no expression of CD34, CD31 and CD45) at passage 2 or 3 (Figure 1A). All MSCs had the ability to differentiate into adipocytes and osteoblasts upon stimulation (Figures 1B-I). However, MSC lines derived from two patients (UPN 2 and 5 in Table I), which were obtained from frozen material, failed to differentiate into the osteoblastic lineage. These two MSC cultures were initiated with relatively low numbers of BMMC ($0.46x10^6$ and $1.4x10^6$, respectively). These MSC were, therefore, not evaluated in the immunomodulatory assays.
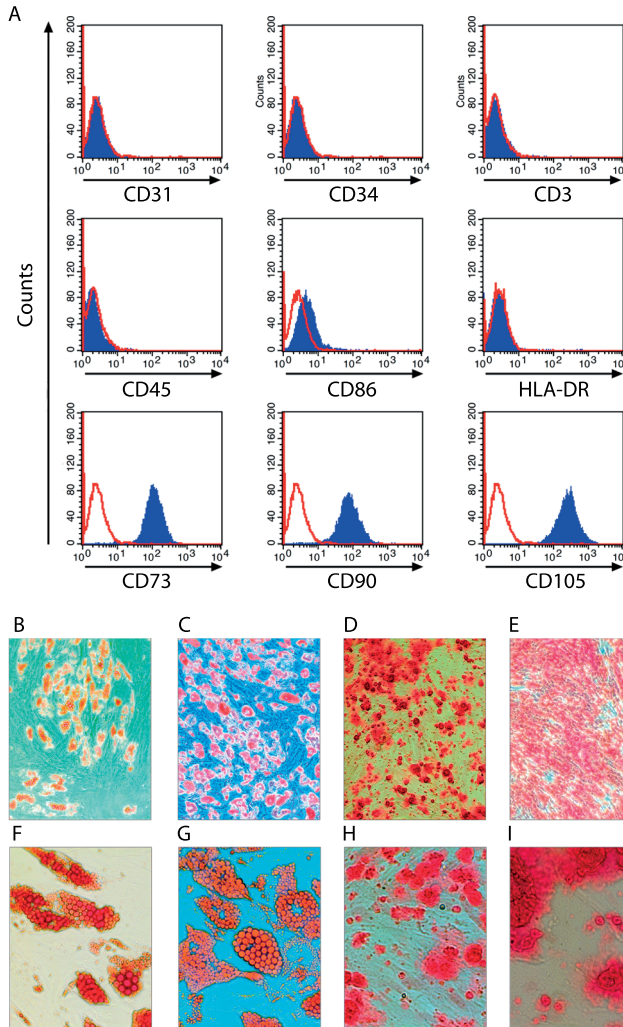
Figure 1. *MSCs of patients with sJIA and healthy controls (HC) show an identical phenotype and differentiation potential.* A) Representative FACS staining of UPN 12 is shown in closed histograms. Open histograms represent the staining intensity with isotype-matched negative control antibodies. B) Differentiation of HC MSC (B,D,F,H) and sJIA MSC (C,E,G,I) to adipocytes (B,C,F,G; stained with Oil-Red-O) and osteoblasts (D,E,H,I; stained with Alizarin Red). Magnification 10x (B,C,D,E) and 40x (F,G,H,I).

Immune modulation of PBMC by MSCs

MSCs down modulated the proliferation of PHA-stimulated PBMC in a dose-dependent manner (Figure 2A). A significant reduction of proliferation was seen at all MSC:PBMC ratios used. No differences were observed between sJIA and HC MSCs investigated in these experiments (Figure 2A). sJIA MSC derived from patients *in vivo* treated with (n=4) and without prednisone (n=4) gave comparable results (data not shown). ^{3}H-thymidine counts did not exceed background levels in MSCs and PBMC co-cultured in the absence of PHA, or in MSCs cultured without PBMC in the presence of PHA.

IFN-γ and TNF-α concentrations were reduced in supernatants of PHA-stimulated HC PBMC co-cultured with MSCs compared with PHA-stimulated PBMC without MSCs. MSCs of HC and patients with sJIA equally suppressed the production of these pro-inflammatory cytokines (Figures 2C/D).

Suppression of autologous PBMC proliferation by sJIA MSCs

PBMC of patients with sJIA were stimulated with PHA and co-cultured either with autologous sJIA MSC or with MSC from two allogeneic HC (Figure 2B). PBMC frozen at the time of BM harvest were used in these experiments. Independent of their origin, MSC suppressed the PBMC proliferation to a similar extent. At an MSC:PBMC ratio of 1:5, the average down-modulating effect of sJIA MSC on autologous sJIA PBMC (Figure 2B) did not differ from their effect on allogeneic HC PBMC (Figure 2A) (25.6% vs. 20.6%; p=0.20).

Influence of immunosuppressive drugs on function of MSCs

Addition of dexamethasone to cultures, but not of indomethacin or adalimumab, to cultures significantly reduced the PHA-induced proliferation of PBMC from HC (p<0.01) (Figure 3A). Despite this effect, MSCs added at culture initiation retained their suppressive capacity in the presence of dexamethasone (Figure 3B). The same observation was evident for the production of cytokines (Figures 3C/D).

In contrast, indomethacin interfered significantly with the immunosuppressive activity of MSCs derived from either sJIA or from HC at an MSC:PBMC ratio 1:5 (Figure 3A/B). However, the effect of indomethacin did not reach significance at an MSC:PBMC ratio ranging from 1:10 to 1:80 (data only shown for 1:40). IFN-γ and TNF-α production appeared to be a more sensitive indicator for the interference of indomethacin with the immunosuppressive activity of MSC, because a significant lower decrease of the cytokine production in the presence of the drug (p<0.05) was not only observed at an MSC:PBMC ratio of 1:5 but also at 1:40 (Figures 3C/D).

Adalimumab did not affect PBMC proliferation (Figures 3A/B). IFN-γ production, however, was clearly decreased in the presence of adalimumab alone and further diminished when MSCs were added to the culture. The effects of these drugs on the immunomodulatory capacity of MSCs were similar for MSCs derived from patients with sJIA and from HC (data not shown). The mean +/- standerd error of the mean (SEM) percentages of proliferation at a ratio of MSC:PBMC of 1:5 were 38.4% +/- 4.9% and 33.5 +/- 1.9% for HC-MSC and sJIA-MSC, respectively.

Suppression of NK cell activation by MSCs

The expression of CD69 and NKG2D on NK cells was up-regulated after 5 days activation with IL-2 (30 IU/mL). The percentage of CD69+ cells (range 26-70%) and the
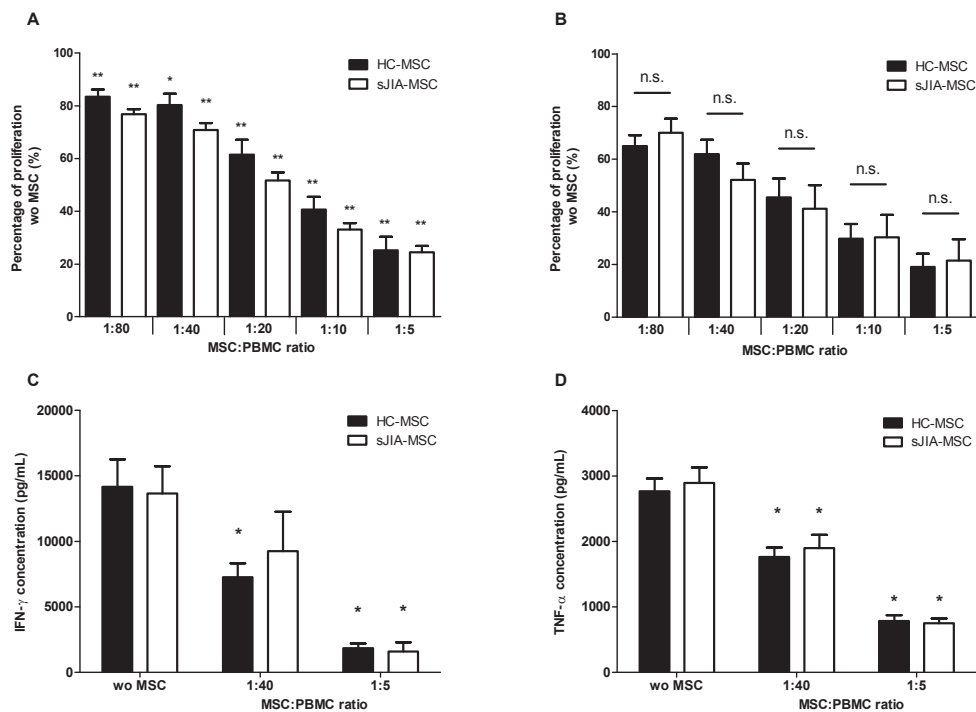
Figure 2. *sJIA and healthy control (HC) derived MSCs suppress activation of patient and control derived PBMC.* sJIA and HC-derived MSCs show no differences in the down modulation of the functional activity of PBMC after PHA stimulation. A) The mean and standard error of the mean (SEM) of proliferation at day 5 are depicted of experiments using HC (n=4) and sJIA (n=8) MSCs as modulators and PBMC of four healthy donors as responders. PHA-induced proliferation in the absence of MSCs was set at 100%. B) Autologous sJIA and allogeneic HC-derived MSCs show no differences in their down-modulating effect on PHA-induced proliferation of sJIA-derived PBMC. The mean and SEM of experiments with six different sJIA PBMC are depicted. The effect of the six autologous sJIA MSCs and of two allogeneic HC MSCs was tested on PBMC of all six patients with sJIA investigated in this experiment. C/D) In co-cultures of PHA-stimulated HC PBMC with sJIA and HC-derived MSCs the (C) IFN-γ and (D) TNF-α concentrations in supernatants taken at day 4 are decreased. The mean concentration of cytokines in cultures of two PBMC donors as responders with or without MSCs from 2 HC and 4 sJIA patients as modulators are shown. *: *p* <0.05 compared to without (wo) MSC. **: *p* <0.01 compared to without MSC.

MFI of NKG2D expression (range 150-297), depending on the healthy donor, were set at 100 for NK cells cultured without MSCs to enable comparison amongst different experiments.

After 5 days of co-culture of NK cells with MSCs, the percentage of CD69+ NK cells and the expression of NKG2D were significantly reduced (*p*<0.05) (Figures 4A/B). No significant differences were observed between MSCs derived from sJIA and HC in down-modulation of IL-2 induced NK cell activation.

The cytotoxic capacity of the cytokine-activated NK cells against Daudi target cells was significantly suppressed after co-culture with MSCs. In contrast, ATG-mediated ADCC of NK cells against EBV-BLCL was not significantly impaired, indicating that the intrinsic cytolytic potential of the NK cells was not affected (Figures 4C-F).

MSCs inhibit the differentiation of monocytes to dendritic cells

The monocyte population isolated by positive CD14 selection contained less than 1% CD1a-positive cells. Upon combined GM-CSF and IL-4 exposure, monocytes differentiated towards immature CD1a$^+$/CD14$^-$ DC (Figure 5A). MSCs strongly inhibited this differentiation, as shown by retainment of CD14$^+$ monocytes accompanied by a reduced appearance of CD14$^-$CD1a$^+$ DC, in a dose-dependent manner (Figure 5B/C). A similar pattern in CD14 and CD1a expression was seen after 2 additional days of
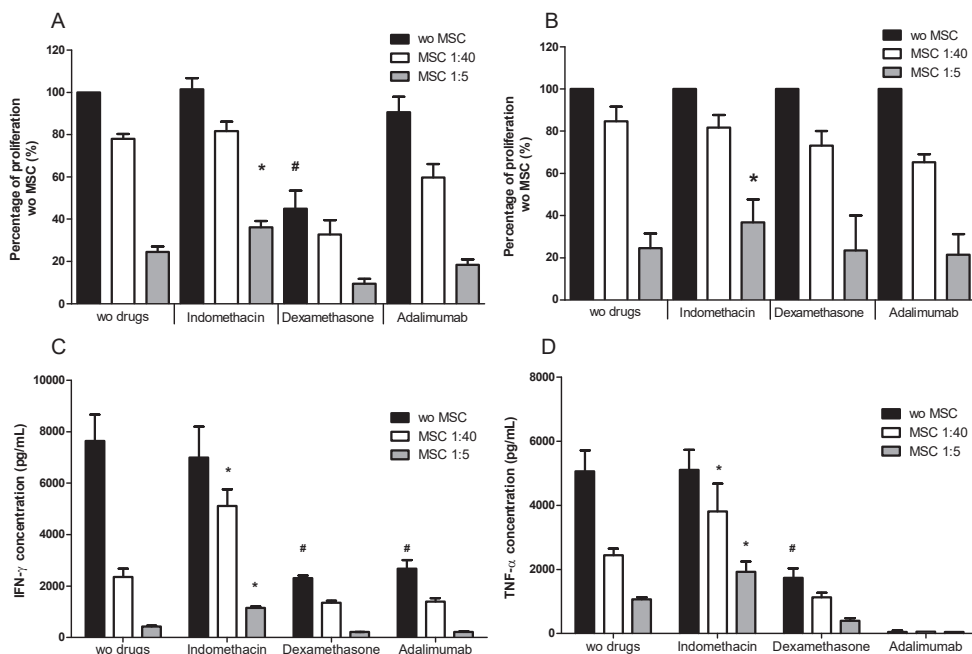
Figure 3. *Indomethacin decreases the immunosuppressive effect of MSCs*. A/B) PHA-induced proliferation of healthy control PBMC in co-cultures with or without MSCs and in the presence or absence of different drugs is shown. A) The proliferation in the culture condition without drugs and without MSCs was set at 100%. B) The proliferation in the culture conditions without MSC but with drug was standardized to 100% for each of the drugs separately. The mean and SEM of 6 different MSC preparations (sJIA = 3; HC = 3) are depicted. C/D) Concentrations (mean +/- SEM) of (C) IFN-γ and (D) TNF-α in the culture supernatants of healthy control PBMC stimulated with PHA and cultured in the absence or presence of MSCs with or without drugs are depicted. One experiment, representative for MSCs derived from 2 HC and 2 patients with sJIA, is shown. *: *p*<0.05 compared with the corresponding MSC:PBMC ratio without drugs; #: *p*<0.05 compared with the condition without MSC and without drugs.
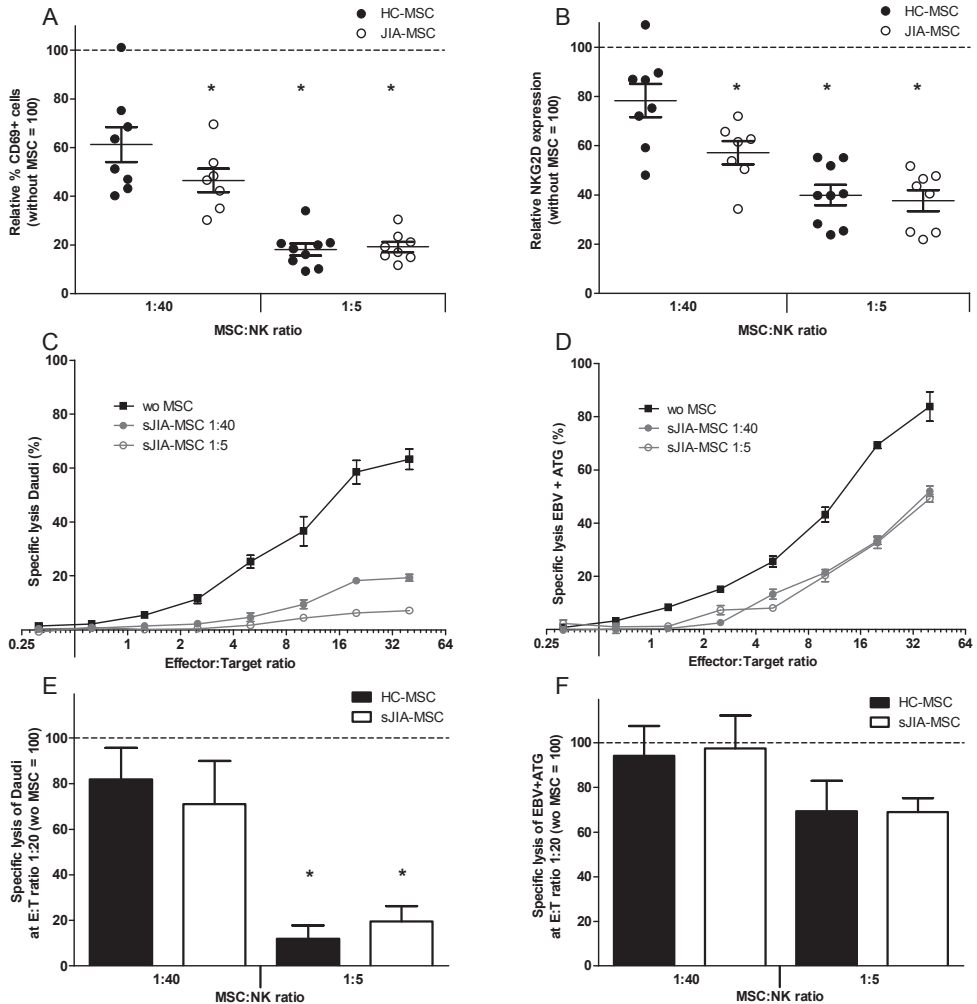
Figure 4. *sJIA derived MSCs suppress NK cell activation and cytotoxicity during co-cultures.* MSCs were co-cultured with NK cells during 5 days in the presence of IL-2 (30 IU/mL). At day 5, activation and cytotoxicity of NK cells were determined using flow cytometry and a chromium release assay, respectively. A/B) The relative percentage of CD69 positivity (A) and the relative MFI of NKG2D expression (B) on NK cells isolated from PBMC of different healthy donors are depicted after co-culture of NK cells of three different donors with MSCs from six patients with sJIA and seven allogeneic healthy controls (HC) at MSC:NK cell ratios of 1:40 and 1:5. Percentage of CD69 positivity and MFI of NKG2D expression of NK cells cultured for 5 days with IL-2 in the absence of MSC was set at 100. The mean +/- SEM is indicated by the horizontal lines. *: $p<0.05$ compared to without MSC. C/D) The killing of (C) Daudi and (D) ATG mediated ADCC of EBV-BLCL by NK cells cultured with or without (wo) MSCs of one sJIA patient is shown. E/F) At an effector:target (E:T) ratio of 1:20, the killing of (E) Daudi and (F) ATG coated EBV-BLCL by NK cells co-cultured with MSCs at the indicated MSC:NK ratios is expressed relative to the killing by NK cells cultured without MSC which was set at 100; The effect of MSCs derived from five patients with sJIA and five HC was analyzed. The bars represent the mean +/- SEM. *: $p<0.05$ compared to without MSCs.

exposure to GM-CSF, IL-4, CD40-ligand and IFN-γ in order to further mature the DC. Monocytes co-cultured with MSC acquired CD163 expression (relative MFI +/- SEM 8.8 +/- 0.3 on day 0) on day 5 (12.7 +/- 1.8 without MSCs and 47.3 +/- 3.5 with HC MSCs or 39.4 +/- 1.6 with sJIA MSC) (data not shown). On day 7, the expression of CD80 and CD86 on the total population was significantly ($p<0.01$ for CD80 and $p<0.05$ for CD86) lower in co-cultures with MSCs (Figure 5D). Comparable results were obtained for either CD1a$^+$ or CD14$^+$ cells (data not shown). MSCs of sJIA patients and HC did not differ in their effect on DC differentiation.
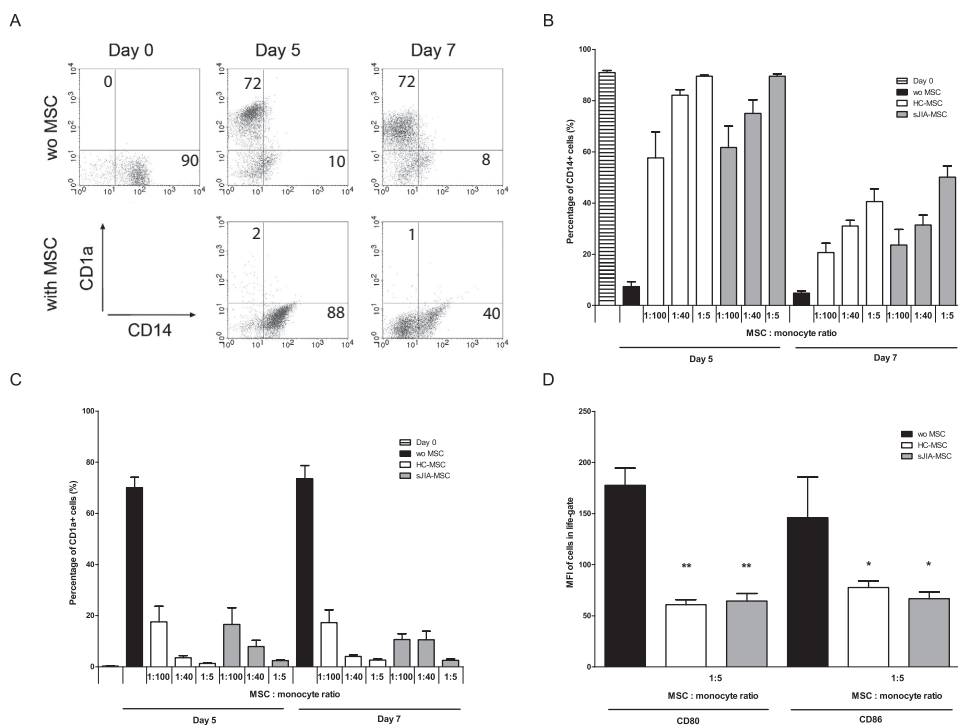
Figure 5. *MSCs of patients with sJIA interfere with differentiation of monocytes to dendritic cells.* Monocytes in co-culture with MSC in the presence of GM-CSF, IL-4, CD40 ligand and IFN-γ show impaired maturation to dendritic cells (DC). Cells were gated on forward scatter/side scatter life-gate. A) Representative FACS plots of combined CD14 and CD1a staining on day 0, day 5 and day 7 are presented. The numbers in plots represent the percentage of gated cells in the different quadrants reflecting CD1a$^+$CD14$^-$ DC and CD1a$^-$CD14$^+$ monocytes, respectively. Monocytes were cultured in the absence or in the presence of MSCs at a MSC:monocyte ratio 1:5. B/C) Mean and SEM of the percentage (B) CD14$^+$ monocytes and (C) CD1a$^+$ DC are depicted. Results were obtained using isolated monocytes from four healthy adult donors co-cultured with allogeneic MSCs generated from BM of three healthy controls (HC) and four patients with sJIA. D) The CD80 and CD86 expression on the total population is significantly decreased in co-cultures with sJIA and HC MSC at day 7. *: $p <0.05$ compared to without (wo) MSC. **: $p <0.01$ compared to without MSC.

Discussion

In this study, the biological and functional characteristics of bone marrow derived MSCs from patients with sJIA were compared with those from paediatric HC. Determination of the immunomodulatory capacity of sJIA-derived MSCs is an essential step before considering the administration of autologous MSCs in patients with sJIA. Previously, aberrant immunomodulation was reported for BM-derived MSCs from severe aplastic anaemia patients.(Bacigalupo, 2005) In addition, synovial fluid-derived MSCs from patients with rheumatoid arthritis had a decreased differentiation capacity compared to MSCs from synovial fluid of patients with osteoarthritis.(Jones, 2010)

The use of autologous MSCs in the treatment of sJIA is preferred to allogeneic MSCs for three reasons. Firstly, knowledge on the administration of allogeneic MSCs in children is limited to immunocompromised patients, such as hematopoietic stem cell transplantation recipients with steroid-resistant aGvHD.(Le Blanc, 2008) Secondly, mice models indicate the occurrence of immune rejection and specific immunological memory induction after administration of allogeneic MSCs in an immunocompetent setting which would disallow a repetitive infusion of MSCs.(Nauta, 2006; Zangi, 2009) Third, more recent data in patients treated for renal graft rejection demonstrate that primed T cells are able to lyse allogeneic MSCs.(Crop, 2011)

Systemic JIA is considered an autoinflammatory disease caused by an inadequate suppression of innate immunity.(Vastert, 2009) Therefore, we evaluated the *in vitro* immunomodulatory capacities of MSCs not only by focusing on T cell function in a model of PHA-stimulated PBMC,(Samuelsson, 2009; Bacigalupo, 2005; Aggarwal, 2005) but also by investigating the effect on NK cells(Spaggiari, 2006) and monocytes. In both adaptive and innate experimental systems, the immunomodulatory effects of MSC of patients and healthy controls were indistinguishable, in accordance with previous reports on patients with autoinflammatory diseases.(Bocelli-Tyndall, 2007; Bernardo, 2009; Larghero, 2008) However, in these studies, only the effect of MSC on lymphocyte proliferation was investigated. We have shown a decreased cytolytic function of NK cells after co-cultivation with MSCs of patients with sJIA and HC. In addition, MSCs of patients and HC inhibited the differentiation of monocytes towards DC, as illustrated by the generation of a low percentage of CD1a-positive cells and decreased levels of CD80 and CD86 expression. In contrast, monocytes cultured with MSCs acquired expression of CD163, a scavenger receptor associated with the suppressive type 2 macrophage.(Sica, 2006)

To enable inclusion of a substantial number of patients, not only fresh BM but also long-term frozen BM of patients with sJIA was applied to culture MSCs. The expansion of MSC was successful for six of eight frozen BM samples. Previously, the use of 1-week cryopreserved BM for the expansion of MSCs was compared to

fresh BM.(Haack-Sorensen, 2007; Casado-Diaz, 2008) In these studies, no differ-
ences were observed in expansion or differentiation capacity. However, we found
an aberrant osteoblast differentiation in the MSCs started from frozen BM of two
sJIA patients. The absence in osteoblast differentiation could be explained by the
relatively low numbers of BMMC at the initiation of MSC cultures compared with these
studies, in which they used 20 or 100x10^6 BMMC, respectively.(Casado-Diaz, 2008;
Haack-Sorensen, 2007) Nevertheless, a possible influence of the disease itself or the
long-term use of drugs can not formally be excluded based on our study results. In
our study, no differences in immunomodulatory capacity were found between MSCs
derived from cryopreserved and fresh BM. This is in contrast to results reported by
Samuelson *et al.*(Samuelsson, 2009) describing a variable effect of cryopreservation.

Despite unsuccessful expansion of MSCs from frozen BM in two patients, the ex-
pansion of MSCs from frozen material is a promising tool for further research on rare
paediatric diseases.

An important issue in administration of MSCs to patients is the continuation of
disease-specific drugs. Previously, calcineurin inhibitors and indomethacin were
shown to suppress the immunomodulatory effect of MSCs, whereas mycophenolic
acid has a synergistic effect with MSCs.(Aggarwal, 2005; Buron, 2009) Consistent
with previous data(Rasmusson, 2005) our findings indicate that indomethacin sup-
presses the immunomodulatory function of MSCs. All of our patients were treated
with non-steroidal anti-inflammatory drugs (NSAID) before aspiration of BM. Appar-
ently, *in vivo* exposure of MSCs to NSAID does not interfere with the suppressive
effect of MSCs after *ex vivo* expansion. Dexamethasone, on the other hand, although
having a suppressive effect on PBMC proliferation by itself, did not affect the sup-
pressive effect of MSCs, consistent with a previous report.(Buron, 2009) Adalimumab,
a TNF-α antagonist, did not affect PBMC proliferation, but significantly decreased
PHA-induced production of IFN-γ. Interestingly, despite the low IFN-γ production in
cultures containing adalimumab, MSCs retain their capacity to reduce proliferation
and IFN-γ secretion in this condition.

It is relevant to evaluate whether patient-derived cells, independent of the course
and actual activity of the disease and past or present medication, are susceptible to
suppression by MSC. Therefore, we determined the effect of patient derived MSCs
on PHA-driven T cell proliferation using autologous PBMC. Patient-derived PBMC
were equally suppressed by MSCs compared to healthy control derived PBMC. Our
data further show a comparable suppression of T cell responses by allogeneic and
autologous MSCs, implying a comparable susceptibility of patient and control PBMC
to MSCs.

Our *in vitro* study did not address the route of administration of these expanded
MSCs, and the optimal use remains to be determined. Most reported clinical studies

of MSC treatment have used intravenous infusions to control systemic inflammatory diseases, and this would also seem to be a logical choice for the future use in children with sJIA. In contrast, however, oligo-articular disease or target joints might benefit from local intra-articular administration. Future prospective clinical trials should address these issues.

In conclusion, this is the first study describing that MSCs derived from a cohort of patients with sJIA have similar immunosuppressive capacities compared to age-matched HC in assays focused on adaptive (T cell) and innate (NK cell and monocyte) immunity. Therefore, the results of this study support the use of autologous MSCs in clinical trials of patients with sJIA.

Chapter 3.

Despite differential gene expression profiles pediatric MDS derived mesenchymal stromal cells display normal functionality *in vitro.*

Calkoen FGJ, Vervat C, van Pel M, de Haas V, Vijfhuizen L, Eising E, Kroes WGM, 't Hoen PAC, van den Heuvel-Eibrink MM, Egeler RM, van Tol MJD, Ball LM.

Abstract

Pediatric myelodysplastic syndrome (MDS) is a heterogeneous disease covering a spectrum ranging from aplasia (RCC) to myeloproliferation (RAEB(t)). In adult-type MDS there is increasing evidence for abnormal function of the bone-marrow micro-environment. Here, we extensively studied the mesenchymal stromal cells (MSC) derived from children with MDS.

MSC were expanded from bone-marrow of 17 MDS patients (RCC: n=10 and advanced MDS): n=7) and pediatric controls (n=10). No differences were observed with respect to phenotype, differentiation capacity, immunomodulatory capacity or hematopoietic support. mRNA expression analysis by Deep-SAGE revealed increased *IL-6* expression in RCC- and RAEB(t)-MDS. RCC-MDS MSCs expressed increased levels of *DKK3*, a protein associated with decreased apoptosis. RAEB(t)-MDS revealed increased *CRLF1* and decreased *DAPK1* expression. This pattern has been associated with transformation in hematopoietic malignancies. Genes reported to be differentially expressed in adult MDS-MSCs did not differ between MSCs of pediatric MDS and controls.

An altered mRNA expression profile, associated with cell survival and malignant transformation, of MSCs derived from children with MDS strengthen the hypothesis that the micro-environment is of importance in this disease. Our data support the understanding that pediatric and adult MDS are two different diseases. Further evaluation of the pathways involved might reveal additional therapy targets.

Introduction

Pediatric myelodysplastic syndrome (MDS) represents a range of disorders characterized by dysplastic morphology comprising in total less than 5% of pediatric hematological malignancies.(Hasle, 2004) The spectrum of MDS ranges from refractory cytopenia of childhood (RCC) to advanced MDS with excess of blasts (RAEB) with increasing risk of leukemic transformation.(Hasle, 2003) Survival has increased from 30 to 60% since hematopoietic stem cell transplantation (HSCT) is applied.(Strahm, 2011; Sasaki, 2001; Woods, 2002) The pathophysiology of MDS is not fully elucidated. However, genetic predisposition, acquired cytogenetic abnormalities and abnormal immune responses have been linked to MDS.(Strahm, 2011; Hasle, 2011) These aspects do not explain the entire range of disease in pediatric or adult MDS. Recently, it has been suggested in adult MDS that impaired interaction between hematopoietic precursor cells and their bone-marrow microenvironment might contribute to the disease.(Zhang, 2012b) In children, no conclusive data is yet available.

Mesenchymal stromal cells (MSC) have been identified as supporting cells of hematopoietic stem cells (HSC) *in vivo* and *in vitro* (Morikawa, 2009; Mendez-Ferrer, 2010; Sugiyama, 2006b) and linked to disease, as aberrant MSC function was shown to contribute to the pathophysiology of malignant disorders in murine models.(Raaijmakers, 2010; Schepers, 2013) Characteristics of MSCs from adult MDS patients have been extensively studied focusing on cytogenetic and molecular abnormalities(Blau, 2011; Lopez-Villar, 2009; Flores-Figueroa, 2008) as well as gene and protein expression.(Marcondes, 2008; Flores-Figueroa, 2008; Santamaria, 2012) In addition, abnormal immunomodulation(Wang, 2013b; Zhao, 2012b; Marcondes, 2008) as well as decreased hematopoietic support(Zhao, 2012b; Ferrer, 2013) by MSCs has been reported in MDS. However, these data remain conflicting with other studies reporting no abnormalities in stromal function.(Flores-Figueroa, 2008; Klaus, 2010; Alvi, 2001) Differences in results may be explained by a variety in MSC expansion protocols and experimental set-up, but also by the heterogeneity of the disease.(Aizawa, 1999) Studies reporting on (cyto)genetics and function of MDS-MSCs have been summarized in the Supplementary Tables S1 and S2.

Pediatric MDS is a very rare disease and publications on the role of stroma in the ontogeny and maintenance of pediatric MDS are limited to a case report on aberrant hematopoietic support by MSCs derived from an MDS patient with trisomy 8,(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.

In the present study we compared MSCs derived from children with RCC and RAEB(t) / MDS-AML to MSCs expanded from age-matched healthy controls. Biological characteristics, *e.g.* differentiation capacity and phenotype were analyzed. MSC function *in vitro* was evaluated by immunomodulatory and hematopoietic assays. In addition, genome wide gene-expression profiles were studied using Deep-SAGE sequencing.

Materials and Methods

Patients and MSC expansion

Children referred to our center for HSCT were included in this study according to a protocol (P08.001) approved by the institutional review boards on medical ethics. Next to bone-marrow of 10 healthy controls (HC, median age 7.4, range 1.1 – 16.4 years) being HSCT donors, bone-marrow of 17 MDS patients (10 RCC, 2 RAEB, 4 RAEBt, 1 MDR-AML was collected at diagnosis and prior to treatment initiation. The WHO classification adapted for children was used for the classification of patients. (Hasle, 2003) MSCs from children with RAEB, RAEBt and MDR-AML were grouped as advanced MDS to enable the comparison between advanced and RCC-MDS. In addition, bone-marrow after HSCT was collected from 9 children (4 RCC, 1 RAEB, 1 RAEBt, 3 MDR-AML) including 6 paired samples (Table 1).

MSCs were expanded and characterized as previously described.(Calkoen, 2013a) Briefly, bone-marrow mononuclear cells (MNC) obtained after Ficoll separation were cultured in DMEM (Invitrogen, Paisley, UK) containing 100 U/mL penicillin/100 μg/mL streptomycin (P/S; Invitrogen) and 10% (v/v) fetal bovine serum (FBS; VWR International, Bridgeport, NJ, USA). Non-adherent cells were removed by refreshing medium twice weekly. Upon reaching confluency MSCs were harvested, pooled and passaged for further expansion resulting in non-clonal MSCs. Phenotype (CD73, CD90, CD105 positive; CD3, CD31, CD34, CD45, CD86, HLA-DR negative) and differentiation capacity towards osteoblasts and adipocytes were investigated at passage 2-3 and 5-7, respectively. All but anti-CD105 (Ancell Corporation Bayport, MN) antibodies were derived from Becton Dickinson Biosciences (BD), San Diego, CA, USA. Culture supernatant was collected after reaching 80% confluency at passage 3-5 for measurement of cytokine production.

Cytogenetics

To exclude common chromosome abnormalities in MSCs and malignant cells, interphase fluorescence in situ hybridization (FISH) for chromosome 7 and 8 was performed on MSCs from patients with known monosomy 7 or trisomy 8 using the

The microenvironment in pediatric myelodysplastic syndrome

Table 1. Characteristics of patients included in the study

UPN	Sex	Age (yrs) at HSCT	Diagnosis	Donor type	Donor Source	Conditioning	Remark MSC	Cytogenetics in hematopoietic cells	Pre-HSCT MSC	Post-HSCT MSC
MSC-MDS001	M	8.9	RAEBt	MUD	BM	Bu, Cy, Mel, rATG		-	Yes	Yes + 7 months
MSC-MDS002	F	13.3	RAEB	MUD	PBSC	Bu, Cy, Mel, rATG		Trisomy 1, monosomy 7, trisomy 8	Yes	Yes + 4 months
MSC-MDS003	F	5.4	RCC	IRD	BM	Bu, Cy, Mel	Only post	Monosomy 7		Yes + 2 months
MSC-MDS005	M	6.1	RCC	ORD	PBSC/MSC	1st: Flu, Thio, rATG, 2nd: Treo, Flu, Campath		-	Yes	Yes + 2 months
MSC-MDS008	F	14.2	MDR-AML	IRD	BM	Bu, Cy, Mel	Only post	-		Yes + 2 months
MSC-MDS009	F	15.8	MDR-AML	ORD	PBSC/MSC	Thio, Treo Flu, rATG	Only post			Yes + 7 months
MSC-MDS011	F	10.2	RCC	MUD	BM	Flu Thio Campath		-	Yes	Yes + 3 months
MSC-MDS015	F	9.3	RCC	MUD	BM	Bu, Flu, rATG		Monosomy 7	Yes	Yes + 2 months
MSC-MDS017	M	14.0	RCC	MUD	BM	Bu, Cy, Mel, rATG		-	Yes	
MSC-MDS018	M	14.6	RAEBt	MUD	BM	Bu, Cy, Mel, rATG		-	Yes	
MSC-MDS019	F	4.8	RCC	MUD	BM	Bu, Cy, Mel, Campath		Monosomy 7	Yes	
MSC-MDS020	M	13.3	RAEB	MUD	BM	Bu, Cy, Mel, Campath		Monosomy 7	Yes	
MSC-MDS021	M	13.3	RAEBt	IRD	BM	Bu, Cy, Mel, rATG		Trisomy 8	Yes	
MSC-MDS022	F	4.1	RCC	MUD	CB	Bu, Cy, Mel, rATG		Monosomy 7	Yes	
MSC-MDS023	F	1.2	RAEBt	MUD	BM	Bu, Mel, ARA-C, rATG		Monosomy 7	Yes	
MSC-MDS024	M	4.1	MDR-AML	IRD	BM	1st: Flu Thio Campath, 2nd: Flu Thio treo rATG, 3rd none		-	Yes	Yes + 3 months
MSC-MDS026	M	17.8	RCC	MUD	BM	Flu, Thio, Campath		-	Yes	
MSC-MDS027	M		RCC	n.a.			No HSCT	-	Yes	
MSC-MDS028	F		RCC	n.a.			No HSCT	-	Yes	
MSC-MDS029	F	17.6	RCC	MUD	BM	Flu, Thio, Campath		-	Yes	

RCC: refractory cytopenia of childhood; RAEB: refractory anemia with excess of blasts; RAEBt: RAEB in transformation; MDR-AML: myelodysplasia related acute myeloid leukemia; HSCT: hematopoietic stem cell transplantation; MUD: matched unrelated donor; IRD: identical related donor; ORD: other related donor; BM: bone marrow; PBSC: peripheral blood stem cells; MSC: mesenchymal stromal cells; CB: cord blood; Bu: busulphan; Cy: cyclofosfamide; Mel: melphalan; rATG: rabbit anti-thymocyte globulin; Flu: fludarabine; Thio: thiotepa; Treo: treosulphan; ARA-C: cytosine arabinoside; n.a.: not applicable.

following probes: Vysis LSI D7S486/CEP7 and LSI IGH/LSI MYC, CEP8, (Abbott Laboratories, Abbott Park, IL,USA).(Bronkhorst, 2011)

Chimerism analysis

Chimerism (donor or recipient origin) was studied by cytosine adenine (CA)-repeat analysis in MSCs cultured from bone-marrow harvested after HSCT as previously described.(Lankester, 2010)

Immunomodulatory assays

The effect of MSCs (30 Gy irradiated) on proliferation of peripheral blood (PB) MNC obtained from adult bloodbank donors (100 000 cells/well) after stimulation with phytohemagglutinin (PHA 2 µg/mL) was analyzed at MSC : PBMC ratios of 1:5 and 1:40. MSC and PB-MNC were co-cultured in RPMI P/S, 10% (v/v) fetal calf serum (FCS) for 5 days with the addition of ^{3}H-thymidine (1 µCi/well; Perkin Elmer, Wellesley, MA, USA) for the last 16 hours to measure proliferation using a β-counter (Perkin Elmer). Experiments were performed in triplicate.

To evaluate the effect of MSCs on antigen presenting cells, monocytes were isolated from PB using positive CD14 selection (Miltenyi, Bergisch Gladbach, Germany) and cultured with IL-4 (40 ng/mL) and GM-CSF (800 IU/mL) (both from Tebu-Bio, Le Perray en Yvelines, France) for 5 days to differentiate towards immature dendritic cells (DC). Cells were harvested or cultured for 2 additional days with IL-4, GM-CSF, IFN-γ (500 U/mL, Boehringer, Mannheim, Germany) and CD40-ligand (0.25 µg/mL Beckman-Coulter, Marseille, France) to generate mature DC. Cells were phenotyped by flow cytometry for the expression of CD14 and CD1a (BD) on day 0, day 5 and day 7 after co-culturing of monocytes and MSCs at MSC : monocyte ratios 1:5, 1:40 or 1:100 or after culturing monocytes without MSCs.

Hematopoietic support

Short-term co-culture assays with MSCs and hematopoietic progenitor cells (HPC) were performed to determine the supportive capacity of MSCs for HPC maintenance and differentiation. Therefore, HPC were isolated from remaining material of G-CSF mobilized stem cell grafts from healthy transplant donors using CD34 positive selection (Miltenyi). Selected cells expressed >90% CD34 after purification. Short-term cultures of 500 CD34 selected cells/well without or with MSCs (CD34 : MSC ratios 1:2 and 1:20) were performed in Stemspan medium (H3000, StemCell Technologies, Vancouver, Canada) with addition of 1% P/S, stem cell factor (SCF, 100 ng/mL, StemCell Technologies) and Flt3-ligand (Flt3-L, 100 ng/mL, StemCell Technologies), because SCF and Flt3-L are not produced by MSCs. Cultures were initiated with 10×10^3 CD34$^+$ cells at a CD34 : MSC ratio of 1:5 for flow cytometry analysis. Half of
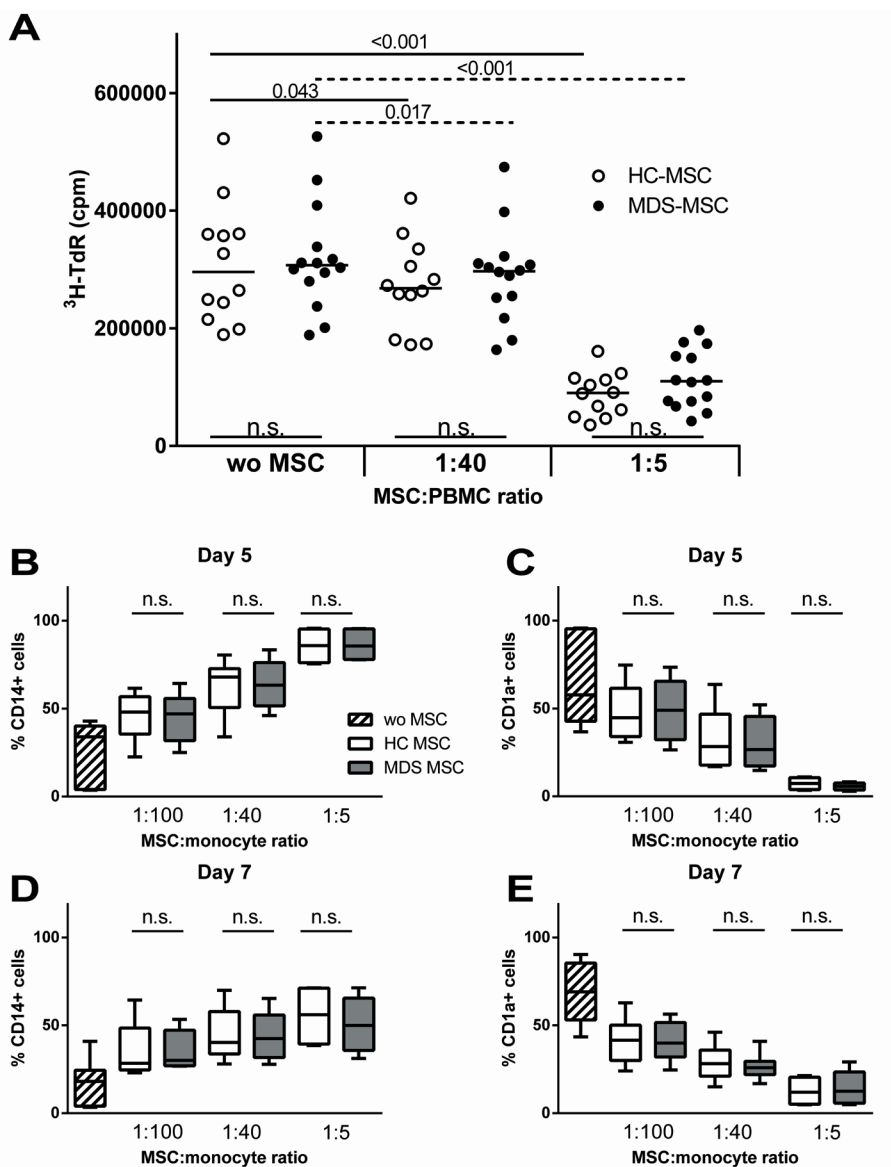
Figure 1. *Immunomodulation by MDS-MSCs*. A. Both healthy control (HC, n=6) and MDS-MSCs (n=5 of which RAEB/RAEBt n=3 and RCC n=2) significantly suppressed PHA-induced PBMC (n=4 healthy adults) proliferation at MSC : PBMC ratios 1:40 and 1:5. No significant differences were observed between MDS patients (black circles) and controls (white circles). wo MSCs: without MSCs. B-E. MSCs suppressed the differentiation of monocytes (CD14$^+$ cells) towards dendritic cells (CD1a$^+$ cells) in a dose-dependent manner (hatched boxes: no MSCs added). No significant differences were observed between HC (white boxes) and MDS-MSCs (black boxes). Boxes represent median and 25-75 percentiles and the whiskers the minimum and maximum values. *P*-values were calculated using Wilcoxon matched-pairs signed rank tests (in A for comparison of different MSC ratios) and Mann-Whitney tests (in A-E for comparison between HC and MDS). n.s.: not significant.

the culture medium was refreshed with the addition of growth factors on day 4, 7 and 11. Proliferation (day 7) and differentiation (day 7 and 14) was assessed using ^{3}H-thymidine during the last 16 hours or flow cytometry, respectively. Antibodies used for flow cytometry were anti-CD34-PE, anti-CD45-FITC, anti-CD38-Percpc5.5, anti-CD45-Percpc5.5, anti-CD14-FITC, anti-CD33-APC, anti-GPA-PE (glycophorin A) and anti-CD13-PE (all antibodies from BD).

In long-term cultures, CD34 selected cells (50 000 cells) were cultured on a conflu-ent MSC layer for 3-5 weeks in the absence of growth factors. Cells were harvested and counted using trypan blue as viability stain.

To determine the functional impact of HPC expansion and differentiation after short-term culture, non-adherent cells were harvested after 7 days of culturing CD34$^+$ cells in the absence or presence of MSCs (CD34 : MSC ratio 1:5), transferred (1000 cells/dish) to methylcellulose containing essential growth factors, *i.e.* SCF, GM-CSF, IL-3, and erythropoietin (EPO) (H4434 StemCell Technologies), and cultured for 14 days (colony-forming unit assay; CFU-assay). Colonies were scored by two independent observers according to standard guidelines for the definition of CFU-GEMM, BFU-e, CFU-GM, CFU-G and CFU-M. Results are depicted as the average of duplicate wells. To determine a more direct effect of MSCs on HPC in CFU-assays, MSCs (30 000 or 150 000 per dish) were added to freshly purified HPC (500 cells/dish) in methylcellulose containing SCF, GM-CSF, IL-3 and EPO (H4434 StemCell Technologies). The direct effect of MSCs on colony formation was also assessed in methylcellulose containing EPO (H4330 StemCell Technologies) only. Cells were harvested and phenotyped after scoring of colonies in the CFU-assay.

Cytokine expression

IL-6 quantification in MSC culture supernatants was performed by ELISA (Sanquin, Amsterdam, the Netherlands) according to the manufacturer's instructions.

Gene expression

Total RNA was isolated at passage 2-3 using a Qiagen RNeasy Minikit (Qiagen, Hilden, Germany). mRNA was profiled using Deep-SAGE sequencing using Illumina technology.(Mastrokolias, 2012) CATG was added to the 5' end of the 17 base pair sequences obtained. Data were mapped against the UCSC hg19 reference genome using Bowtie for Illumina (version 1.1.2) without the permission of one mismatch and suppression of reads if more than one best match existed. Tags aligned to the same gene were summed for further analysis. Gene information was added to the sequences with the biomaRt package in R (version 2.16.0). The expression data will be published online in the Gene Expression Omnibus (GEO).
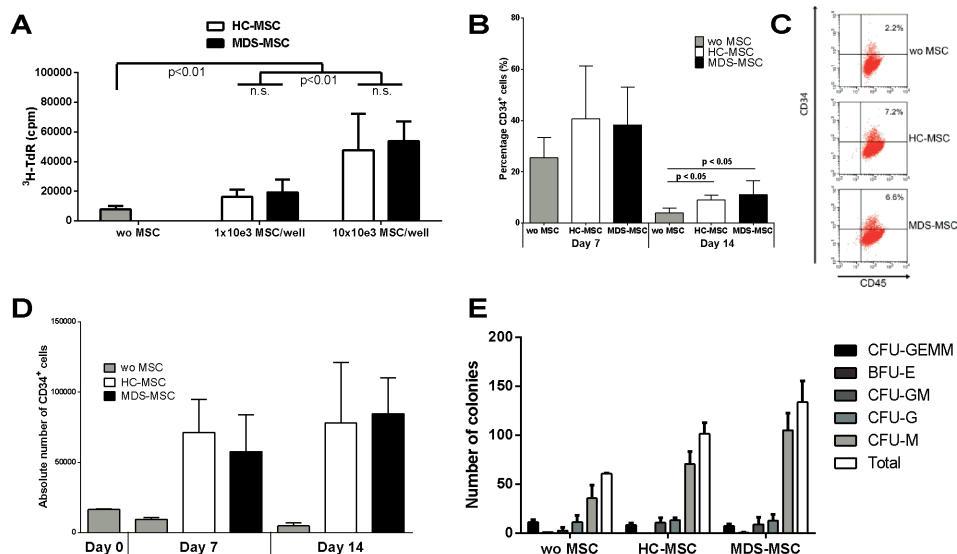
Figure 2. *MDS-MSCs support maintenance and differentiation of hematopoietic progenitor cells (HPC).*
A. CD34$^+$ cells (500 cells) of HSCT donors were cultured in the absence (wo MSCs) or presence of MSC (CD34 : MSC ratios 1:2 and 1:20) obtained from MDS patients or healthy controls (HC) in the presence of SCF and Flt3-L. Proliferation of HPC at day 7 was assessed by ^{3}H-thymidine incorporation. B-C. CD34$^+$ expression declined overtime, but MSCs supported the expansion of HPC (CD34$^+$ cells) and a higher percentage of CD34$^+$ cells was retained in comparison with cultures in the absence of MSCs. Results shown are from cultures at a CD34$^+$: MSC ratio of 1:5 starting with 10x10^3 HPC. Flow cytometry data presented in C are obtained after 14 days of culture and show plots of CD34 versus CD45 expression and the percentage of CD34$^+$ cells within the CD45$^+$ cell population. D. Whereas the absolute number of HPC decreased significantly in the absence of MSCs, the absolute number of HPC increased in co-cultures with MSCs (CD34$^+$: MSC ratio 1:5). Data depicted in A, B and D represent at least 2 independent experiments of 5 HC-MSCs and 8 MDS-MSCs (grey bars: without (wo) MSCs, white bars: healthy control (HC)-MSCs, black bars: MDS-MSCs). E. Non-adherent cells harvested at day 7 were transferred to methylcellulose to test their capability of colony formation. Graphs represent the total number of CFU and indicated CFU types in the CFU-assay resulting from the investigation of 4 MDS-MSCs and 3 HC-MSCs present during the initial HPC and MSCs co-culture. wo MSCs: without MSCs. Bars depict the mean with standard deviation. *P*-values were calculated using Mann-Whitney tests. n.s.: not significant.

Expression of genes of interest was validated using independent biological samples by RT-PCR after generation of cDNA (cDNA synthesis kit, Roche, Basel, Switzerland) using the listed primers (Table S3), as previously described.(Mastrokolias, 2012) Expression levels were calculated relative to expression of the housekeeping genes *GAPDH* and *HPRT1*.

Statistical analysis

Graphpad 6 (Prism, La Jolla, CA) was used for data-analysis. Mann-Whitney and Wilcoxon matched-pairs signed rank tests were performed to compare different groups. Differential gene expression analysis was performed in R (version 2.15.0), using the EdgeR (version 3.2.4) and Limma (version 3.16.7) data analysis packages.(Robinson, 2010; R Development Core Team, 2012; Smyth, 2005) Correction for multiple testing was performed according to Benjamini and Hochberg.(Hochberg, 1990) Adjusted *p*-values <0.05 were considered statistically significant.

Results

Expansion and characterization of MSCs

MSCs were successfully cultured from bone-marrow of all patients and controls. Expanded MSCs expressed CD73, CD90 and CD105, whereas the cells did not express lineage markers (Supplementary Figure S1A). MSCs differentiated towards adipocytes and osteoblasts when cultured in culture media supporting these distinct directions of differentiation (Figure S1B-E). In one *de novo* RCC patient (UPN: MDS026) no osteoblast differentiation was established. Post-HSCT derived MSCs were of complete patient origin in all children that were analyzed (n=6). Monosomy 7 was not detected in MSCs from any bone marrow harvested prior to HSCT from children with monosomy 7 (n=6). The MSC lines generated from the two patients with trisomy 8 in the hematopoietic cell compartment tested negatively for trisomy 8.

Immunomodulation

MSCs down modulate functions of various cell types involved in innate and adaptive immunity.(Le, 2012) The effect of MSCs of pediatric MDS patients and healthy children on T cell proliferation and monocyte differentiation was investigated. MSCs suppressed the PHA-induced PB MNC proliferation in a dose-dependent manner. No differences in suppressive capacity were observed between MDS-MSC and healthy control (HC-)MSC (Figure 1A).

To investigate the suppressive effect of MSCs on DC maturation, MSC-monocyte co-cultures were performed. The purified monocyte fraction was > 95% CD14^{+} and <1% CD1a^{+} at the start of MSC-monocyte co-culture. After 5 days of culture with GM-CSF and IL-4, the monocytes lost CD14 expression and gained CD1a, characteristic for DC. This process further progressed from day 5 to 7 during maturation of DC in the presence of GM-CSF, IL-4, IFN-γ and CD40-ligand. MSCs of controls and MDS patients showed inhibition of the differentiation at day 5 and 7 at various MSC : monocyte ratios (Figure 1B-E). No differences between both groups were observed.
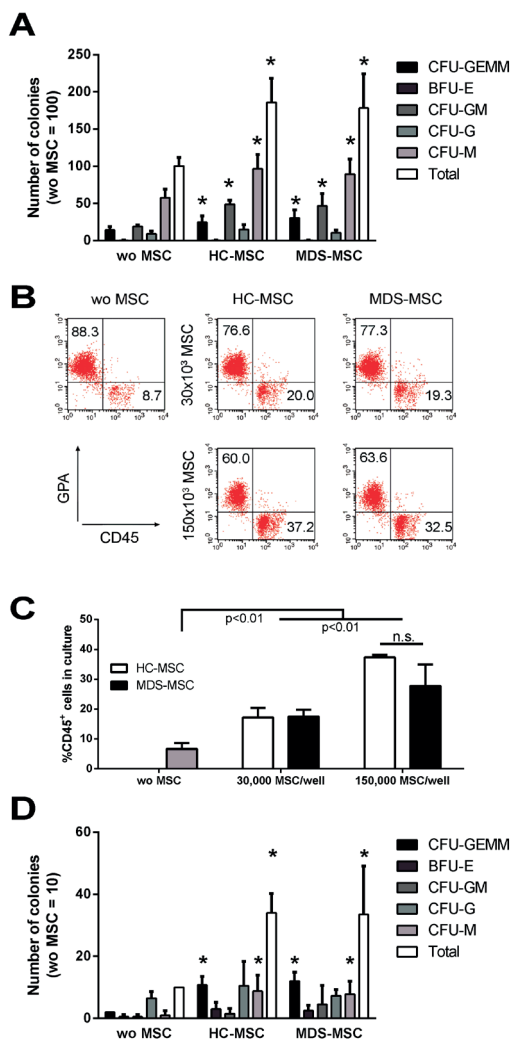
Figure 3. *MSCs of MDS patients support colony formation in CFU-assays.* A-C. MSCs (30 000 cells, or 150 000 cells when indicated) of healthy controls (HC-MSCs), of MDS patients (MDS-MSCs) or no MSC (wo MSCs) were added to freshly isolated HPC (CD34^{+} cells, 500 cells) of HSCT donors at initiation of colony forming unit assays (CFU-assay). Cultures contain exogenous SCF, GM-CSF, IL-3 and EPO. Addition of MSCs support the increase the number of colonies (MDS n=6, HC n=4). To compare data from different experiments, the number of colonies in cultures without MSCs (range: 83-105) was set at 100. Colonies were scored and harvested for phenotyping at day 14 after culture initiation. The percentage (B) and number (C) of CD45^{+}GPA^{−} cells is increased in CFU-assays in the presence of MSCs, irrespectively whether MSCs were derived from MDS patients or healthy controls (HC). D. MSCs from MDS patients (n=3) and healthy controls (HC; n=3), co-cultured with HPC in the presence of EPO only, significantly increase the formation of CFU-GEMM and CFU-M, but not of the other colony types (read-out CFU-assay at day 14). wo MSCs: without MSCs. Bars represent mean and standard deviation. *P*-values were calculated using Mann-Whitney tests. *: statistically different ($p < 0.05$) from culture without (wo) MSCs. n.s.: not significant.

RAEB(t)-MSC and RCC-MSC showed comparable suppressive effects in both assays (data not shown).

Expansion of hematopoietic progenitor cells

As part of the stromal bone-marrow compartment, MSCs play an important role in the regulation of hematopoiesis through interaction with HPC.(Mendez-Ferrer, 2010) The effect of MSCs of pediatric MDS patients and healthy children on expansion and differentiation of CD34$^+$ HPC was investigated in various *in vitro* culture systems.

HPC (CD34$^+$ cells) proliferation was enhanced in the presence of MSCs in a dose-dependent manner after stimulation with SCF and Flt3-L for 7 days (Figure 2A). The percentage of cells expressing CD34 declined over time, but the level of this decline is significantly less in cultures containing MSCs (Figure 2B-C). In co-cultures of HPC with MSCs, CD38 expression, associated with activation but also with loss of stemcellness,(Calloni, 2013; Chillemi, 2013) was significantly increased at day 14 on both CD34$^+$ and CD34$^-$ cells (CD34$^+$ cells, mean and SD: without MSCs: 18.8 +/- 1.5%; plus HC-MSCs: 46.8 +/- 8.9%; plus MDS-MSCs: 52.0 +/- 5.0%. CD34$^-$ cells: without MSCs 25.9 +/- 2.9%; plus HC-MSCs: 54.9 +/- 9.4%; plus MDS-MSCs: 61.5 +/- 5.3%). In cultures with MDS-MSCs, differentiation towards myeloid-lineage cells was not enhanced in comparison with cultures with HC-MSCs or without MSCs (CD14$^+$ cells as a percentage of CD45$^+$ cells, mean and SD: without MSC: 16.2 +/- 2.0%; plus HC-MSCs 16.0 +/- 2.5%; plus MDS-MSCs: 19.0 +/- 2.2%).

The absolute number of CD34$^+$ cells decreased significantly when HPC were cultured in the absence of MSCs. In contrast, in the presence of MSCs, the HPC (CD34$^+$) population expanded from day 0 to day 7. From day 7 to day 14 the CD34$^+$ numbers remained unchanged compared to day 7 (Figure 2D).

Non-adherent cells (1000 cells), harvested from HPC cultures with or without MSCs at day 7, were transferred to methylcellulose for CFU-C analysis. Cells that have previously been co-cultured with MSCs gave rise to higher numbers of CFU-C, compared to HSC that have been cultured in the absence of MSCs (Figure 2E). This is in accordance with the increased CD34$^+$ numbers that were observed (Figure 2D). No differences were found in the types of colonies that were formed and this was independent of the presence or absence of MSCs. Overall, the impact of MSCs in these various assays of HPC function was similar for MDS patients and healthy controls, as well as for RAEB(t) versus RCC patients (data not shown).

Maintenance of hematopoietic progenitor cells

To exclude the influence of exogenous growth factors on HPC expansion and the capacity to mount colonies, 50x10^3 CD34$^+$ cells were seeded on confluent MSC layers and cultured for 3-5 weeks without the addition of growth factors. When cultured in
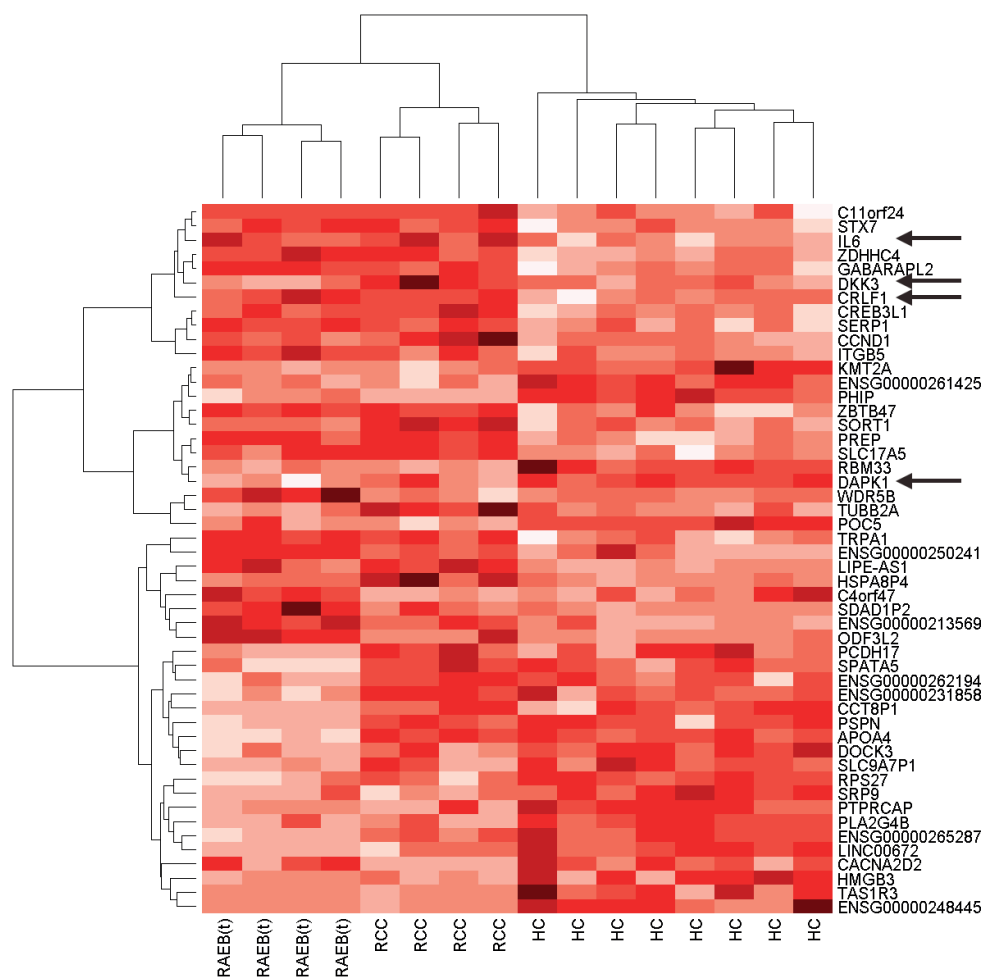
Figure 4. *Heat map depicting clustering of MDS derived MSCs.* Gene expression was analyzed using LIMMA software. MSCs of different groups (RAEB(t)-MDS, RCC-MDS and healthy controls (HC)) showed hierarchical clustering in a heat map of differentially expressed genes. Color intensity of the squares correlates with increased gene expression.

the absence of MSCs, all CD34$^+$ cells died. In contrast, the CD34$^+$ cells were maintained when cultured in the presence of MSCs. The number of viable cells harvested following 3-5 weeks of culture did not differ between MDS-MSCs (n=4) and HC-MSCs (n=2) (Figure S2A). Non-adherent cells harvested after 3 weeks of culture in the presence of HC-MSCs or MDS-MSCs formed similar numbers of colonies in CFU-assays (range 12-27 vs 8-32, respectively) (Figure S2B).

Support of colony formation

To study the direct influence of MSCs on colony formation by freshly isolated HPC, MSCs (30×10^3 or 150×10^3 cells/well) were added to purified HPC (500 cells/well) in methylcellulose containing growth factors, *i.e.* SCF, GM-CSF, IL-3, and EPO. The total number of colonies at day 14 was increased by the addition of MSCs (p=0.01). A significant increase was seen in CFU-GEMM, CFU-GM and CFU-M (Figure 3A). The proportion of CFU-GM colonies was increased when cells were cultured in the presence of MSCs compared to CFU-C assays in the absence of MSCs. In accordance with this, the percentage of $CD45^+GPA^-$ myeloid cells was increased in the non-adherent cell population (colonies) harvested at day 14 from cultures containing MSCs (Figure 3B-C). No differences in the supportive effect on colony formation and HPC differentiation were observed between MDS-MSCs (n=6) and HC-MSCs (n=4). When HPC were cultured in the presence of HC-MSCs (n=3) or MDS-MSCs (n=3) in methylcellulose with erythropoietin and without GM-CSF, SCF and IL-3 the number and size of colonies was significantly increased in comparison with cultures without MSCs (Figure 3D). In conclusion, HC-MSCs and MDS-MSCs have similar effects on colony formation *in vitro*.

Gene and protein expression

In functional assays no evidence was obtained for a disturbed MSC function in children with MDS. However, these functional studies are limited and the results do formally not exclude the possible existence of biologically relevant differences between MDS-MSCs en HC-MSCs. To further investigate this Deep-SAGE was performed on total RNA identifying the expression of all mRNA from the 3'-end.

MSCs derived from RCC (n=4), RAEB(t) (n=4) and healthy controls (HC, n=8) were analyzed. A median of 15.9×10^6 reads (range $9.5 \times 10^6 - 30.6 \times 10^6$) was obtained. Between 59.3% and 68.4% (median 65.6%) of reads were aligned to the genome using Bowtie; with a median of 55.4% (min 45.3%; max 57.6%) of the reads being mapped to an exon.

A heat map reflecting the top 50 differentially expressed genes demonstrates clustering of healthy control, RCC and RAEB MSCs (Figure 4). The gene expression profile of RCC clustered more towards healthy controls than RAEB(t). After correction for multiple testing, *IL6* and dickkopf 3 homologue (*DKK3*) were significantly higher expressed in RCC-MSCs compared to HC-MSC (p=0.002 and p=0.005, respectively). Death-associated protein kinase 1 (*DAPK1*) expression was decreased (p=0.049) in RAEB(t)-MSCs compared to HC-MSCs, whereas cytokine receptor-like factor 1 (*CRLF1*) and *IL6* expression were increased (p=0.009 and p=0.048, respectively). Differential expression of *IL6*, *DKK3*, *DAPK1* and *CRLF1* was confirmed by RT-PCR (Figure 5A). In addition, the IL-6 concentration in culture supernatants of MSCs from bone-marrow

obtained at diagnosis was significantly increased in all MDS cases (n=10) compared to supernatants of healthy control MSCs (n=8) (p <0.001; Figure 5B).

Deep-SAGE and RT-PCR showed that IL-6 expression was elevated in MSCs of both RCC and RAEB(t) obtained prior to HSCT. Of note, after HSCT, *IL6* and *DAPK1* expression levels in MSCs were comparable to HC-MSCs (Figure 5C). IL-6 concentration in MDS-MSCs culture supernatant was, although lower than in MSC samples generated
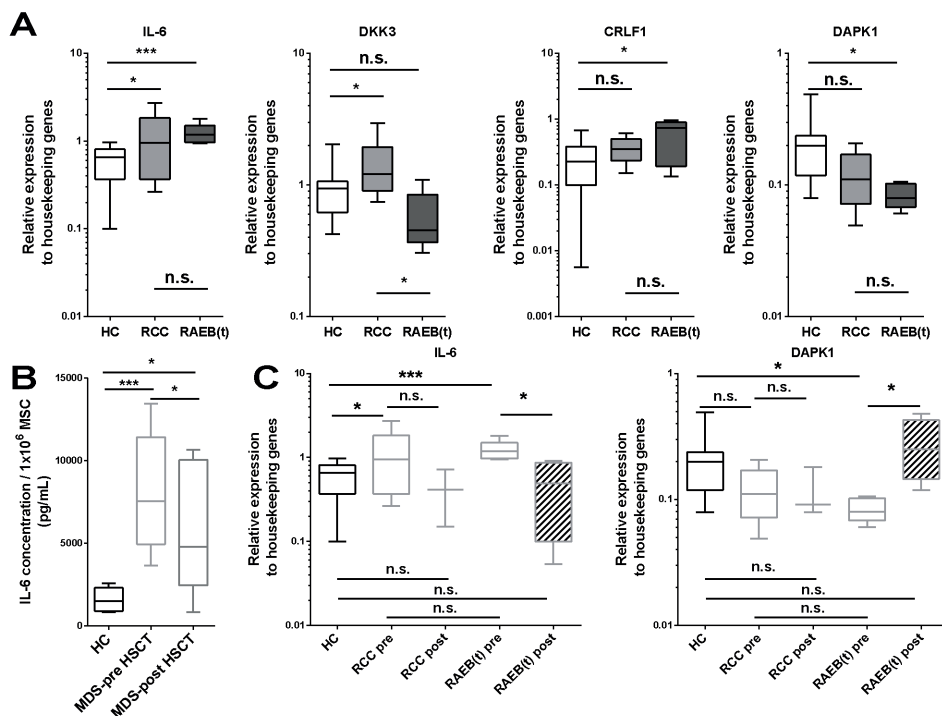
Figure 5. *Differential mRNA expression by MSCs of MDS patients.* A: Aberrant gene expression detected by Deep-SAGE was confirmed by RT-PCR. *IL-6* expression was increased in MSCs of both RCC and RAEB(t) MDS patients at diagnosis compared to MSCs of healthy controls (HC). In contrast, *DKK3* was specifically increased in RCC-MDS, whereas *CRLF1* and *DAPK1* were significantly altered in RAEB(t)-MDS derived MSCs. Data were normalized using GAPDH and HPRT1 as house-keeping genes (HC-MSCs n=10, RCC-MSCs n=7 and RAEB(t)-MSCs n=4). B: Increased *IL-6* gene expression in MSCs of MDS patients pre-HSCT (n=10) compared to HC-MSCs (n=8) was confirmed by ELISA quantifying IL-6 secreted by MSCs in culture supernatant. IL-6 concentration was still elevated in supernatant of MSCs generated from bone-marrow obtained after HSCT, although there is a trend to normalization (n=7). C: Gene expression analysis demonstrated a normalized mRNA expression in MDS-MSCs after HSCT compared to HC-MSCs for *IL-6* and *DAPK1.* RCC: refractory cytopenia; RAEB(t): refractory anemia with excess of blasts (in transformation); HC: healthy control; DKK3: dickkopf 3 homologue; CRLF1: cytokine receptor-like factor 1; DAPK1: death-associated protein kinase 1; HSCT: hematopoietic stem cell transplantation; Boxes represent median and 25-75 percentiles with whiskers marking the range; Mann-Whitney statistical tests were performed to compare the different groups; *: *p* <0.05; **: *p* <0.01; ***: *p* <0.001. n.s.: not significant.

from bone-marrow taken pre-HSCT, still significantly higher than in supernatant of HC-MSC (Figure 5B). *DKK3* and *CRLF1* expression in MSCs expanded after HSCT from RCC and RAEB(t) patients, respectively, was not significantly altered compared to MDS-MSCs before HSCT or to HC-MSCs (data not shown).

Potential candidate genes based on their reported differentially expression in adult MDS-MSCs, i.e. *AURKA*, *AURKB*, *SCF*, *G-CSF* and *GM-CSF* (Zhao, 2012a; Santamaria, 2012; Oliveira, 2013; Ferrer, 2013), were specifically analyzed and no differences were observed comparing RCC-MSCs, RAEB(t)-MSCs and HC-MSCs.

Similarly, a comparable expression level of *CXCL12*, *Dicer1* and *Drosha* in pediatric MDS-MSCs versus HC-MSCs was confirmed by RT-PCR (data not shown).

Discussion

The spectrum of pediatric MDS ranges from aplasia to myeloproliferative disease. The pathophysiology of the disease has been attributed to different cytogenetic abnormalities.(Gohring, 2010) Previous studies in mice and in human adults have linked the interaction of hematopoietic progenitors and the micro-environment to the progression of disease in several hematopoietic disorders.(Zhang, 2012b; Schepers, 2013) In adult MDS specific alterations in the MSCs have been reported as summarized in Table S1. Data on pediatric MDS are limited (Table S2). In this study, we compared the MSC characteristics and function in children with different types of MDS with healthy controls. Differences were neither observed with respect to the differentiation capacity of MSCs, their immunomodulatory capacity using T cell proliferation and monocyte differentiation to dendritic cells as read-out, nor regarding their impact on maintenance and differentiation of hematopoietic progenitor cells. In addition, cell viability in co-cultures was equally increased by both groups of MSCs, as assessed by trypan-blue staining (data not shown).

However, evaluation of total mRNA expression profiles demonstrated gene expression differences between MSCs derived from pediatric MDS patients and controls. Cytogenetic abnormalities present in the hematopoietic cells could not be detected in the stromal compartment, and, therefore, this cannot explain the differential gene expression. The partial normalization of *IL6* and *DAPK1* expression in MSCs after HSCT in these patients demonstrates that the expression differences can be reversed. Of note, using chimerism analysis, the presence of donor MSCs in the expanded cells has been excluded.

Differential gene expression between pediatric MDS in general and healthy controls was most prominent for *IL6*. This gene has previously been reported to be over-expressed in adult MDS and in one child with MDS and a constitutional trisomy

8.(Zhao, 2012b; Narendran, 2004) In contrast, other studies in adults did not show differential *IL6* expression between MDS patients and healthy controls.(Zhao, 2012b; Klaus, 2010; Flores-Figueroa, 2002; Flores-Figueroa, 2008) *IL6* has been described to increase myeloid differentiation via *STAT3* activation and support multiple myeloma cell growth and survival.(Minami, 1996; Zhang, 2010; Csaszar, 2013; Gunn, 2006) *STAT3* up-regulation was not observed in this pediatric MDS cohort. In addition, IL-6 is one of the cytokines responsible for bone-remodeling in inflammatory and malignant disease.(Ara, 2010; Dayer, 2010) Suppression of monocyte to dendritic cells differentiation is dependent on IL-6. (Melief, 2013) However, we did not observe a correlation between the degree of the suppressive effect of MSCs and the level of *IL-6* expression, suggesting that IL-6 is not the sole factor hampering this differentiation.

Besides *IL6* (RCC- and RAEB(t)-MDS)*, DKK3* (RCC-MDS)*, CRLF1* and *DAPK1* (RAEB(t)-MDS) were differentially expressed by MSCs of healthy controls versus MDS. *DKK3* and *CRLF1* have been associated with increased cell survival by suppressing apoptosis in MSCs and neuroblastoma cells, respectively.(Song, 2006; Looyenga, 2013) Differential expression of these genes did not correlate with MSC expansion rates (data not shown). In addition, increased CRLF1 in combination with IL-6 has been described in idiopathic pulmonary fibrosis causing inflammation, but suppression of fibrosis. (Kass, 2012) *DAPK1* down-regulation, associated with malignant transformation, has been described in the hematopoietic cells in adult RAEB(t)-MDS potentially attributing to aberrant methylation.(Raval, 2007; Qian, 2010; Wu, 2011; Claus, 2012; Karlic, 2013) We demonstrate a similar expression profile in our MSCs, with expression in RCC-MDS being similar to HC-MSC, and down-regulation in RAEB(t)-MSCs. After successful HSCT, *DAPK1* expression was normalized. The allogeneic HSCT procedure leading to elimination of derailed cells and restoration of hematopoiesis through donor HPC might contribute to normalization of the stromal environment in the hematopoietic niche, including MSCs of recipient origin.

Analysis of total mRNA expression profiles not only revealed differences between RCC and RAEB(t)/MDR-AML in children, but also enabled us to specifically focus on genes previously reported to be differentially expressed in adult MDS. Genes of interest included micro-RNAs reported by Santamaria *et al.*(Santamaria, 2012) as well as genes encoding cytokines, their receptors, chemokines and adhesion molecules. In contrast to what has been described for adult MDS, *AURKA*, *AURKB*, *SCF*, *G-CSF*, *GM-CSF*, *CXCL12*, *Dicer1* and *Drosha* were not differentially expressed in our cohort of pediatric MDS patients compared to healthy controls. Lack of differences in expression levels of *Dicer1*, *Drosha* and *CXCL12* was further confirmed by RT-PCR (data not shown). This supports the current understanding that pediatric and adult MDS are two different diseases as previous studies have highlighted the differences between adult and pediatric MDS, *e.g.* in response to treatment and rarity and prognostic

value of (epi-) genetic mutations in the hematopoietic compartment.(Glaubach, 2014; Hasle, 2004; Hirabayashi, 2012) Besides IL-6, genes included in the clustered analysis did not encode for molecules known to be involved in MSC signaling as reviewed by Le Blanc and Mougiakakos.(Le, 2012)

Our MDS cohort is heterogeneous containing RCC as well as advanced MDS patients. Bone-marrow post HSCT was not available in all cases, because of informed consent was limited to bone-marrow sampling on clinical indication namely relapse risk and non-engraftment. Correlation of mRNA expression in MSCs obtained at diagnosis with MSCs at MDS relapse after HSCT was not feasible due to low sample numbers in combination with limited numbers of relapse after HSCT.

Our findings demonstrate differences in mRNA expression between pediatric MDS and age-matched healthy control derived MSCs. This is in accordance with published data on MSCs derived from adults with MDS, however, as expected, not all abnormalities described in adults were present in pediatric MDS. In addition, different expression levels of specific genes were not associated with functional aberrations in assays pointing to immunomodulation and hematopoiesis, potentially caused by compensatory mechanisms or insufficient sensitivity of our tests. Growth differences between MSC precursors and use of non-clonal MSC populations may lead to loss of information and, thereby, to potential loss of differences between pediatric MDS and healthy control derived MSCs. Unfortunately, data on the interaction of MSCs with MDS patient derived hematopoietic stem cells was limited by the available material. However, preliminary data do not reveal differences in co-cultures of MDS-RAEB HPC with MDS patient or healthy control derived MSCs.

Studying the pathogenesis of MDS has been complicated by the poor engraftment of human MDS HPC in immunodeficient mice.(Thanopoulou, 2004) However, co-transplantation of stromal cells and intramedullary transplantation of hematopoietic cells has led to increased engraftment.(Kerbauy, 2004) Knockout models resulting in an MDS-like phenotype or the of use of scaffolds with patient-derived MSCs to resemble the human bone-marrow microenvironment might be instrumental in further exploring the potentially functional implications of these differences in future studies.(Groen, 2012; Walkley, 2007; Raaijmakers, 2010)}

In conclusion, our data show that the gene expression profile is different in MSCs of children with MDS. It remains to be elucidated whether the abnormalities are a cause or a consequence of the disease. Normalization of the aberrant gene expression seen in patients derived MSCs after allogeneic HSCT is an argument favoring the latter possibility. Induced abnormalities in the MSCs by dysplastic cells might be targets to sustain response to therapy.

Acknowledgements:

The microenvironment in pediatric myelodysplastic syndrome

Supplementary data

Supplementary Figure 1. *Phenotype and differentiation capacity of MDS-MSC.* A: MSC of MDS patients (*e.g.* UPN MDS015, passage 2) express the characteristic phenotype of MSC: CD73+, CD90+ and CD105+ and negative for the indicated lineage-specific markers. Red line indicates the staining intensity obtained with a negative isotype-matched control. B-E: MSC of healthy controls (B, D HC012, passage 5) and MDS patients (C, E MDS002, passage 7) differentiate towards osteoblasts (B, C Alizarine red staining, magnification 10x) and adipocytes (D, E Oil-Red-O staining, magnification 40x).
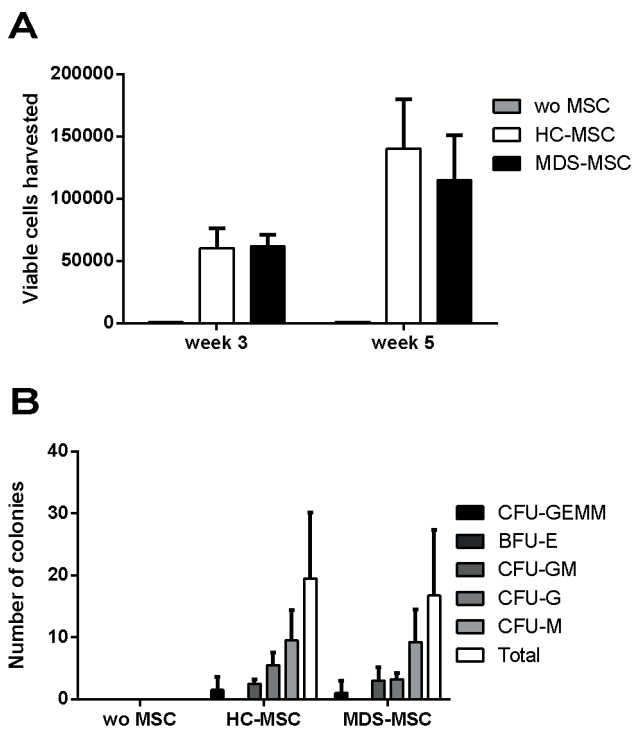
Supplementary Figure 2. *MSC of MDS patients maintain hematopoietic cells in the absence of growth factors.* CD34$^+$ cells (HPC, 50 000 cells) of HSCT donors were seeded on a confluent layer of MSC (white bars: healthy control (HC)-MSC, black bars: MDS-MSC) or in wells without (wo) MSC (grey bars) and cultured for 3 to 5 weeks. A. Viability of non-adherent cells obtained after harvesting was assessed by trypan blue staining. No viable cells were present in culture conditions without MSC. B. Non-adherent cells harvested after 3 weeks of co-culture with healthy control (HC)-MSC (n=2) or MDS-MSC (n=4) were tested in CFU-assays (1500 cells/dish) to investigate their differentiation capacity. Results are depicted as the mean and standard deviation.

Supplementary Table 1. Studies describing characteristics and function of MDS derived MSC in adults

Author	Ref	Year	Study set-up	MSC characterization	Cytogenetic abnormalities	Protein expression	Gene expression	Immuno-modulation	Hematopoietic support	Technique for analysis of hematopoiesis
Blau	(164)	2011	43 MDS 51 AML + 36 controls	Yes	5 out of 43 MDS using karyotype	NA	NA	NA	NA	NA
Lopez-Villar	(165)	2009	36 MDS + 15 controls	Yes, decreased growth	all cases using array-CGH	low CD105	NA	NA	NA	NA
Flores Figueroa	(166)	2008	16 MDS + 6 controls	negative selection; then characterized	8 out of 12 using karyotype	IL-1b increased; IL-6 a.o. similar	NA	NA	no differences	5 week culture
Blau	(212)	2007	18 MDS 13 AML 7 controls	Yes	7 out of 16 using karyotype	NA	NA	NA	NA	NA
Flores Figueroa	(213)	2005	11 MDS + 5 controls	Negative selection; then characterized	5 out of 9 using karyotype; all MDS MSC hypodiploid	NA	NA	NA	NA	NA
Song	(214)	2012	22 MDS + 7 controls	Yes	15 out of 22 using karyotype	NA	NA	NA	NA	NA
de Oliveira	(189)	2013	60 MDS	yes, decreased growth	18/60 using karyotype	NA	AURKA AURKB increased (especially in abnormal karyotype)	NA	NA	NA
Marcondes	(167)	2008	13 MDS + 6 controls	Yes	NA	Increased TNF and IL32	IL-32 increased	NA	NA	NA
Lubkova	(215)	2011	5 MDS + 7 controls	Yes	NA	decreased VCAM1	NA	NA	NA	NA
Santamaria	(168)	2012	33 MDS + 25 controls	Yes	NA	NA	Dicer Drosha SBDS decreased; effect on miRNA	NA	NA	NA
Flores Figueroa	(191)	2002	25 MDS + 8 controls	Yes	NA	TNF-a increased IL-6 normal	NA	NA	NA	NA

Author	Ref	Year	Study set-up	MSC characterization	Cytogenetic abnormalities	Protein expression	Gene expression	Immuno-modulation	Hematopoietic support	Technique for analysis of hematopoiesis
Hirayama	(216)	1993	8 AA + 7 MDS + 9 controls	passage 0	NA	large variation	large variation	NA	NA	NA
Aanei	(217)	2012	20 MDS + 8 controls	yes, decreased growth	NA	NA	decrease in CD29, CD49e, CD44, CD31, CD106	NA	NA	NA
Aanei	(218)	2011	9 MDS + 4 controls	use passage 1 CD73 enrichment; in MDS more morphological changes + decrease proliferation	NA	increase in focal adhesion proteins	NA	NA	NA	NA
Wang	(169)	2013	31 MDS + 8 controls	yes	NA	NA	NA	low risk MDS less suppression of DC maturation, TGF-b most important	NA	NA
Zhao	(170)	2012	29 MDS + 10 controls	yes	normal karyotype	more IL-6; less TGFb1 (for low risk MDS) HGF	NA	strong save from apoptosis, less PHA suppression, less induction of T-reg (low risk MDS only)	NA	NA
Zhao	(188)	2012	14 MDS + 8 controls	yes + clonal expansion	normal karyotype	NA	decreased SCF, G-CSF, GM-CSF; increased IL-6	MLR + PHA; impaired suppression;	decreased support	5 week culture
Han	(219)	2007	15 RA-MDS + 12 controls	yes, but negative selection	normal karyotype	NA	TGFb1+3 + FasL decrease; TGFb2 increase	impaired suppression of PHA + MLR; less suppression of cell cycle; increase saving from apoptosis	NA	NA

Author	Ref	Year	Study set-up	MSC characterization	Cytogenetic abnormalities	Protein expression	Gene expression	Immuno-modulation	Hematopoietic support	Technique for analysis of hematopoiesis
Ferrer	(171)	2013	20 MDS + 6 controls	yes, altered growth morphology and differentiation	NA	decreased adhesion proteins and SDF1a	SDF1 ANG1 SCF lower	normal supperssion of PHA induced proliferation	reduced support by MDS MSC	CAFC assays 6 weeks
Varga	(220)	2007	10 MDS + 15 controls	Yes	NA	NA	NA	NA	decreased support	CAFC assays 6 weeks
Tennant	(221)	2000	16 RA + 7 RARS + 5 RAEB + 2 RAEBt + 2 CMML	passage 0	NA	NA	Increased Epo, G-CSFr TNFr TPOr decrease IL-1b	NA	decreased in MDS	5-7 week culture
Flores Figueroa	(222)	2012	various	direct biopsy IHC	NA	increased CXCL12	NA	NA	NA	NA
Coutinho	(223)	1990	10 MDS 4 controls	passage 0	NA	NA	NA	NA	similar support	7 week culture
Soenen-Cornu	(224)	2005	12 MDS + n controls	Yes	FISH no abnormalities	NA	NA	NA	supportive but no controls	2 and 5 week culture
Klaus	(172)	2010	13 MDS + 20 controls	acquired during culture in 4 patients and 1 control	yes	TNFa, IL1b, IL-6 VEGF SDF1a similar	NA	similar PHA suppression	NA	NA
Alvi	(173)	2001	103 MDS + 12 controls	use of BM biopsy not MNC	NA	NA	NA	NA	normal support	2 week culture
Aizawa	(174)	1999	11 RA + 12 controls	passage 0	NA	NA	NA	NA	decreased support, but large variation between MDS patients	5 week culture

MDS: myelodysplastic syndrome; MSC: mesenchymal stromal cells; AML: acute myeloid leukemia; NA not applicable; CGH: comparative genomic hybridisation; AA: aplastic anemia; MLR: mixed lymphocyte reaction; RA: refractory anemia; CAFC: cobblestone area-forming cells; RARS: RA with ring sideroblasts; RAEB: RA with excess of blasts; RAEBt: RAEB in transformation; CMML: chronic myelomonocytic leukemia; IHC: immunohistochemistry.

Supplementary Table 2. Studies describing characteristics and function of MDS derived MSC in children

Author	Ref	Year	Study set-up	MSC characterization	Cytogenetic abnormalities	Protein expression	Gene expression	Immuno-modulation	Hematopoietic support	Technique for analysis of hematopoiesis
Narendran	(175)	2004	case-report	yes	constitutional trisomy 8	Increased TGF-b, VEGF, IL-6, LIF; decreased SCF	NA	NA	increased stromal support of HPC and leukemic cells	NA
Borojevic	(176)	2004	7 MDS patients	yes	NA	NA	OSF2, SPARC, COL1A2, THBS1 increased	NA	decreased maturation and absolute numbers	NA
Roela	(177)	2007	6 MDS 3 controls	yes	NA	NA	different patterns of endocytosis and protein secretion related genes	NA	NA	NA

MDS: myelodysplastic syndrome; MSC: mesenchymal stromal cells; HPC hematopoietic progenitor cells; NA not applicable.

Supplementary Table 3. Primer design for RT-PCR

CRLF1-forward	CCAGAGAAACCCGTCAACAT
CRLF1-reverse	TCCTGGCCATACCACCTAAG
DAPK1-forward	AGCCCGTCATGATCTACCAG
DAPK1-reverse	CTCCGAGTGAGGAGGTTCAG
IL-6-forward	GAAAGCAGCAAAGAGGCACT
IL-6-reverse	TTTCACCAGGCAAGTCTCCT
DKK3-forward	TGGGGAAATGTGGAGAAGAG
DKK3-reverse	GAGCAACACTGCTGGATGAA
Dicer1-forward	GAGGCGTGTCTTGAAAAAGC
Dicer1-reverse	AGTTAGGACTGCGGAAAGCA
DROSHA-forward	AATGGATGCGCTTGAAAAAT
DROSHA-reverse	TGCATGCCCTCCTTTATTTC
CXCL12-forward	AGAGCCAACGTCAAGCATCT
CXCL12-reverse	CTTTAGCTTCGGGTCAATGC
HPRT1-forward	TGACACTGGCAAAACAATGCA
HPRT1-reverse	GGTCCTTTTCACCAGCAAGCT
GAPDH-forward	GGCCTCCAAGGAGTAAGACC
GAPDH-reverse	AGGGGAGATTCAGTGTGGTG

Chapter 4.

Gene-expression and *in vitro* function of mesenchymal stromal cells are affected in juvenile myelomonocytic leukemia.

Abstract

An aberrant interaction between hematopoietic stem cells and mesenchymal stromal cells has been linked to disease and shown to contribute to the pathophysiology of hematologic malignancies in murine models. Juvenile myelomonocytic leukemia is an aggressive malignant disease affecting young infants. Here we investigated the impact of juvenile myelomonocytic leukemia on mesenchymal stromal cells. Mesenchymal stromal cells were expanded from bone marrow samples of patients at diagnosis (n=9) and after hematopoietic stem cell transplantation (n=7; from 5 patients) and from healthy children (n=10). Cells were characterized by phenotyping, differentiation, gene expression analysis (of controls and samples obtained at diagnosis) and in vitro functional studies assessing immunomodulation and hematopoietic support. Mesenchymal stromal cells from patients did not differ from controls in differentiation capacity nor did they differ in their capacity to support in vitro hematopoiesis. Deep-SAGE sequencing revealed differential mRNA expression in patient-derived samples, including genes encoding proteins involved in immunomodulation and cell-cell interaction. Selected gene expression normalized during remission after successful hematopoietic stem cell transplantation. Whereas natural killer cell activation and peripheral blood mononuclear cell proliferation were not differentially affected, the suppressive effect on monocyte to dendritic cell differentiation was increased by mesenchymal stromal cells obtained at diagnosis, but not at time of remission. This study shows that active juvenile myelomonocytic leukemia affects the immune response related gene expression and function of mesenchymal stromal cells. In contrast, the differential gene expression of hematopoiesis-related genes could not be supported by functional data. Decreased immune surveillance might contribute to the therapy resistance and progression in juvenile myelomonocytic leukemia.

Introduction

The bone-marrow (BM) niche represents the supportive environment for hematopoietic stem cells (HSC).(Mendez-Ferrer, 2010; Morikawa, 2009) Mesenchymal stromal cells (MSCs), being precursors to osteoblasts, adipocytes and chondrocytes and a cellular constituent of the niche, are crucial for maintenance of quiescent HSC. (Sugiyama, 2006b) MSCs, or differentiated sub-populations of these cells, are used *in vitro* as a model for the BM microenvironment. Soluble factors as well as direct cell-to-cell contact have been described to play a role in normal MSC-HSC interaction. (Greenbaum, 2013; Wang, 2010)

Hematopoietic malignancies such as leukemia originate in the BM. Although leukemic blast cells can be detected throughout the body during disease, the leukemic stem cells are thought to remain in the BM, and more specifically in the hematopoietic stem cell niche.(Ayala, 2009) It is widely accepted that malignant cells have a negative impact on the normal hematopoiesis causing anemia and thrombocytopenia. However, the effect of the malignant cells on the BM microenvironment has not been studied extensively.

Recent studies in mice have demonstrated that myeloid neoplasms affect the normal niche structure.(Schepers, 2013; Zhang, 2012b; Arranz, 2014) These alterations contribute potentially to the formation of the leukemic niche in which leukemic stem cells are difficult to target by conventional chemotherapy or irradiation.(Kurtova, 2009) Studies describing MSC characteristics in human myeloproliferative neoplasms are mostly limited to adult patients, demonstrating conflicting results with regard to genetic abnormalities, gene-expression and MSC function.(Zhao, 2012b; Raaijmakers, 2012; Klaus, 2010; Flores-Figueroa, 2008)

Juvenile Myelomonocytic Leukemia (JMML) is an aggressive leukemia occurring in young children, predominantly in infants between 0 and 4 years of age. Patients usually present with hepatosplenomegaly, fever and monocytosis.(Niemeyer, 1997) Monosomy 7 is the most common karyotype abnormality detected in 25% of cases, and numerous leukemogenic mutations have been identified mainly involving the RAS-RAF-ERK pathway, *e.g. PTPN11*, *K-RAS* and *c-CBL*.(Niemeyer, 2008; de Vries, 2010) Hematopoietic stem cell transplantation (HSCT) is the standard first line treatment. Unfortunately, the one year relapse rate ranges between 30 and 50%.(Locatelli, 2005; Locatelli, 2015)

We hypothesize that the aggressive and therapy resistant characteristics of JMML may point towards an altered BM microenvironment based on previous experimental data suggesting support of a neoplasm by the stromal compartment.(Zhang, 2012b; Arranz, 2014) In the current study we aimed to identify stromal factors involved in the support of JMML. MSCs of patients with JMML were obtained at diagnosis and after

HSCT and expanded from BM. These patient derived MSCs were compared to healthy pediatric donor derived MSCs by investigating their capacity of immunomodulation and hematopoietic support and their gene expression profiles.

Methods

A detailed version of the Methods section is available online at the Haematologica website.

Patients

Children referred to our center for HSCT were included in this study according to a protocol approved by the institutional review board (P08.001). BM of 9 consecutive children with JMML was collected prior to treatment initiation. In addition, BM after HSCT was collected from 5 of these 9 children. The patients were classified following previously described criteria.(de Vries, 2010; Loh, 2011) BM samples were sent to the EWOG-MDS-reference center in Freiburg, Germany for mutation analysis. BM samples of healthy pediatric HSCT donors (n=10) were used as control group (HC). Informed consent was obtained from all parents. This study was conducted according to the Declaration of Helsinki.(World Medical Association, 2013)

MSC expansion and characterization

MSC were expanded from fresh BM and characterized as previously described.(Calkoen, 2013a) Monosomy 7 by FISH and chimerism by variable number of cytosine adenine (CA) repeat analysis were determined in expanded MSC before and after HSCT, respectively.(Lankester, 2010; Bronkhorst, 2011) MSC gene expression and function was investigated using MSCs obtained at passage 2-3 and 3-5, respectively.

Functional assays

The effect of MSCs on proliferation of peripheral blood mononuclear cells (PBMCs) was investigated in co-cultures stimulated with phytohemagglutinin (PHA) by measuring ^{3}H-thymidine incorporation.

The suppressive effect of MSC on NK cell activation was determined by stimulation of purified NK cells with IL-2 in the absence or presence of MSCs for 5 days. NK cells were harvested and activation was measured by flow cytometry investigating DNAM-1, NKp30 and NKp44 expression.

To evaluate the effect of MSCs on antigen presenting cells, monocytes were isolated from PBMCs and cultured with growth factors for 5 or 7 days to differentiate towards

immature dendritic cells (DC) or mature DC, respectively. Cells were phenotyped for the expression of CD14 and CD1a after co-culture.

Short-term co-culture assays with MSCs and hematopoietic progenitor cells (HPC) were performed to determine the supportive capacity of MSCs for HPCs maintenance and differentiation. Therefore, HPCs were isolated from healthy transplant donors using CD34 positive selection. Proliferation (day 7) and differentiation (day 7 and 14) were assessed using ^{3}H-thymidine incorporation and flow cytometry, respectively.

To determine a direct effect of MSCs on HPC differentiation into colony-forming-units (CFU), MSCs were added to freshly purified HPCs in methylcellulose containing growth factors and cultured for 14 days (CFU-assay).

Gene expression

Total RNA was isolated from MSCs and mRNA was profiled using Deep-SAGE (serial analysis of gene expression) sequencing using Illumina technology.(Mastrokolias, 2012) Data were mapped against the UCSC-hg19 reference genome using Bowtie, with permission of one mismatch and only retaining unique mappings.

Expression of genes of interest was validated using independent biological samples by RT-qPCR after generation of cDNA using the listed primers (Supplementary Table 1), as previously described.(Mastrokolias, 2012) Expression levels were calculated relative to the average expression of two housekeeping genes.

Statistical analysis

Mann-Whitney and Wilcoxon matched-pairs signed rank tests were performed to compare different groups in functional assays and in the validation of mRNA expression. Differential gene expression analysis was performed in R, using EdgeR, Globaltests and Limma data analysis packages.(Robinson, 2010; R Development Core Team, 2012; Smyth, 2005) Correction for multiple testing was performed according to Benjamini and Hochberg.(Hochberg, 1990) STRING software was used for analysis of protein interactions.(Franceschini, 2013) Adjusted *p*-values <0.05 were considered statistically significant.

Results

Patients

MSCs were successfully expanded from all 9 patients with JMML (Table 1) at diagnosis and 10 healthy controls (HC). In addition, 7 BM samples collected after HSCT, derived from 5 of the 9 JMML patients included, were used for MSC expansion (Table 1). These MSCs were of patient origin as tested by CA-repeat analysis. Median age

at diagnosis was 2.1 years (range 0.5 - 3.5), whereas the age of healthy controls ranged between 1.1 and 16.4 years (median 7.4). Children with different genetic mutations were included as shown in Table 1. Monosomy 7, present at diagnosis in two children, was not detected in the MSCs at diagnosis.

MSC expansion and characterization

All expanded MSC populations fulfilled the criteria proposed by Horowitz *et al.* (Horwitz, 2005), *i.e.* differentiation towards adipocytes and osteoblasts, a characteristic phenotype (CD73^{+}, CD90^{+}, CD105^{+}, HLA-DR^{-} and no expression of lineage markers) and adherence to plastic. Representative examples of differentiation of

Table 1. Patient characteristics

UPN	Sex	Age at diagnosis (months)	Chromosomal abnormalities and gene mutation(s)	Donor and graft type	Conditioning	MSC post HSCT (time BM sample, months after HSCT)	Relapse (months after HSCT)
JMML001	F	8	None	MUD/ PBSC	Thio, Flu, Treo, ATG	Yes (6[1])	No
JMML002	M	9	c-CBL	MUD/ BM	Flu, Bu, ATG	Yes (4[1])	No
JMML003	M	32	RAS (exon 1 c35 G>A)	MUD/ BM	Bu, Cy, Mel, ATG	Yes (3[1])	No
JMML004	M	35	PTPN11 (exon 3 c182 A>T)	IRD/BM	Bu, Cy, Mel	Yes (4[1], 7[1], 17[2])	Yes (12, 17)
JMML005	F	26	Monosomy 7 + NF1 gene: (2033delC en R416x)	MUD/ BM	Bu, Cy, Mel, ATG	No	No
JMML006	F	6	None	MUD/ CB	Flu, Treo, ATG	No	No
JMML007	M	43	PTPN11 gene (exon 13 1508 G>C 503 G>A)	MUD/ CB	Flu, Bu, ATG	Yes (5[2])	Yes (5)
JMML008	F	35	PTPN11 gene (exon 13 1504 T>C 502 S>P)	MUD/ BM	Bu, Cy, Mel, ATG	No	No
JMML009	M	12	Monosomy 7/RAS	MUD/ PBSC	Flu, Bu, ATG	No	No

Donor: MUD: matched unrelated donor; IRD: identical related donor. Graft type: BM: bone-marrow; CB: cord blood; PBSC: peripheral blood stem cells. Conditioning: Thio: thiotepa; Flu: fludarabine; Treo: treosulfan; ATG: anti-thymocyte globulin; Bu: busulfan; Cy: cyclophosphamide; Mel: melphalan. [1]: Bone-marrow (BM) sample after HSCT was obtained after remission induction; [2]BM sample after HSCT was obtained at relapse.

MSCs prior to and after HSCT are shown in Figure 1A-B. Besides these criteria, MSCs have been described to suppress PHA-induced PBMC proliferation. MSCs derived from patients and controls did not differ in their immunosuppressive capacity of PHA-induced PBMC proliferation (Figure 1C).

Hematopoietic support

MSCs have been described to play an important role in the tight regulation of hematopoiesis in the bone marrow. We investigated the capacity of JMML-MSCs derived at diagnosis to support hematopoiesis *in vitro*. HPC proliferation induced by SCF and Flt3-L was enhanced in the presence of MSCs and dependent on the HPC : MSC ratio (Figure 2A). JMML-MSCs and healthy control MSCs did not differ in this respect. Despite increased HPC proliferation, the percentage of CD34$^+$ cells after 14 days of
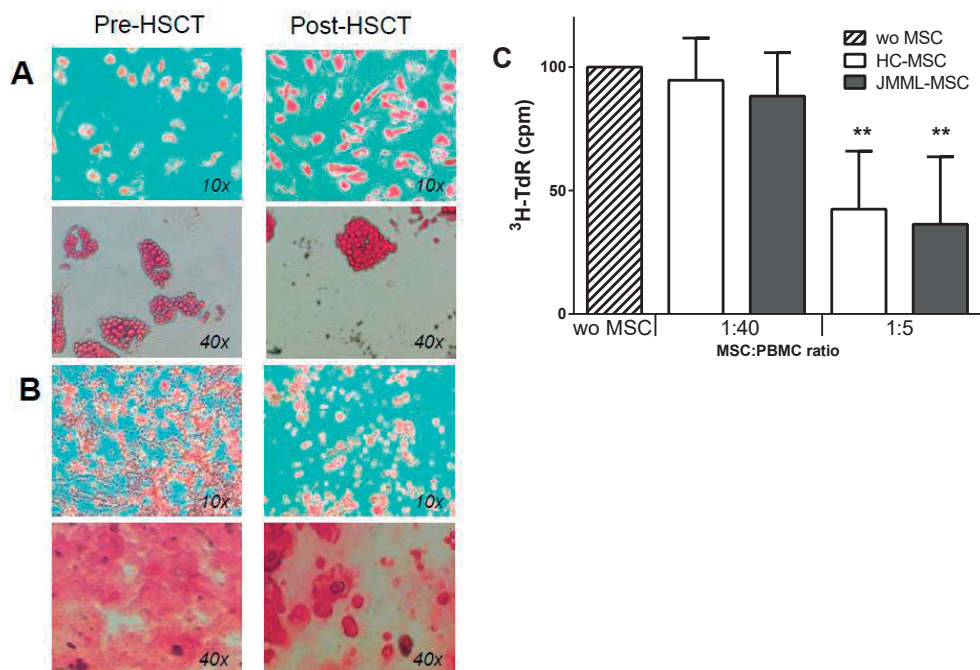
Figure 1. *Expansion and characterization of MSCs from JMML patients at diagnosis and post-HSCT and healthy controls.* MSCs were expanded and differentiation capacity towards adipocytes (A) and osteoblasts (B) was evaluated. MSCs were cultured with differentiation factors for 3 weeks and Oil Red O and Alizarine Red was used to stain fat and calcium deposition, respectively (examples are representative for MSCs of 9 JMML patients at diagnosis and 5 JMML patients after HSCT). C. Healthy Control (HC)-MSCs and JMML-MSCs showed a comparable dose-dependent suppressive effect on PBMC proliferation after PHA stimulation. Boxes indicate the mean proliferation at the indicated PBMC:MSC ratio relative to the proliferation induced without (wo) MSCs. Error bars represent standard deviation. Experiments were performed with n=7 JMML-MSCs and n=4 HC-MSCs. Statistics were performed using Mann-Whitney tests: **: *p*<0.01.

culture was significantly better maintained in the presence of MSCs (Figure 2B). Cells that lost CD34 expression expressed lineage markers such as CD14. The frequency of CD14$^+$ cells amongst CD45$^+$ lineage-committed cells was significantly higher after 7-14 days of culture of CD34$^+$ cells with MSCs (Figure 2C). The supportive effect by MSCs was comparable between MSCs of JMML patient and MSCs of healthy controls.

To evaluate the effect of MSCs on functional differentiation of HPCs, MSCs were added to semi-solid cultures of isolated HPCs in methylcellulose containing growth-factors. Both the total number of CFU and the different types of colonies increased in the presence of MSCs (Figure 2D). The percentage of CD45$^+$CD235a$^-$ cells was increased after culture with MSCs suggesting support of differentiation and prolif-eration of myeloid cells at the cost of CD45$^-$CD235a$^+$ erythroblasts in this culture condition (Figure 2E). Indeed, also in this culture configuration the percentage of monocytes (CD14$^+$ cells) was significantly increased in the presence of both control as well as JMML-MSCs (Figure 2F). Also in these differentiation assays, no difference was observed between JMML-MSCs and healthy control MSCs.

Gene expression analysis

Because the functional tests executed so far did not reveal differences between JMML and healthy control (HC) MSCs, we explored possible differences in mRNA expression profiles by performing Deep-SAGE sequencing, a next generation sequencing-based approach with higher sensitivity than traditional microarrays (*de novo* JMML n=8; HC n=8).(Hoen 't, 2008) The median number of obtained reads that fulfilled quality control criteria was 15.9x10^6 reads (range 11.4x10^6 - 30.6x10^6). A median of 65.6% of all reads aligned uniquely to the reference genome (range 59.3% - 68.4%). The percentage of the aligned reads mapping to an annotated exon was 84.5% (range: 74.7% - 86.3%).

The differentially expressed genes (n=162; *p*<0.001) are listed in Supplementary Table 2. A heat-map of the top 100 differentially expressed genes shows clustering of the JMML and HC derived MSCs (Supplementary Figure 1). After correction for multiple testing, in total 43 genes were differentially expressed between JMML and HC-MSCs (criterion: false discovery rate (FDR) < 0.05, see Supplementary Table 2) The top 6 genes (Dickkopf-1 (*DKK1*), ectonucleotide pyrophosphatase/phosphodies-terase 2 (*ENPP2*), DEAD (Asp-Glu-Ala-Asp) box helicase 17 (*DDX17*), monooxygenase DBH-like 1 (*MOXD1*), Adenomatosis Polyposis Coli Down-Regulated 1-Like (*APCDD1L*) and tumor necrosis factor alpha-induced protein 2 (*TNFAIP2*)), that were expressed at intermediate to high levels (> 100 reads per million sequenced reads, in both patients and controls) were selected for RT-qPCR validation (Figure 3).

In addition, three out of the 162 differentially expressed genes were previously reported to be involved in MSC function and were validated using RT-qPCR (*CXCL12,*
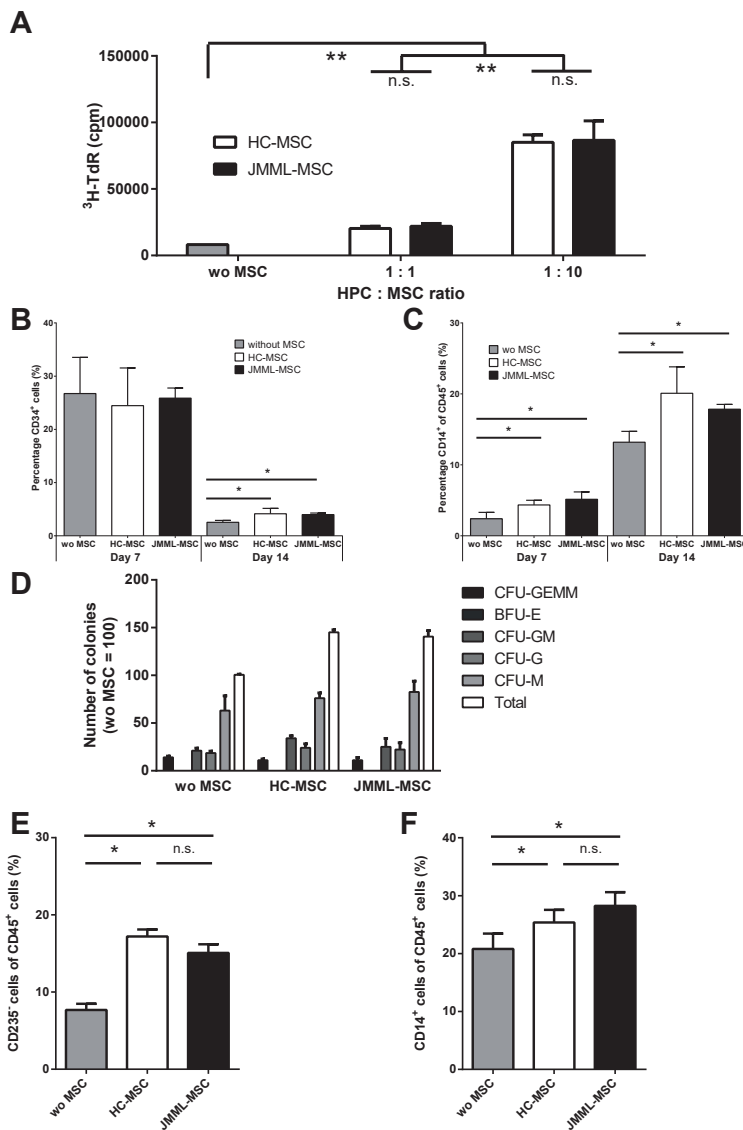
Figure 2. *JMML-MSCs support the proliferation and differentiation of HPCs in vitro.* A. Both in the presence of JMML patient and healthy control (HC) derived MSCs the proliferation of CD34+ cells (HPCs) was increased after 7 days of culture. MSCs alone did not show ^{3}H-thymidine incorporation (data not shown). B. HPCs lost the expression of CD34 after 14 days of culture; however, in the presence of MSCs (HPC : MSC ratio 1:5) the decline in CD34 expression was diminished. C. HPCs acquired lineage markers, *e.g.* CD14, but no differences were seen between JMML-MSCs and HC-MSCs. D. A significantly (*p*<0.05) increased number of colonies was seen in CFU-assays in the presence of MSCs (HPC : MSC ratio 1:60). E-F. Cells harvested after colony formation contained increased percentages of CD235a negative and CD14 positive cells within the CD45+ cell population. Boxes indicate the mean and error bars represent standard deviation. Experiments were performed with n=4 JMML-MSCs and n=2 HC-MSCs. Statistics were performed using Mann-Whitney tests: *: *p*<0.05; **: *p*<0.01; n.s.: non-significant.

CXCR7 and *IL-6*, Figure 3G, 3F and 3H, respectively*). CXCL12* (Figure 3G), previously reported to be of importance in HSC - MSC interaction and mobilization of HSCs, was found to be significantly decreased in JMML-MSCs.(Sugiyama, 2006b) Whereas the commonly involved receptor *CXCR4* was not differentially expressed, expression of the alternative receptor *CXCR7* was significantly decreased in JMML-MSCs (Figure 3F). String analysis of the top differentially expressed genes (data not shown) revealed that many of the genes upregulated in JMML-MSCs are associated with the IL-1 superfamily (*IL-1β*, *IL-6* (Figure 3H), *PTHLH*, *CLU*, *ATF3*, *PENK*, *RGS3* and *RGS16*). *DKK1* expression (Figure 3B), related with osteolysis, was also increased. In contrast, expression of genes in the leptin pathway was decreased (*LEP*, *LEPR*, *KISS1*, *SLC25A27*, *RXRA* and *CBLB*). Results of gene-ontology ($p<0.001$ after Holms correction and at least 100 covariates) are listed in the Supplementary Table 3. Pathways related to immune responses and protein ubiquination, involved in cell regulation of cellular interaction,(Hammond-Martel, 2012) were predominantly affected.

Normalization of gene expression in JMML-MSCs after HSCT

Genes of interest, identified by Deep-SAGE, were studied in patient samples after HSCT. BM obtained at remission was available for MSC expansion of 4 patients, 5 samples in total. RT-qPCR was used to study gene expression in these samples. *ENPP2*, *DDX17*, *CXCR7*, *CXCL12* and *TNFAIP2* expression was decreased in JMML-MSCs at diagnosis, however, expression was restored to the level of HC-MSC in samples after HSCT (Figure 3A, D, F, G, I). *DKK1*, *MOXD1* and *IL-6* expression was increased in JMML-MSCs at diagnosis, but normalized in JMML-MSCs post-HSCT (Figure 3B, C, and H). *APCDD1L* (Figure 3E), a paralog of the WNT inhibitor *APCDD1*,(Shimomura, 2010) was the only gene studied, of which the abnormal (increased) expression at diagnosis remained unchanged after HSCT. BM at time of relapse post HSCT was only available in two patients and therefore not included in the gene-expression analysis.

Immunomodulation

Differential expression of *IL-6* and other genes in the IL-1 superfamily suggests a differential effect of JMML patients derived MSCs on the innate immune system. Escape from NK cell surveillance is an important survival mechanism in tumorigenesis. However, HC-MSCs and JMML-MSCs derived from BM obtained at diagnosis suppressed NK cell activation to a similar extent (Figure 4A).

The suppressive effect of MSCs on monocyte to dendritic cell (DC) differentiation has been described to be IL-6 dependent.(Melief, 2013) Therefore, we analyzed the monocyte to DC differentiation in the absence and presence of MSCs at different MSC : monocyte ratios. The addition of both sources of MSCs resulted in decreased differentiation to immature DCs as measured by a lower percentage of cells express-
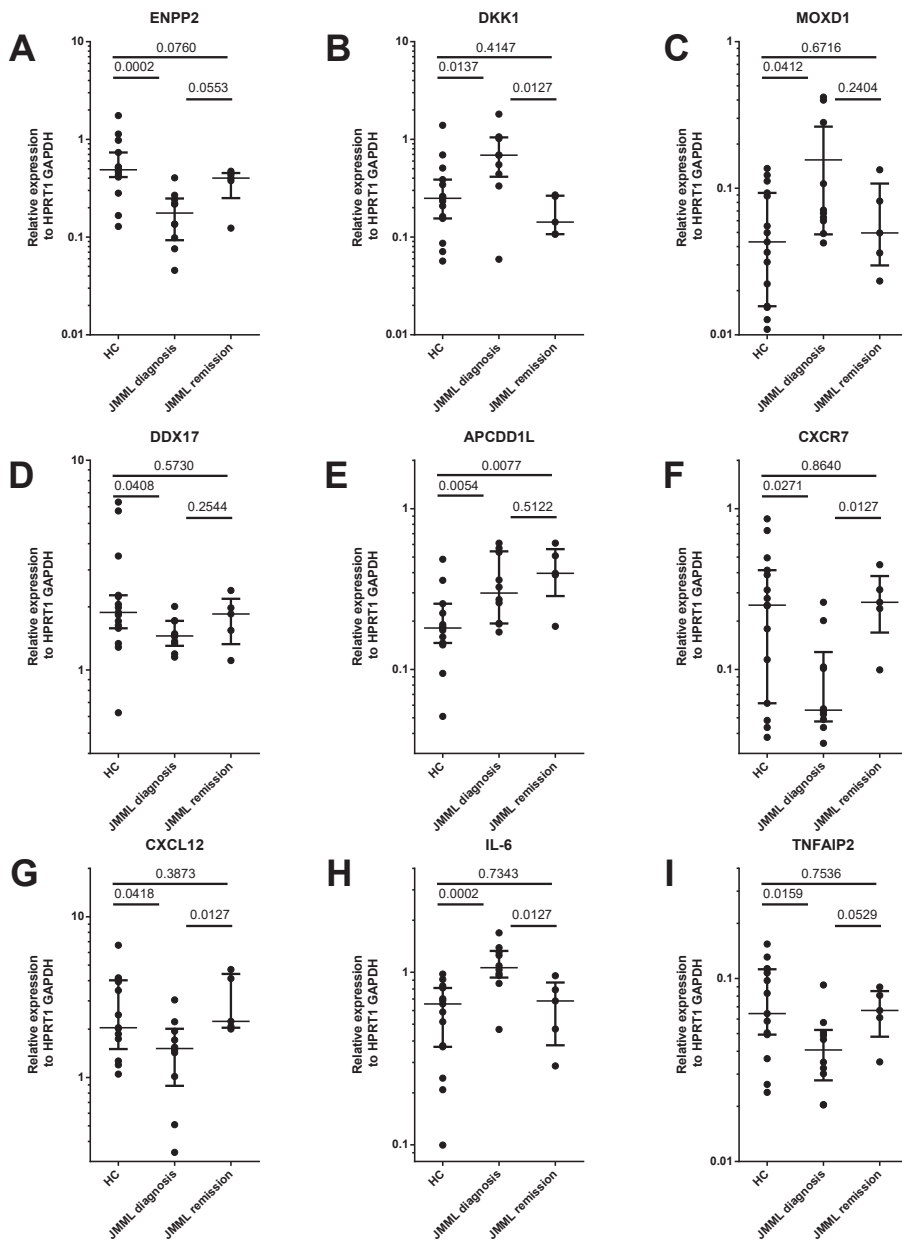
Figure 3. *Gene expression of differentially expressed genes in MSCs of JMML patients at time of diagnosis and after HSCT.* Genes identified by Deep-SAGE sequencing were validated by RT-qPCR in JMML-MSCs expanded from bone-marrow samples obtained prior to (n=9) and after HSCT at time of remission (n=5) and in MSCs derived from healthy donors (HC, n=10). Expression levels were expressed relative to that of the average of the housekeeping genes *HPRT1* and *GADPH*. The results are indicated as median (horizontal line) and interquartile range (whiskers) on a logarithmic scale. Statistics were performed using Mann-Whitney tests.

ing the DC marker CD1a on day 5 of co-culture; these cells remained CD14$^+$ (Figure 4B-C). JMML-MSCs had a significantly stronger suppressive effect on monocyte to immature DC differentiation than HC-MSCs. This was supported by the observation that CD163 expression on CD14$^+$ cells, a marker associated with anti-inflammatory monocytes and macrophages, was higher after co-culture with JMML-MSCs (MFI: 49.3 +/- 7.4 SEM) compared to co-culture with HC-MSCs (MFI: 26.6 +/- 4.7 SEM; *p* = 0.03, data not shown).
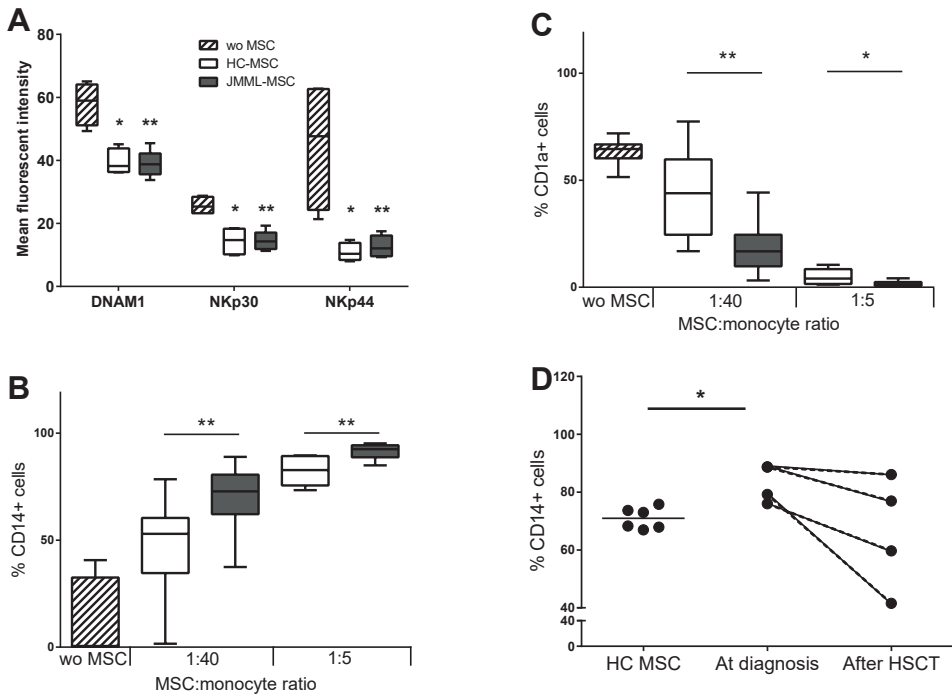
Figure 4. *JMML-MSCs expanded from bone-marrow at diagnosis have an increased suppressive effect on monocyte to immature dendritic cell differentiation but not on NK cell activation.* A. NK cell activation induced by IL-2 (30 IU/mL), measured by mean fluorescence intensity (MFI) of staining for DNAM-1, NKp30 and NKp44 expression on day 5, was suppressed in the presence of MSCs of healthy controls (HC) and JMML patients. MSC: NK cell ratio 1:5 (JMML-MSCs n=4 and HC-MSCs n=4). B-C. An increased suppressive effect of JMML-MSCs was observed on the differentiation of monocytes (CD14$^+$ cells, B) to immature dendritic cells (CD1a$^+$ cells, C) during 5 days of stimulation with IL-4 and GM-CSF (JMML-MSCs n=6, grey boxes, and HC-MSCs n=6, white boxes). D) JMML-MSCs derived from bone-marrow after hematopoietic stem cell transplantation (HSCT) showed a trend towards a lower suppressive effect on the monocyte to immature DC differentiation than JMML-MSCs derived from bone-marrow obtained at diagnosis. Paired samples of JMML-MSCs at diagnosis and after HSCT (n=2) and HC-MSCs (n=3) were investigated using monocytes from two donors as target populations (MSC : monocyte ratio 1:40). Results in panel A-C are depicted as mean (horizontal line) with interquartile distance (boxes) and range (whiskers). Statistics were performed using Mann-Whitney tests in panel A-D. *: *p*<0.05; **: *p*<0.01; wo: without MSC.

The differential suppressive effect of JMML-MSCs compared to HC-MSCs disappeared after two additional days in culture with growth-factors (IFN-γ and CD40-L) to support the differentiation of immature DCs to mature DCs (data not shown).

Gene-expression, *i.e. IL-6*, in MSCs obtained after HSCT was comparable to healthy controls. Therefore, the effect of JMML-MSCs expanded from BM after HSCT on monocyte to immature DC differentiation was studied. The percentage of CD14^{+} cells in co-cultures of monocytes with JMML-MSCs derived from BM obtained after HSCT during remission was variable and not different from that in co-cultures with HC-MSCs. In addition, MSC derived during remission showed a trend to a lower percentage of CD14^{+} cells compared to the percentage observed in co-cultures with JMML-MSC at diagnosis.(Figure 4D).

Discussion

A genetic mutation in the RAS-pathway is identified in 90% of JMML patients.(de Vries, 2010) However, the etiology of therapy resistance and the high relapse rate after HSCT remains unknown. The aggressive nature of the disease in combination with the high burden of malignant cells may lead to a disturbance of the BM micro-environment.

As a model for the BM microenvironment, we used BM derived MSCs. In our study, MSCs of JMML patients had a comparable differentiation capacity compared to MSCs of healthy controls. In contrast to previously reported studies in adult MDS, adipocyte and osteoblast differentiation was not adversely affected.(Zhao, 2014) This is in line with the unaffected differentiation capacity of MSCs derived from BM of children with MDS.(Calkoen, 2015) However, gene expression analysis revealed increased *DKK1* expression in JMML derived MSCs. *DKK1*, related with osteolysis and *WNT* signaling, has been described to be upregulated in MSCs derived from patients with multiple myeloma.(Corre, 2007) Identification of this protein, also expressed by multiple myeloma cells,(Qian, 2007) led to therapeutic strategies using antibodies or vaccination against *DKK1* with a beneficial effect in murine models.(Heath, 2009; Qian, 2012)

The control group differed from the patients regarding age at BM collection. However, data were validated using selected controls aged 1 to 4 years (n=4). We did not observe an age dependent effect on gene-expression in our patient or control MSCs.

In this study, the suppressive effect of JMML-MSCs on differentiation from monocytes to immature DCs was significantly stronger compared to MSCs of healthy controls. The increased *IL-6* expression by JMML-MSCs suggests a causal relation because IL-6 has been described to be essential in the suppressive mechanism.(Melief, 2013) Previously, pediatric MDS derived MSCs showed an even stronger increase in

IL-6 expression, whereas no effect on monocyte to DC differentiation was observed. (Calkoen, 2015) Inhibition of differentiation towards professional antigen presenting cells might contribute to the escape of JMML cells from the immune system and might explain the usually progressive course of this disease. NK cells are involved in the innate defense against malignant transformed cells. Whereas IL-6 production has been reported to suppress NK cell cytotoxicity against neuroblastoma and lymphoblastoid cells, facilitating tumor escape,(Xu, 2013; Tanner, 1991) the suppressive effect of MSCs on NK cell activation was comparable between JMML-MSCs and HC-MSCs. In previous studies, we demonstrated a correlation between the suppressive effect of MSCs on NK cell activation marker expression, *i.e.* DNAM-1, NKp30 and NKp44, and cytotoxicity.(Calkoen, 2013a)

   JMML clinically presents with anemia and thrombocytopenia in combination with monocytosis and leukocytosis. Previously, the misbalance in hematopoiesis in patients with hematologic malignancies was suggested to be caused by factors excreted by malignant cells.(Aoyagi, 1994) In addition, disturbed support of hematopoiesis by MSCs might contribute to dysplasia in these patients. *CXCL12* expression was decreased in JMML-MSCs as was also previously shown in studies on MSC from adult CML and pediatric ALL patients.(van den Berk, 2014; Zhang, 2012b) The CXCL12-CXCR4 interaction is an important mechanism in hematopoietic support and decreased *CXCL12* expression has been linked to dysplasia in mice and adult chronic myeloid leukemia.(Zhang, 2012b; Arranz, 2014) Our *in vitro* hematopoiesis experiments did not reveal differences in support of proliferation and differentiation of HPCs between JMML and control derived MSCs. Both JMML and healthy control MSCs have a strong supportive effect on hematopoiesis involving multiple pathways, *e.g.* CXCL12, G-CSF and SCF. Therefore, aberrant expression of one of these molecules could be compensated by other pathways in our *in vitro* experiments. In this study, we used *in vitro* and polyclonal expanded MSCs and as a consequence differences *in vivo* between healthy controls and JMML derived MSCs might be lost during culture. However, Zhang *et al.* demonstrated sustained decreased expression of *CXCL12 in vivo and* in *in vitro* expanded MSCs.(Zhang, 2012b) Further *in vivo* characterization of the MSCs in mice models modelling the hematopoietic niche with human MSCs, such as previously used in *e.g.* multiple myeloma, might help to identify more subtle differences.(Groen, 2012)

   The different gene expression profile observed in JMML-MSCs versus HC-MSCs is more likely the consequence than the cause of the disease, with JMML cells interacting with the stromal compartment. This conclusion is supported by the observation that after allogeneic HSCT, in which hematopoietic progenitor cells become of donor origin, gene expression profiles in MSCs normalized in paired samples analysis. Of

note, the MSCs after HSCT remain of patient origin, suggesting that the aberrant MSC gene expression pattern before HSCT is induced by JMML cells.

In conclusion, our data demonstrate an effect of JMML cells on MSCs during active disease. *In vitro* hematopoietic support and maintenance of CD34+ HPCs by JMML-MSCs were not affected despite differential gene expression. However, immunosuppression was affected via a stronger decrease of monocyte to immature DC differentiation by JMML-MSC.

Acknowledgements:

Supplementary data

Methods for online publication

Patients

Children referred to our center for HSCT were included in this study according to a protocol approved by the institutional review board (P08.001). Bone marrow of 9 children with JMML was collected prior to treatment initiation. In addition, bone marrow after HSCT was collected from 5 of these 9 children. The patients were classified following the criteria described by Loh *et al.*(Loh, 2011) Bone marrow samples were sent to the JMML-reference center in Freiburg, Germany for genetic analysis. Bone marrow samples of healthy pediatric hematopoietic stem cell donors (n=10) were used as control group (HC). Informed consent was obtained from the children and/or their parents or guardians. This study was conducted according to the Declaration of Helsinki.(World Medical Association, 2013)

MSC expansion and characterization

MSCs were expanded and characterized as previously described.(Calkoen, 2013a) Briefly, bone marrow mononuclear cells (MNC) obtained after Ficoll separation were cultured in DMEM (Invitrogen, Paisley, UK) containing 100 U/mL penicillin/100 µg/mL streptomycin (P/S; Invitrogen) and 10% (v/v) fetal bovine serum (FBS; VWR International, Bridgeport, NJ, USA). Non-adherent cells were removed by refreshing medium twice weekly. Upon reaching confluence MSC were harvested and passaged for further expansion. Phenotype (CD73, CD90, CD105 positive; CD3, CD31, CD34, CD45, CD86, HLA-DR negative) and differentiation capacity towards osteoblasts and adipocytes were investigated at passage 2-3 and 5-7, respectively. All but anti-CD105 (Ancell Corporation Bayport, MN) antibodies were derived from Becton Dickinson Biosciences (BD), San Diego, CA, USA.

To investigate common chromosome abnormalities in MSCs and malignant cells, interphase fluorescence in situ hybridization (FISH) for chromosome 7 was performed on MSC from patients with known monosomy 7 using the Vysis LSI D7S486/CEP7 (Abbott Laboratories, Abbott Park, IL,USA) probe.(Bronkhorst, 2011) Chimerism of MSCs obtained after HSCT (donor or recipient origin) was studied by variable number of cytosine adenine (CA)-repeat analysis in MSCs cultured from bone marrow harvested after HSCT as previously described.(Lankester, 2010) MSC function was investigated using MSCs obtained at passage 3 to 5 and MSC gene expression was analyzed using MSCs obtained at passage 2 to 3.

Immunomodulatory assays

Effect of MSCs on PHA-induced PBMC proliferation

The effect of MSCs (30-Gy irradiated) on proliferation of peripheral blood mono-nuclear cells (PBMC) obtained from adult bloodbank donors (100.000 cells/well) after stimulation with phytohemagglutinin (PHA, PeproTech, London, UK, 2 µg/mL) was analyzed at MSC : PBMC ratios of 1:5 and 1:40. MSCs and PBMC were co-cultured in RPMI P/S, 10% (v/v) fetal calf serum (FCS) for 5 days with the addition of ^{3}H-thymidine (1 µCi/well; Perkin Elmer, Wellesley, MA, USA) for the last 16 hours to measure pro-liferation using a β-counter (Perkin Elmer). Experiments were performed in triplicate.

Effect of MSCs on NK cell activation

The suppressive effect of MSCs on NK cell activation was determined using NK cells isolated from PBMC of bloodbank donors with manual MACS cell separation technol-ogy and negative selection (Miltenyi Biotec, Bergisch Gladbach, Germany). NK cell purity (CD56$^+$CD3$^-$ cells, using anti-CD3-PerCPC5.5 and anti-CD56-APC (Immunotech, Marseille, France)) was over 95%. NK cells (0.1 x 10^6/well) were cultured with (0.02 x 10^6/well) or without MSCs in the presence of IL-2 (30 IU/mL, Chiron Corporation, Emeryville, CA, USA) for 5 days. NK cells were harvested and activation was measured by flow cytometry investigating the mean fluorescence intensity (MFI) of DNAM1 (BD), NKp30 (Immunotech) and NKp44 (Immunotech) expression using PE-labelled antibodies.

Effect of MSCs on monocyte differentiation

To evaluate the effect of MSCs on antigen presenting cells, monocytes were iso-lated from PBMC of blood bank donors using positive CD14 selection (Miltenyi Biotec) and cultured with IL-4 (40 ng/mL) and GM-CSF (800 IU/mL) (both from Tebu-Bio, Le Perray en Yvelines, France) for 5 days to differentiate towards immature dendritic cells (DC). Cells were harvested or cultured for 2 additional days with IL-4, GM-CSF, IFN-γ (500 U/mL, Boehringer, Mannheim, Germany) and CD40-ligand (0.25 µg/mL Beckman-Coulter, Marseille, France) to generate mature DC. Cells were phenotyped by flow cytometry for the expression of CD14 and CD1a (both antibodies from BD) on day 0, day 5 and day 7 after co-culturing of monocytes and MSC at MSC : monocyte ratios 1:5 or 1:40 or after culturing monocytes without MSCs.

In vitro hematopoietic support of MSCs

Short-term co-culture assays with MSCs and hematopoietic progenitor cells (HPC) were performed to determine the supportive capacity of MSCs for HPC maintenance and differentiation. Therefore, HPC were isolated from remaining material of G-CSF

mobilized stem cell grafts from healthy transplant donors using CD34 positive selection (Miltenyi). After purification, >90% of selected cells expressed CD34. To investigate the effect of MSCs on proliferation of CD34^{+} cells, short-term cultures of 1000 CD34 selected cells/well without or with MSCs (MSC : CD34^{+} cell ratios 1:1 and 10:1) were performed in Stemspan medium (H3000, StemCell Technologies, Vancouver, Canada) with addition of 1% P/S, stem cell factor (SCF, 100 ng/mL, StemCell Technologies) and Flt3-ligand (Flt3-L, 100 ng/mL, StemCell Technologies), because SCF and Flt3-L are not produced by MSCs. To investigate the effect of MSCs on differentiation of CD34^{+} cells, cultures were initiated with 10×10^3 CD34^{+} cells at a CD34^{+} cell : MSC ratio of 1:5. Half of the culture medium was refreshed with the addition of growth factors on day 4, 7 and 11. Proliferation (day 7) and differentiation (day 7 and 14) were assessed using ^{3}H-thymidine during the last 16 hours or flow cytometry, respectively. Antibodies used for flow cytometry were anti-CD13-PE, anti-CD14-FITC, anti-CD33-APC, anti-CD34-PE, anti-CD45-FITC, anti-CD45-Percpc5.5, anti-CD163-PE and anti-CD235a-PE (all antibodies from BD).

To determine a direct effect of MSCs on HPC differentiation into colony-forming units (CFU), MSC (30.000 per dish) were added to freshly purified HPC (500 cells/dish) in methylcellulose containing SCF, GM-CSF, IL-3 and EPO (H4434 StemCell Technologies), and cultured for 14 days (CFU-assay). Colonies were scored by two independent observers according to standard guidelines for the definition of CFU-GEMM, BFU-e, CFU-GM, CFU-G and CFU-M. Results are depicted as the average of duplicate dishes. After scoring of colonies in the CFU-assay, cells were harvested and phenotyped for the expression of CD45, CD14 and CD235a by flow cytometry as described above.

Gene expression

Total RNA was isolated from MSCs obtained at passage 2 to 3 using a Qiagen RNeasy Minikit (Qiagen, Hilden, Germany). mRNA was profiled using Deep-SAGE sequencing using Illumina technology.(Mastrokolias, 2012) CATG was added to the 5' end of the 17 base pair sequences obtained. Data were mapped against the UCSC hg19 reference genome using Bowtie for Illumina (version 1.1.2) with the permission of one mismatch and suppression of reads if more than one best match existed. Tags aligned to the same gene were summed for further analysis. Gene information was added to the sequences with the biomaRt package in R (version 2.16.0).

Expression of genes of interest was validated using independent biological samples by RT-qPCR after generation of cDNA (cDNA synthesis kit, Roche, Basel, Switzerland) using the listed primers (Supplementary Table S1), as previously described.(Mastrokolias, 2012) Expression levels were calculated relative to expression of the housekeeping genes *GAPDH* and *HPRT1*.

Statistical analysis

Graphpad 6 (Prism, La Jolla, CA) was used for data-analysis. Mann-Whitney and Wilcoxon matched-pairs signed rank tests were performed to compare different groups in functional assays. Validation of gene-expression amongst the different groups was compared using Mann Withney tests. Differential gene expression analysis was performed using the following data analysis packages in R (version 2.15.0): EdgeR (version 3.2.4) for data normalization, Globaltests (version 5.12.0) for gene-ontology, and Limma (version 3.16.7) for correction of multiple testing performed according to Benjamini and Hochberg.(Robinson, 2010; R Development Core Team, 2012; Smyth, 2005; Hochberg, 1990) STRING version 9.1 software was used for analysis of protein interaction (string-db.org).(Franceschini, 2013) Adjusted *p*-values <0.05 were considered statistically significant.

Supplementary Table 1. Primer design for RT-PCR

ENPP2-forward	CAGCATCATCACCAGCTGTC
ENPP2-reverse	ATTGCAGCTCTCCTCGTTGT
DKK1-forward	TCCGAGGAGAAATTGAGGAA
DKK1-reverse	CCTGAGGCACAGTCTGATGA
MOXD1-forward	TGCTGAGTGGTCGATTCAAG
MOXD1-reverse	TGCAGGGAAGAGGAAGAAGA
DDX17-forward	TCACAGAGCTCTAGCCAGCA
DDX17-reverse	CAGTCTGCCCCATGTAACCT
APCDD1L-forward	GCAGCTCAGCTTTCCTGAGT
APCDD1L-reverse	CCCGGGAAAACTGGATTTAT
CXCR7-forward	GGCTATGACACGCACTGCTA
CXCR7-reverse	CTCATGCACGTGAGGAAGAA
CXCL12-forward	AGAGCCAACGTCAAGCATCT
CXCL12-reverse	CTTTAGCTTCGGGTCAATGC
IL-6-forward	GAAAGCAGCAAAGAGGCACT
IL-6-reverse	TTTCACCAGGCAAGTCTCCT
TNFAIP2-forward	CCTATTGCCGTGACAGGTTT
TNFAIP2-reverse	CTCCAGAAGGAGTGCAGGAC
HPRT1-forward	TGACACTGGCAAAACAATGCA
HPRT1-reverse	GGTCCTTTTCACCAGCAAGCT
GAPDH-forward	GGCCTCCAAGGAGTAAGACC
GAPDH-reverse	AGGGGAGATTCAGTGTGGTG

Supplementary Table 2. Differential expression between healthy control and JMML MSC

Ensembl gene id	Gene symbol	p-value	FDR	Average expression Healthy control	Average expression JMML	Analyzed[1]	Function[2]
ENSG00000130592	LSP1	4.67E-10	8.14E-06	1483	479	No available primer set	Adhesion
ENSG00000136960	ENPP2	5.65E-10	8.14E-06	365	168	Yes	Cell proliferation, chemotaxis
ENSG00000127951	FGL2	2.02E-09	1.94E-05	4	111	<100	Unknown
ENSG00000125851	PCSK2	1.91E-07	1.38E-03	206	7	<100	Protein processing
ENSG00000185215	TNFAIP2	3.52E-07	1.71E-03	479	154	Yes	Inflammation
ENSG00000138135	CH25H	3.57E-07	1.71E-03	66	6	<100	Cholesterol and lipid metabolism
ENSG00000010610	CD4	5.18E-07	2.13E-03	85	11	<100	Immunology
ENSG00000125538	IL1B	8.59E-07	3.09E-03	3	56	<100	Inflammation
ENSG00000230750	SDAD1P2	9.63E-07	3.09E-03	0	10	<100	Pseudogene
ENSG00000164764	SBSPON	1.88E-06	5.38E-03	1	89	<100	Polysaccharide binding
ENSG00000107984	DKK1	2.05E-06	5.38E-03	268	783	Yes	WNT inhibitor
ENSG00000079931	MOXD1	2.41E-06	5.79E-03	130	414	Yes	Copper binding, dopamine activity
ENSG00000065485	PDIA5	4.44E-06	9.31E-03	757	1729	No function	Unknown
ENSG00000226920		4.52E-06	9.31E-03	9	0	<100	Unknown
ENSG00000100201	DDX17	6.27E-06	1.20E-02	3361	1300	Yes	RNA processing
ENSG00000198768	APCDD1L	6.68E-06	1.20E-02	683	1265	Yes	Membrane protein
ENSG00000183092	BEGAIN	7.44E-06	1.26E-02	1	24	<100	Neuronal cell body
ENSG00000143882	ATP6V1C2	1.01E-05	1.62E-02	406	710	No	ATPase
ENSG00000123500	COL10A1	1.23E-05	1.79E-02	142	4	<100	Ossification

Supplementary Table 2. Differential expression between healthy control and JMML MSC (continued)

Ensembl gene id	Gene symbol	p-value	FDR	Average expression Healthy control	Average expression JMML	Analyzed[1]	Function[2]
ENSG00000138735	PDE5A	1.24E-05	1.79E-02	279	667	No	Phosphodiesteras
ENSG00000108950	FAM20A	1.39E-05	1.91E-02	1248	461	No function	Unknown
ENSG00000082684	SEMA5B	1.73E-05	2.14E-02	0	14	<100	Axon growth
ENSG00000079215	SLC1A3	1.77E-05	2.14E-02	312	79	<100	Neurotransmission
ENSG00000181195	PENK	1.78E-05	2.14E-02	1547	18076	Based on one outlier	Neuropeptide activity
ENSG00000133106	EPSTI1	2.40E-05	2.76E-02	219	63	<100	Epithelial stroma interaction
ENSG00000239648	MTND1P3	2.69E-05	2.98E-02	127	24	<100	Pseudogene
ENSG00000206066	IGLL3P	3.00E-05	3.14E-02	0	9	<100	Pseudogene
ENSG00000234745	HLA-B	3.05E-05	3.14E-02	0	12	<100	Immunology
ENSG00000196139	AKR1C3	3.23E-05	3.21E-02	423	114	No	Aldo/keto reductase
ENSG00000118515	SGK1	3.55E-05	3.41E-02	1083	1929	No	Stress response
ENSG00000170323	FABP4	3.83E-05	3.49E-02	1	76	<100	Fatty acid metabolism
ENSG00000120885	CLU	4.15E-05	3.49E-02	2981	24019	Based on one outlier	Protein binding
ENSG00000163975	MFI2	4.17E-05	3.49E-02	138	522	No	Glycoprotein
ENSG00000114423	CBLB	4.37E-05	3.49E-02	535	244	No	Proto-oncogene
ENSG00000003137	CYP26B1	4.40E-05	3.49E-02	3	43	<100	Lipid metabolism
ENSG00000198113	TOR4A	4.52E-05	3.49E-02	63	186	<100	Protein folding
ENSG00000254366		4.58E-05	3.49E-02	11	0	<100	Unknown
ENSG00000170801	HTRA3	4.59E-05	3.49E-02	171	57	<100	Protease
ENSG00000164484	TMEM200A	5.36E-05	3.85E-02	359	671	No function	Unknown

Supplementary Table 2. Differential expression between healthy control and JMML MSC (continued)

Ensembl gene id	Gene symbol	p-value	FDR	Average expression Healthy control	Average expression JMML	Analyzed[1]	Function[2]
ENSG00000227825	SLC9A7P1	5.36E-05	3.85E-02	5	0	<100	Pseudogene
ENSG00000255198	SNHG9	5.48E-05	3.85E-02	0	21	<100	RNA gene
ENSG00000196352	CD55	7.09E-05	4.78E-02	142	281	No	Complement activation
ENSG00000104332	SFRP1	7.12E-05	4.78E-02	105	795	No	WNT pathway
ENSG00000095380	NANS	8.43E-05	5.53E-02	1821	2588	No	
ENSG00000248874	C5orf17	8.97E-05	5.54E-02	8	0	No	
ENSG00000236318		9.17E-05	5.54E-02	0	12	No	
ENSG00000118855	MFSD1	9.37E-05	5.54E-02	1048	1467	No	
ENSG00000161980	POLR3K	9.42E-05	5.54E-02	477	703	No	
ENSG00000079385	CEACAM1	1.03E-04	5.89E-02	0	16	No	
ENSG00000046604	DSG2	1.04E-04	5.89E-02	6	0	No	
ENSG00000154188	ANGPT1	1.12E-04	5.95E-02	1424	668	No	
ENSG00000171819	ANGPTL7	1.14E-04	5.95E-02	1	46	No	
ENSG00000166592	RRAD	1.15E-04	5.95E-02	80	174	No	
ENSG00000101000	PROCR	1.15E-04	5.95E-02	379	1050	No	
ENSG00000106823	ECM2	1.16E-04	5.95E-02	1290	542	No	
ENSG00000078725	BRINP1	1.20E-04	6.05E-02	1544	510	No	
ENSG00000109323	MANBA	1.30E-04	6.47E-02	607	948	No	
ENSG00000091483	FH	1.59E-04	7.73E-02	539	762	No	
ENSG00000117152	RGS4	1.63E-04	7.73E-02	1369	3147	No	
ENSG00000104412	EMC2	1.67E-04	7.73E-02	1010	1378	No	

Supplementary Table 2. Differential expression between healthy control and JMML MSC (continued)

Ensembl gene id	Gene symbol	p-value	FDR	Average expression Healthy control	Average expression JMML	Analyzed[1]	Function[2]
ENSG00000140750	ARHGAP17	1.67E-04	7.73E-02	649	918	No	
ENSG00000187720	THSD4	1.70E-04	7.73E-02	85	209	No	
ENSG00000106809	OGN	1.72E-04	7.73E-02	4	57	No	
ENSG00000010278	CD9	1.77E-04	7.78E-02	266	748	No	
ENSG00000145908	ZNF300	1.80E-04	7.78E-02	45	8	No	
ENSG00000119899	SLC17A5	1.86E-04	7.78E-02	246	370	No	
ENSG00000116337	AMPD2	1.87E-04	7.78E-02	919	1268	No	
ENSG00000185803	SLC52A2	1.87E-04	7.78E-02	1303	1840	No	
ENSG00000171067	C11orf24	1.89E-04	7.78E-02	1874	2677	No	
ENSG00000117616	RSRP1	1.97E-04	8.02E-02	1894	730	No	
ENSG00000131732	ZCCHC9	2.01E-04	8.04E-02	369	188	No	
ENSG00000143333	RGS16	2.07E-04	8.17E-02	50	311	No	
ENSG00000165409	TSHR	2.13E-04	8.29E-02	66	10	No	
ENSG00000197406	DIO3	2.21E-04	8.50E-02	1	37	No	
ENSG00000103342	GSPT1	2.35E-04	8.90E-02	1312	1835	No	
ENSG00000138834	MAPK8IP3	2.42E-04	9.05E-02	325	137	No	
ENSG00000228300	C19orf24	2.51E-04	9.20E-02	246	376	No	
ENSG00000112096	SOD2	2.68E-04	9.66E-02	247	446	No	
ENSG00000127418	FGFRL1	2.84E-04	9.98E-02	933	1639	No	
ENSG00000137497	NUMA1	2.96E-04	9.98E-02	919	444	No	
ENSG00000108602	ALDH3A1	3.01E-04	9.98E-02	3	53	No	
ENSG00000250569	NTAN1P2	3.01E-04	9.98E-02	5	0	No	

Supplementary Table 2. Differential expression between healthy control and JMML MSC (continued)

Ensembl gene id	Gene symbol	p-value	FDR	Average expression Healthy control	Average expression JMML	Analyzed[1]	Function[2]
ENSG00000163798	SLC4A1AP	3.06E-04	9.98E-02	425	588	No	
ENSG00000036672	USP2	3.08E-04	9.98E-02	21	63	No	
ENSG00000164414	SLC35A1	3.15E-04	9.98E-02	110	45	No	
ENSG00000145020	AMT	3.15E-04	9.98E-02	145	41	No	
ENSG00000183421	RIPK4	3.15E-04	9.98E-02	17	68	No	
ENSG00000184465	WDR27	3.15E-04	9.98E-02	303	114	No	
ENSG00000104549	SQLE	3.31E-04	1.02E-01	710	1004	No	
ENSG00000157168	NRG1	3.36E-04	1.02E-01	325	106	No	
ENSG00000170498	KISS1	3.36E-04	1.02E-01	5	0	No	
ENSG00000231290	APCDD1L-AS1	3.37E-04	1.02E-01	56	122	No	
ENSG00000185633	NDUFA4L2	3.39E-04	1.02E-01	642	1629	No	
ENSG00000163682	RPL9	3.55E-04	1.05E-01	56	13	No	
ENSG00000164344	KLKB1	3.56E-04	1.05E-01	10	0	No	
ENSG00000101843	PSMD10	3.64E-04	1.05E-01	1141	1499	No	
ENSG00000136826	KLF4	3.65E-04	1.05E-01	2246	996	No	
ENSG00000103196	CRISPLD2	3.67E-04	1.05E-01	749	223	No	
ENSG00000149547	EI24	3.90E-04	1.10E-01	732	988	No	
ENSG00000112981	NME5	4.01E-04	1.12E-01	169	62	No	
ENSG00000102471	NDFIP2	4.07E-04	1.13E-01	357	515	No	
ENSG00000099889	ARVCF	4.14E-04	1.14E-01	495	237	No	
ENSG00000162804	SNED1	4.20E-04	1.14E-01	621	246	No	
ENSG00000123572	NRK	4.28E-04	1.14E-01	31	3	No	

Supplementary Table 2. Differential expression between healthy control and JMML MSC (continued)

Ensembl gene id	Gene symbol	p-value	FDR	Average expression Healthy control	Average expression JMML	Analyzed[1]	Function[2]
ENSG00000227502	LINC01268	4.28E-04	1.14E-01	5	0	No	
ENSG00000132688	NES	4.40E-04	1.16E-01	58	179	No	
ENSG00000163347	CLDN1	4.44E-04	1.17E-01	22	77	No	
ENSG00000184924	PTRHD1	4.54E-04	1.17E-01	1405	1880	No	
ENSG00000198682	PAPSS2	4.57E-04	1.17E-01	2291	3753	No	
ENSG00000164106	SCRG1	4.77E-04	1.20E-01	1223	2416	No	
ENSG00000143248	RGS5	4.78E-04	1.20E-01	63	221	No	
ENSG00000229729		4.88E-04	1.20E-01	5	0	No	
ENSG00000148450	MSRB2	4.89E-04	1.20E-01	360	182	No	
ENSG00000187514	PTMA	4.96E-04	1.21E-01	210	60	No	
ENSG00000139874	SSTR1	5.04E-04	1.21E-01	5	35	No	
ENSG00000147408	CSGALNACT1	5.05E-04	1.21E-01	53	238	No	
ENSG00000184408	KCND2	5.38E-04	1.28E-01	7	0	No	
ENSG00000114857	NKTR	5.65E-04	1.33E-01	623	302	No	
ENSG00000170266	GLB1	5.76E-04	1.33E-01	1040	1386	No	
ENSG00000105248	CCDC94	5.76E-04	1.33E-01	1	9	No	
ENSG00000155363	MOV10	5.80E-04	1.33E-01	180	80	No	
ENSG00000105426	PTPRS	5.92E-04	1.34E-01	401	210	No	
ENSG00000196616	ADH1B	5.94E-04	1.34E-01	18	238	No	
ENSG00000130600	H19	5.99E-04	1.34E-01	92	452	No	
ENSG00000136244	IL6	6.14E-04	1.36E-01	1658	2495	Yes	
ENSG00000125430	HS3ST3B1	6.21E-04	1.37E-01	122	49	No	

Supplementary Table 2. Differential expression between healthy control and JMML MSC (continued)

Ensembl gene id	Gene symbol	p-value	FDR	Average expression Healthy control	Average expression JMML	Analyzed[1]	Function[2]
ENSG00000110328	GALNT18	6.31E-04	1.38E-01	90	192	No	
ENSG00000179091	CYC1	6.43E-04	1.39E-01	2716	3739	No	
ENSG00000174109	C16orf91	6.45E-04	1.39E-01	220	310	No	
ENSG00000129250	KIF1C	6.49E-04	1.39E-01	945	1228	No	
ENSG00000254187		6.58E-04	1.39E-01	0	5	No	
ENSG00000261857	MIA	6.65E-04	1.40E-01	8	62	No	
ENSG00000179403	VWA1	6.78E-04	1.41E-01	227	96	No	
ENSG00000251349	MSANTD3-TMEFF1	6.78E-04	1.41E-01	275	388	No	
ENSG00000087494	PTHLH	6.92E-04	1.42E-01	17	62	No	
ENSG00000185608	MRPL40	6.98E-04	1.43E-01	799	1038	No	
ENSG00000258498	DIO3OS	7.05E-04	1.43E-01	4	72	No	
ENSG00000171813	PWWP2B	7.41E-04	1.49E-01	159	69	No	
ENSG00000150627	WDR17	7.72E-04	1.54E-01	1	15	No	
ENSG00000102760	RGCC	7.75E-04	1.54E-01	56	127	No	
ENSG00000231231	LINC01423	7.84E-04	1.55E-01	72	8	No	
ENSG00000121039	RDH10	7.96E-04	1.56E-01	136	290	No	
ENSG00000154174	TOMM70A	8.16E-04	1.59E-01	814	1047	No	
ENSG00000184232	OAF	8.20E-04	1.59E-01	188	294	No	
ENSG00000177542	SLC25A22	8.33E-04	1.59E-01	277	391	No	
ENSG00000186469	GNG2	8.38E-04	1.59E-01	108	196	No	
ENSG00000145337	PYURF	8.47E-04	1.59E-01	2593	3524	No	

Supplementary Table 2. Differential expression between healthy control and JMML MSC (continued)

Ensembl gene id	Gene symbol	p-value	FDR	Average expression Healthy control	Average expression JMML	Analyzed[1]	Function[2]
ENSG00000021762	OSBPL5	8.56E-04	1.59E-01	171	75	No	
ENSG00000156042	CFAP70	8.59E-04	1.59E-01	87	12	No	
ENSG00000153291	SLC25A27	8.60E-04	1.59E-01	175	59	No	
ENSG00000197635	DPP4	8.62E-04	1.59E-01	179	397	No	
ENSG00000151929	BAG3	8.90E-04	1.63E-01	658	870	No	
ENSG00000145901	TNIP1	9.05E-04	1.65E-01	1059	1366	No	
ENSG00000103449	SALL1	9.11E-04	1.65E-01	0	9	No	
ENSG00000239382	ALKBH6	9.16E-04	1.65E-01	52	11	No	
ENSG00000137965	IFI44	9.20E-04	1.65E-01	155	62	No	
ENSG00000229563	LINC01204	9.53E-04	1.70E-01	5	0	No	
ENSG00000113643	RARS	9.71E-04	1.71E-01	1951	2540	No	
ENSG00000145476	CYP4V2	9.81E-04	1.71E-01	194	87	No	
ENSG00000151376	ME3	9.84E-04	1.71E-01	118	45	No	
ENSG00000144476	ACKR3	9.89E-04	1.71E-01	618	231	Yes = CXCR7	
ENSG00000107159	CA9	9.97E-04	1.71E-01	3	23	No	

p-value of the average of HC and JMML-MSC; average reads expressed as the number of reads per 10^6 reads analyzed
[1] Analyzed: this column indicates if the gene was further analyzed using RT-qPCR, based on FDR, expression threshold >100 copies and function.
[2] Function: a short discription, not intended to be exhaustive, of the function based on gene-ontology and www.genecards.org
HC: healthy control; JMML: juvenile myelomonocytic leukemia; FDR: false discovery rate with threshold $p<0.05$

Supplementary Table 3. GO-term analysis

GO-term	Holm's correction	Alias	p-value	Covariates
GO:0002252	0.0001	immune effector process	8.65E-09	305
GO:0002253	0.0005	activation of immune response	3.27E-08	235
GO:0050778	0.0005	positive regulation of immune response	3.29E-08	283
GO:0007186	0.0006	G-protein coupled receptor signaling pathway	3.98E-08	520
GO:0043254	0.0011	regulation of protein complex assembly	6.57E-08	152
GO:0007005	0.0015	mitochondrion organization	9.59E-08	201
GO:0051091	0.0015	positive regulation of sequence-specific DNA binding transcription factor activity	9.66E-08	161
GO:0002250	0.0027	adaptive immune response	1.67E-07	148
GO:0002460	0.0028	adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	1.77E-07	133
GO:0035270	0.0037	endocrine system development	2.33E-07	120
GO:0002449	0.0038	lymphocyte mediated immunity	2.42E-07	113
GO:0002443	0.0040	leukocyte mediated immunity	2.51E-07	148
GO:0016567	0.0041	protein ubiquitination	2.60E-07	513
GO:0031396	0.0043	regulation of protein ubiquitination	2.70E-07	160
GO:0051260	0.0061	protein homooligomerization	3.84E-07	205
GO:0031625	0.0083	ubiquitin protein ligase binding	5.30E-07	134
GO:0044389	0.0083	small conjugating protein ligase binding	5.30E-07	134
GO:0031398	0.0089	positive regulation of protein ubiquitination	5.64E-07	121

GO: gene-ontology
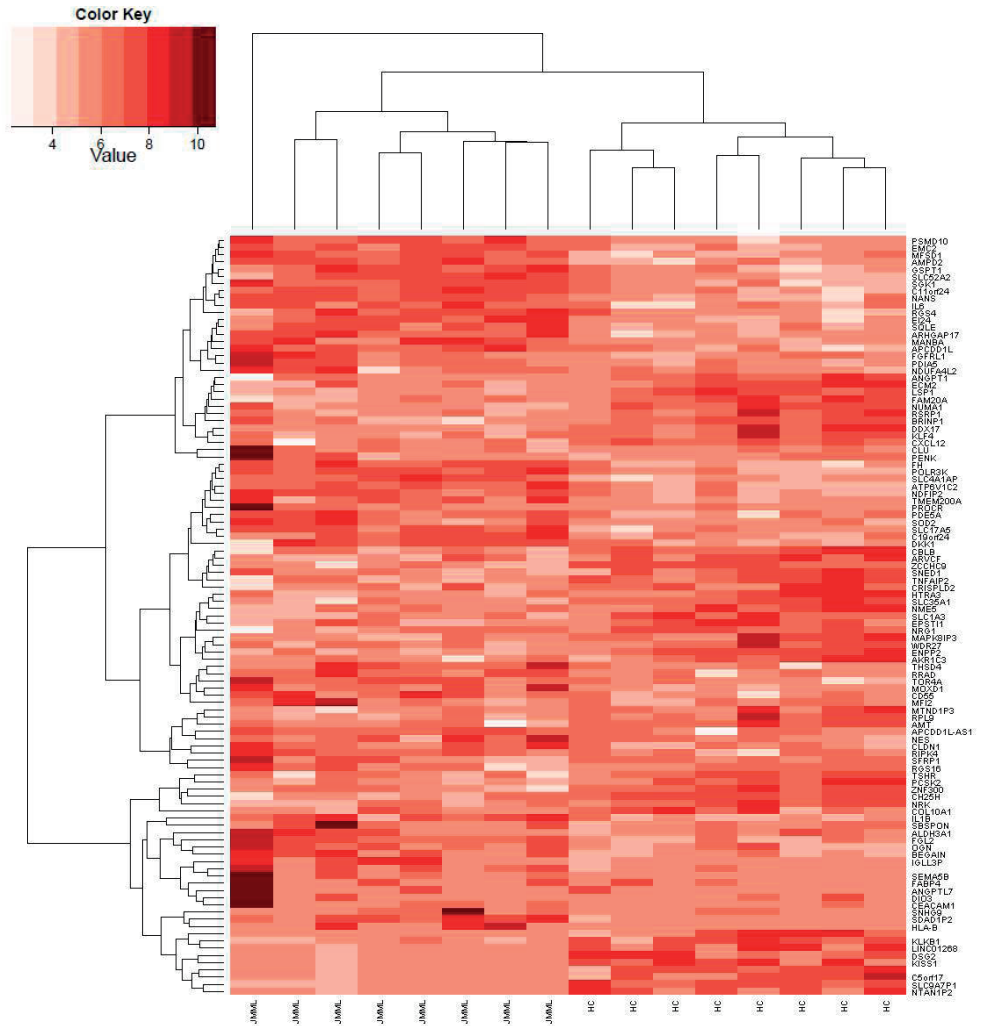
Supplementary Figure 1. *JMML and healthy control (HC) MSCs have a different gene-expression profile.* In this heat-map the 100 differentially expressed genes detected by Deep-SAGE sequencing are depicted demonstrating clustering of healthy control (HC) derived bone-marrow (BM) MSC (n=8) and MSCs derived from BM of JMML patients at diagnosis (n=8). Darker colors correspond with increased expression.

Chapter 5.

Gastrointestinal acute graft-versus-host disease in children: histology for diagnosis, mesenchymal stromal cells for treatment and biomarkers for prediction of response.

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Abstract

Steroid non-responsive acute graft-versus-host disease (aGvHD) following hematopoietic stem cell transplantation carries a poor prognosis. Various groups have reported beneficial effects of mesenchymal stromal cell (MSC) infusion as salvage treatment. Response to treatment is typically evaluated using the diagnostic clinical criteria for aGvHD. In this study we report the usefulness of additional gastrointestinal biopsy specimens paired with serum biomarkers. In a cohort of 22 pediatric patients, persistent or recurrent diarrhea was seen in 18 children treated with MSC infusion for steroid-refractory aGvHD. To exclude other causes of gastrointestinal pathology, patients were biopsied for histological analysis. Eight out of 12 patients exhibited residual tissue damage and villous atrophy, but no active aGvHD. Serum biomarkers have been identified as an alternative tool for monitoring the response to aGvHD treatment. The value of biomarkers for inflammation, tissue and endothelial cell damage was evaluated in our cohort. Although predictive for response to treatment and survival, these markers lack the necessary specificity and sensitivity to predict response to MSC therapy. Objective endpoints for clinical trials on the treatment of steroid-refractory aGvHD remain to be defined. Thus, we recommend including biopsies and biomarkers to discriminate between ongoing aGvHD and post inflammatory malabsorption.

Introduction

Acute graft-versus-host disease (aGvHD) is a major complication after allogeneic hematopoietic stem cell transplantation (HSCT) mediated by alloreactive donor T lymphocytes targeting skin, gut and liver.(Couriel, 2004a; Goker, 2001) Gastrointestinal (GI) aGvHD is clinically graded according to volume of diarrhea, severity of abdominal pain and presence of paralytic ileus.(Ball, 2008c) Children with the most severe forms, namely grade III-IV aGvHD, have substantially reduced survival, especially those refractory to initial treatment with systemic steroids.(Deeg, 2007; Davies, 2009)

Mesenchymal stromal cells (MSCs) are non-hematopoietic cells capable of inducing immunosuppressive and anti-inflammatory effects.(Aggarwal, 2005; Krampera, 2003) MSCs were first administered to a child with steroid refractory aGvHD in 2014, leading to resolution of symptoms.(Le Blanc, 2004) Subsequent studies reported response rates to MSC therapy for aGvHD ranging from 30% to 80% with a trend toward better response in children.(Le Blanc, 2008; Kebriaei, 2009; Prasad, 2011; von Bonin, 2009; Lucchini, 2010) These studies used different end-points to document the response to MSC infusion, such as best ever response(Le Blanc, 2008) or response on day 28 to 32 after treatment initiation.(Kebriaei, 2009; Prasad, 2011; von Bonin, 2009; Lucchini, 2010) One as-yet unpublished study, that randomized patients for treatment with either MSCs and steroids or with steroids only did not confirm these observations.(Martin, 2010)

Nonetheless, in all studies to date irrespective of outcome, response to MSCs treatment has been based upon assessment of clinical parameters, originally developed for the diagnosis of aGvHD.(MacMillan, 2002) As such, multiple MSC infusions have been administered to patients with persistent diarrhea in the absence of confirmatory histological evidence of GI aGvHD. However, persistent diarrhea has been described after resolution of GI aGvHD documented by histology.(Ball, 2008b) In addition, studies of the effect of MSC infusion in other inflammatory bowel disease, such as Crohn's disease, included endoscopic evaluation after MSC infusion as an endpoint of treatment response.(Ciccocioppo, 2011; Duijvestein, 2010) This indicates that the assessment of serial gastrointestinal biopsies might be necessary to determine for evaluating the response to experimental interventions in these patients. Obtaining biopsy specimens might be feasible in all cases, however, and thus alternative predictors of response should be explored.

Serum biomarkers, including IL-8, TNF receptor 1 (TNFr1), IL-2 receptor alpha (IL-2rα), and hepatocyte growth factor (HGF), have been identified as supportive markers to substantiate the diagnosis and predict response to treatment and outcome of aGvHD.(Paczesny, 2009; Paczesny, 2013) In addition, serum concentrations of tissue-specific biomarkers, including regenerating islet derived 3 alpha (REG3α),(Ferrara, 2011) soluble cytokeratin 18 (CK18) fragments (sCK18f)(Luft, 2007) and thrombo-

modulin (sTM)(Luft, 2011) for GI aGvHD and elafin(Paczesny, 2010) for skin aGvHD, have been correlated with local tissue damage. However, the usefulness of these markers has not been investigated systematically or compared with serial biopsy specimens during MSC treatment of steroid-refractory aGvHD.

In our center, a substantial pediatric cohort has been treated with MSCs for steroid-refractory aGvHD involving the gut in an institutional ethically approved non-randomized study. GI biopsy specimens were obtained at the onset of aGvHD and after MSC infusion. The present study focused on the relevance, safety and feasibility of performing GI endoscopy to obtain biopsy specimens during experimental trials on aGvHD, and investigated the added value of different biomarkers in objectively evaluating the effect of MSC infusion.

Patients and materials

Patients

Children diagnosed with grade III/IV steroid refractory aGvHD after allogeneic hematopoietic stem cell transplantation (HSCT) were treated with MSCs (protocol LUMC-MEC: P05-089). For the purpose this study, steroid-refractory aGvHD was defined as progression of symptoms at 72 hours or no improvement or worsening at 7 days after initiation of methylprednisolone (2 mg/kg/day). All eligible pediatric patients (n=22), age 0-18 years, who underwent HSCT in the pediatric stem cell transplant unit of Leiden University Medical Center between January 2005 and November 2012 were included in this study. Five of these patients were previously reported in a multicenter analysis of outcome of MSC therapy for aGvHD.(Le Blanc, 2008) Patients referred to our center solely for MSC infusions only were not included in this analysis. General patient and transplant characteristics are summarized in Table 1. Median duration of follow-up of survivors after HSCT was 379 days (range, 242 to 2708 days).

MSC expansion, characterization and quality control have been described previously.(Le Blanc, 2008) Patients received third party, bone marrow-derived expanded MSCs in doses ranging from 1.0 to 2.0x10e6 MSCs/kg at a median of 12 days after initiation of steroids. A median of 2 (range, 1 to 3) MSC infusions was given. Subsequent MSC infusions were administered in 18 patients for persistence or after recurrent symptoms during reduction of pharmaceutical aGvHD therapies at a median of 14 days (range, 7 to 42 days), with the majority (14 of 18) receiving a subsequent infusion within 28 days after the first MSC infusion. No patient received additional pharmacological immunosuppression after MSC infusion. Six patients with a partial response who remained steroid-dependent, received a third MSC infusion at a median of 50 days (range, 24 to 97 days) after the first MSC infusion.

Table 1. Characteristics of 22 children mith severe gastrointestinal aGvHD

Characteristic		Value
Follow-up (days; median; range)		344 (121 - 2587)
Diagnosis	ALL/AML	9
	Other hematologic malignancies	6
	Nonmalignant disorder	7
Relapse after HSCT		3
Age at HSCT median (range)		6.3 (0.7 - 18.1)
Sex (M/F)		12/10
Donor	HLA-identical related	7
	Matched Unrelated donor	5
	Mismatched Unrelated donor	10
Stem cell source (BM/PBSC/CB)		13/4/5
Donor sex (M/F)		12/10
Conditioning	TBI based MAC	5
	Busulphan based MAC	14
	Other MAC	2
	RIC	1
Serotherapy	+ rATG/Campath	13/3
GvHD prophylaxis	CsA	7
	CsA + MTX	10
	CsA + prednisolon (1 mg/kg)	5
aGvHD organs involved	GI only	2
	GI and skin	10
	GI and liver	5
	GI, skin and liver	5
aGvHD grade	III	14
	IV	8
aGvHD GI stage	2	2
	3	16
	4	4
Median days between start aGvHD and first MSCs		12 (5 - 59)
Other 2nd line therapy	Total	6
	Steroids 3-5 mg/kg	5
	MMF / Tacrolimus	3

ALL acute lymphatic leukemia; AML acute myeloid leukemia; HSCT hematopoietic stem cell transplantation; HLA human leukocyte antigen; BM bone marrow; PBSC peripheral blood stem cells; CB cord blood; TBI total body irradiation; MAC myeloablative condtioning; RIC reduced intensity conditioning; rATG rabbit antithymocyte globulin; GvHD Graft-versus-Host Disease; CsA cyclosporin A; GI: gastrointestinal; MTX methotrexate; MSCs mesenchymal stromal cells; MMF mycophenolate mofetil.

Endoscopy, biopsies and pathology report

The diagnosis of GI aGvHD was histologically confirmed in 19 patients according to published criteria,(Washington, 2009) at 5 days after the first MSC infusion in 1 child and prior to the start of MSC therapy in the other 18 children. Endoscopic procedures to obtain biopsy specimens were performed by 2 experienced pediatric gastroenter-ologists. Five patients underwent 2 endoscopic procedures before MSC infusion, 2 of whom underwent an additional endoscopy after negative aGvHD results obtained by proctoscopy. Biopsy specimens were obtained from the rectum (n=14), the lower GI tract (n=5), or upper and lower GI tracts (n=5). One patient refused endoscopy and in 2 patients no biopsy specimens were obtained during endoscopy because of mas-sive inflammation and ulceration and an increased risk of perforation. All 3 of these patients met the clinical criteria for aGvHD. A liver biopsy was performed in 1 child to confirm liver involvement. Increased bilirubin levels in combination with confirmed GI and or skin aGvHD was considered toindicate hepatic aGvHD in 9 patients in whom other underlying liver pathology (e.g. concomitant viral infections, veno-occlusive disease or drug toxicity) had been excluded.

Patients with persistent or recrudescent of diarrhea after MSC infusion underwent endoscopic to obtain biopsy specimens. Histological examination of these specimens was performed by 2 pathologists with access to clinically relevant details, using apoptosis, necrosis and denudation as characteristics for aGvHD.(Washington, 2009) An expert panel with extensive experience in pediatric GI pathology evaluated all biopsy results. In case of viral infections detected through standard PCR monitoring of serum or plasma for viral reactivation,(Echavarria, 2001) additional staining was performed to exclude viral enteritis.

Definitions

Clinical grading of aGvHD was done according to the adapted Seattle criteria.(Ball, 2008c) The response to MSC treatment was documented at 28 days after the first MSC infusion, as suggested by MacMillan *et al.*.(MacMillan, 2010) A complete response (CR) was defined clinically as resolution of all symptoms of aGvHD; partial response (PR) as improvement by at least 1 overall grade and no response (NR) as worsening of disease or no improvement.

Two different time-points related to the course and initial treatment of aGvHD before MSC infusion were defined: (1) onset of GI aGvHD, and (2) start of systemic methylprednisolone administration.

Biomarkers

Serum samples were collected and stored at -20°C under protocols approved by the local ethical committee (LUMC-MEC P01-028/P03-061). Serum biomarker concentra-

tions were measured in samples obtained before conditioning, on the day of HSCT, and weekly starting 2 weeks before the onset of aGvHD. Additional samples were obtained immediately prior to the first MSC infusion, and weekly thereafter for 4 weeks, followed by 2 weekly sampling for another 4 weeks.

Systemic biomarkers (TNFr1, IL-2rα, HGF and IL-8), organ specific markers (CK18, sCK18F and REG3α), and sTM were monitored in all patients using enzyme linked immunosorbent assays (ELISA) in accordance with the manufacturer's instructions. Manufacturer details, serum dilutions, and lower and upper detection limits are provided in supplementary Table 1 (Table S1).

Statistical analysis

Survival analysis and non-relapse mortality (NRM) were modeled with log-rank Mantel-Cox tests. The Mann-Whitney test was used to compare serum biomarker concentrations among groups with differing response to treatment. Receiving operating curves (ROC) with area under the curve (AUC) were constructed to define the discriminatory value of the biomarkers. Differences in serum biomarker concentrations at various time points were assessed with the paired Wilcoxon signed-rank test. The chi-squared test was performed on categorical data. Graphpad 5 (Prism, La Jolla, USA) and SPSS 20 (IBM, NY, USA) were used for the statistical analyses.

Results

Effect of MSCs and clinical evaluation of included patients

All 22 patients had worsening of symptoms or stable disease after the start of systemic steroid administration (2 mg/kg/day). Only 6 patients received additional immunosuppression with high-dose steroids (5 mg/kg/day), tacrolimus, or mycophenolate mofetil as second-line therapy before MSC infusion; however, no improvement of skin, liver or gut symptoms was seen. The remaining children received only MSC infusion as additional immunosuppressive therapy.

The response documented at day 28 after the first MSC infusion was CR in 11 patients (50%), PR in 6 patients (27%) and NR in 5 patients (23%). Overall survival was significantly better in patients with CR(p=0.027) compared with those with PR and NR (Figure 1A). Patients with CR had a significantly lower NRM (p=0.01) than those with PR and NR, as reported previously (Figure 1B).(Le Blanc, 2008) Response rates categorized according to aGvHD grade, GI stage, and organs affected are summarized in Table S2. The response rate for GI aGvHD was not dependent on the overall aGvHD grade, the stage of GI GvHD, or involvement of other organs. Children with CR were more likely to undergo HSCT for a malignant disease and with a graft from an

HLA-identical sibling donor, and as such, significantly more children with CR received either no serotherapy or serotherapy with ciclosporin A only (Table S3).

Monitoring of viral load using PCR in serum revealed infection in 14 patients, including 7 cases of cytomegalovirus infection, 7 cases of adenovirus infection, and 5 cases of Epstein-Barr virus infection. The presence of viremia did not influence the response to MSC treatment. Three patients who achieved CR relapsed from their underlying malignancy at days +332, +358, and +511 after HSCT, including 2 of 4 children who underwent HSCT for relapsed or refractory acute myeloid leukemia or acute myeloid leukemia/myelodysplastic syndrome and in 1 of 5 children who underwent HSCT for high-risk or relapsed acute lymphoblastic leukemia.
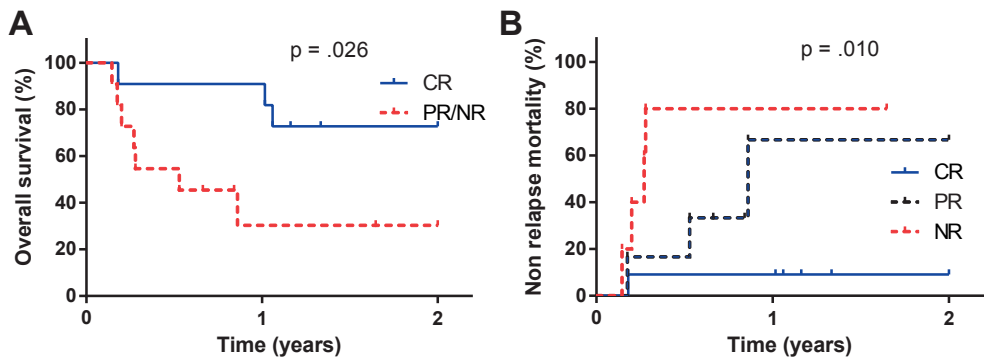

Figure 1. *Clinical response to MSC salvage therapy.* Response to MSC treatment of children with steroid refractory aGvHD of the gastrointestinal (GI) tract was documented clinically on day 28 after first MSC infusion. Patients with CR have significantly better overall survival (A) and (B) lower non-relapse mortality. Log-rank Mantel-Cox tests were performed for statistical analysis. CR: complete response; PR: partial response; NR: no response.

GI biopsy specimens collected after MSC infusion are highly informative

Persistent diarrhea was observed in 18 children after the first MSC infusion. According to the study protocol, these patients were eligible for additional MSC infusions. Twelve of these patients underwent GI endoscopy (3 lower GI and 9 lower and upper GI) with biopsy at a median of 33 days (range, 19 to 59 days) after the first MSC infusion. The clinical decision making process for these patients is summarized in the flowchart in Figure 2A. In 4 out of these 12 patients, GI histology showed active aGvHD. These 4 patients received additional MSC infusions, and 3 subsequently achieved CR.

At the start of the study, persistent diarrhea was an indication for subsequent MSC infusions, and 4 children received MSCs accordingly despite reports of negative histological findings received later. Four other patients did not receive additional
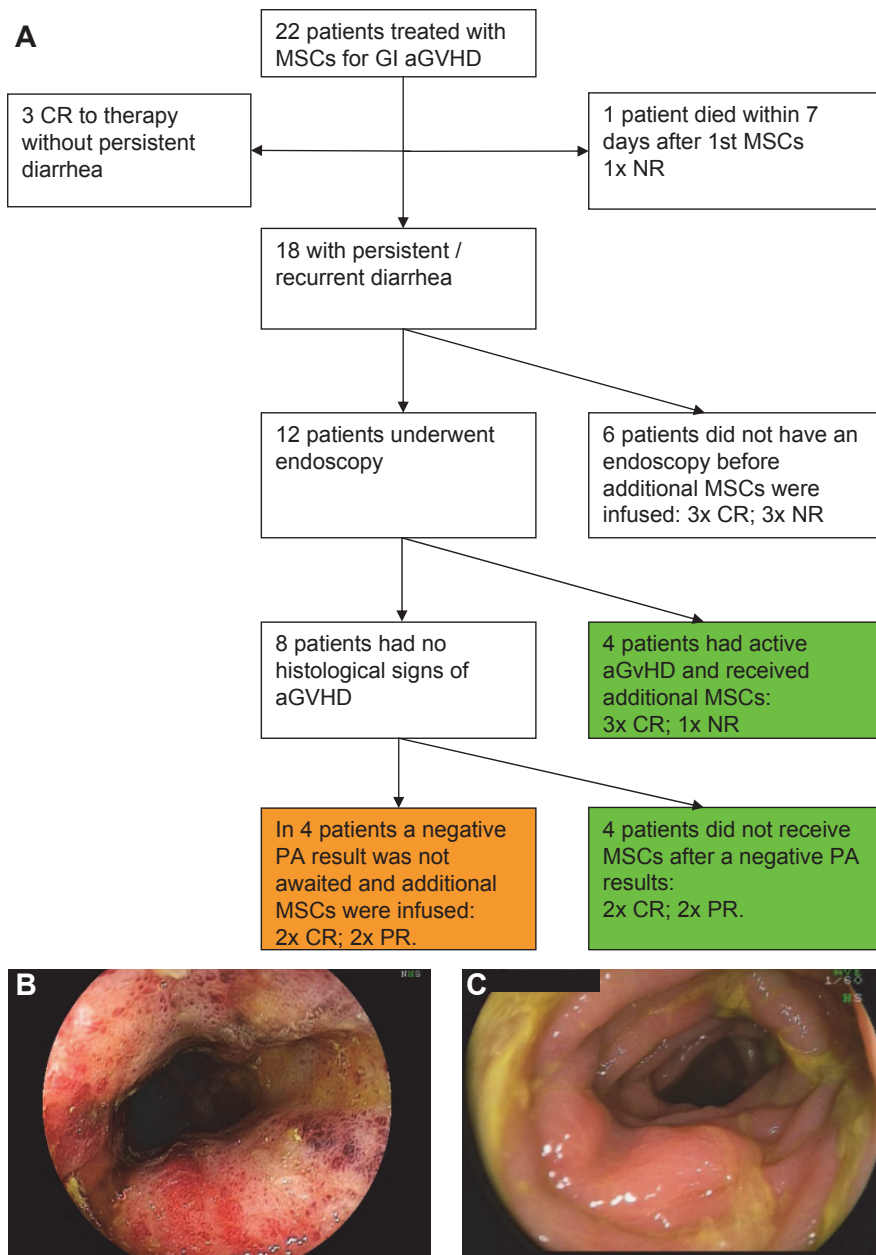
Figure 2. *Flow diagram of patients with persistent symptoms.* A) The decision to infuse additional MSCs was guided by the results obtained by endoscopy with biopsies in children with persistent or recurrent diarrhea. B) Endoscopic evaluation of grade IV aGvHD prior to MSC infusion showing hemorrhage, denudation of the epithelium and ulceration. Histological examination was consistent with aGvHD. C) Repeat endoscopy 22 days after MSC infusion showed evident healing of mucosa despite persistent diarrhea. Histological examination did not reveal active GvHD. CR: complete response; PR: partial response; NR: no response.

MSC infusions based on negative findings on endoscopy (Figure 2B-C). Accordingly, the study protocol was amended to provide subsequent MSC infusion only to patients with histopathological features of aGVHD.

A protocol violation and 2 refusals led to MSC infusion without prior endoscopic assessment, and these 3 patients achieved CR. Three other patients were deemed unfit owing to worsening symptoms with multiple comorbidities and died from ongoing aGvHD despite additional MSC infusions.

One patient with documented *Klebsiella* bacteremia deteriorated after the endoscopic procedure despite appropriate antibiotic therapy. He died from multiple organ failure with no evident aGvHD in postmortem samples. No infectious complications associated with the procedure or any documented side effects, such as hemorrhage, GI perforation, or intra operative complications, occurred in any of the other patients

Serum biomarkers concentrations are increased at the onset of aGvHD

The serum concentrations of the different biomarkers at the 2 different time points related to the course and initial treatment of aGvHD were compared with those measured at 7 days before these respective time points. All of the biomarkers in the panel for aGvHD defined by Paczesny *et al.*(Paczesny, 2009) were increased significantly during the progression of aGvHD to grade II or higher requiring systemic steroid therapy (Figure S1A-D). In addition, levels of CK18, sCK18F and REG3α were significantly elevated at the onset of GI symptoms (Figure S1E-G). In agreement with findings reported by Luft *et al.*(Luft, 2011), sTM serum concentrations were not increased at this time point (Figure S1H). Before HSCT all but 1 patient had an sTM concentration above the cutoff of 8 ng/mL, which has been proposed to be predictive for NRM (Figure S2).(Luft, 2011)

GI biomarkers display different patterns amongst patients

Longitudinal analysis of the data obtained for the various GI biomarkers revealed large interindividual variations. Five children with extensively documented and histology confirmed GI aGvHD had a REG3α concentration <50 ng/mL. Response to MSC infusion was not related to the different patterns shown in Figure S3A-C.

No increase in CK18 and sCK18F concentrations were seen despite the occurrence of GI aGvHD (Figure S3E and H). A subset of patients with elevated CK18 and sCK18F exhibited a steep decline in serum concentrations at the start of MSC infusion (Figure S3D and G, respectively). Interestingly, no patients who achieved CR demonstrated this phenomenon (Figure S3F and I).

Although higher concentrations of sCK18F have been reported in patients with liver involvement,(Harris, 2012) we were unable to confirm this finding in patients with (n=10) or without (n=12) liver involvement (data not shown).

TNFr1 concentration at onset of aGvHD is predictive for response to MSC infusion

In this analysis, we compared serum concentrations of all biomarkers between the patients who achieved CR (n=11) and those who achieved PR/NR (n=11). TNFr1 concentration at the start of systemic corticosteroid therapy were lower in patients who achieved CR at day 28 after first MSC infusion (p=0.049) (Figure 3A). A TNFr1 cutoff of 4100 pg/mL was predictive for CR and PR/NR, with a sensitivity of 63.6% (95% confidence interval (CI), 30.8% to 89.1%) and specificity of 72.7% (39.0% to 93.9%) at onset aGvHD and a sensitivity of 60.0% (95% CI: 26.2% to 87.8%) and specificity of 81.9% (48.2% to 97.7%) at day 7 after first MSC infusion, as defined by ROC-curves (Figure 3A). Concentrations of other biomarkers (e.g., IL-2rα and REG3α) did not differ between patients with CR and those with PR/NR (Figure 3B-C).

At the onset of GI aGvHD and the start of MSC therapy, the ratio of sCK18F to total CK18 was similar in the CR and PR/NR groups (ratio ±0.4). After initiation of MSC therapy, the sCK18F : CK18 ratio, including all values with sCK18F > 200 U/L, was significantly lower in the PR/NR group (Figure 3D).

Serum biomarkers predict survival

We compared serum concentrations of the various biomarkers at the different time points between patients who were alive (n=14) and those who were not alive (n=8) at 1 year after HSCT. Relapse-related death did not occur during the first year after HSCT in this cohort. Patients with aTNFr1 concentration below the median level at the start of systemic corticosteroids had significantly better survival (p=0.012) compared with those with a concentration above the median level (Figure S4).

At day 28 after the first MSC infusion, HGF, TNFr1, IL-8 and REG3α concentrations were significantly increased in patients not alive at 1 year post HSCT (Figure 4A-D). Applying the median values as cutoff levels (HGF: 2063 pg/mL, TNFr1: 3980 pg/mL, IL-8: 80 pg/mL, and REG3α: 36 pg/mL), TNFr1 and REG3α concentrations were predictive of survival censored for relapse (Figure 4A-D). No significant differences in IL-2rα, CK18 or sCK18F concentrations were observed between the 2 groups (p=0.941, p=0.086, p=0.881, respectively; data not shown).

Biomarker concentrations are illustrative at time of biopsy

The diagnostic value of biomarkers was compared with the histochemical evaluation of GI biopsy specimens. Independently of the timing of endoscopy, IL-2rα, REG3α, CK18 and sCK18F concentrations were significantly higher in sera obtained from patients in whom GI aGvHD was histopathologically confirmed compared with sera from patients in whom aGvHD was histopathologically excluded (Figure 5A-B). Separate analyses of serum biomarkers at the time of biopsy taken after MSC infu-
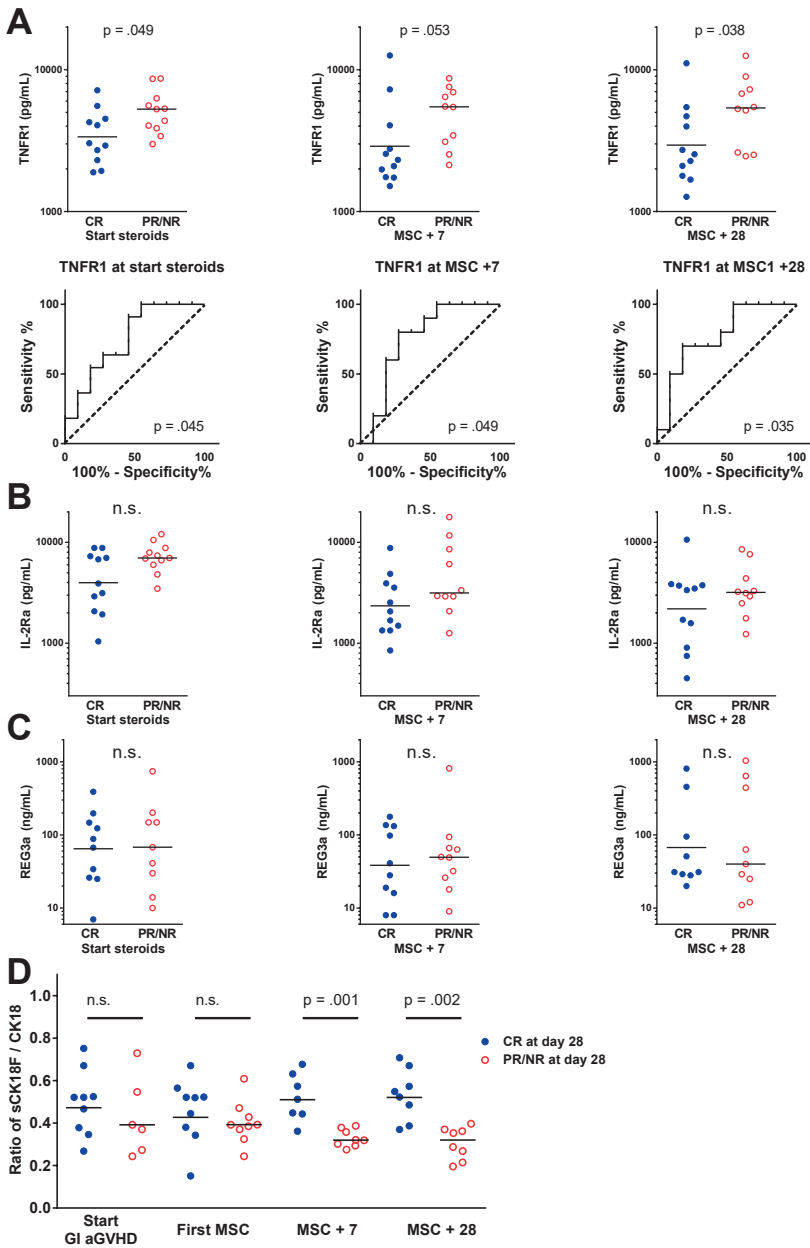
Figure 3. *TNFr1 discriminate between patients with a CR and PR/NR upon MSC treatment.* At the start of systemic corticosteroids and 28 days after infusion of the first MSCs serum TNFr1 concentrations (A), but not IL-2rα (B) and REG3α (C), are significantly lower in patients with a complete response (CR) on day 28 compared to patients with a partial (PR) or no response (NR). Receiver operating characteristics (ROC) are shown at the different time-points for TNFr1 (A). (D) Ratio of sCK18F versus CK18 after MSCs discriminates between CR and PR/NR. P-values for the different time-points using area under the curve (A) and Mann-Whitney tests (A-D) are depicted.
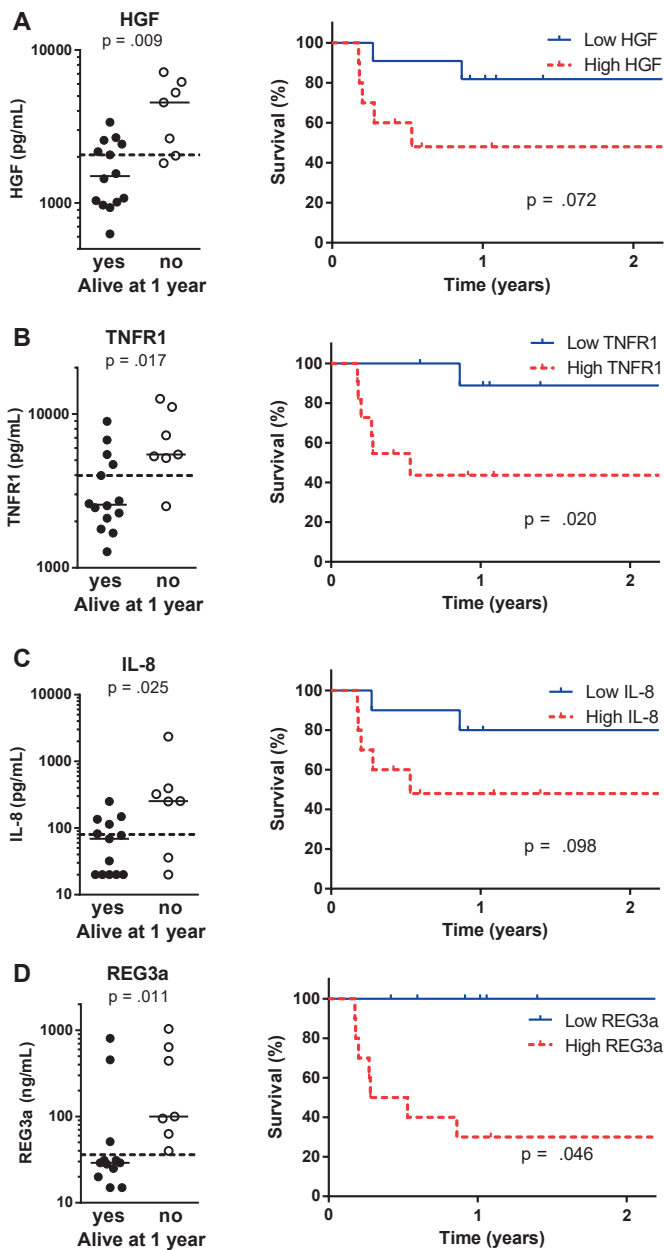
Figure 4. *Serum biomarkers 28 days after the first MSCs are predictive for survival.* (A-D) HGF, TNFr1, IL-8 and REG3α concentrations in available samples at day + 28 after the first MSC infusion are increased in patients not alive one year after transplantation. Using the median as a cut-off value, patients with low TNFr1 and low REG3α concentrations have a significantly better survival. The dotted horizontal line in the left panels represents the median of all values. Closed, half open and open symbols were used for CR, PR and NR at day 28 after MSC infusion, respectively. Statistics were performed using Mann-Whitney (left panels) and Log-rank (right panels) tests.
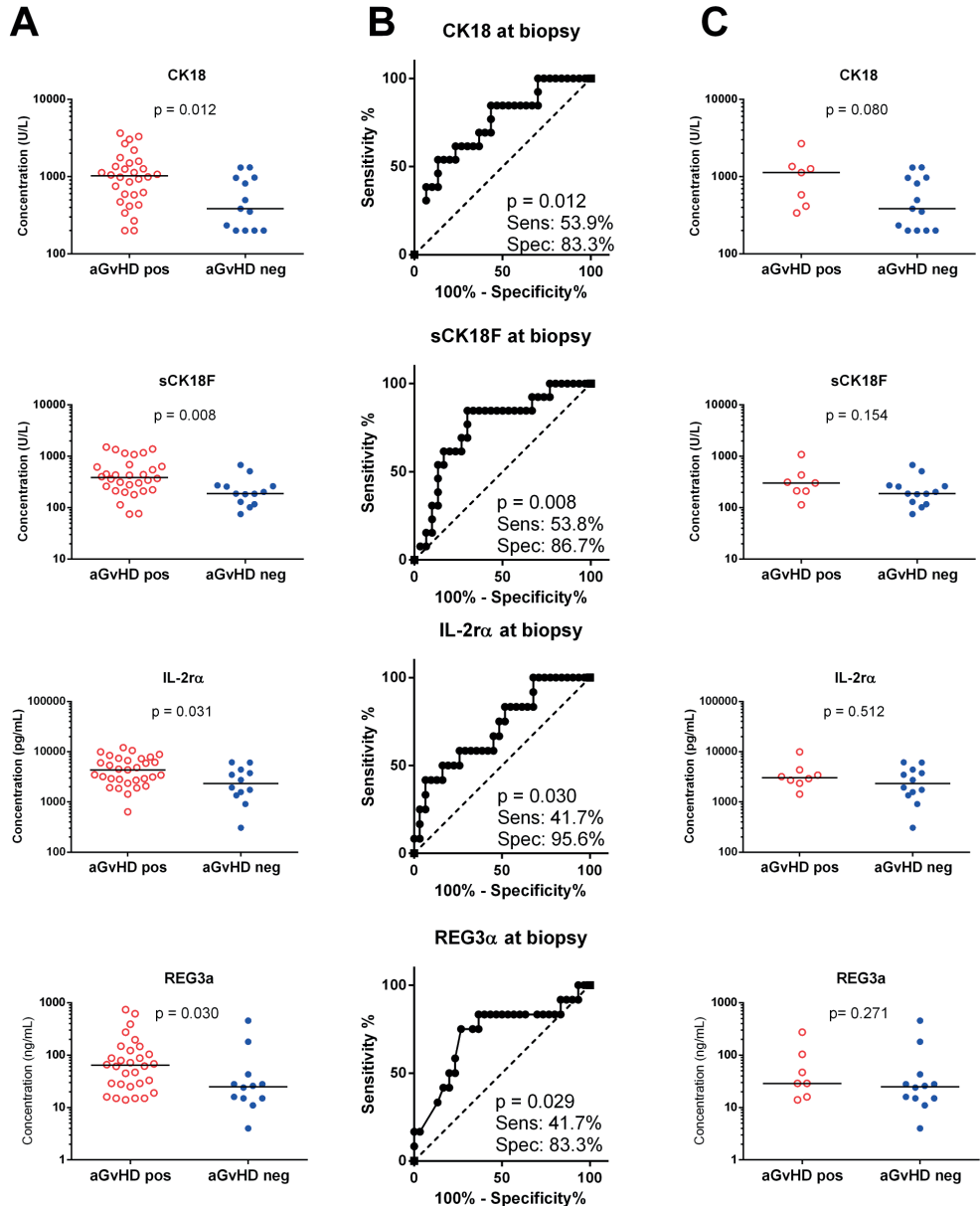
Figure 5. *Concentrations of serum biomarkers at time of gastrointestinal biopsies.* (A) Biomarker concentrations in serum are higher at the time of histological confirmed aGvHD compared to concentrations in sera taken at the time of negatively scored biopsies. A total of 45 time-points at which combined biopsy and serum samples were available was evaluated independently whether this was before or after the first MSC infusion. (B) Sensitivity and specificity are shown by ROC curves if specificity was at least 80%. (C) Focusing on paired biopsy and serum samples obtained after MSC infusion, biomarker concentrations do not differ significantly between samples collected at the time of a positive or negative biopsy. Depicted p-values were derived from Mann-Whitney (A and C) or area under the curve (B).

sion showed a trend toward lower CK18 and sCK18F levels (p=0.08 and p=0.15, respectively) in biopsy-negative GI aGvHD (n=13 sample dates) compared with biopsy positive ongoing aGvHD (n=8) (Figure 5C).

Discussion

Here we report the largest single-center cohort of children treated with MSCs for aGvHD to date in whom sequential GI biopsy specimens and serum biomarkers were analyzed to evaluate response to treatment. Five patients included in this study have been reported in a previous study on safety of MSC administration(Le Blanc, 2008) that did not focus on the relevance of biopsy specimens and serum biomarkers. Response to MSCs was scored at day +28 after the first MSC infusion, which was recently proposed as the optimum endpoint for evaluating response to treatment. (MacMillan, 2010) Six patients without CR received a third MSC infusion after day +28 and 5 of these patients subsequently achieved complete resolution of symptoms. Not unexpectedly, when the efficacy of MSC infusion was analyzed as the best-ever response, CR rates were higher compared with the response at day +28 (CR 16 vs 11, PR 2 vs 6, NR 4 vs 5).

Ideally, diagnosis of aGvHD as defined by the Seattle clinical criteria ideally should be confirmed by a biopsy of the target organ.(Ertault-Daneshpouy, 2004) Previous studies of MSC therapy in children lack confirmatory biopsy data. The present study indicates that obtaining GI biopsies is safe and feasible in the majority (86%) of patients with extensive aGvHD. This is especially relevant in patients entered in experimental clinical studies of MSCs, extracorporeal photopheresis(Messina, 2003; Perfetti, 2008), anti-TNF(Couriel, 2004b; Busca, 2007), and mammalian target of rapamycin inhibitors(Hoda, 2010).

Irrespective of the treatment under investigation, published data continue to define response based solely on clinical criteria established primarily for diagnosis. These criteria can be unreliable in GI aGvHD, especially in children, given that postinflammatory villous atrophy resulting from extensive damage of the GI epithelium is a recognized complication after severe aGvHD(Ball, 2008b). In light of this potential bias, the patients included in our cohort underwent both diagnostic and post-treatment assessment of the GI tract, including biopsy analysis in patients with persistent clinical symptoms, to reliably document their response to MSCs salvage treatment. A high incidence (18 of 21 cases) of persistently profuse diarrhea after first MSC infusion was documented. On biopsy analysis, the majority of children (8 of 12) demonstrated no histological evidence of active aGvHD. Non-GvHD villous atrophy and introduction of oral feeding might have caused malabsorption and subsequent osmotic diarrhea.

This distinction between persistent aGvHD and diarrhea from other causes allowed for the pertinent use of MSC infusion and subsequently altered clinical management of these patients. The reliability of this approach is supported by our ability to successfully reduce immunosuppression in patients without aGvHD after MSC infusion, whereas in the majority of patients with ongoing aGvHD, additional MSC infusions resulted in complete resolution of the disease. Moreover, feeding regimens could be tailored in children with post-inflammatory villous atrophy.

Owing to ethical committee and safety concerns, GI biopsies were obtained only for clinical indications and unlike serum biomarkers, they were not available at fixed time points. Because the timing of biopsy varied, an analysis based on histological definitions on day +28 after MSC infusion was not possible.

Despite its demonstrated usefulness and safety profiles, endoscopy with biopsy is an invasive procedures general anesthesia in children. In a large multi-center study, especially in centers lacking pediatric gastroenterology expertise, obtaining biopsies might not be feasible. Thus, we determined the value of biomarkers in relation to response as determined by clinical and pathological findings. The biomarkers studied in our patient cohort confirm the previously reported increase of inflammatory and GI specific biomarkers at the onset of aGvHD.

Measurement of CK18 and sCK18F measured in serum at the time of biopsy has the potential to serve as an alternative diagnostic tool. Levels of both total protein and cleaved fragments were significantly increased at the time of positive GI biopsy compared with negative biopsy. The sensitivity (53.8% in the present study; 95% CI 25.1% to 80.8%) and specificity (86.7% in the present study; 95% CI 69.3% to 96.2%) of the CK18 test remain to be validated in larger cohorts of patients, however. In addition, biomarkers not reaching statistic significance in our cohort should be evaluated as potential alternatives to serial biopsies in future studies. Our observed decrease in the sCK18F : CK18 ratio in patients not responding to MSC infusion suggests the occurrence of apoptosis-independent cell death after MSC infusion in these patients. This is in line with observations by Jitschin *et al.* in patients with steroid-refractory aGvHD or hemorrhages treated with MSC.(Jitschin, 2013)

In accordance with von Bahr *et al.*, we observed a lower IL-2rα concentration at day 7 after MSC infusion, which was not correlated with the response to MSCs.(von Bahr, 2012) This is reminiscent of the previously described decline in IL-2rα concentrations in both patients responding to steroids and those refractory to steroids.(Luft, 2007) In contrast, Dander *et al.* reported a correlation of biomarkers IL-2rα and TNFr1 with the response to MSC treatment in 10 patients with aGvHD or chronic GvHD.(Dander, 2012) However, the timing of the measurement with regard to the sequential number of MSC infusions in that study is unclear.

Selected biomarkers have demonstrated predictive value in terms of the response to treatment and survival at multiple time points before and after MSC infusion. Although these data are based on a limited number of patients, they support the stratification in clinical trials based on biomarkers as suggested by Paczesny *et al.* at the time of diagnosis or start of salvage therapy.(Paczesny, 2013) However, the wide variation in biomarker patterns observed in our patient cohort after MSC treatment does not allow for the substitution of a biomarker panel fo biopsy analysis to evaluate response and guide clinical decision making in individual patients. Our remain to be validated in large multicenter studies, which are currently under development.

REG3α levels were lower in our patient cohort compared with the American cohort reported by Ferrrara *et al.*, but in line with levels reported in Regensburg (Germany) and Kyushy (Japan) cohorts described in the same paper.(Ferrara, 2011) Unlike the patients in the American cohort, all of the patients in our cohort received selective GI decontamination with polymyxin neomycin and amphotericin B or total GI decontamination with piperacillin, tazobactam and amphotericin B.(Vossen, 1990; Ferrara, 2011) REG3α is secreted in response to bacteria, and the lower REG3α concentrations in our cohort might be replated to the suppression of bacterial microflora.(Ayabe, 2000) Even at the lower levels of REG3α, the concentrations were correlated with aGvHD and were predictive for survival.

Along with serum biomarkers, stool and urine biomarkers also have been proposed.(Rodriguez-Otero, 2012; Landfried, 2011) These materials were not available for analysis in the present retrospective study. Collection of uncontaminated urine from children with aGvHD at multiple time points is difficult, and analysis of stool samples for the measurement of procalcitonin in a pilot study in our center was complicated by insufficient material in young children (data not shown).

The present study demonstrates the importance of critical and intensive monitoring of patients included in an experimental study. Before the introduction of MSC therapy, experience with children surviving severe aGvHD was limited. A new phenomenon, persistence of severe villous atrophy after resolution of aGvHD causing severe protracted diarrhea, was identified. Endoscopy with biopsy remains the golden standard for differentiating between active aGvHD and postinflammatory villous atrophy. In the later phase of this trial, additional MSC infusions were provided based only on clinical symptoms in combination with histological results.

Further recommendations for steroid-refractory aGvHD that allow for comparative observation among various modalities of treatment are needed. Response criteria and timing of assessment of response remain to be defined, preferably through consensus of the transplant organizations, such as the Center for International Blood and Marrow Transplantation Research and the European Group for Blood and Marrow Transplantation. Based upon our findings reported here, these criteria should include

biopsy analysis and serial serum biomarker concentrations at diagnosis and after treatment in patients with persistent clinical symptoms.

Acknowledgements

Supplementary data

Table S1: ELISA antibodies used

	Supplied by	Serum dilution	Lower detection limit	Upper detection limit	units
HGF	R&D, Minneapolis, MN, USA	2	250	16000	pg/mL
IL-8	R&D, Minneapolis, MN, USA	5	20	8335	pg/mL
IL-2Rα	R&D, Minneapolis, MN, USA	5	156	10000	pg/mL
TNFR1	R&D, Minneapolis, MN, USA	20	250	16000	pg/mL
Cytokeratin M30	Peviva, Bromma, Sweden	1	75	1000	U/L
Cytokeratin M65	Peviva, Bromma, Sweden	1	200	5000	U/L
REG3α	MBL International, Woburn, MA, USA	10	16	1000	ng/mL
Trombomodulin	Sanquin, Amsterdam, the Netherlands	5	1	50	ng/mL

Table S2: Response at 28 days after first MSCs

		CR/PR/NR
Overall	Prior to MSC	11 / 6 / 5
Grade	III	6 / 6 / 2
	IV	5 / 0 / 3
Stage GI	2	1 / 1 / 0
	3	7 / 5 / 4
	4	3 / 0 / 1
Organs affected	GI	2 / 0 / 0
	GI + skin	2 / 4 / 4
	GI + Liver	3 / 2 / 0
	GI + liver + skin	4 / 0 / 1

CR: complete response; PR: partial response; NR: no response; GI: gastrointestinal

Table S3: Differences between CR and PR/NR group

	CR	PR/NR	*P*-value
N	11	11	
HSCT for malignancy	10	5	*P* = .022
Age (years)*	12.52 (1.3 - 18.1)	4.08 (0.7 - 16.2)	*P* = .071
Donor HLA-id related	6	1	*P* = .022
10/10 or 6/6 matched	9	3	*P* = .010
Donor source BM vs other	8	5	*P* = .193
time HSCT to onset aGVHD (days)*	47 (12 - 100)	28 (6 - 46)	*P* = .061
time onset aGVHD to first MSC (days)*	14 (7 - 59)	9 (0 - 31)	*P* = .411
Liver involvement	7	3	*P* = .087
Skin involvement	6	8	*P* = .375
Prophylaxis CsA only	7	0	*P* = .001
Conditioning TBI	3	2	*P* = .611
Serotherapy yes	5	11	*P* = .004
MSC as third line therapy	3	3	*P* = 1.00
Total number of MSC infusions	20	26	*P* = .135

CR: complete response; PR: partial response; NR: no response; BM: bone-marrow; HSCT: hematopoietic stem cell transplantation; aGVHD: acute graft-versus-host disease; CsA: ciclosporin A; TBI: total body irradiation; * median is shown with range in brackets.
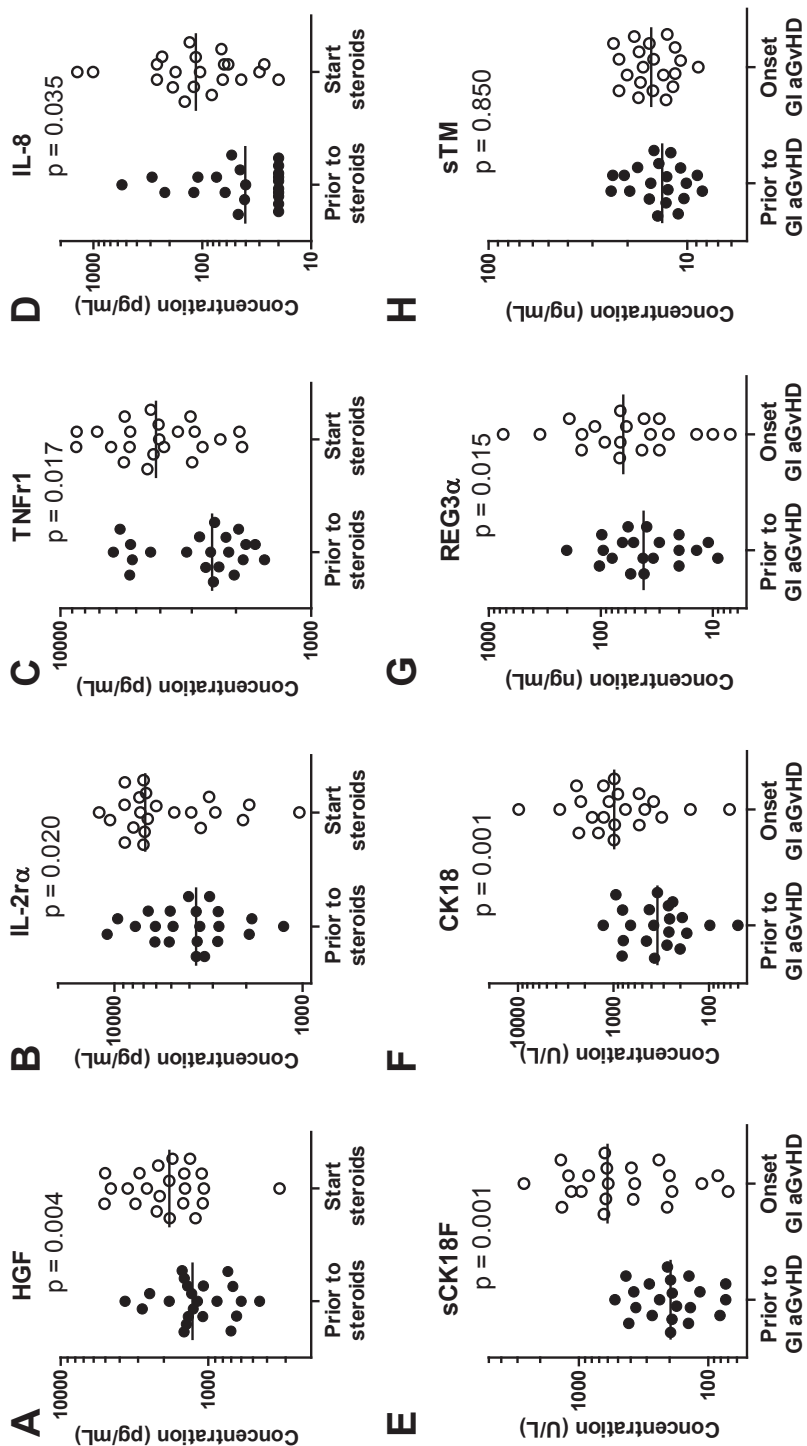
Figure S1. *Serum biomarkers are increased around the onset of severe aGvHD.* (A-D) Serum biomarkers are increased at the start of systemic corticosteroids compared to concentrations measured between 6-12 days prior to start of steroids. (E-G) Organ specific biomarker concentrations are increased in serum samples collected at the onset of gastrointestinal (GI) aGvHD compared to samples collected between 6-12 days prior to onset of GI aGvHD. (H) Soluble thrombomodulin (sTM) concentrations did not change around the onset of GI aGvHD. Wilcoxon paired signed rank test; horizontal lines represent median values.

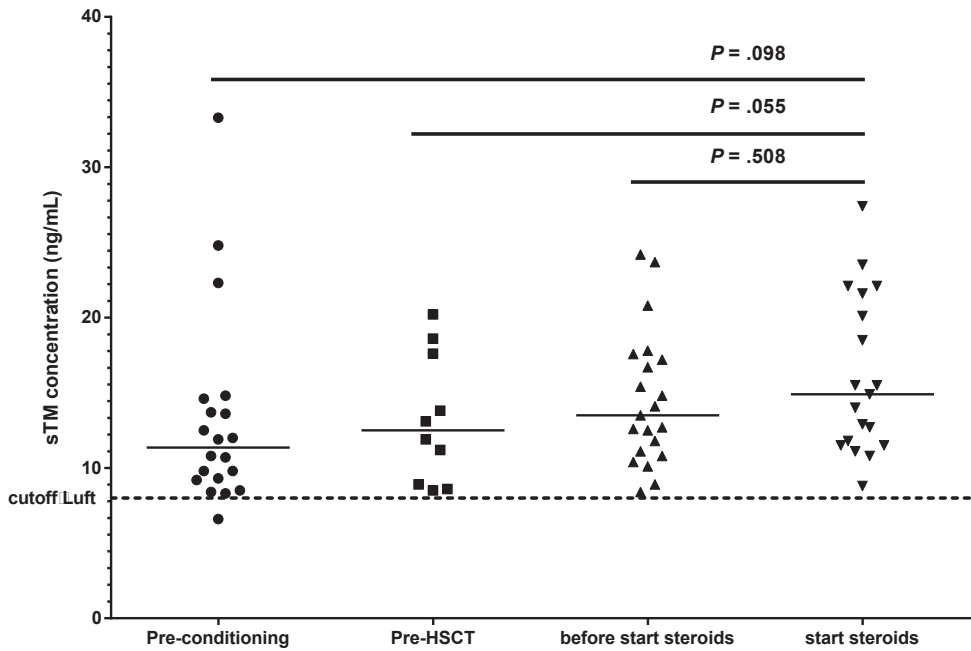**Figure S2.** *Soluble trombomodulin (sTM) concentrations at different time points.* sTM concentrations were higher than the cut-off value (dashed line) used by Luft *et al.*(17) at all time-points starting before conditioning. Samples included before start of steroids were collected 6-12 days prior to start of steroid treatment. Wilcoxon signed rank test; horizontal lines represent median values.
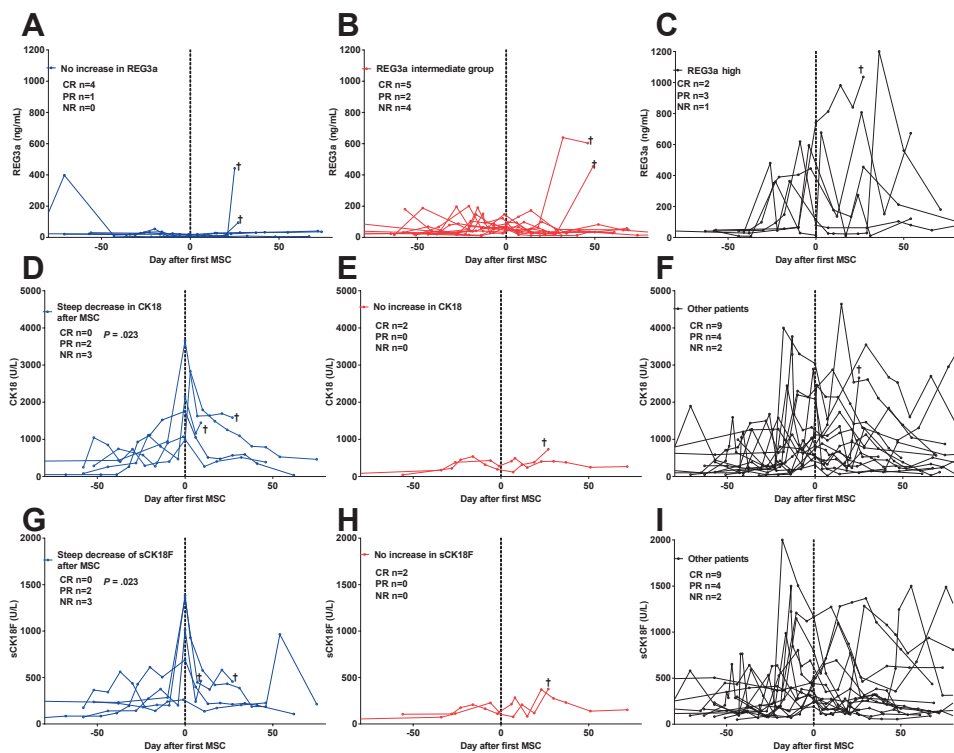
Figure S3. *Longitudinal patterns of gastrointestinal biomarkers.* Patients are categorized according to low (A), intermediate (B) or high (C) levels of REG3α at the time of first MSC infusion. Different patterns were observed in CK18 (D-F) and sCK18F (G-I) concentrations. 5 patients showed a steep decline of CK18 and sCK18F after MSC infusion (D, G) and two patients had no increase in CK18 (<1000 U/L) or sCK18F (<400 U/L) (E, H). The remaining patients had an increase, but no rapid, consistent, decrease after MSC infusion (F, I). P-values were calculated using chi-squared tests. † indicate samples collected peri-mortem not taken into account in this analysis. CR: complete response; PR: partial response; NR: no response.
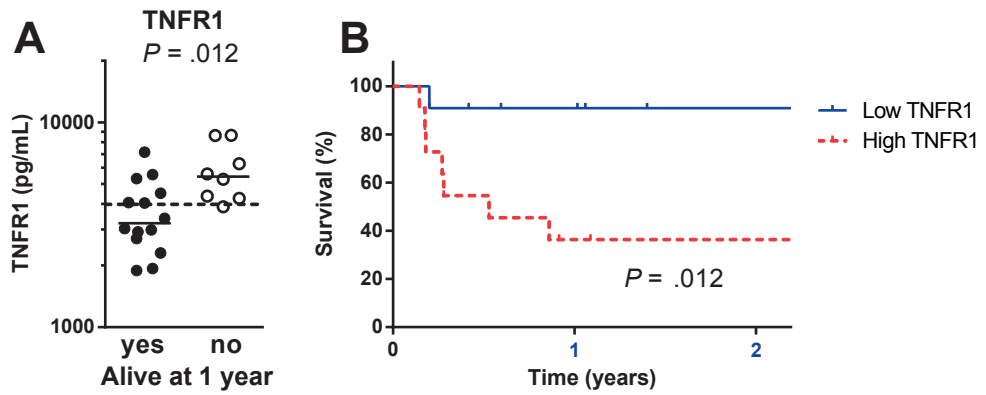
Figure S4. *TNFr1 concentrations at start of aGvHD are predictive for survival.* (A) Patients alive at 1 year after HSCT have lower TNFr1 concentrations at start of systemic steroids. (B) Patients with low TNFr1 concentrations have significantly better survival. Wilcoxon signed rank test; horizontal line represents median value.

Chapter 6.

Mesenchymal stromal cell therapy is associated with increased adenovirus-associated, but not cytomegalovirus-associated mortality in children with severe acute Graft-versus-Host Disease.

Abstract

Beneficial effects of mesenchymal stromal cells (MSCs) in patients with severe steroid-refractory acute graft-versus-host disease (aGvHD) have been reported. However, controversy exists about the effect of MSCs on virus-specific T cells. We evaluated 56 patients with grade II-IV aGvHD who responded to steroids (n=21), or were steroid-refractory receiving either MSC (n=22) or other second-line therapy (n=13). Although the overall incidence of cytomegalovirus (CMV), Epstein-Barr virus and human adenovirus (HAdV) infections was not significantly increased, HAdV infection was associated with decreased survival in children treated with MSCs. Therefore, we investigated *in vitro* the effects of MSCs on virus-specific T cells. Both CMV-specific and, to a lesser extent, HAdV-specific T cell activation and proliferation were negatively affected by MSCs either after induction of a response in peripheral blood mononuclear cells (PBMC) or after restimulation of virus-specific T cell lines. In patient-derived PBMC, CMV-specific proliferative responses were greatly decreased on first line treatment of aGvHD with systemic steroids and slowly recovered after MSC administration and tapering of steroids. HAdV-specific T cell proliferation could not be detected. In contrast, the proportion of CMV and HAdV-specific effector T cells, measured as interferon-γ-secreting cells, remained stable or increased after treatment with MSCs. In conclusion, although *in vitro* experimental conditions indicated a negative impact of MSCs on CMV- and HAdV-specific T cell responses, no solid evidence was obtained to support such an effect of MSCs on T cell responses *in vivo*. Still, the susceptibility of steroid-refractory severe aGvHD patients to viral reactivations warrants critical viral monitoring during randomized controlled trials on second-line treatment including MSCs.

Introduction

Mesenchymal stromal cells (MSCs) are multipotent non-hematopoietic cells that can be easily expanded *in vitro*. In culture, MSCs remain genetically stable and their low immunogenicity makes them suitable candidates for immunotherapy.(Dominici, 2006; Bernardo, 2007c) In *in vitro* studies MSCs have been shown to suppress proliferation and activation of T lymphocytes, B lymphocytes, natural killer cells, and monocytes.(Sotiropoulou, 2006; Traggiai, 2008b; Krampera, 2003; Melief, 2013) Although cell-cell interaction and various soluble factors have been reported to mediate the *in vitro* (Gieseke, 2010; Meisel, 2004; Selmani, 2008; Mougiakakos, 2011) immunomodulatory effect of MSCs, the mechanism(s) underlying the *in vivo* (Ren, 2008; Gonzalez, 2009) suppressive capacity of MSCs remains further to be elucidated.

In clinical studies, MSCs showed beneficial effects in patients with steroid-refractory acute graft-versus-host disease (aGvHD), autoimmune disorders and auto-inflammatory diseases.(Sun, 2009; Le Blanc, 2008; Garcia-Olmo, 2005) Although dampening of alloreactive or autoimmune-driven inflammatory reactions is frequently observed after MSC infusion, overall down modulation of immune responses might increase the risk of viral infections. Infectious complications are a major cause of morbidity and mortality after allogeneic hematopoietic stem cell transplantation (HSCT).(Broers, 2000; Boeckh, 2003; Walls, 2003) In part, this is related to the delayed immune reconstitution following HSCT; however, infections can also be exacerbated by additional immunosuppression, such as systemic steroids given for the treatment of aGvHD.(Cantoni, 2010)

Published data on the effect of MSCs on virus-specific T cells are somewhat conflicting. Karlsson *et al.*,(Karlsson, 2008) reported no effect of MSCs on the two major viral pathogens in HSCT related to T cell expansion and cytotoxicity specific to cytomegalovirus (CMV) and Epstein-Barr virus (EBV) specific. However, in a recent study, von Bahr *et al.*(von Bahr, 2012) commented on high peak levels of CMV DNA load shortly after MSC infusion, suggesting a suppressive effect of MSCs on CMV-specific immunity. No data are available on the effect of MSCs on human adenovirus (HAdV) infections, which occur at a high frequency (up to 40%) in pediatric graft recipients(Flomenberg, 1994; Howard, 1999; Baldwin, 2000) and are lethal in up to 50% of cases with disseminated infection.(Walls, 2003; Kojaoghlanian, 2003; Leen, 2005; van Tol, 2005b)

At the Leiden University Medical Center, a large cohort of children with steroid-refractory aGvHD has been monitored closely after receiving MSC infusions. In the present study, the prevalence, course and outcome of viral infections after HSCT in this cohort were documented. These data were compared with a cohort consisting of children developing aGvHD responding to steroids and with a group of historic

controls with steroid-refractory aGvHD who did not receive MSCs as second/third line treatment.

Because both viral infections and aGvHD often occur coincidentally, it is important to further investigate the impact of MSCs on virus-specific T cell responses. Thus, we studied the influence of MSC in co-cultures with T lymphocytes at different stages of differentiation, with T cells naturally present among peripheral blood mononuclear cells (PBMC) and with *in vitro* expanded virus-specific T cell lines. We focused on CMV and HAdV, because of the potentially severe clinical impact of these viruses in pediatric HSCT. To analyze the *in vivo* effect of MSC on virus-specific T cell responses, PBMC of patients treated with MSCs after HSCT were longitudinally investigated for their reactivity against CMV and HAdV.

Material and methods

Patients and definitions

All patients (n=22) treated with MSC for steroid-refractory aGvHD grade II-IV from 2004 until 2012 according to an ethical approved protocol (number LUMC-MEC: P05-089) were included in the current study. Patients received 1 to 3 third-party, bone marrow derived, MSC infusions consisting of 1-2 x 10^6 MSCs per kg recipient body-weight, as previously described.(Le Blanc, 2008; Calkoen, 2013b) Full resolution of symptoms at 28 days after the first MSC infusion was defined as complete response (CR). Partial response (PR) was defined as at least one grade improvement and no response (NR) was defined as stable disease or worsening of symptoms. Viral status of CMV, EBV and HAdV was routinely monitored by polymerase chain reaction on plasma samples. For the purpose of the study, but in contrast to the cutoff of log 3.0 copies per milliliter commonly used to define a disseminated infection, viral infection or reactivation (referred to in this paper as "infection") was defined as the presence of at least log 2.3 copies per milliliter, in two samples taken with a time interval of at least 3 days. This allowed the inclusion of all patients with viral infections. Monitoring frequency in the first two months after HSCT varied between weekly and every 2 weeks thereafter until immune recovery (defined as ≥300 CD3^{+} T cells per milliliter of blood) was observed. Pre-emptive treatment with ganciclovir (CMV), rituximab (EBV) or cidofovir (HAdV) was initiated on detection of log ≥3 viral DNA copies per milliliter at two or more consecutive time points. Viral infections resolving before onset of severe aGvHD (defined as start of systemic steroid therapy) or occurring more than 90 days after the first MSC infusion were not taken into account.

Control cohorts consisted of patients with grade II-IV aGvHD who either responded to steroids only (HSCT in the period 2004 to 2012, n=21) or were steroid-refractory

but received second- or third-line treatment other than MSCs (historic controls: HSCT performed in the period 1994 to 2004, n=13). Patient and transplant characteristics of the study cohort and both control groups are summarized in supplementary Table 1 (Table S1).

Patient materials

PBMC collected weekly prior to and after MSC infusion as well as PBMC stored after routine immunophenotyping post HSCT (ethical approved protocols LUMC-MEC P01-028 and P03-061) were used for this study. Whenever possible, PBMC were investigated at the following time-points: before the start of systemic steroids, before the first MSC infusion, 7-14 days after first MSC infusion, 7-14 days after subsequent MSC infusions and 180 and 365 days after the first MSC infusion. Cryopreserved PBMC of patients after HSCT were used after thawing and resting for 4 hours at 37°C, 5% CO_2 in RPMI 1640 (Invitrogen, Paisley, UK) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin (P/S; Invitrogen), 10% human serum (HS, Sanquin, Amsterdam, the Netherlands).

MSC isolation and culture for in vitro experiments

Fresh bone marrow samples of 10 healthy pediatric stem cell donors were used for MSC expansion. Parental donor and age appropriate pediatric donor informed consent forms were signed in all cases. The study, approved by the ethical committee of the Leiden University Medical Center (LUMC-MEC P08-001), was performed in accordance with the Declaration of Helsinki.(World Medical Association, 1997) After Ficoll separation, bone marrow mononuclear cells were plated in polystyrene culture flasks at a density of 0.16×10^6 cells/cm^2. Cells were cultured in Dulbecco's modified Eagle medium with Glutamax (DMEM; Invitrogen) supplemented with P/S and 10% fetal bovine serum (FBS; VWR International, Bridgeport, NJ). Medium was refreshed every three to four days. Cultures were harvested at 80% confluency by treatment with trypsin (Invitrogen), replated and maintained for maximally 6 passages at 37°C and 5% CO_2. All MSC cell lines were phenotypically characterized at their second or third passage using antibodies against CD3, CD45, CD86, human leukocyte antigen-DR (HLA-DR), CD31, CD34, CD73 and CD90 (all Becton Dickinson Biosciences (BD), San Diego, CA). CD105 was obtained from Ancell Corporation (Bayport, MN). The osteoblast and adipocyte differentiation potential was evaluated on cells at passage 4 to 6 as described previously.(Bernardo, 2007b; Calkoen, 2013a) After 3 weeks, fat vacuoles in adipocytes and calcified depositions in osteoblast were stained with Oil-Red-O (Sigma, St. Louis, MI) or Alizarin Red (MP Biomedicals, Solon, OH), respectively.

PBMC stimulation

PBMC were stimulated with methylene blue photoinactivated HAdV (multiplicity of infection: 100) or 11 amino acids overlapping 15-mer peptide pools with HAdV hexon Peptivator (0.6 nM Miltenyi Biotec, Bergisch Gladbach, Germany) or CMV pp65 peptides (1.0 nM, Department of Immunohematology, Leiden University Medical Center, the Netherlands).(Zandvliet, 2011) Phytohemagglutinin (PHA, PeproTech, London, UK) and interleukin-2 (IL-2 Novartis International, Basel, Switzerland) were used at the indicated concentrations. PBMC were stimulated directly with exogenously added peptides or with peptide-loaded mature dendritic cells (mDC) generated *in vitro* from purified autologous monocytes. In brief, monocytes were isolated from PBMC (3.0 x 10^6/mL) by 2 hours plastic adherence and cultured for 6 days in RPMI 10% fetal calf serum containing 800 U/mL of granulocyte-macrophage colony-stimulating factor (GM-CSF; TebuBio, Le Perray-en-Yvelines, France) and 40 ng/mL of IL-4 (PeproTech). Immature DC (iDC) were harvested and cultured (1.0 x 10^5/mL) with similar concentrations GM-CSF and IL-4 combined with 0.25 ng/mL CD40-ligand (Beckman-Coulter, Marseille, France) and 500 U/mL of interferon-γ (IFN-γ; Boehringer, Mannheim, Germany) for 2 days.

Generation of virus-specific T cells

PBMC from CMV-seropositive healthy adult Sanquin Blood Bank donors and from donors previously screened for a measurable proliferative response to HAdV peptides were selected for the generation of CMV- and HAdV-specific T cell lines, respectively, as previously described.(Veltrop-Duits, 2006) Briefly, PBMC were stimulated with peptide-loaded mDC for 12 days in the presence of IL-2 (10 IU/mL) and IL-7 (5 ng/mL, Peprotech). T cell lines were harvested on day 12 and restimulated with 30-Gray-irradiated peptide-loaded autologous PBMC and IL-2 and IL-7 for an additional 16 days, after which the virus-specific T cell lines were harvested and cryopreserved.

Co-culture experiments

PBMC (100,000 cells per well) were co-cultured with 20,000 or 2,500 (30-Gray irradiated) MSCs or without MSCs in 96-well plates. Virus-specific T cells (20,000 cells per well) were co-cultured with 20,000, 4,000 or 500 (irradiated) MSCs or without MSCs. PBMC directly stimulated with virus-derived peptides were cultured for 7 days. PBMC stimulated with viral peptide-loaded mDC or virus-specific T cell lines were cultured for 5 days after defining the optimal culture duration in preliminary experiments. Negative controls with cells cultured with either unpulsed mDC or with no exogenously added viral peptides were included. Proliferation was assessed by addition of ^{3}H-thymidine (1 μCi per well; Perkin Elmer, Waltham, MA, USA) for the last 16 hours of culture. IFN-γ concentration in culture supernatants was measured by

enzyme-linked immunosorbent assay (Sanquin) performed according to the manufacturer's instruction.

Flow cytometry

Monoclonal antibodies (mAbs) used to characterize the T cells proliferating in culture were: anti-CD3 PerCPC5.5 (Becton Dickinson (BD), Mountain View, CA), anti-CD4 APC (Beckman Coulter Immunotech, Marseille, France), anti-CD8 PE (BD), anti-CD8 APC (Beckman Coulter). Prior to stimulation, cells were labeled with carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) to discriminate between proliferating and non-proliferating cells. To determine activation and the differentiation stages of virus-specific T cell lines, the mAbs anti-HLA-DR FITC (BD), anti-CD45RA PE (Beckman Coulter) and anti-CCR7 FITC (R&D, Minneapolis, MN) were applied. T cell differentiation stages were defined as "naïve": $CD45RA^+CCR7^+$; "central memory": $CD45RA^-CCR7^+$; "effector memory": $CD45RA^-CCR7^-$ and "end-stage effector cells": $CD45RA^+CCR7^-$.

Virus-specific T cell stimulation of patient-derived PBMC

Disseminated EBV reactivations in three HSCT patients were successfully treated with rituximab; therefore, functional studies focused on the detection of CMV- and HAdV-specific T cells. To detect IFN-γ-secreting cells, ELISpot plates (Millipore, Billerica, MA) were coated with anti-IFN-γ antibody (Mabtech, Stockholm, Sweden) overnight. PBMC (0.2×10^6 cells per well) were stimulated with CMV or HAdV peptides with or without a low dose of IL-2 (3 IU/mL) for 20 hours. PBMC cultured without stimuli or with low dose IL-2 only were used as negative controls. PBMC stimulated with PHA (10 ug/mL) plus a high dose of IL-2 (50 IU/mL) was used as positive controls.

IFN-γ was detected by anti-IFN-γ-biotin antibody (Mabtech) and visualized by streptavidin-alkaline phosphatase (Sigma, St Louis, MO) and its substrate 5-chromo-4-chloro-3-indolyl phosphate (BCIP; Sigma). IFN-γ spots were counted with a fully automated, computer-assisted, video-imaging analysis system (BioSys 5000, Karben, Germany). Virus-induced ELISpot results were compared to background levels observed in cultures without viral peptides. Values of more than 10 spots per 200,000 cells and at least two times higher than background were considered positive. All conditions were performed in duplicate.

Proliferation of 1×10^5 PBMC per well was assessed by ^{3}H-thymidine incorporation performed overnight after 6 days of stimulation with CMV or HAdV peptides with a low dose of IL-2 (3 IU/mL). PHA (2 ug/mL) plus a high dose of IL-2 (50 IU/mL) stimulation for 5 days adding ^{3}H-thymidine at day 4 was used as a positive control.

For comparison of the different time-points, viability of PBMC was determined after thawing and four hours of resting using trypan blue. In addition, PHA-induced proliferation and IFN-γ production were considered measures of general functionality

of the thawed cells. In cases of negative PHA proliferation time points were excluded from the analysis.

Statistical analysis

Survival analysis was modeled with Log-rank Mantel-Cox tests. Paired Wilcoxon signed-rank tests were used to compare subsets prior to and after MSC infusion and for evaluation of the *in vitro* effect of MSCs. Chi-squared tests were performed on categorical data. Graphpad 6 (Prism, La Jolla, USA) was used for data analysis, and *p*-values <0.05 were considered statistically significant.

Results

Clinical results

One-year survival after HSCT in the patients treated with MSCs was higher (62.9%) than in historic controls (33.3%) but lower than in children responsive to steroids (90.5%), and did not reaching statistical significance. The latter group, being less severely affected regarding the grade and gut involvement of aGvHD, differed significantly from the MSC group (Table S1). Patients with MSCs as second-line therapy (n=16) showed a better one-year survival compared with patients with MSCs as third line therapy (n=6; 73.9% vs 33.3%, respectively p=0.049). One-year survival in the latter subgroup was comparable to historic controls with steroid-refractory aGvHD not treated with MSCs (n=13; 38.5%).

In 14 of the 22 consecutive children treated with MSCs for steroid-refractory aGvHD grade III-IV viral infections were present either at the onset of severe GvHD or occurred within a period of 90 days after the first MSC infusion (Table 1). Seven patients had a CMV infection, seven patients had an HAdV infection, and five patients had an EBV infection; multiple viral infections occurred in 5 out of 22 enrolled patients. Overall survival at 1 year after HSCT, censored for relapse, was higher (but not significantly) in patients without viral infection (Figure 1A). Survival in historic controls (n=13, six children with an infection) with steroid-refractory aGvHD receiving no MSCs showed the same trend (Figure 1B; HAdV, n=4; CMV, n=3; EBV, n=3). In contrast, the one-year survival in the control cohort with steroid-responsive patients was comparable for children with (n=7) and without (n=14) a viral infection (Figure 1C; HAdV, n=3; CMV, n=4; EBV, n=3). Although, not statistically significant (p=0.09), there was a trend to a higher percentage of viral infections and a longer duration of viremia in the MSC-treated children compared to patients responsive to steroids only (data not shown).

In contrast to CMV and EBV infections, HAdV infection was significantly associated with higher nonrelapse mortality (Figure 1D). HAdV infections which became appar-

Table 1. Steroid-unresponsive children receiving MSCs: patient characteristics and viral infections

UPN	MSC as 2nd line	MSC response[1]	Follow-up post HSCT	Cause of Death	Virus	Anti-viral treatment	Before steroids	Start steroids	MSC1	MSC1 + 30	MSC1 + 60	MSC1 + 90
2	No[2]	NR	Death (day + 99)	CMV + GvHD	CMV	(Val)ganciclovir	+	+	+	+	+	NA
11	Yes	CR	Alive (day + 1378)		CMV	(Val)ganciclovir	+	+	+	+	+	+
12	Yes	CR	Death (day + 371)	Relapse	CMV	(Val)ganciclovir	-	+	+	-	-	-
15	Yes	NR	Alive (day + 601)		CMV	(Val)ganciclovir	+	+	+	+	+	+
					EBV		+	+	+	-	-	-
17	Yes	PR	Death (day + 314)	Respiratory insufficiency,	CMV	(Val)ganciclovir	+	+	-	+	+	+
				Aspergillus infection, HAdV, CMV	HAdV	Cidofovir	+	-	-	+	-	-
19	Yes	CR	Alive (day + 487)		CMV	(Val)ganciclovir	+	-	+	-	+	-
					EBV	RTX	+	- (RTX)	-	-	-	-
20	Yes	PR	Death (day + 193)	interstitial pneumonitis -> chronic GvHD	CMV	(Val)ganciclovir	+	+	-	+	+	+
					HAdV	Cidofovir	-	-	-	-	-	+
1	No[2]	CR	Death (day + 66)	Klebsiella pneumonia	HAdV	Cidofovir	-	-	-	+	NA	NA
4	No[2]	CR	Death (day + 1498)	Line infection; pneumonia	HAdV	Cidofovir	+	+	+	+	+	+
5	No[2]	PR	Death (day + 64)	MOF, HAdV	HAdV	Cidofovir	-	-	-	+	NA	NA
6	No[2]	NR	Death (day + 102)	MOF, HAdV,	HAdV	Cidofovir	-	-	-	-	+	NA
				klebsiella, EBV	EBV	RTX	-	-	+	- (RTX)	+	na
7	Yes	NR	Death (day + 53)	EBV, HAdV and ongoing aGvHD	HAdV	Cidofovir	-	-	+	+	NA	NA
					EBV	RTX	-	-	+ (RTX)	NA	NA	NA
3	No	CR	Alive (day+ 2708)		EBV		-	-	+	+	-	-
10	Yes	PR	Alive (day + 1497)		EBV	RTX	-	-	+	+	- (RTX)	-

[1]: response of aGvHD at 28 days after the first MSC infusion;

[2]: MSCs were applied as third line treatment. Abbreviations: + or -, indicative of a viral DNA load greater than or equal to log 2.3 copies per milliliter; aGvHD, acute Graft-versus-Host disease; CMV, cytomegalovirus; CR, complete response; EBV, Epstein-Barr virus; HAdV, human adenovirus; HSCT, hematopoietic stem cell transplantation; MMF, mycofenolate mofetil; MOF, multiorgan failure; MSC, mesenchymal stromal cell; NA, not applicable; NR, no response; PR, partial response; RTX, rituximab; UPN, unique personal number.

ent after start of MSC therapy were associated with a poor outcome; none of these patients (n=6) including two patients with recurrence of CMV were alive at 1 year after HSCT, whereas five of six patients with a CMV (n=5) or HAdV (n=1) infection present before and persisting during aGvHD were alive at 1 year after HSCT (Table 1). Six patients in the MSC cohort, who all were from the first 3 years of the inclusion period,
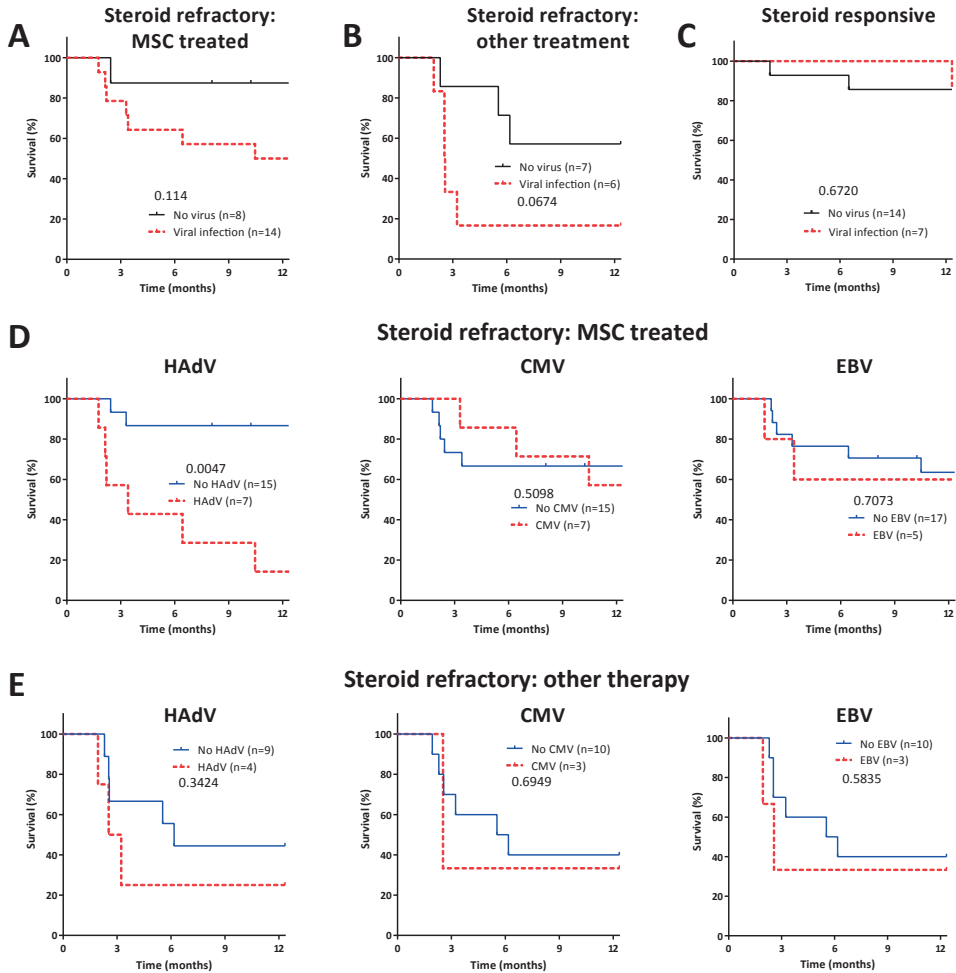

Figure 1 *HAdV infections negatively affect the survival of patients treated with MSCs for steroid refractory aGvHD.* A-C: The one-year survival of patients in relation to viral infections (HAdV, CMV and/ or EBV) is depicted for steroid-refractory patients receiving MSCs (A) or other second-line therapy (B) and for steroid-responsive patients (C). D-E: HAdV, but not CMV or EBV was significantly associated with poor survival in patients treated with MSCs (D). This was not observed in steroid refractory patients receiving other therapies (E). Differences were assessed using the Log-rank Mantel-Cox test.

received other second-line treatment prior to MSC infusion. All these patients had a viral infection either prior to (n=3) or after MSC (n=3) infusion (Table 1).

In the steroid-refractory patients receiving other second-line therapy and no MSCs, CMV was present prior to initiation of steroid therapy, whereas HAdV reactivated after start of aGvHD (Table e). None of the three viruses was associated with decreased survival, as seen for HAdV in the MSC cohort (Figure 1E).

The observed association between infections appearing after MSC infusion and decreased survival in these patients warranted further research into the possible effect of MSC infusion on anti-viral immunity.

In vitro effect of MSC on virus-specific T cells

To determine the putative negative effect of MSCs on HAdV-specific T cells, PBMC obtained from healthy adult donors with demonstrable precursor frequencies of viral-specific T cells (data not shown) were stimulated with inactivated HAdV in the presence or absence of MSCs. MSCs suppressed the proliferative response, but not the IFN-γ production in these co-cultures (Figure 2A-B). To exclude bystander immune activation induced by TLR ligands or other non-specific virus-derived stimuli, experiments were also performed using synthetic, good manufacturing practices-grade, 15-mer viral peptides. In cultures of PBMC stimulated with HAdV hexon-derived peptides, proliferation and IFN-γ production were low, and no suppressive effect of MSCs was observed in either assay (Figure 2C-D). In contrast, after stimulation with synthetic CMV pp65-derived peptides, MSCs showed an inhibitory effect on proliferation and IFN-γ production (Figure 2C-D). Phenotypic analysis of T cells in CMV-stimulated cultures demonstrated a suppressive effect of MSCs on both CD4^{+} and CD8^{+} T cells. This was assessed in proliferation assays using CFSE staining and in assays measuring the percentage of cells expressing HLA-DR after antigen-specific activation (Figure 2E-F).

Stimulation of PBMC with peptide-loaded autologous monocyte-derived dendritic cells

Conflicting results were obtained when investigating the interaction of MSCs and HAdV-specific T cells after employing two ways of stimulation and using two different read-out systems. Consequently, we decided to stimulate PBMC from healthy adult donors with peptide-loaded autologous mDC. MSCs suppressed PBMC proliferation after stimulation with CMV-peptides or HAdV-peptide-loaded mDC (Figure 3A). IFN-γ production was suppressed by MSCs in co-cultures after stimulation with CMV PepTivator with or without IL-2, (although not significantly, p=0.06), but not after stimulation with HAdV PepTivator (Figure 3B). Altogether, these data suggest that the height of the response generated *in vitro* is of importance for the detection of a suppressive effect of MSCs.
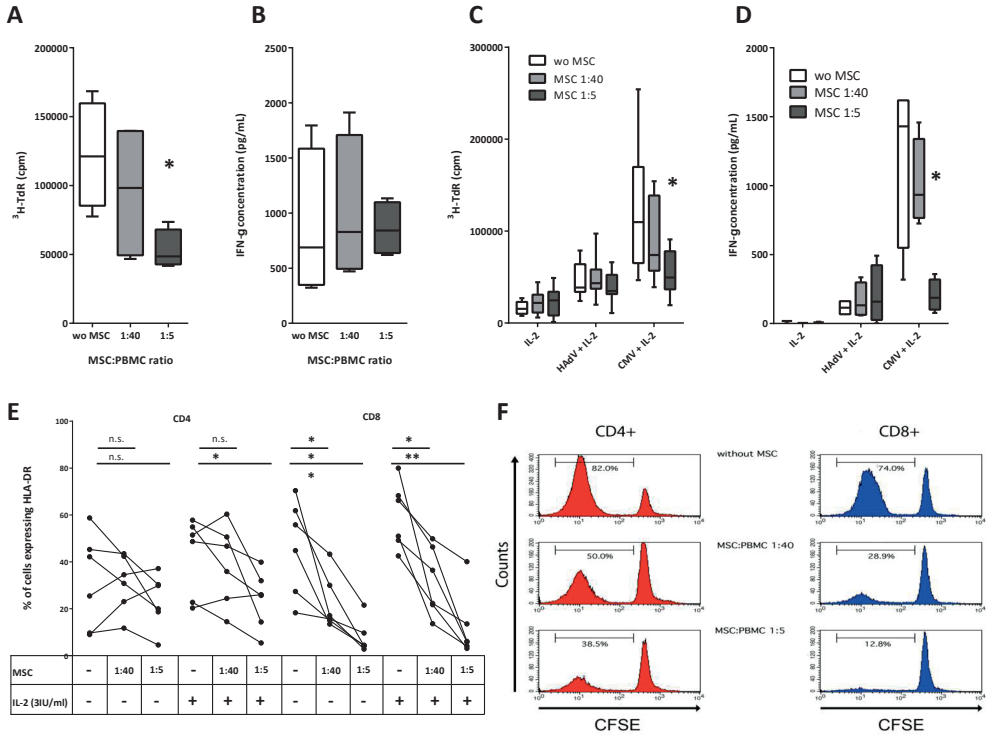
Figure 2. *MSCs suppress PBMC proliferation.* A-B: In cultures of PBMC stimulated with inactivated HAdV, proliferation at day 7 (A) was suppressed by the addition of MSCs, whereas IFN-γ concentration in supernatant obtained at day 6 (B) was not affected (n=4). C-D: After stimulation of PBMC with CMV (pp65 peptides) and IL-2 (3 IU/mL) proliferation (C) and IFN-γ concentrations (D) were lower in conditions with than without (wo) MSCs. Activation with HAdV (hexon peptides) plus IL-2 was lower compared to CMV plus IL-2 and was not affected by the addition of MSCs. E-F: In cultures of PBMC stimulated with CMV pp65 peptides, MSCs affected activation and proliferation of CD4+ and CD8+ T cells as demonstrated by decreased HLA-DR expression (E) and decreased CFSE dilution (F, representative picture of n=4 after stimulation with CMV pp65 peptides only), respectively. Box plots represent 4 independent experiments. Boxes represent median with standard deviation. Whiskers indicate minimum and maximum values. *: p<0.05; **: p<0.01 using paired Wilcoxon signed-rank tests.

Inhibitory effect of MSCs on virus-specific T cell lines

To combat viral infection, activation and clonal expansion of memory T cells is of importance. Consequently, the effect of MSCs on virus-specific T cell lines was investigated. T cell lines raised against HAdV-derived peptides consisted mainly of CD4+ T cells. The ratio of CD4+ to CD8+ T cell in CMV-specific T cell lines varied (Figure S1A). The percentage of T cells expressing HLA-DR was significantly higher in CMV T cell lines than in HAdV T cell lines, regardless of the ratio of CD4+ to CD8+ T cells (Figure S1B). All cell lines were predominantly of effector memory phenotype (>50%) characterized by the lack of CCR7 and CD45RA expression (Figure S1C-D).
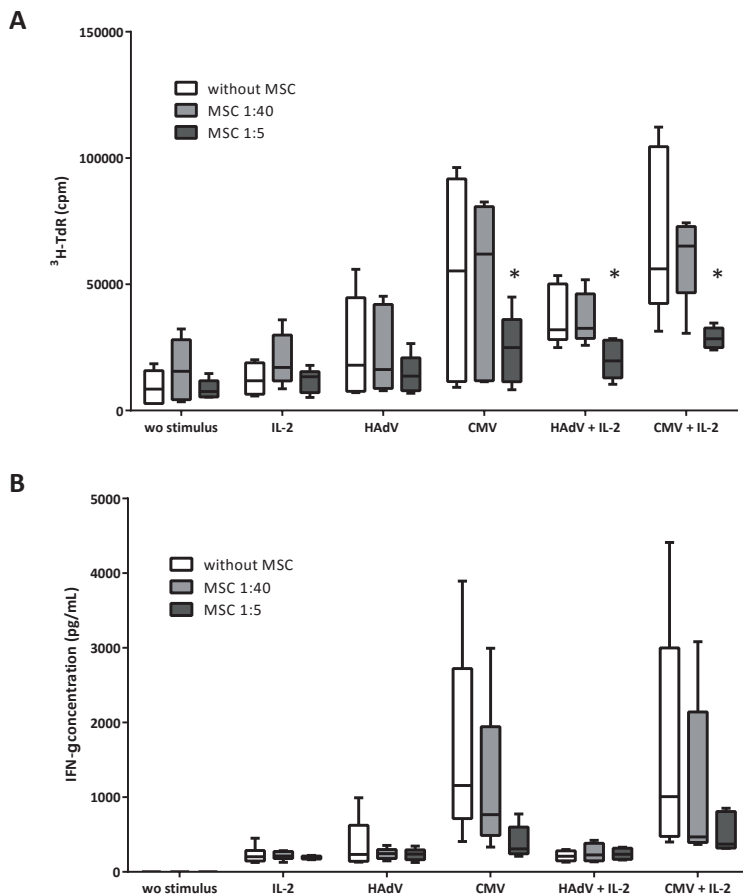
Figure 3. *Effect of MSCs on proliferation of and IFN-γ production by PBMC stimulated with peptide loaded autologous mature dendritic cells (mDC).* A: Proliferation after stimulation of PBMC with hexon peptides-loaded (HAdV) or pp65 peptides loaded (CMV) mDC was increased in co-cultures of MSC and PBMC at a ratio 1:5. In this set-up, a significant reduction in proliferation was observed. B: IFN-γ production remained relatively low for HAdV compared with CMV, and no suppression was documented after stimulation with HAdV hexon-derived peptides. Box plots represent data of six different experiments. Boxes represent median with standard deviation. Whiskers indicate minimum and maximum values. *: p<0.05 using paired Wilcoxon signed-rank tests. IL-2: 3 IU/mL.

Viral peptide-induced proliferation and IFN-γ production were suppressed in co-cultures of HAdV- and CMV-specific T cell lines with MSCs (Figure 4A-B). HLA-DR expression on T cell lines stimulated with HAdV and IL-2 was decreased when T cells were activated in the presence of MSCs (Figure 4C). In contrast, the high percentage of HLA-DR-expressing cells on CMV-specific T cells was not altered (data not shown). Although T cell lines raised against HAdV consisted mainly of CD4^{+} T cells, both CD4^{+} and CD8^{+} T cell-dominated expansions were observed upon restimulation of such

T cell lines; in both cases suppression by MSCs was documented (Figure 4D-E). In conclusion, *in vitro* MSCs have a suppressive effect on proliferation and activation of effector memory-type T cells.

Virus-specific responses in HSCT patients

Sufficient frozen material collected at multiple time points was available from 8 of the 14 children with viral infections in the cohort treated with MSCs for steroid-refractory aGvHD. At the start of MSC therapy two of the seven patients with CMV infection had an undetectable viral DNA load (UPN17 and UPN20; Table 1 and Figure 5A). In the period of 90 days after start of MSC therapy, these patients showed persistent recurrence of viremia. In two of the five patients with CMV virema at the start of MSC therapy, the viremia resolved in this 90 days time window (UPN12 and UPN19) and persisted in the other three patients (UPN02, UPN11 and UPN15) (Table 1 and Figure 5A).
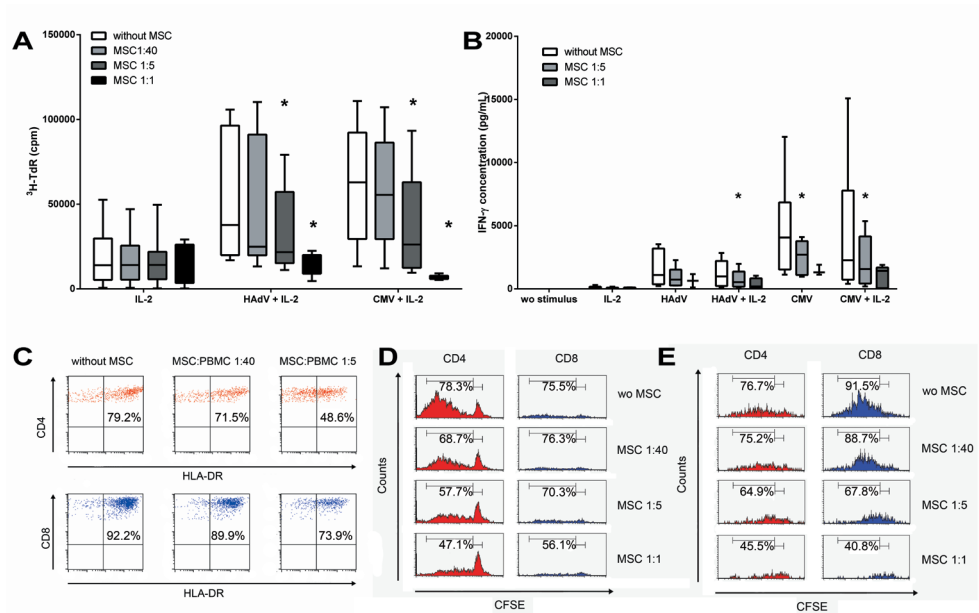

Figure 4. *Effect of MSCs on proliferation, IFN-γ production and HLA-DR expression by virus-specific T cell lines.* A-B: MSCs suppressed proliferation of and IFN-γ production by virus-specific T cell lines (n=6) stimulated with HAdV hexon peptides or CMV pp65 peptides plus IL-2 (3 IU/mL). C: HLA-DR expression was lower in HAdV-specific T cell lines stimulated with HAdV and IL-2 in co-cultures with MSCs. A representative experiment of three cell lines is shown. D-E: Dose-dependent suppression by MSCs of proliferation of both CD4+ (D) and CD8+ (E) T cells after stimulation with HAdV plus IL-2 measured by CFSE dilution was observed. Boxes represent median with standard deviation. Whiskers indicate minimum and maximum values. *: p<0.05 using paired Wilcoxon signed-rank tests.

In six of the seven patients with CMV infection, the frequency of IFN-γ producing cells was determined in the week before, 1-2 weeks after and 28 days after MSC infusion. These six patients received MSCs as second-line therapy. Both patients with
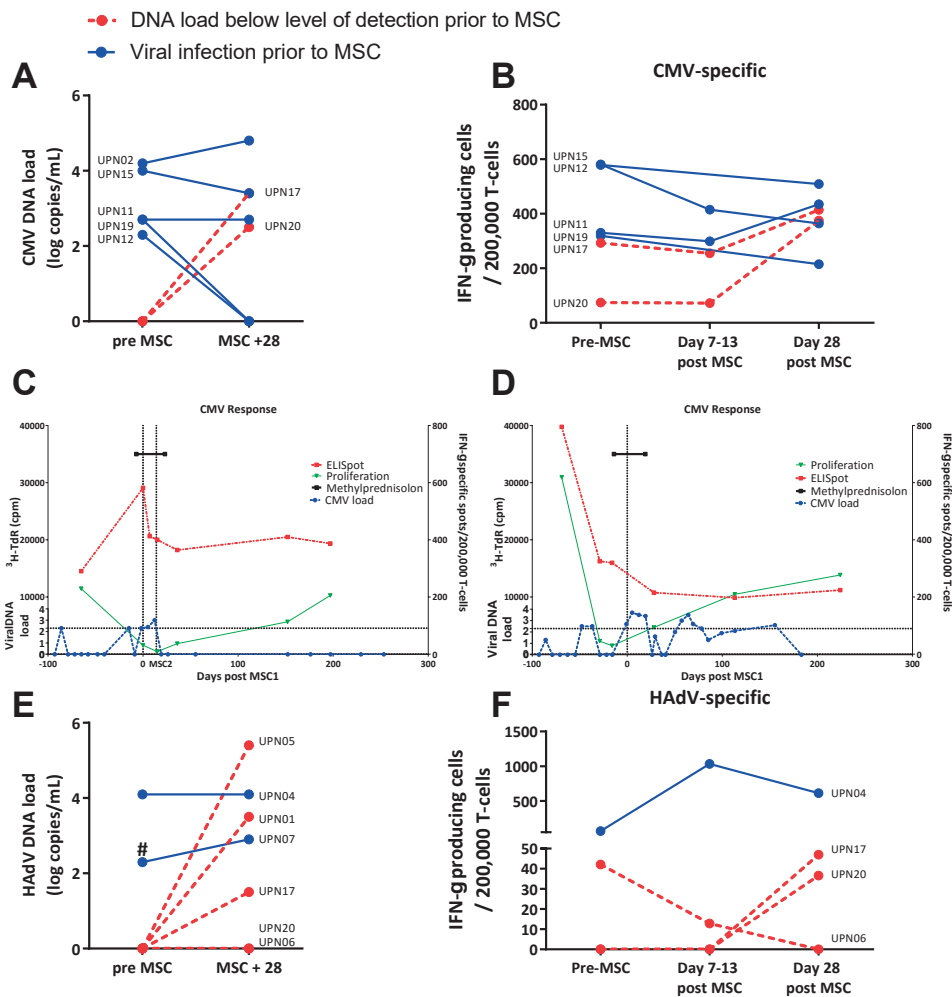
Figure 5 *Viral DNA loads increase around start of aGvHD despite the persistence of IFN-γ producing virus-specific T cells in patients treated with MSCs.* A-B: At 28 days after MSC infusion, CMV DNA loads were increased in two patients, UPN17 and UPN20, (A), however, the number of CMV-specific IFN-γ-producing cells was not affected (B). C-D: In two children UPN12 (C) and UPN19 (D), CMV-specific proliferation could be detected by ^{3}H-thymidine incorporation. Proliferation was decreased after start of steroids. E-F: In 5 patients HAdV DNA loads were detected at 28 days after MSC infusion (E). Plasma HAdV DNA load became positive in UPN06 and UPN20 at 49 days and 106 days, respectively, after MSC infusion. Despite the presence of IFN-γ producing cells (UPN04)(F), the load did not decrease. #: viral DNA load detected three days prior to MSC infusion. UPN01, UPN02, UPN05, and UPN07: no PBMC were available for ELISpot assay.

Table 2. Steroid-unresponsive children receiving other second-line therapy: patient characterization and viral infections

UPN	Second line treatment	aGvHD resolution	Follow-up post HSCT	Cause of Death	Virus	Anti-viral treatment	Before steroids	Start steroids	Steroids +14	Steroids +30	Steroids +60	Steroids +90
31	Thalidomide	Yes	Alive (day +5,463)		CMV		+	-	-	+	-	-
					HAdV		-	-	+	-	-	-
					EBV		-	-	+	+	+	-
32	Prednisolone	No	Death (day +97)	aGvHD, HAdV	HAdV		-	-	-	-	+	NA
33	MMF	No	Death (day +77)	aGvHD, line infection	EBV		-	-	-	+	+	NA
34	MMF	No	Death (day +76)	liver failure, HAdV	CMV		+	+	+	+	+	NA
					HAdV		-	-	-	+	+	NA
35	anti-CD25	Yes	Death (day + 58)	aGvHD, liver failure, HAdV	HAdV		-	-	-	+	NA	NA
					EBV		-	+	+	-	NA	NA
36	MMF	No	Death (day +76)	Respiratory failure, MOF	CMV	(Val) ganciclovir	+	+	+	+	NA	NA

Abbreviations: + or2, indicative of a viral DNA load greater than or equal to log 2.3 copies per milliliter; aGvHD, acute Graft-versus-Host disease; CMV, cytomegalovirus; EBV, Epstein-Barr virus; HAdV, humanadenovirus; HSCT, hematopoietic stem cell transplantation; MMF, mycofenolate mofetil; MOF, multiorgan failure; NA, not applicable; UPN, unique personal number.

recurrent viremia after MSC treatment (UPN17 and UPN020) had low numbers of IFN-γ-producing cells detected by ELISpot in the 2 weeks after receiving MSCs and showed an increase in IFN-γ-producing cells at 28 days after MSC infusion (Figure 5B). In the four evaluable patients with detectable CMV DNA loads before receiving MSCs, the number of IFN-γ-producing cells did not change significantly after MSC infusion and was not associated with the course of the CMV DNA load, which remained stable in two cases and diminished in the other two cases. Two children had a late development of aGvHD (at 83 days and 92 days, respectively, after HSCT), and CMV-specific proliferation was observed prior to the start of systemic steroids. Both showed a decrease of proliferation after start of steroids (Figure 5C-D) with recurrence of CMV viremia prior to MSC infusion. Both had CR after MSC infusion which allowed tapering of steroids. CMV-induced proliferation restored slowly in these patients. Another child with CR showed CMV-specific proliferation from 53 days after MSC infusion onward (data not shown). In the other three children (PR, PR, and NR to MSC, respectively) virus-specific proliferation was not detectable despite the continued presence of IFN-γ-producing cells (data not shown).

In four of the seven patients with HAdV infection, the presence of IFN-γ-producing cells upon *ex vivo* HAdV stimulation of PBMC could be analyzed longitudinally. In all four patients tested IFN-γ-producing T cells were detected: in two children before and after MSC infusion, installed as third-line therapy, and in the other two only after initiation of MSC therapy as second-line treatment (Figure 5F). The patient with high numbers of HAdV-specific IFN-γ-producing T cells (UPN04) experienced a persistent viremia from before the start of systemic steroids onward; however, this patient is the only survivor at one year. The other patients developed a HAdV infection after MSC infusion, and those occurred late for UPN06 (49 days after HSCT) and UPN20 (106 days after HSCT) (Table 1 and Figure 5E). HAdV-specific proliferation was not detectable in any of the patients.

Discussion

Numerous studies have demonstrated the feasibility of MSC therapy for steroid-refractory aGvHD after allogeneic HSCT and have indicated its potentially beneficial effects.(von Bonin, 2009; Prasad, 2011; Le Blanc, 2004; Kebriaei, 2009; Ball, 2013) Response rates in these nonrandomized studies varied but were higher than in reported historic controls, in which various highly immunosuppressive medications were applied as second- or third-line treatment. Patient numbers included so far were too small to draw firm conclusions not only with respect to efficacy but also about the potential occurrence of adverse events such as leukemia relapse and viral infections.

Currently available data on the incidence of infections are focused on CMV.(von Bahr, 2012) The incidence of HAdV infections, which is especially relevant to children after HSCT (Chakrabarti, 2002; Flomenberg, 1994; Walls, 2003), has not been addressed. In addition, the *in vitro* effect of MSCs on HAdV-specific T cells has not been reported.

In a pediatric cohort treated with MSCs for steroid-refractory, severe, aGvHD in the Leiden University Medical Center, the occurrence of HAdV infection after MSC treatment was associated with decreased survival. This was not seen in children with aGvHD grade II-IV who were responsive to steroids or in children receiving second-line therapy other than MSCs. A likely explanation is the relatively shorter duration of immune suppression in the patients responding to steroids. The difference between steroid-refractory children receiving MSCs versus other second-line treatment might point to an effect of MSCs; however, the effect might be influenced by the comparatively high number of children (10 of 22) in the MSC cohort receiving a mismatched graft. Of note, six out of seven children with an HAdV infection in this cohort were transplanted with a mismatched graft, thereby affecting 6 out of 10 children transplanted with such a graft, in line with previously reported data.(van Tol, 2005a) Graft modulation, either T cell depletion or CD34$^+$ enrichment did not differ between the two groups.

One-year survival in patients with steroid-refractory aGvHD receiving second- or third-line therapy other than MSCs (historic controls) was significantly lower than in patients receiving MSCs as second-line therapy (38.5% vs. 73.9%), and was comparable to that of children receiving MSC as third-line therapy (33.3%), which can be attributed to ongoing severe aGvHD. In a study on the use of monoclonal antibodies for the treatment of steroid-refractory aGvHD in children, 9 of 22 patients had viremia (13 episodes of viral reactivation: 5 CMV, 4 EBV and 4 HAdV) compared with viremia in 14 out of 22 children (19 episodes of viral reactivation: 7 CMV, 5 EBV and 7 HAdV) included in our MSC-treated cohort.(Rao, 2009) We deliberately choose log 2.3 copies per milliliter as a cutoff for infection and reactivation, regardless of the virus involved, and chose to include patients with controlled viral infections. This might explain the somewhat higher viremia rate in our study cohort. In addition, when applying the presence of a concentration of log 3.0 copies per milliliter in at least two consecutive plasma samples - the widely accepted criterion for a disseminated infection – the rate of one-year survival for patients with (n=11) and without (n=11) viral dissemination in our MSC cohort is statistically different (36.4% vs 91.0%; respectively; p=0.01).

This is the first study describing the interaction of MSCs with HAdV-specific T cells. Karlsson *et al.* have published experimental data indicating the absence of an *in vitro* effect of MSCs on CMV- and EBV-specific T cells.(Karlsson, 2008) These data are in contrast to results from other studies demonstrating a downmodulating effect of MSCs on the autologous EBV-B- lymphoblastoid cell line-induced proliferation

of EBV-specific T cells.(Kang, 2005; Sundin, 2006) The latter observation is in line with our findings of a negative impact of MSCs on proliferation of both PBMC and virus-specific T cell lines after stimulation with CMV pp65 or HAdV hexon derived peptides loaded mDC or PBMC, respectively. The *in vitro* suppressive effect of MSCs on virus-induced T cell proliferation and IFN-γ production was MSC-dose dependent and most evident at high ratios of MSC to target cells. This and the fact that the relevant *in vivo* effect of MSCs most probably occurs at the sites of infection and tissue damage might explain that no increase in CMV-related disease is observed. Of note, suppression of proliferation was not observed in co-culture experiments using MSC and T cell clones specific for CMV, HAdV or control HY minor histocompatibility antigen (data not shown).

In a recent study in mice, the suppression of the induction of an ovalbumin-specific T cell response *in vivo* after infusion of MSCs was explained by a decreased homing of ovalbumin-pulsed dendritic cells to the lymph nodes.(Chiesa, 2011) An indirect effect of MSCs on lymphocyte activation and proliferation via monocytes has been suggested previously.(Groh, 2005) In our MSC cohort, survival of patients with infections already present at the time of MSC infusion was significantly higher than that of patients with an onset of infections after the initiation of MSC therapy. A negative impact of MSC on antigen-presenting cells involved in the induction of a response *in vivo* might be an explanation for the differences in lethality of HAdV and CMV infections occurring prior to and after MSC infusion, respectively. However, based on our data, we cannot exclude a differential effect of immunosuppressive drugs on the initiation of a response versus an ongoing response.

Using CMV pentamers, Karlsson *et al.* were the first to show that the percentage of virus-specific T cells was stable prior to and after MSC infusion in two patients. (Karlsson, 2008) Lucchini *et al.* came to a similar conclusion based on the analysis of IFN-γ-producing cells in two patients after MSC infusion.(Lucchini, 2012) However, one of these patients showed a decrease in the number of IFN-γ-secreting cells and a recurrence of CMV viremia after multiple MSC infusions. In addition, 11 of 24 patients had a viral reactivation that occured after initiation of MSC therapy, including three patients with HAdV infection. Interestingly, no viral-infection-related death occurred in this patient group. Our data confirm the previously reported observation that MSC infusion did not affect the number of circulating CMV-specific T cells and extended this to HAdV-specific T cells. Of note, the level of IFN-γ-producing cells amongst peripheral blood T cells did correlate with the course of the CMV viremia or with the HAdV viremia, suggesting that more in-depth analysis of T cell subpopulations combined with other functional aspects might be of interest.

A major difficulty in interpreting data of *ex vivo* analysis of virus-specific T cells in MSC-treated patients is the immune suppression by methylprednisolone preced-

ing MSC infusion and continued thereafter. A decreased number of CD8^{+} T cells after initiation of methylprednisolone has been reported previously,(Aubert, 2001; Cwynarski, 2001) in addition to decreased cytokine production without reduction of T cell counts.(Ozdemir, 2002) Previous studies did not comment on the influence of methylprednisolone tapering on recovery of immunity. We demonstrated a similar number of IFN-γ-producing cells in PBMC prior to and after initiation of aGvHD treatment, whereas the proliferative capacity was hampered from the start of steroid infusion. This analysis could only be performed in two patients with sufficient numbers of PBMC prior to MSC 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.

Although our *in vitro* data demonstrate a suppressive effect of MSCs on T cell proliferation, the effect of methylprednisolone *in vivo* is likely to be stronger. Consequently, aiming for a fast reduction of methylprednisolone after MSC infusion seems to be of utmost importance in these patients following objective documentation of resolvement of the aGvHD symptoms.(Calkoen, 2013b) Interestingly, infections already present at the start of aGvHD did not negatively affect the outcome. Thus, patients with active viral infections should not be excluded from future randomized controlled trials on MSCs as second-line therapy of steroid-refractory aGvHD; however, critical viral monitoring is advised.

Conclusion

In this study, we show an association between HAdV infections occurring after MSC infusion and decreased survival in patients treated for severe steroid-refractory aGvHD. In addition, our *in vitro* data demonstrate a suppressive effect of MSCs on proliferation and activation of, among others, HAdV-specific T cells, whereas no solid evidence was obtained to support a negative impact of MSCs on antiviral T cell driven immune responses *in vivo*. However, the beneficial effect of MSC therapy on steroid-refractory aGvHD is strongly supported by the observation that CR or PR was established at 28 days after start of MSC therapy in 10 of the 14 children with a virus infection. In conclusion, the results of the present study indicate that the benefits of MSCs outweigh the potentially increased risk of infections. Randomized controlled trials, currently under development, will not only further address the efficacy of MSC treatment but should also represent the ideal platform to further investigate potential side-effects of MSC, specifically with regard to viral reactivation.

Acknowledgements

Supplementary data
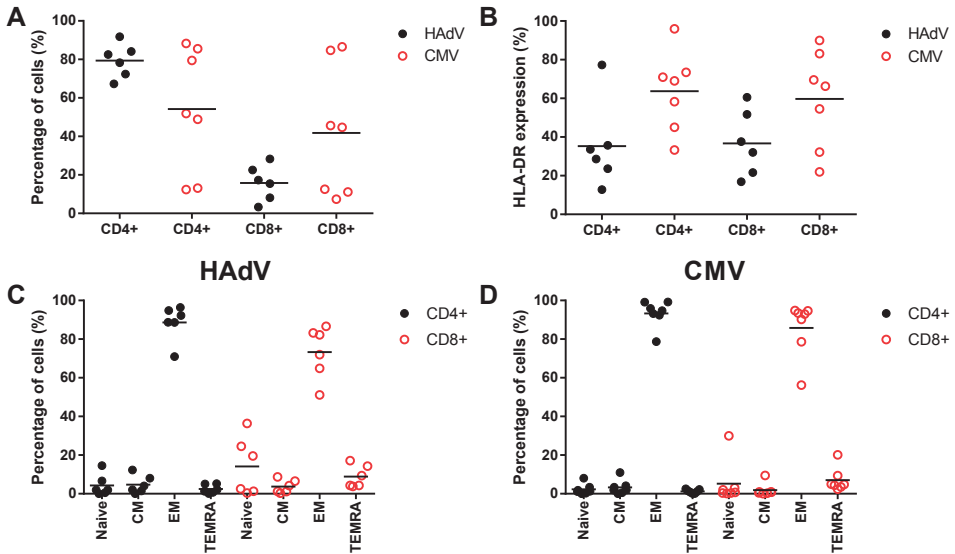
Figure S1: *Phenotype of virus-specific T-cell lines on day 28 after culture initiation.* T-cell lines raised against HAdV (hexon) or CMV (pp65) derived peptides were characterized by immunophenotyping. A: The percentage of CD4+ and CD8+ T-cells in the HAdV- and CMV-specific T-cell lines is depicted. B: The percentage of cells expressing HLA-DR on either CD4+ or CD8+ T-cells was higher in CMV-specific T-cell lines compared to HAdV-specific T-cell lines. C-D: Both HAdV-specific (C) and CMV-specific (D) T-cell lines consisted mainly of effector memory (EM) cells (CD45RA−CCR7−). Naïve, central memory (CM) and end stage effector (TEMRA) cells were defined as CD45RA+CCR7+, CD45RA−CCR7+ and CD45RA+CCR7− cells, respectively.

Supplementary Table 1. Patient's characteristics

		Steroid refractory aGvHD; MSC	Steroid refractory aGvHD; no MSC	Steroid responsive aGvHD
N		22	13	21
Follow-up (days; median; range)		379 (53-2708)	166 (58-5463)	1123 (61-2575)*
Diagnosis	ALL/AML	9	2	13
	Other hematologic malignances	6	6	4
	Nonmalignant disorder	7	5	4
Age at HSCT median (range)		6.3 (0.7-18.1)	5.2 (0.6-14.3)	9 (0.4-17.1)
Sex (M/F)		12/10	9/4	16/5
Donor	HLA-identical related	7	2	9
	Matched Unrelated	5	6	10
	Mismatched Unrelated	10	2	2*
	Other related	0	3	0
Stem cell source (BM/PBSC/CB)		13/4/5	9/4/0	17/3/1
Graft manipulation (none/TCD/CD34 selection)		20/1/1	10/2/1	21/0/0
Donor sex (M/F)		12/10	6/7	7/14
Conditioning	TBI based MAC	5	5	9
	Busulfan based MAC	14	7	10
	Other MAC	2	0	1
	RIC	1	1	1
Serotherapy	+ rATG/Campath	13/3	5/3	12/2#
GvHD prophylaxis	CsA	7	2	6
	CsA + MTX	10	9	13
	CsA + prednisolon (1 mg/kg)	5	0	1
	CsA + MMF	0	1	0
	none	0	1	1
Relapse		3	0	3
Median days between first MSC and start aGvHD (range)		12 (5-59)		
aGvHD organs involved	skin only	0	1	10***
	gut only	2	0	2
	gut and skin	10	8	6

Supplementary Table 1. Patient's characteristics (continued)

		Steroid refractory aGvHD; MSC	Steroid refractory aGvHD; no MSC	Steroid responsive aGvHD
	gut and liver	5	0	0
	skin and liver	0	0	3
	gut, skin and liver	5	4	0
aGvHD grade	II	0	7***	16***
	III	14	3*	5*
	IV	8	3	0**
aGvHD stage gut	0	0	1	11***
	1	0	5**	7*
	2	2	1	0
	3	16	3*	3***
	4	4	3	0
aGvHD treatment	Steroids up to 2 mg/kg	22†	13	21†
2nd line therapy	Total	6††	13***	0*
	Steroids 3-5 mg/kg	5	3	0
	MMF / Tacrolimus	1/2	6/0	0/0
	thalidomide / aCD25	0	3/1*	0

ALL acute lymphatic leukemia; AML acute myeloid leukemia; HSCT hematopoietic stem cell transplantation; HLA human leukocyte antigen; BM bone marrow; PBSC peripheral blood stem cells; CB cord blood; TCD T-cell depletion; TBI total body irradiation; MAC myeloablative conditioning; RIC reduced intensity conditioning; rATG rabbit anti-thymocyte globulin; aGvHD acute Graft-versus-Host Disease; CsA cyclosporin A; MTX methotrexate; MSC mesenchymal stromal cells; MMF mycophenolate mofetil. # one patient received Campath and rATG. † one patient received only 1 mg/kg prednisone. †† MSC were given as third-line therapy. Mismatched donor defined as: 5/6 (CB) or 9/10 HLA allele-matched (BM/PBSC). Grading and staging of aGvHD was according to the adapted Seattle criteria. (Ball LM, Egeler RM *BMT* 2008) Both control groups were compared to the MSC-group using Chi-squared and unpaired students T-tests: * p<0.05; ** p<0.01; *** p<0.001.

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.

General discussion and future perspectives

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 allo-geneic 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 Administra-tion (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

General discussion and future perspectives

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 hemato-poietic 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 af-fecting 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 expres-sion 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 dis-turbed 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.
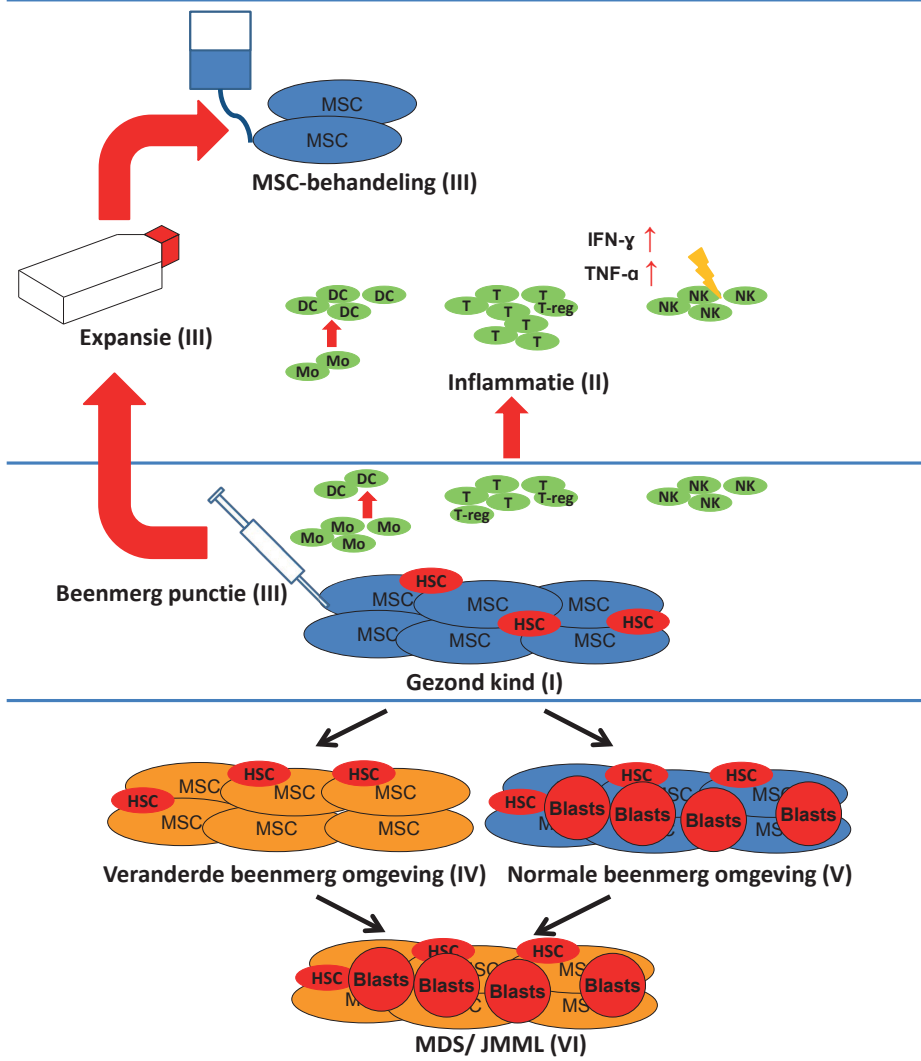
Figuur 1. *Een overzicht van de verschillende onderwerpen in dit proefschrift.* I: MSCs zorgen bij gezonde kinderen voor ondersteuning van hematopoëse en een stabiel immuunsysteem. II: Tijdens momenten van ontsteking, zoals bij acute *graft-versus-host* ziekte, ontstaat er een toename in geactiveerde *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.

Chapter 8.

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References

Chapter 9.

Dankwoord
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Dankwoord

Wat een mooi moment om eindelijk iedereen te mogen bedanken die heeft bijgedragen aan de totstandkoming van dit proefschrift. Onderzoeken doe je samen, en samen is niet alleen.

Mijn promotor, Maarten Egeler, beste Maarten, wat een positiviteit, wat een motivatie. Je bent in de afgelopen jaren niet altijd dichtbij geweest, maar na elke bespreking ging ik weer met nieuwe kracht aan de slag om weer een stuk verder te komen met deze puzzel. Jouw enthousiasme en praktische inslag zal ik altijd met mij meedragen. Ciao!

Mijn co-promotor, Lynne Ball, beste Lynne, wat een eer om jouw project over te nemen. Alle uren samen achter de computer om mijn eerste versies om te toveren tot volwaardige manuscripten. Wat heb ik veel van je geleerd. Met jouw brede kennis en tomeloze energie kwamen we iedere keer tot nieuwe inzichten. Een aantal van deze ideeën zijn in dit proefschrift beland. Nog veel werk te doen de komende jaren.

Mijn co-promotor, Maarten van Tol, beste Maarten, veel dank! Je hebt van mij een onderzoeker gemaakt. Gedetailleerd en precies, kritisch en perfectionistisch, altijd alles snel bekeken (wel vaak een kwartiertje te laat). Tussendoor de sport uitslagen doornemen zorgde voor de nodige ontspanning om de laatste uren van de dag door te komen.

Carly, uren zaten we naast elkaar in de flowkast, bij de FACS of met Maarten en Lynne aan tafel. Zonder jou waren er geen labresultaten in dit boekje gekomen. Jij had altijd alles op orde en de experimenten nog wat uitbreiden kon altijd worden geregeld. Je hebt veel geduld met me gehad, dank daarvoor.

Het kinder immunolab, Els, Anja, Susy, Monique, Kitty, Janine, Gertjan, Jacqueline, Gerda, Astrid en Marco, een goede sfeer is de basis voor hard werken. Jullie hebben allemaal bijgedragen aan het onderzoek door hulp bij de experimenten of door (meestal) constructief commentaar.

Jens, Willemijn en Harmke, mijn mede-promovendi, het is zo belangrijk om mensen om je heen te hebben die in hetzelfde schuitje zitten. Samen om 16:00 uur een blikje drinken of na het werk biertjes drinken, we hebben het allemaal nodig gehad. Het was een top tijd.

Helene en Melissa, dank voor jullie adviezen en het opbouwende commentaar, het was inspirerend om met jullie te overleggen. Lisanne, Else, Leon (SASC) en Peter-Bram, jullie hebben mij een andere wereld aan de andere kant van de brug laten leren kennen. Zonder jullie was het mij nooit gelukt om verder te komen met de RNA experimenten. Beste Marry, dank voor het geloof in dit project en de ingang die jij

ons gaf in EWOG-MDS. Beste Monique, dank voor het uitwisselen van kennis tussen de Rotterdamse en Leidse MSC groep, een ware stimulans.

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Vrienden, bij inspanning hoort ontspanning, maar zoals jullie van mij gewend zijn is inspanning ook vaak ontspanning. Schaatsen, hockey, tennis, fietsen of gewoon een biertje aan de bar. Het gaat nooit vervelen. Dank voor al deze momenten van ontspanning.

Coentjes, lieve pap en mam, jullie zijn altijd geïnteresseerd, stimulerend en betrokken. Veel dank daarvoor. Os en Emm, altijd weer veel gezelligheid, sportiviteit en een nuchtere blik, vroeger in Teteringen en nu weer alle drie in Amsterdam.

Lieve Claire, met jou mijn leven delen is een feestje. Er zijn heel wat vrije uurtjes gaan zitten in dit proefschrift, dat kon alleen maar omdat de overgebleven vrije uurtjes zo ontspannen waren. Thuis is ook altijd echt gezellig thuis met jou. Lieve Ilou, na een dag werken, jou lachend aan zien komen rennen, 'papa!' roepend is de beste motivatie voor mijn werk binnen de kindergeneeskunde.

Curriculum vitae

Friso G.J. Calkoen was born on April 5th 1983 in Rotterdam, the Netherlands. He grew up in the villages of Terheijden and Teteringen. He graduated school from the Stedelijk Gymnasium Breda in 2001 (cum laude). That same year he continued his education at Victoria University in Wellington, New Zealand where he studied Biomedical Sciences. He went on to study Medicine at the Leiden University Medical Center in 2002. He worked as an allocation officer at Eurotransplant during his studies. In his third year of Medicine he was awarded an Excellent Student award and started a research project at the department of Pulmonology of the Leiden University Medical Center under supervision of prof. dr. P.S. Hiemstra. He investigated the role of natural killer T cells in asthma. After completion of this project and finishing his final medical internship at the pediatric stem cell transplantation unit of the Leiden University Medical Center, Friso received his medical degree in 2009.

During his final internship he was introduced to the immunological research at the Department of Pediatrics by dr. L.M. Ball and dr. A.C. Lankester. Subsequently, prof. dr. R.M. Egeler invited him to start as a PhD student and he started the work described in this thesis focussing on the role of mesenchymal stromal cells in pediatric disease (promotor prof. dr. R.M. Egeler, co-promotores dr. L.M. Ball and dr. M.J.D. van Tol). He received the Young Investigator Award of the European Society for Pediatric Gastroenterology, Hepatology and Nutrition to present his work at the 2013 congress in Londen, United Kingdom. Moreover, the work of this thesis was presented at the EBMT congresses in Vienna 2010 and Geneva 2012, the ISEH congress in Amsterdam 2012, the IBMTR congress in Salt Lake City 2013 and the NVK congresses in Veldhoven in 2010 and 2015 (as late breaking abstract). Friso was a warded a Sequencing Grant of the Leiden University Medical Center to continue the research described in this thesis.

In 2013 Friso started as a resident in pediatrics at the Juliana Children's Hospital, The Hague, the Netherlands. This is where he started as a pediatrician in training under supervision of dr. F. Brus in 2014. He will continue his pediatric residency in the Willem-Alexander Children's Hospital, Leiden University Medical Center in April 2016 (dr. W.J.W. Kollen).

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