Clinical and laboratory features of mesenchymal stromal cells in pediatric stem cell transplantation

Ball, L.M.

Part 5

Summary and General Discussion

"My aim is not to teach the method that everyone ought to follow in order to conduct his reason well, but solely to reveal how I have tried to conduct my own"

- Rene Descartes (1596-1650)
  Discours de la methode 1637

Chapter 10 Summary and General Discussion

10.1 Summary

This thesis describes the clinical use and laboratory findings of bone marrow (BM) derived*, ex-vivo* expanded human Mesenchymal Stromal Cells (MSCs) in pediatric stem cell transplantation. Advances in modern transplant technologies have permitted the use of "alternative donors", namely haploidentical peripheral blood stem cells (PBSCs) and umbilical cord blood (UCB), as a source of donor hematopoietic stem cells. However, associated problems of graft dysfunction, delayed immune reconstitution as well as steroid refractory acute graft versus host disease, reduce the effectiveness of these forms of allogeneic hematopoietic stem cell transplantation (HSCT).

MSCs located in the bone marrow form the matrix or stromal network, essential for the support of normal hematopoietic stem cell growth and development. Methods to expand MSCs sufficient for clinical uses have recently become available. *Ex vivo* expanded MSCs have been shown to be hypo-immune, in as much that they do not possess MHC class II antigens or co-stimulatory molecules and they do not induce stimulate T cell proliferation in a mixed lymphocyte reaction (MLR). *In vitro* experiments have demonstrated a wide range of interactions of MSCs with various cells of both the innate and adaptive immune system, which overall results in the induction of an anti-inflammatory state. The exact mechanisms operational *in vivo* are unknown but cell to cell contact and the production of soluble factors seem to be important.

The ability of MSCs to support hematopoietic stem cell growth coupled with their potent immune suppressive effects mean that they are ideal candidate cells to explore in the context of overcoming some of the problems of allogeneic HSCT, especially those specifically associated with the use of alternative donors. In this thesis, we therefore attempted to address the most relevant of these issues that, given the known properties of MSCs, would allow us to assess the possible usefulness of MSCs as an innovative strategy to resolve them.

Chapter 2 addresses the outcome of highly enriched CD34+ selected haploidentical peripheral blood stem cell transplantation in children. No published information was available regarding transplant centers' experience with haploidentical transplantation and the influence that exerted on transplant related mortality (TRM) associated with the procedure, including graft failure, graft versus host disease (GvHD) rates and overall survival.[a] We showed in a single center retrospective analysis, that haploidentical stem cell transplantation is a feasible solution for children requiring HSCT, who otherwise have no suitable donor. However, delays in immune reconstitution, graft rejection and infections were major problems associated with, but not exclusive to, this form of HSCT. In our analysis large does of CD34+ selected cells did not overcome the risk of graft rejection. MSCs have been described to accelerate hematological recovery in adults undergoing allogeneic bone marrow transplantation, but their role in overcoming graft failure in the haploidentical setting was unknown. In light of this observation we decided to investigate the role of MSCs in overcoming graft rejection in the haploidentical setting. We devised a phase I/II study under the auspices of the European Group for Blood and Marrow Transplantation (EBMT) Development Committee, which studied the effect of co-transplantation of haploidentical bone marrow derived *ex-vivo* expanded MSCs with haploidentical G-CSF mobilized peripheral blood stem cell transplantation.

Chapter 3 describes the results of the feasibility and safety of MSCs infusions in the haploidentical peripheral blood stem cell setting. A total of 13 children were recruited from two participating centers (Leiden and Pavia) and compared to 52 consecutive non selected historical controls from both centers. Graft failure was evident in 11/52 (20%) of the control group compared to 0/13 (0%) in the study group receiving MSCs (p=0.06). Lymphocyte recovery was accelerated in the study group compared to controls (p=0.005), with increased numbers of NK cells, evident in the first month following transplantation, accounting for this observation. Acute GvHD was reduced in the study group but was not significantly different from the control group. We concluded that co-transplantation of haploidentical MSCs was feasible and safe in this setting and was not associated with an increased risk of infection or relapse in children undergoing transplantation for malignant disease.

In chapter 4, we report the outcome of a similar EBMT developmental committee phase I/II study undertaken in children undergoing umbilical cord blood stem cell transplantation (UCBT). From reported literature and our own unit's clinical experience (unpublished), UCBT has the disadvantage of delayed hematopoietic and immunological recovery related to the low numbers of infused CD34+ cells of UCB compared to bone marrow grafts. Animal models suggest that fetal lung derived MSCs can accelerate hematological recovery following UCBT. We felt our experience with haploidentical PBSC and MSC co- transplantation warranted an exploratory study in children undergoing UCBT. Children undergoing UCBT for malignant disease (n=13) were recruited from 3 centers (Pavia, Leiden and Stockholm) and co-transplanted with parental derived haploidentical BM derived MSCs. This study cohort was compared to 39 matched historical controls. Although no differences in hematological recovery were observed between the groups, requirement for additional granulocyte colony stimulating factor (G –CSF) administration was less in the study cohort compared to the controls (23% versus 69%, respectively; p=< 0.05). Overall rates of acute GvHD did not significantly differ between study and control cohorts but severe (grade III-IV) did (0% versus 26%). Although overall survival was not significantly reduced by the addition of MSCs, early transplant related mortality (TRM) showed a reduction, related to the decrease in death due to fatal GvHD.

Chapter 5 addresses the concerns that MSCs, once considered immune privileged, may in a murine model induce T cell mediated rejection of donor hematopoietic stem cells. We report the clinical outcome of two children undergoing unrelated UCBT with paternal haploidentical MSC co- transplantation, who initially rejected their cord transplant. They were later successfully re- transplanted using the father as the donor for both haploidentical PBSCs and MSCs. Despite exposure to paternal derived MSCs during the initial transplant procedure they did not subsequently generate specific antibodies against the father, suggesting in immunosuppressed transplant patients multiple infusions of MSCs are not immunogenic and do not induce rejection.

Chapter 6 reviews the present knowledge regarding the patho-physiology of acute graft versus host disease. This chapter describes in details the complex interactions between donor and recipient that initiate the clinical syndrome of acute GvHD. An

understanding of the patho-physiology allows for the rational development of a phase I/II multi-center study of MSC treatment for GvHD refractory to conventional steroid therapies.

In chapter 7 we describe the outcome of an EBMT Developmental Committee phase II study of MSCs in patients (both adult and children) with severe, grade II-IV, steroid refractory acute GvHD.
Patients were recruited from five centers (Stockholm, Leiden, Pavia, Genoa and Adelaide) and included a total of 55 patients (30 adults and 25 children). MCS were administered in patients with severe grade of GvHD who were unresponsive to steroids, and/or further attempts at immune suppression. Prior to receiving MSCs, the majority of patients (60%) had received 2nd line treatments, 25% 3rd line treatments and 10% > 3rd line treatments, in addition to steroids, without documented response. 80% of children responded to MSCs infusions either with complete resolution (CR) of partial resolution (PR) of their symptoms. In adults the response was less (60%), which difference was not, however, statistically significant. The estimated probability of survival 2 years after HSCT was 35% for the whole cohort, with adults doing worse than children (26% versus 45% p=0.06). The 2-year probability of survival in complete responders (52%) was significantly better than that in the patients with partial or no response (16% p=0.018). We concluded that MSCs cells derived from bone marrow might be a safe and effective treatment for patients with severe, acute GVHD who do not respond to corticosteroids and other immunosuppressive therapies. The collaborating centers adopted a common protocol for the expansion of mesenchymal stem cells. As there were no center differences we concluded that the use of a common protocol allowed for the reproducibility of the results. The results obtained from this study has allowed for the development of a randomized multi-center study of the effectivity of MSCs infusion in the treatment of GvHD, again under the auspice of the EBMT Developmental Committee.

Chapter 8 describes in detail the outcome of one of the pediatric patients included in the above study and highlights the need for long term follow up in this patient cohort. Although potential tissue repair effects of MSCs have been suggested by some authors, we describe a child successfully treated with MSC infusions for severe acute

steroid refractory GvHD, who failed to repair gastric mucosal damage incumbent on severe gastro-intestinal GvHD and coexistent adenoviral enteritis. This has led to the initiation of a detailed clinical and physiological study in childhood survivors of severe GvHD.
MSCs exert a broad immune suppressive effect in vitro. However, it is unknown whether or not there are differential effects of MSCs on alloreactive T-cells and virus-specific T-cell responses.

Chapter 9 describes the results of collaboration between Leiden and Great Ormond Street Hospital, London of *in-vitro* investigations into the interaction of MSCs with different virus-specific T-cell populations, namely cytomegalovirus (CMV) and Epstein Barr virus (EBV). Alloreactive T-cells were, as expected from the results of published literature, highly susceptible to suppression by MSCs, whereas in contrast we demonstrated that MSCs do not appear to suppress virus-specific T-cell functions *in vitro*. Children with severe acute GvHD treated with high dose steroids, may (in addition) develop virus re-activation. A number of patients may receive both MSCs as treatment for GvHD and virus-specific T-cells as treatment for the viral re-activation. The results suggest that the antiviral effect of such specific T cells would not be affected by the concomitant infusion of MSCs.

Finally, chapter 10 contains the summary and discussion and chapter 11 the future directions of potential research into the use of MSCs in pediatric diseases.

10.2 General discussion

Allogeneic hematopoietic stem cell transplantation has become am established treatment for selected children with life-threatening diseases.[1] Initially limited by the availability of human leukocyte antigen (HLA) matched sibling donors, advances in modern transplant medicine allows for the use of both HLA matched and mismatched donors, either from family and unrelated sources. To facilitate the search for a suitable donor, a large world wide network of donor registries has been established, which presently contain over 13 million registered volunteer donors.[2] Despite these immense advances and the significant numbers of suitable donors indentified in a timely fashion, there still is a need for alternative options for those children, who despite these measures, are left without a suitable donor. For these children, alternative donor sources can be explored, namely haploidentical stem cells[3] (usually from a parent) or a related or unrelated cord blood.[4]
Despite the limitations of alternative donor HSCT, for many children there is no other possible means of attempting cure of their underlying disease. As such transplant physicians must strive to improve the outcome of these transplants in order to maximize their potential and reduce the accompanying associated risks. If the complications of these forms of HSCT can be reduced or overcome to allow parity of results with "suitable donor HSCT" then the considerable advantages associated with haploidentical or cord blood HSCT could potentially outweigh their risks, with the results that these transplants may become more widely applicable.

Generally it is true to say the more disparate the HLA match, the more complicated the stem cell transplantation procedure becomes. Graft manipulation to avoid lethal GvHD is necessary but intense donor T cell depletion together with residual recipient T cells that may escape conditioning, lead to an increased risk of graft failure and delayed immune reconstitution.[5]

In chapter 2 we addressed this issue and showed a relatively good malignant free survival (41%), comparable to published data.[6,7] However, the high rate of graft rejection, despite infusing very large doses of stem cells, reduced the overall

effectiveness of this procedure. Graft rejection in the haploidentical setting was not restricted to our unit, but has been widely published.[8-10]
In our series children rejected early and there was significant mortality due to fatal viral reactivation following second attempt transplantation.

The early *in vivo* studies in humans with MSCs are concentrated mainly within the fields of hemato-oncology and stem cell transplantation and focused on the ability of MSCs to promote and sustain engraftment of hematopoietic stem cells. [11-15] The co-infusion of MSCs had been shown to support hematological recovery in adult patients undergoing either autologous or allogeneic matched related bone marrow transplantation.[13-15]
There was no data available on the role of MSCs to overcome graft rejection, although it seemed to us reasonable given the physiological role of MSCs in hematopoietic stem cell support, to hypothesize that they could be a valuable adjunct in the haploidentical PBSCT setting. If MSCs were to overcome graft failure then there was a considerable opportunity to improve on the historical results of transplantation from HLA disparate donors.

In chapter 3 we demonstrated that it was feasible and safe to administer haploidentical bone marrow derived expanded MSCs to children undergoing haploidentical PBSCT. Study patients (n=13) received haploidentical bone marrow derived *ex vivo* expanded MSC at a dose of 1-2 x 10^6/kg recipient weight. Additionally they received haploidentical G-CSF mobilized peripheral blood stem cells, four hours after the infusion of MSCs, with a target dose of 20 x 10^6/kg CD34+. The outcome in terms of feasibility, toxicity, engraftment, rates of infection, acute GvHD and relapse were compared to 52 non selected historical controls, who received haploidentical PBSCs alone. Epidemiologically there was no difference between the study patients and the controls other than the administration of MSCs. There was a trend to a statistically significant improvement in sustained engraftment (p=0.06) without increasing the rate of infections, relapse or transplant related toxicities in children receiving MSCs compared to controls. Despite these encouraging results, the study was limited in as much that children were assigned to the study in a non-randomized fashion and compared to historical controls. However, since the initial publication, a

total of 32 children have been included and to date none have documented graft failure.

To determine the efficacy of this approach we would ideally undertake a prospective, randomized study of MSCs co-transplantation in children undergoing haploidentical PBSCT. However calculations revealed that a minimum of 100 patients per arm would be necessary to statistically prove efficacy of this approach. This form of transplant is relatively limited both in numbers of children undergoing the procedure and as to the centers experienced in this method of transplantation, and where MSCs are also available for co-infusion. Therefore, regrettably, we concluded that it would not be feasible to carry out a randomized multi- center study.

Although the immune recovery in the initial patient group was not intrinsically different from the historical controls we demonstrated in chapter 3 a short lasting but significant increase in the absolute number of natural killer (NK) cells immediately post haploidentical PSBCT in the study patients receiving MSCs compared to the historical controls. We plan to further evaluate the increased NK numbers following MSCs co- transplantation with haploidentical PBSCT to determine the role, if any, MSCs co-transplantation has on the donor-derived alloreactive NK cells with measurable anti-leukemic activity in children undergoing haploidentical PBSCT.

Umbilical cord blood (UCB) offers a readily available source of stem cells with minimal risk to the donor and can expand the available HLA matches especially in ethnic minorities who are poorly represented in the volunteer donor banks. Since the first publication, advances in understanding of the biological characteristics of UCB and the clinical application in transplantation has been widely reported and reviewed.[4, 16-24] The use of cord blood is an accepted source of stem cells mainly for pediatric patients, but increasingly so in adults. Numerous studies have demonstrated that cord blood shows a higher proportion of primitive hematopoietic progenitors, with superior in vitro proliferative responses than those detected in bone marrow.[25-28] These laboratory observed characteristics are supported by the fact that recipients of human UCBT demonstrate reliable engraftment of myeloid and lymphoid cell lineages after myeloablative conditioning with greater than one log less nucleated cell dose than that used for recipients of bone marrow. However, the lower number of cells contained within UCBT compared to those obtained by bone

marrow harvest is associated with delayed engraftment/immune reconstitution when compared to standard bone marrow or peripheral blood stem cell transplantation. It was reported that the co-transplantation of fetal lung derived MSCs enhances engraftment of CD34+ cells in NOD/SCID mice, especially evident when the total numbers of CD34+ cell in the graft was low.[29] We thus designed a study in collaboration with the Department of Pediatric Hematology Oncology Fondazione IRCCS Policlinico S. Matteo, University of Pavia, Italy, to ascertain whether or not haploidentical bone marrow derived *ex vivo* expanded MSCs would be mirror this finding and enhance engraftment in children undergoing either related or unrelated UCB transplantation.

In chapter 4 we report our findings namely, that it was feasible and safe to administer MSCs in the context of children undergoing UCB for leukemic disease, with no additional toxicities compared to matched historical controls. In contrast to the pre-clinical studies and our findings in haploidentical PBSCT, there was no difference in rates of engraftment or rejection between the study patients (n=13) and controls (n=39). The only epidemiological difference between the two groups was that the controls received G-CSF more often than those included in the study arm (69% v 23%; p= < 0.05). Despite these disappointing results, it became evident on further analysis that there was a significant decrease in the rates of severe acute GvHD in children receiving MSCs and UCBT compared to controls. (0% v 25%; p=0.058). Disease free survival at 2 years was 92% in the study patients and 63% in the controls. Although this did not reach statistical significance there were no deaths due to GvHD in the study arm compared to 3 in the control group.

A characteristic of UCB is the reduced alloreactive response as compared to BMT. The median T cell dose is lower by comparison (8×10^6 kg^{-1}), but is still capable of inducing GvHD, especially in the setting of HLA disparity. Functional differences play a role in the reduced levels of GvHD observed following successful UCBT. These may include higher levels of naïve CD4+CD45RA+ cells, lower alloantigen and mitogen induced cytokine production and responses, a polyclonal T cell repertoire, increased susceptibility to tolerance induction, and differences in NK cell and dendritic cell biology.[28,30-32]

Published data from most of the transplant registries show that despite the infusion of class I or II disparate UCB grafts, the incidence of acute GvHD has been lower than previously reported in matched unrelated donor bone marrow or partially matched family donor marrow allografts.[18,33-35] The incidence of grade I-II and grade III-IV acute GvHD in children ranges between 30-50% and 10-20%, respectively. The rates of acute GvHD observed in our historical control group conformed to these published data obtained from large cohorts of children undergoing UCB transplantation.
Thomson et al.[36] reported on the immune reconstitution following URD UCBT in 27 children. Lymphocyte recovery occurred at 2 months for NK cells, 6 months for B cells, 9 months for CD8+ T-cells and 12 months for CD4+ T-cells. The authors concluded that this was similar to other stem cell sources. Niehues et al.[37] reported similar findings. Factors favoring T cell recovery were the use of a related donor, higher nucleated cell dose, and positive recipient CMV serology. Recovery was delayed by acute GvHD.
Klein et al. measured T-cell receptor excision circles (TREC) values, which were abnormal up to 12 months following URD UCBT. In adults normal levels were not detected until 18 months post UCBT. Recently other studies have suggested that the T-cell repertoire following UCBT as well as TREC values are low 12 months after UCBT but are comparable to those measured following BMT recipients.[38]
The data of immune reconstitution following UCB transplantation with co-transfusion of MSCs has yet to be analyzed. The lack of severe acute and extensive chronic GvHD observed in our study cohort and obfuscating the need for additional immune suppression, may well positively influence the kinetics of the immune reconstitution, despite the lack of enhanced hematopoietic stem cell engraftment.
There have been concerns raised that reduction in GvHD could also lead to a reduction in GvL effect,[39] related to the down regulation of alloreactive donor T cells by MSCs. We have not documented any significant increased risk of relapse in either our haploidentical PBSCT or UCBT study cohorts. However the numbers included in both studies were small and although matched as far as possible for disease status at transplant, the controls were included on an historical rather than a randomized basis.
The potential of co-infused haploidentical MSCs to reduce significant life-threatening GvHD is an important consideration for children undergoing UCBT for non-malignant disease, such as metabolic disorders or inborn errors of metabolism. For children without malignant disease there is no advantage to the development of GvHD as

there is no requirement of allogeneic T cell reactivity to reduce tumor load. On the contrary, a reduction in TRM due to GvHD could play a significant role in overall survival. As such a randomized study with the use of MSCs versus placebo with UCBT could be undertaken in children with benign indication for HSCT with the incidence of acute GvHD and survival as primary endpoints. A possible alternative would be to explore the use MSCs in the context of double cord transplantation, where acute GvHD rates are reportedly higher than in single cord transplants.[40]

MSCs are poor antigen presenting cells and do not express MHC class II or co-stimulatory molecules. Accordingly, expanded MSC's do not stimulate T cell proliferation in Mixed Lymphocyte Reactions (MLR) and are able to down regulate alloreactive T cell responses when added to mixed lymphocyte cultures.[41] They were once considered immune privileged but reports that, in a murine model of reduced intensity allogeneic stem cell transplantation, MSCs can elicit an immune response,[42] has led to the acceptance that they should be clinically considered as hypo-immune, especially in an immunocompetent host. Concerns have since been raised that in patients undergoing MSC co-transplant procedures, multiple exposure to donor-derived MSCs may induce rejection of donor hematopoietic stem cells.

In chapter 5 we present two unusual clinical cases of children undergoing unrelated UCB transplantation with co-infusion of paternal MSCs. They both rejected the umbilical hematopoietic stem cells but did not produce antibodies directed against cells of paternal origins. They successfully underwent subsequent haploidentical PBSCT and MSCs co-infusion, with the father acting as the donor. Long term engraftment with 100% paternal donor cells was established. This report gives some reassurances that children undergoing complex transplant procedures , especially if receiving myeloablative conditioning, can safely receive multiple MSCs infusions without jeopardising the hematopoietic stem cell engraftment potential of the MSCs donor, should that later be required. However in immunocompetent patients the risk that MSCs may induce an immune response has not been assessed.

GvHD can be described as a three-phase process.[43] Firstly, the initiation of damage to host tissue, as occurs with the conditioning of the patient. This induces the release of inflammatory cytokines, including IL-1, TNFα, and IFN- γ. [44-46]
The second step is the triggering and activation of donor-derived T-cells by recipient and donor antigen presenting cells as well as the inflammatory cytokines.[47] Activated T-cells result in the production of IL-2 and IFN- γ (or T_H1 response).[48] IL-2 controls and amplifies the allogeneic immune response.[49]
Finally, the effector phase is characterized by activated donor T-cell mediated cytotoxic damage against host cells through Fas-Fas ligand interaction,[50,51] and perforin-granzyme[51] and TNFα mediated apoptotic pathwyas.[52] The latter plays a central role in the pathophysiology, stimulating cytokine production (IL-1, IL-6 IL-10, IL-12, and TNFα). This dysregulation leads to the clinical manifestations of aGvHD.[43,46,53]
Residual host antigen presenting cells (APCs) may be implicated in the initiation phase of GvHD and their localization may be relevant to organ-specific manifestations of GvHD.[54-56]
HLA differences between donor and recipient are the major predictor of acute GvHD.[57] Other factors implicated include recipient age,[58] gender mismatch between donor and recipient,[58] minor histocompatibility in otherwise identical HSCT,[59] donor age,[60] source and dose of stem cells (PBSCT greater risk than BM),[61] intensity of conditioning, and GvHD prophylaxis.[62]
Administration of unmanipulated doses of donor derived lymphocytes (so-called DLI) for the treatment of mixed chimerism or relapse of solid malignancies has an increased risk of GvHD, especially following reduced intensity conditioning. [47,63-66] Again, this is related to dose and timing following HSCT.

In light of the published *in-vitro* immune suppressive properties of MSCs, we decided to review the current literature regarding the pathophysiology of acute GVHD and presented our findings in <u>chapter 6.</u> In this way we hoped to develop a rational for the use of MSCs in children with steroid refractory GvHD.

Despite advances in pre-transplant immune suppression and donor HLA typing methods, acute GvHD remains a significant cause of transplant related mortality and morbidity following allogeneic HSCT.[43]

The initial management of acute GvHD comprises of steroid treatment. This may be combined with Cyclosporine (CSA)[67,68] or tacrolimus.[69-71] The majority of centers utilize methyl prednisolone at 2.0 mg/kg/day. Approximately 50% of patients will remit or improve with this treatment but the remaining patients require second-line treatment, which to date remains unsatisfactory.[67]

There is presently no consensus as to salvage treatment in steroid refractory GvHD. Acute GvHD is considered steroid refractory when there is no response to methyl prednisolone at 2.0 mg/kg/day within 5-7 days, or when there is progressive disease at 72 hours after initiation of treatment with this dose.[43]

Numerous agents have been reported as second line treatment and continue to be evaluated. Whatever there initial effects, they have not fulfilled their expectations and have had little impact on overall survival, which remains dismal.

Arai et al. reported the use of ATG in a population of 69 steroid refractory patients but only 4 patients survived.[72] Similarly, Khoury et al, reported 90% of patients in a study of ATG were dead at 40 days after the start of treatment, mainly due to infection.[73]

Monoclonal antibodies such as anti- CD25 (daclizumub),[74,75] anti-TNFα (infliximab)[76,77] and anti-CD3 (visilizumab)[78] have been used in clinical studies, with varying degrees of success. However, associated increased infections such as moulds and EBV reactivation have been problematic. Many authors regard these drugs to have failed their original expectations for the majority of patients requiring salvage treatment.

New modalities of treatment are under study such as the toxin fusion product (denileukin diftitox), a fusion of IL-2 and diphtheria toxin, which targets the IL-2 receptor over-expressed on activated T-lymphocytes.[79]

To date there is no evidence of improvements in overall survival for any of these agents in the treatment of steroid refractory GvHD.

The infusion of third party MSC's was described which effectively eradicated steroid refractory acute GvHD that had failed all other attempts at treatment.[80] The hypothesis of action was that MSC's demonstrate powerful immune-modulatory

functions that can effectively down-regulate T cells and thus diminish or eradicate GvHD. [41,81-83]

In chapter 7 we report the findings of a phase I/II multicenter analysis of 55 patients (30 adults and 25 children) treated with MSCs infusions for severe grade II-IV steroid refractory acute GvHD. Five centers (Stockholm, Leiden , Pavia, Genoa and Adelaide) participated in the study. Patients varied in the time to MSCs infusions and the type of previous immunosuppression administered before receiving MSCs. The majority of patients (60%) had received 2nd line treatments, (25%) 3rd line treatments and 10% > 3rd line treatments, in addition to steroids, without documented clinical or pathological response. MSCs were expanded according to the EBMT consortium protocol, with identical batches of FBS. A median dose of bone-marrow derived *ex vivo* expanded MSCs of 1.4 (range 0.4-9) x10^6 cells/kg was administered. Twenty-seven patients received 1 infusion, 22 patients received 2 infusions and 6 patients received 3 to 5 infusions. In light of the rapid progression of symptoms necessitating prompt treatment, MSCs were mainly derived from third-party HLA-mismatched donors (n=69), the rest being obtained from either from HLA-identical sibling donors (n=5) or haploidentical donors (n=18).
As was evident in our other clinical protocols no toxicities were documented associated with the infusion of MSCs, even in the most seriously ill patients treated. Thirty patients showed a complete response (55%) and 8 showed improvement, the overall response rate being 69%. The response rate was higher in children (80%) than in adults (60%; p=0.28). Although not significant, the better response of children contributed to the improved overall survival. Patients with a complete response had a lower TRM 1 year after MSCs infusion as compared to partial or non-responders (37% versus 72%; p=0.002) and a higher overall survival 2 years after hematopoietic stem cell transplantation (52% versus 16%; p=0.018). Nearly half the patients survived (n=21) and 8 patients developed chronic GvHD. The study was hampered by the fact that the patients were heterogeneous not only in terms of timing of MSCs infusions but also to the variable immune suppressive regimens administered prior to MSCs. Although compared to historical controls and recent literature, the survival rates seem favorable, the efficacy of MSCs in the treatment of acute GvHD remains to be proven.

To address this issue, the EBMT MSC consortium has designed and a double blind randomized study between 2nd line treatment with or without allogeneic MSCs infusions, in patients with steroid refractory severe acute GvHD. Patients having failed steroid treatment will be allocated to receive 2nd line therapy with either MSCs (1-2 x 10^6/kg recipient weight) at day 0 and day +7 versus 2nd line therapy and placebo. A follow up of two years will determine not only efficacy but also document any long term side effects such as increased risk of TRM, relapse or increased infection rates.

The results of a randomized studies undertaken using the commercially available product Prochymal®, in patients with acute GvHD have recently been released (but not yet published).[84]
Protocol 265 was designed to evaluate Prochymal as a first-line agent for the treatment of acute GvHD in combination with steroid therapy. The majority of patients in this trial were suffering from skin GvHD, which responded significantly better to steroids than had been previously reported in controlled trials. This high response rate to standard of care diminished the potential for Prochymal to demonstrate an effect. (45% vs. 46%, n=192).
In the more severe, steroid-refractory GvHD setting (protocol 280), the benefit of adding Prochymal to second-line therapy was evaluated. Prochymal approached statistical significance (40% vs. 28%, p=0.087, n=179) for the primary endpoint (durable complete response) in the per-protocol patient population .Additionally, Prochymal significantly improved response rates to liver (76% vs. 47%,p=0.026, n=61) and gastrointestinal GvHD(88% vs. 64%,p=0.018, n=71), for which there is currently no known reliable therapy. Notably, the Prochymal cohort had more severe GvHD (27% of Prochymal patients had grade IV GvHD, the most severe form, vs. 16% of placebo patients, p=0.05).
In pediatric patients, Prochymal showed a strong trend of improvement in response rates (86% vs. 57%, p=0.094, n=28).

These results are seemingly at variance with the findings of the multi-center feasibility study (chapter 7). However the proposed randomized EBMT study has important conceptual differences. Firstly, not all MSCs products can be considered the same. There are no details as to the propriety expansion protocols as compared to the

published data from the EBMT consortium. It is known that expansion methods including the number of passages can alter the immunological proprieties of MSCs.[85,86] The EBMT recognizing this problem has developed a strict expansion protocol which includes permitted reagents especially the use of tested FCS batches. In the Prochymal study, 52 centers entered 192 patients, with some centers only treating one patient. Lack of sufficient center experience, especially of such critically ill patients, can negatively affect outcome of clinical studies. Similarly, centers with little exposure to the problem of GvHD will have difficulties in assessing response. We have shown that clinical symptoms of gastrointestinal GvHD i.e. diarrhea and abdominal pain may persist following MSCs treatment. This is due villous atrophy and persistent malabsorption, even when histologically there is no evidence of active acute GvHD (unpublished data). Thus it is important that response to treatment wherever possible is documented with histological confirmation rather than relying on clinical symptoms alone.

To avoid such clinical bias, the EBMT protocol is limited to large experienced centers (undertaking at least 20 allogeneic transplants per year).

Rather than negate the necessity of the proposed EBMT study, the commercial results reinforce the need for a well conducted collaborative study with the aforementioned safeguards to avoid unnecessary bias and determine the true efficacy of MSCs in controlling refractory GvHD.

The ability of MSCs not only to differentiate into various tissues but also to induce both structural[87] and functional[88] tissue repair, led Ringden et al to implicate the infusion of MSCs in patients being treated for refractory GvHD in the coincidental tissue repair of gut, lung and bladder.[89]

This led us to review the clinical outcome and follow up histopathology of our pediatric patients entered into the multicenter study. Graft versus host disease of the gastrointestinal tract in our clinical experience is often associated with concurrent adenoviral infection. This leads to widespread destruction and loss of integrity of the gastrointestinal (GI) mucosa. Despite documented resolution of acute GvHD following MSCs infusions our patient commonly exhibited protracted gastro-intestinal dysfunction, until mucosal regeneration occurred (data not presented).

In chapter 8 we report a young girl in whom this period of regeneration was associated with widespread fibrosis with no evidence of tissue repair. In contrast to the patients reported by Ringden et al. she was treated with alternative immune suppression before MSCs infusions. She also developed protracted GI adenoviral infection simultaneously with her severe GvHD and the combination likely contributed to her long-term outcome. As donor and recipient were female, we were unable to determine MSCs of donor orgin in biopsy material by dual staining fluorescent in-situ hybridization (FISH) for X and Y chromosomes. Unlike the Swedish group, we therefore could not document trafficking of MSCs to the site of tissue damage.

This case highlights the potential sequlae that previously, in light of the associated high mortality rates were not appreciated in survivors of severe and widespread GvHD, especially young children.

We have therefore implemented a multicenter pediatric study to determine the health related quality of life (HRQoL) in children treated for acute GvHD following HSCT. We will utilise the PedsQL which, is a modular approach to measuring HRQoL in healthy children and adolescents and those with acute and chronic health conditions.[90] The PedsQL integrates both generic core scales and disease-specific modules into one measurement system. The study will recruit children receiving MSCs infusions for refractory GvHD as well as those successfully treated with steroids alone. The control group will be children having undergone HSCT without GvHD manifestations, matched as far as possible for age, sex and type of transplant and underlying disease. In this way we hope to study the impact of MSCs infusions on the perception of health both by the child and their parents,

Infectious complications especially virus reactivation is a major problem in children undergoing unrelated and alternative donor HSCT owing to the delays in immune reconstitution.Besides potentially lethal infections with cytomegalovirus (CMV) and Epstein Barr virus (EBV), human adenoviruses (HAdV) are increasingly recognized as life-threatening pathogens.

Between 40-70% of HSCT recipients who are CMV seropositive or have a seropositive donor develop CMV reactivation.[91-93] Reactivation occurs more frequently when patients are further immunosuppressed during treatment of GvHD.[93] EBV

reactivation may result in post-transplant lympho-proliferative disease (PTLD) and occurs in 11-26% of HSCT patients where selective T-cell depletion has been used for prevention of GvHD.[94-96] Anti-viral T-cell effector functions are essential for preventing viral reactivation and progression to virus-associated disease. T cells as well as NK cells may play a role in recognition and killing of HAdV-infected cells, while cytokines and neutralizing antibodies may limit the spread of the infection. As has been shown for CMV and EBV reactivations, [97-100] immunological recovery after HSCT seems also of great importance for clearance of HAdV infections.[101-105] Adoptive cellular immunotherapy with virus-specific T cell clones or lines has been successfully applied as either prophylactic treatment in patients at risk for viral reactivation or as pre-emptive treatment of patients with reactivation based on the appearance of a viral DNA load in the circulation or established EBV lympho-proliferative disease (CMV,[106-108] EBV,[108-110] and HAdV[108,111]). The pediatric unit in Leiden is actively pursuing specific T cell therapies as a post transplant treatment strategy for children with viral reactivations.

MSCs exhibit a broad immune suppressive effect in vitro.[83] Children undergoing treatment with MSCs infusions for refractory acute GvHD may also, in light of the additional steroid immune suppression, suffer viral reactivation(s).[93] The use of MSCs in these circumstances theoretically could negatively influence survival by down regulating endogenously developed or exogenously infused virus-specific T-cell functions. Therefore in the later part of the thesis we studied the effect of MSCs *in vitro,* specifically related to the interactions of MSCs with virus-specific T-cells.

In chapter 9 we describe the differential effects of MSCs on alloreactive T cells compared to CMV and EBV virus-specific T cell responses.
Alloreactive T-cells were, as expected, highly susceptible to suppression by MSCs *in vitro* as has been widely reported. In contrast, CMV-specific immunity was not affected by MSCs. Potentially this difference may be explained by differential effects of MSCs on naïve and memory T cells. However we demonstrated that MSCs suppress proliferation of both $CD45RO^+$ memory and $CD45RA^+$ naïve T-cells in response to allogeneic dendritic cells (DC) or phytohemagglutinin (PHA) stimulation.

MSCs suppressed proliferation of PBMC in response to EBV particles but we found no suppression of proliferation in established EBV-cytotoxic T lymphocyte (CTL) lines. This could be explained by differences in cell types contained in PBMC and EBV-CTL lines. Proliferation of EBV-specific T-cell clones with reactivity against immuno-dominant EBV-antigens was not influenced by MSCs. Furthermore, MSCs had no effect on the function of established EBV-CTL to proliferate and produce IFN-γ in response to EBV antigens and to lyse EBV-infected targets. Besides potentially lethal infections with cytomegalovirus (CMV), human adenoviruses (HAdV) are increasingly recognized as pathogens causing life-threatening infections in patients after HSCT. HAdV infections were especially noticed at a high frequency (up to 40%) in pediatric graft recipients,[112-114] and being lethal in up to 50% of cases with disseminated infection.[115-117] Very little is known about the specificity of immunosuppression by MSC and, in particular, the interaction of MSC with virus-specific T cells and specific T cell subsets. As both GvHD and viral reactivation may occur coincidentally it is important to explore any potential interaction between specific cellular treatments that may be co-administered. To this end, we have initiated investigations into the effect of MSCs on proliferation of virus-specific CD4+ [(HAdV) and CD8+ (CMV] T-cells/clones as well as CD4+ and CD8+ minor-HLA (HY) specific T-cell clones *in vitro*. We hope to be able to demonstrate a differential ability of 3rd party MSCs isolated from normal healthy donors to inhibit proliferation of viral peptide or HY peptide stimulated specific T cell subsets.

Our preliminary results demonstrated that although MSCs as expected suppressed proliferation of PMBCs in response to polyclonal phytohemagglutinin (PHA) stimulation, they did not suppress either CD4+ or CD8+ T cells, irrespective of the specific stimulus used. Confirmation experiments are ongoing as well as the possible requirements for APCs or other cells in this setting. Therapeutically infused virus-specific T cell lines although predominantly CD4+ or CD8+, vary in the proportion of these specific cell subtypes. Subsequently, we will investigate the interaction of MSCs with expanded virus-specific T cell lines prepared for potential therapeutic use in order to determine any possible interactions which may be clinically relevant. Finally, we will explore the effect of MSCs on the induction of virus-specific T-cell responses in cultures of PBMC, i.e., on the initiation phase of activation of virus-specific effector and memory T cells.

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