Cover Page

Universiteit Leiden

The handle http://hdl.handle.net/1887/37810 holds various files of this Leiden University dissertation.

Author: Garde, Mark Paul van der
Title: Different strategies to improve the use of the umbilical cord and cord blood for hematopoietic and other regenerative cell therapies
Issue Date: 2016-02-09

Different strategies to improve the use of the umbilical cord and cord blood for hematopoietic and other regenerative cell therapies

Proefschrift

ter verkrijgen van de graad van Doctor aan de Universiteit Leiden op gezag van Rector Magnificus prof. mr. C. Stolker volgens besluit van het College voor Promoties ter verdediging op dinsdag 9 februari 2016, klokke 16:15 uur door

Mark van der Garde

geboren te Goes in 1979

The research described in this thesis was conducted at the Jon J van Rood Center for Clinical Transfusion Research of the Sanquin Blood Supply Foundation and the Leiden University Medical Center (department of Immunohematology and Blood Transfusion and the Experimental Animal Center)

The studies were funded by the Sanquin Blood Supply Foundation, grants PPCOC 06-030 and PPOC 08-009

All rights reserved. No part of this material may be reproduced or utilized in any form or by any means, electronic, mechanical, photocopying, recording or otherwise, without the permission of the author of this thesis

Promotiecommissie

Promotoren: Prof. dr. Jaap Jan Zwaginga en prof. dr. Anneke Brand

Co-promotor: Dr. Yvette van Hensbergen

Overige leden: Prof. Dr. Fred Lackenburg (Universiteit Leiden), Prof. Dr. Anske van der Bom (Universiteit Leiden), Prof. Dr. Jan Cornelissen (Erasmus Universiteit Rotterdam), prof. dr. Suzanne Watt (University of Oxford, UK)

Table of contents

Introduction **001**

Chapter 1: **023**
Direct comparison of Wharton Jelly and bone marrow derived mesenchymal stromal cells to enhance the engraftment of CB $CD34^+$ transplants

Chapter 2: **047**
Thrombopoietin treatment of one graft in a double cord blood transplant provides early platelet recovery while contributing to long term engraftment in NSG mice

Chapter 3: **071**
Cryopreservation of cord blood $CD34^+$ cells before or after thrombopoietin expansion differentially affects early platelet recovery in NOD SCID mice

Chapter 4: **089**
No synergistic effect of co-transplantation of MSC and ex vivo TPO expanded CD34+ cord blood cells on platelet recovery and bone marrow engraftment in NOD SCID mice

Chapter 5: **109**
Dual use of cord blood for both hematopoietic stem cell transplantation and other therapies

General Discussion **123**

Summary (English) **139**

Samenvatting (Nederlands) **143**

Acknowledgements **147**

Publication list **151**

List of acronyms **153**

Introduction

Cord blood transplantation

Hematopoietic stem cell transplantation (HST) is used as a treatment for patients with blood related disorders. In Europe, around 40,000 HST are performed annually. [1] Depending on the disorder, its stage and earlier treatment response, HST is performed with autologous hematopoietic stem cells (HSC) or allogeneic HSC. HSC can be obtained via direct bone marrow aspiration (BM grafts) or by (nowadays much more common) mobilizing the HSC via injecting the patient or donor with G-CSF and subsequent harvest of the cells by apheresis (mobilized peripheral blood stem cell grafts, PBSC grafts). Autologous transplantation is mostly used for lymphoproliferative disorders such as myeloma and (non)-Hodgkin's lymphoma. Allogeneic HST, accounting for around 42% of all HST, is mostly used for patients with inherited disorders like hemoglobinopathies, inborn errors of metabolism where allogeneic hematopoiesis will correct the defect. Otherwise allogeneic HST is needed for patients with e.g. leukemia where the so called graft versus leukemia effect (GvL) mediated by donor lymphocytes is the only cure left. If a patient requires an allogeneic transplantation, the search for a suitable donor is first done within the immediate family of the patient. The suitability of a donor depends on the number of HLA-A, HLA-B, HLA-C, HLA-DRB1 and DQ alleles that match between the donor and the patient. The number of matched alleles is associated with overall survival after HST, [2-4] and transplantations are preferably done with fully matched (10/10 or 9/10) donor stem cells. The chance of having a suitable family donor is relatively small, and therefore around 70% of the patients require an alternative donor. Although recently haploidentical related donors are increasingly used as alternative donors, [5-7] the majority of patients still rely on unrelated volunteer donors. The search for an unrelated donor is done via international bone marrow registries such as Bone Marrow Donor Worldwide (BMDW [8]) or via national coordinating centers such as Europdonor [9] the bone marrow registry for the Netherlands. BMDW is an international collaboration of most accredited bone marrow registries in the world. Bone marrow registries maintain a database of HLA typed donors that can be accessed by the affiliated transplantation centers. However, despite over 25 million registered donors worldwide, often no HLA-matched donor may be found, particularly for non-Western European patients. [10] In the United States for approximately 50% of the patients with a European descent a 10/10 HLA matched donors could be found, but for patients with a non-European descent this percentage dropped to just over 20%. [11]

When there is no HLA matched donor available, patients can be transplanted with umbilical cord blood (CB). CB was identified as a source for hematopoietic stem cells (HSC) over 2 decades ago, [12] followed by the first successful transplantation of CB HSC in a patient with Fanconi's anemia in 1989. [13] The immune cells in CB are more immature and are therefore less potent in causing graft versus host disease (GvHD) as compared to transplantation with bone marrow (BM) or mobilized peripheral blood stem cell (PBSC) grafts. [14-16] CB therefore allows for more mismatches in HLA alleles between the donor and the patients. CB is generally matched for HLA-A/B on a serological level and HLA-DRB1 on a high resolution level. Another advantage of CB is the fact that CB cells are cryopreserved and banked, allowing for quality control measurements and making a CB graft more readily available for transplantation. In contrast, the timespan that is needed to select an unmatched donor and the final availability of its mobilized peripheral blood graft is several weeks since the donor has to be counseled for donor and patient safety aspects and take a course of stem cell mobilizing agents such as G-CSF over a period of 5 days, prior to harvesting of the cells with apheresis.

Currently there are over 600,000 CB units stored worldwide and CB transplantations (CB HST) account for approximately 6% of all HST performed in Europe. [1] However, CB units

contain a relatively low number of stem cells compared to BM or PBSC grafts [17] and no additional T-cells for donor lymphocyte infusion (DLI) can be obtained after transplantation. This translates into several disadvantages when compared to transplantation of BM or PBSC grafts. CB HST is associated with an increased risk of graft failure and hematopoietic engraftment is delayed, which lengthens the median time to neutrophil and platelet recovery. [16,18] Furthermore, immune reconstitution is delayed [19,20], making infections, especially in the first 3 months after transplantation, the largest cause of non-transplant related mortality in CB recipients.
While the last 20 years the overall survival after cord blood transplantation improved significantly, [21-23] the total costs for treatment of a hematological malignancy with a CB graft HST remain much higher than treatment with a PBSC graft. Including a one year follow up period, the total cost for a CB graft HCT amounts to € 254,000, compared to € 171,000 for transplantation of unrelated PBSC grafts. [24] While the cost for selection and harvesting of a graft between unrelated allogeneic PBSC grafts and CB grafts is approximately equal, around € 65,000 per graft, the hospital admission period of patients that receive CB transplants in this respect is on average twice as long compared to patients that receive a PBSC graft because of the previously mentioned delay in peripheral blood cell recovery [24]. This shows that also from this cost effectiveness point of view, it is extremely important to improve the engraftment capacity of cord blood cells.

Methods to improve the outcome of cord blood transplantation
The overall survival after cord blood transplantation is related to two factors: the degree of matching for HLA-A, B and DRB1 between the cord blood unit and the patient and the total nucleated cell dose or HSC dose of a CB graft. [25-27] While equally matched BM and CB HST showed an equal probability of neutrophil and platelet recovery, [18] this probability drops considerably when the CB unit has 1 or 2 mismatches (i.e. a 5/6 or 4/6 match), especially when the unit has a low cell dose. [18,25] Patients transplanted with fully 6/6 matched CB grafts have the best chance of survival, followed by recipients of 1 allele mismatched CB grafts with a medium cell dose (TNC=$2.5x10^7$ – $4.9x10^7$ cells/kg) and recipients of 2 allele mismatched CB grafts with a high cell dose (TNC>$5.0x10^7$ cells/kg). [25] Interestingly, recipients of 2 allele mismatched CB grafts with a high cell dose engrafted sooner than recipients of a 1 allele mismatched graft with a medium cell dose.
The outcome of CB HST can thus be improved by better HLA matching, between the CB unit and the patient, and the transplantation of high doses of cells. Moreover, more extensive HLA typing for e.g. HLA-C has been shown to lead to better recovery and reduced non-relapse mortality. [28] Interestingly, in cord blood transplantation the HLA pattern of the parents also seems to play a role. During pregnancy, fetal cells are exposed to non-inherited maternal antigens (NIMA) which can induce tolerance towards these antigens. [29] Matching for donor NIMAs can increase the number of suitable donors and reduce transplant related mortality and thereby improve the outcome of transplantation. The inherited paternal antigens (IPA) might also play a role in the outcome of transplantation. [30] During pregnancy the mother can develop B- and T-cell immunity against these antigens. Cord blood can contain maternal anti IPA cells that can enhance a strong Graft versus Leukemia response and reduce relapse. Despite the large number of banked units, it is difficult to find a 6/6 matched CB unit for a patient and further matching for e.g. HLA-C and NIMA provides an even bigger challenge. Alternative methods are therefore being explored to improve the outcome of CB HST. The most obvious alternative approaches are 1) to increase the number of cells that are transplanted into the patient and 2) to enhance the engraftment of the CB cells by co-transplantation of accessory cells.

Method 1: Increasing the cell dose of a CB graft by improving the number and quality of engrafting cells in a CB graft

There are three ways to increase the cell dose of a cord blood graft: A**)** increasing the number of cells available for transplantation after thawing of the cord blood unit, B**)** transplantation of two cord blood units and C**)** the ex vivo expansion of cord blood cells.

A) Increasing the number of cells available for transplantation after thawing of the cord blood unit: improving processing and storage of CB. There are a number of steps from the collection of the cord blood to infusion of the final product in the patient. All of these affect the number of cells that are eventually transplanted. Over the years progress has been made in optimizing these processing steps. First of all, the volume that can be collected from the umbilical cord and placenta is highly variable and dependent on the method of collection. Intra-uterine collection, i.e. collection before placental delivery, significantly improves the volume of cells that is collected, [31,32] and has therefore become the preferred method of collection. Most cord blood banks have therefore set minimal criteria with respect to the volume and total nucleated cell (TNC) count of a CB unit. This avoids the cost of processing and banking a cord blood unit that has a low potential of successful engraftment. Secondly, the logistics of the banking process also plays an important role in the quality of the CB unit. In the Netherlands, the collection of CB is done in a number of peripheral hospitals after which the blood is transported to a centralized processing facility. The time and conditions between collection and eventual cryopreservation, however, strongly affects the viability of the CB cells. Processing and freezing the CB between 12-24 hours after collection in this respect significantly improves the viability of the cells after thawing. [33,34] Before cryopreservation the red blood cell (RBC) fraction and the plasma containing platelets are removed as much as possible. The most important reason for this is to reduce the volume which reduces the freezer space and thereby the cost of banking the unit. Reducing the RBC content of the graft also seems to coincide with less side effects after transplantation by reducing high concentrations of free hemoglobin released by RBC that do not survive the freezing and thawing process which can lead to significant transfusion reactions. [35]

This CB volume reduction can however be realized with several methods. While initially CB was reduced by hydoxyethyl starch sedimentation, [36] this method is now mostly replaced by automatic devices as the SEPAX and AXP that have a higher TNC and CD34+ cell yield and are more compliant with GCP regulations. [37] Thirdly, the method of cryopreservation of the cord blood unit also affects the post-thaw viability. Important factors in this respect are the use of a cryoprotectant such as dimethylsufoxide (DMSO), the freezing rate and storage temperature. [38] Most CB banks therefore adapted their freezing process to a widely accepted standard in which CB is frozen in 10% DMSO in an automated controlled rate freezer (1-2ºC/min) and stored in the vapor phase of liquid nitrogen at -150ºC. [36]

Finally, after thawing, the cord blood unit the DMSO content can be decreased lowered by washing the cells before transplantation. However, this post thaw wash step, besides certainly is not being practical, leads to a loss of cells [39] and removal of DMSO does not improves the speed of engraftment. [40]

B) Transplantation of two cord blood units to increase the number of transplanted cells. Although the improvement of processing parameters has led to a significant increase in the number of cells in banked cord blood units, cell numbers in a single unit are often still too low for adults or larger children. For these patients, two CB units can be combined (double cord blood transplantation strategies). While both units initially contribute to earlier engraftment, the eventual hematopoiesis is, remarkably, mostly determined by only one of the units. [41,42] This suggests that the increase of the cell dose provided by the second unit is only needed to boost the initial engraftment, but not for contributing to the long term reconstitution of the hematopoietic cells. Although again cell dose might play a role, single unit dominance, is most likely caused by immunological disparities between both grafts. A study that analyzed *in vitro* cultures with mononuclear cells from two cord blood units showed that an immune-mediated dominance for one of the two CB units could be documented in most combinations that were evaluated. [43] As expected, T cells seem to play an important role in this process, but it is unclear which subsets of T cells are involved. Analysis of the cells of the dominant unit 14-28 days after transplantation showed that a CD8+CCR7- population could be responsible for establishing dominance. [44] This effect can be seen earlier than 14 days as well: Somers et al. found that CD4+ T cells are most likely to play a role in the establishment of dominance, [42] while Newell et al. suggest that the CD3 content, i.e. all T cells, in the peripheral blood as early as 7 days after transplantation can predict the dominant unit. [45]
Although combining two units has made cord blood transplantation available for adult patients, the outcome of transplantation is still inferior to BM or PBSC grafts, as shown by delayed platelet and neutrophil recovery. [23,46] Moreover, more complex matching requirements not only between both CB units and the recipient but also between the two cord blood units complicate the availability of acceptable donor SCT. Furthermore, a higher risk of acute grade II- IV and extensive chronic graft versus host disease is observed with double cord blood transplantation. [47,48]

C) Increasing the number of cells in a graft by *ex vivo* culture expansion of CB cells
Another method to increase the number of cells that are available for transplantation is the expansion of CB cells in ex vivo culture in a defined medium that is supplemented with cytokines and small molecules. The composition of the expansion medium has a major influence on the number and types of cells that emerge after culture; Most expansion protocols use a combination of Stem Cell Factor (SCF), thrombopoietin (TPO), Flt3 ligand (Flt3L), interleukin-3 (IL-3), interleukin 6 (IL-6), Granulocyte-Macrophage Colony-Stimulating factor (GM-CSF) and/or Granulocyte Colony-Stimulating Factor (G-CSF). [49] Addition of other cytokines such as insulin growth factor binding protein and angiopoietin like 5 can further improve the expansion and engraftment. [50]
More recently there has been a focus on the addition of small molecules to the expansion medium. These small molecules can act either directly on signaling molecules responsible for survival, proliferation and differentiation of the stem cells in CB or improve the homing and engraftment of the CB cells after transplantation. Apoptosis of the cells during culture can for instance be prevented by caspase and calpain inhibitors, [51] or by the addition a copper chelator such as tetraethylenepentamine (TEPA) [52]. An example of direct interaction with a signaling pathway is the addition of delta like ligand-1 (DLL-1) to the culture medium. [53] DLL-1 interacts with the Notch pathway, a highly preserved signaling pathway in most multicellular organisms that regulates cell proliferation and differentiation. [54] Other molecules such as histone deacetylase inhibitors [55,56] and DNA methylases [57] modulate the epigenetic regulation of transcription.

Small molecules have also been shown to enhance the homing of the stem cells to the bone marrow. Fucosylated cells e.g. have a better ability to home to the BM than non-fucosylated cells. [58] Incubation with fucosyltransferase-VI increases the number of fucosylated cells and improves engraftment in immune deficient mice. Another method is to increase CXCR4 expression, the receptor for, SDF-1α, the main chemoattractant of the BM, for HSC, by incubation of the cells with prostaglandin E2. [59-61]
The culture conditions that are used for expansion aim to mimic the conditions in the BM niche to a certain extend. In this niche the HSC are surrounded by supporting cells such as mesenchymal stromal cells (MSC), adipocytes and osteoblasts. [62] Factors that are secreted by these cells regulate the self-renewal, proliferation and differentiation of the HSC. [63,64] Ex vivo culture on a monolayer of BM stromal cells can improve HSC expansion [65] and specific subtypes of MSC [66] or conditioned medium of other BM cells such as adipocytes [67,68] and osteoblasts [69] have also shown to improve the expansion of HSC.
Ex vivo expansion of cord blood cells has been employed in clinical trials. In 2002, a first study was published in which part of the transplanted cord blood unit was expanded with SCF, G-CSF and megakaryocyte growth and differentiation factor. [70] This study showed the safety and feasibility of ex vivo expanded cells and since 2002, a number of clinical trials have been conducted.
The Shpall group from the MD Anderson Cancer Center has conducted two clinical trials with different expansion strategies. In the first trial they expanded CB HSC with TEPA, a copper chelator, [71] that, by binding to free copper ions can prevent the differentiation of HSC in culture. [52] A proportion of a CB unit was expanded with SCF, Flt3-L, IL-6, TPO and TEPA and infused with the non-expanded part of the unit, leading to a median fold TNC expansion of 219 and an increase in CD34+ cells including the CD34+ cells from the non-expanded part of the unit of six. In this study 9 out of 10 patients engrafted and 100 day survival was 90%. However, there was no improvement in median neutrophil and platelet engraftment (median 30 and 42 days respectively) compared to results from studies with unmanipulated single CBT.
The other strategy the Shpall group used was the expansion of CB HSC with stromal support. [72] This expansion strategy led to a much lower fold TNC expansion that seen with the TEPA trial, i.e. 12.2 fold, but the fold CD34+ cells expansion was 30.1. In this study the expanded cells were transplanted with an unmanipulated cord blood unit and compared to historical controls of dCBT recipients. The patients receiving expanded cells had a clinically significant reduction in the number of days to neutrophil engraftment (15 days vs 24 days in historical controls) and to a lesser extend a reduction in the days to platelet engraftment (44 days vs. 49 days). These promising results have led to initiation of a phase 3 clinical trial which is currently recruiting participants. [73]
The clinical trial that evaluated the expansion with DLL-1 also showed promising results with respect a reduction in the time to neutrophil engraftment. [74] In this trial patients were again transplanted with an expanded unit and an unmanipulated unit. The average fold expansion was 562 (TNC) and 164 (CD34+). The median time to neutrophil engraftment was reduced with 10 days from 26 days to 16 days, platelet engraftment was not mentioned in the publication. Another molecule that interacts with the same signaling pathway (the Aryl hydrocarbon receptor (AHR) pathway), has shown to be even more potent in expanding CB. Ex vivo culture with Stemregenin 1 (SR-1), an AHR inhibitor, led to a 328 fold expansion CD34+ cells, and dCBT recipients that received a unit that was expanded with SR1 had significantly reduced times to both neutrophil and platelet engraftment. [75]
Finally, culture with nicotinamide, a SIRT1 inhibitor has been equally potent in reducing the time to neutrophil engraftment. [76] In the phase 1 trial neutrophil engraftment was

reduced from 25 days to 13 days. Platelet engraftment was again only marginally reduced from 37 to 33 days.

CB expansion with TPO to improve platelet engraftment

Stem cell expansion has also been done with TPO as a single growth factor. Although the fold expansion of the total nucleated cells and CD34+ cells is considerably lower as seen with the expansion of cells with a cocktail of cytokines, [77] this single cytokine strategy has several advantages. TPO as a single growth factor might prevent the differentiation of HSC, [78] thereby preserving the long term repopulating capacity of the graft. In addition, the largest part of the differentiated cells in culture is committed to a megakaryocyte lineage, [79,80] i.e. platelet precursors. These precursors can provide accelerated platelet recovery after transplantation of such transplants and is therefore of special importance since delayed and insufficient platelet recovery is one of the main persisting problems in CBT. Even after dCBT platelet recovery is still delayed compared to PBSC transplantation, median platelet recovery (>50,000 plt/µl PB) takes approximately 2-4 weeks longer and only 50-75% of the patients have a normal platelet count in the peripheral blood 6 months to 1 year after transplantation. [46,81,82]
A low platelet count increases the risk of hemorrhage and mortality and adequate levels of platelets are therefore important for the survival of the patient. [83] Approximately ten percent of dCBT recipients die of hemorrhage, compared to almost absent cases of hemorrhage related death in patients transplanted with BM or PBSC grafts. [46] The platelet count in the peripheral blood of the patient 100 days after transplantation is an important prognostic factor for 4-year survival. [84] However, low 100 day platelet counts are mostly associated with GvHD, infections or veno-occlusive disease and the etiology of the causal association of a low platelet count and mortality is difficult to determine. Finally, delayed platelet recovery and the required platelet transfusions contribute to the already high cost of CBT. Our group has previously shown that expansion of CB CD34+ cells using TPO as a single cytokine that can improve platelet engraftment after CB HST in immune deficient mice models. The (pre)megakaryocytes in the TPO expanded transplants not only induced faster platelet recovery compared to mice that received un-manipulated CD34+ cells and culture of BM cells from these mice harvested 7 days after transplantation generated higher numbers of megakaryocyte colonies, [85] but importantly, BM engraftment and lineage differentiation of the human cells was also similar between both groups. Improvement in platelet recovery therefore was not at the expense of BM engraftment. After TPO expansion, the cultured cells can be divided into 3 major populations, residual CD34+ cells, lineage negative cells (Lin-) and cells expressing megakaryocyte/platelet markers CD61+ or CD41+. [86] Remarkably, while as expected, the remaining CD34+ cells were responsible for stable BM engraftment and hematopoietic recovery, not the CD62+ cells but the Lin- cells were responsible for the early platelet recovery. This suggests that, repopulation effectivity always need to be confirmed by in vivo transplantation experiments and cannot directly be related to the lineage markers that are found.

Method 2: Co-transplantation of CB cells with accessory cells to improve the engraftment of CB cells

Co-transplantation of so called accessory cells is long known to improve engraftment and repopulation of HSC. Such accessory cells can be mesodermal stem cells such as MSC, [87] third party HSC, but also differentiated hematopoietic cells such as (apoptotic irradiated) leukocytes, even when they are from xenogenic origin. [88] The mechanism behind the improvement of engraftment is not fully known, but will be dependent on the type of cell that is used. The cells e.g. might assist is the repair of the bone marrow after

myeloablative conditioning, assist in homing and engraftment of the CB HSC or have an immunological effect. Several co-transplantation strategies can be discerned.

Co-transplantation of CB and third party HSC or regulatory T cells

As mentioned above, in double cord blood transplantation, the non-persisting / non-dominant unit is thought to give a boosting effect on the engrafting dominant unit. This can also be achieved by co-infusion of HSC from a third party e.g. haploidentical adult donor. To avoid graft versus host disease, only immuno-adsorption-isolated HSC, or T cell depleted PBSC grafts are co-infused with the CB cells [89]. Although this third party HSCs will disappear with the developing T-cell immunity of the immunocompetent CB transplant, the increased number of infused HSC seems to be able to temporarily bridge the slow repopulation of the CB.

Regulatory T cells (T-regs) are a subset of T cells that play a role in the tolerance to self-antigens and the suppression of disproportionate immune responses. [90] These cells are capable of preventing and/or suppressing GvHD in patients that are transplanted with mismatched HSC, [91] but they do not seem to enhance engraftment.

Co-transplantation of mesenchymal stromal cells

MSC are multipotent cells originally identified in the bone marrow as cells that support HSC maintenance and hematopoiesis. [92,93] MSC are able to differentiate into mesodermal cells including adipocytes, chondrocytes and osteoblasts [94] and have unique immunomodulatory properties. [95] Both these potentially marrow regenerative and immune modulatory properties have been employed to improve HST, [96-103] although the primary reason for co-infusion is most often the prevention or treatment of GvHD.

In animal models co-transplantation of MSC has been shown to improve the engraftment of HSC [87,104-108]. However, the underlying mechanisms for this enhancement are unknown and it is not clear whether the co-transplanted MSC home to the bone marrow. Both local support of engraftment by e.g. replacing the damaged stromal cells, releasing pro-angiogenic factors [109] or releasing growth factors [110] as well as more systemic mechanisms such as immunomodulation [95] have been suggested. Homing studies of MSC to the bone marrow, needed for more localized effects, have shown variable results. Two studies did not find MSC in the BM of the mice after transplantation. [87,107] MSC were found in the lung, suggesting sequestration of the MSC by a lung barrier. However, after intra-cardiac injection, thereby bypassing the pulmonary circulation, MSC could not be demonstrated in the BM either. This general entrapment of MSC in the lungs is corroborated by a study by Schrepfer *et al*, that used in vivo imaging and found most MSC in the lungs five minutes after transplantation [111] but another study did find MSC co-localized with CB CD34+ cells in the pelvis of the mice after intravenous transplantation. [105] Strong evidence for a local or direct effect of the MSC on engraftment, however, is lacking. As mentioned above, in double cord blood transplantation, single unit dominance is most likely caused by a systemic immunological effect. In a murine double CB transplantation study co-transplantation of MSC led alleviation of single unit dominance, [105] showing that the engraftment of the non-dominant unit can be enhanced by the co-transplantation of MSC. Another study in which CB MNC cells were transplanted, has shown that both MSC co-transplantation and lineage depletion of the MNC alleviated single donor dominance and improved overall engraftment. [107]

Clinical studies in which MSC were co-transplanted to enhance engraftment are so far hard to interpret since these studies were either safety or feasibility studies, [112,113] or studies that compared neutrophil and platelet recovery with historical controls. [114,115] The study of Ball *et al.* did however show an improvement in leukocyte recovery and less graft

failure. [114] Recent pilot studies in which MSC were co-transplanted with cord blood HSC showed an improvement in platelet engraftment when compared to controls that were transplanted with CB HSC alone. [116,117] Wu *et al.* found a median time to platelet engraftment of 32 days (69 days in the control group) and Lee *et al.* a median time of 47 days (57 days in the control group)., However, the median time to MSC induced platelet engraftment was still delayed compared to the median engraftment time of a (PBSC) graft from a matched unrelated donor. In a comparison study by Chen *et al.* in which recipients of a matched unrelated donor graft (MUD) were compared with recipients of double CB (dCB) transplantations, the median time to platelet engraftment was 19 days for the MUD recipient versus 42 days for the dCB recipients. [118]

The use of CB for non-hematopoietic purposes
Publically stored CB is exclusively banked for the purpose of allogeneic HST, but CB also contains cell populations with non-hematopoietic regenerative potential. Private CB banking initially also aimed for using the CB to treat (future) hematological malignancies of the child itself or a family member, but the chance of using the CB for this purpose is small; approximately only 1 in 2700 individuals would benefit from these privately banked units. [119] The prospect of an alternative use of these non- hematopoietic cells for later in life degenerative conditions is therefore used to gain interest for private banking initiatives. CB banking for anonymous allogeneic use is often not cost-efficient. An arrangement to store hematopoietic stem cells for the purpose of allogeneic banking with maintenance of non-hematopoietic regenerative cells for private autologous use, could lead to a financial boost to increase in public anonymous CB banking. The non-hematopoietic capacity of CB was first shown by Nieda et al. in 1997, who cultured cells from CB with endothelial characteristics such as von Willibrand factor and PECAM. [120] These cells were cultured from the CD34+ enriched fraction which also harbors the HSCs. Erices et al. cultured adherent cells from CB resulting in an osteoclast or mesenchymal cell like phenotype [121]. Over the next decade a variety of cell types have been cultured from CB, including endothelial cells (EC) [122], mesenchymal stromal cells (MSC), [123] neural cells, [124] island beta cells [125] and cardiomyocytes. [126] The functionality of these cells has been shown on various levels, some cells were only characterized in vitro as the proposed cell type, Some of these CB derived cells were successfully applied in regenerative cellular therapy such as vascular repair and wound healing, [127,128] or as therapy for immune related diseases. [129-131]
Currently the regenerative potential of CB is restricted to the private setting and thus excludes the use of cord blood for allogeneic HSC purpose. If the cells that generate the non-hematopoietic tissues can be separated from the hematopoietic cells, a 'dual' banking system can be established which would compensate partly for the cost for public anonymous banking. Before "dual" public-private banking can be considered and sincerely offered, several conditions must be fulfilled. The first and main requirement is that the separation of precursors for repair purpose should leave the hematopoietic potential uncompromised. Second, the regenerative potential of CB should have an advantage compared to other (adult) sources of regenerative cells. Third, the methods to separate the hematopoietic precursors should be cost-effective and not increase the cost of CB for hematopoietic transplantation. Separation of the two fractions could be realized with selective immuno-separation on the basis of markers that identify MSC, EPC or the hematopoietic cells. [132] Endothelial cells e.g. can be identified by the expression of vascular endothelial growth factor receptor 2 (VEGF-R2) and VE Cadherin (CD144) and have been identified in fresh cord blood, but the frequency of these cells is very low and highly variable between donors. [133,134] MSC can be identified by a combination of distinct makers

and properties set by the International Society for Cellular Therapy (ISCT). [135] Besides the ability to adhere to plastic and to differentiate into 3 mesodermal linegaes, MSC should express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14, CD19 and HLA-DR. Efforts to identify MSC like cells in CB based on marker expression alone have shown cell types that express certain markers, but confirmation by culture experiments is still needed to confidently state that these cells are indeed MSC. [136] Both MSC and EPC are derived from the non-hematopoietic CD45 negative fraction of CB. [134,137] Separating the CD45 negative cells from the hematopoietic fraction might therefore preserve the possibility to culture both cell types. Selection of hematopoietic cells from the CB on the basis of CD45 expression might be an alternative although not all hematopoietic stem cells need to express CD45+.

Other (extra-) embryonic tissues

Other fetal tissues such as bone marrow, kidney, liver and lungs as well as extra embryonic tissues such as the placenta, the umbilical cord itself, the amniotic membrane and amniotic fluid contain much higher numbers of non-hematopoietic stem cells. [138-140] However the use of embryonic stem cells is accompanied with several ethical constraints and is therefore not a desirable source. Extra-embryonic tissues on the other are more interesting because of their availability as waste products and the resulting lack of ethical constraints.

The advantage of combined collection, HLA typing, screening for infections and banking of CB together with (a selection of) these tissues or their stem cell fraction, is not hard to envision and could in this respect also reduce costs. The umbilical cord as example has shown to be a good source for MSC since the conditions for isolations are less strict and the number of MSC that can be grown from a single collection are much higher than those of MSC isolated from CB [141] Of all extra embryonic tissues the umbilical cord seems to be the most promising candidate for cell culture since the collection of this tissue is easy and the isolation of stem cells from this tissue is relatively straightforward. The placenta is much larger in volume and partly consists of tissue that is derived from the mother. The collection of the amnion and amniotic fluid is difficult to collect during delivery, which creates a burden for the mother.

Objective of this thesis

This thesis focuses on ways to improve CB application from both the perspective of the patient (i.e. better engraftment) as well as from the cost perspective (i.e. wider applicability). To this aim, we investigated (combinations of) methods for the improvement of CB engraftment in a murine engraftment model for human hematopoietic cells. Besides our focus on the PB recovery of platelets and CD45+ cells, we furthermore looked at practical aspects of the application of CB such as the possibility of banking expanded cells, banking CB for other purposes than HST and the use of other extra-fetal (embryonic) tissues such as the umbilical cord.

Chapter 1: Co-transplantation of MSC from the Wharton's Jelly of the umbilical cord.

MSC that are used for therapeutic purposes are usually derived from adult BM, [142] but MSC can be isolated from a variety of other tissues as well such as adipose tissue, [143] compact bone, [144] amniotic fluid, [145] umbilical cord blood, [121] placenta [146] or the umbilical cord. [147] The harvest of MSC from the BM of healthy donors involves a certain risk and discomfort for the donor and is therefore ethically less desirable. Instead we first studied the umbilical cord blood as alternative MSC source. However, the number of MSC in CB is limited compared to BM [148] and isolation is difficult compared to other sources. [149] Furthermore, isolation of MSC from the CB would not allow the additional use of the unit

for HST. The umbilical cord (UC) itself was subsequently studied. UCs consists of an umbilical vein and arteries surrounded by Wharton's Jelly (WJ), which is a rich source of MSC. [147] The already iterated advantage of the umbilical cord (UC) is that after birth it can be regarded as waste. Therefore, after the collection of the cord blood from the placenta and the UC, the UC itself can be stored in a sterile container and used for the isolation of MSC. Furthermore, the cost of the collection of the UC is low while collection logistics, HLA typing and screening of infectious diseases of the donor can be shared with what is needed for the CB.
We therefore set up a collection system for UCs in collaboration with the cord blood bank (Sanquin Navelstreng Bloedbank, NBB) and modified an existing protocol for the expansion of MSC that does not involve the use of enzymes. [150] As definite proof, we analysed the capacity of the WJ derived MSC to enhance CB CD34+ cell mediated recovery of human platelets and CD45+ cells and BM engraftment in an immune deficient mouse model and compared this with the enhancement seen after the co-transplantation of BM MSC together with CB CD34+ cells.

Chapter 2: Combining TPO expansion of CB CD34+ cells with double cord blood transplantation.
Because of concerns about the long term engraftment capacity of expanded cells, manipulated CB grafts are usually transplanted with an unmanipulated graft. We therefore set out to analyze the influence of these co-transplantations with respect to the separate contributions to the platelet and CD45+ cell recovery in the peripheral blood of the mouse and the engraftment in the bone marrow after 20 weeks (long term engraftment). For this, we used selected HLA mismatches between recipients and CB donor cells which allowed for the detection of each graft by HLA specific antibodies. We specifically looked at the contribution of the expanded cells or unmanipulated cells when transplanted together, and compared this with the PB recovery and engraftment of mice that were transplanted with only unmanipulated CD34+ cells from 2 different CB units.

Chapter 3: TPO expansion of CB CD34+ cells: should we use existing, banked CB units or expand fresh CB units?
As mentioned, our lab previously developed a protocol for the expansion of CB CD34+ cells with only TPO as a growth factor. [79,80,85,86] Building on that strategy, cryopreserved CD34+ cells were expanded and again transplanted into immune deficient mice. In this setting we investigated whether expansion before cryopreservation would have an advantage over the expansion of cryopreserved cells. We therefore compared the quality of expanded cells that were cultured from fresh CB CD34+ cells with cells that were expanded from cryopreserved CD34+ cells. We next analyzed the 6 weeks engraftment capacity of the cells in a NOD SCID model by looking at the difference in platelet recovery in the peripheral blood and the general engraftment of human cells in the bone marrow,.

Chapter 4: Combining TPO expansion of CB CD34+ cells with the co-transplantation of MSC
As mentioned above, TPO expanded cells can improve the platelet recovery in the peripheral blood while MSC co-transplantation improves the overall engraftment of CB cells. We therefore combined both approached and studied the engraftment potential of TPO expanded cells co-transplanted with or without MSC in NOD SCID mice.

Chapter 5: Dual use of cord blood for both hematopoietic stem cell transplantation and other cellular therapies

So far CB is mostly banked for the purpose of HST, but as said, CB also contains non hematopoietic cells with an additional potential for cellular therapies. We therefore analyzed the possibility to separate the hematopoietic and non hematopoietic (stem) cells, We separated these non hematopoietic cells from the hematopoietic compartment of the CB unit and studied their function in vitro and their influence on the engraftment of CB CD34+ cells in vivo. This dual banking could stimulate in the future collection and storage in parallel with private banks, reducing the cost for publically stored CB units.

References

1. Passweg J, Baldomero H, Bregni M et al. Hematopoietic SCT in Europe: data and trends in 2011. Bone marrow transplantation 2013;**48**: 1161-7.
2. Flomenberg N, Baxter-Lowe LA, Confer D et al. Impact of HLA class I and class II high-resolution matching on outcomes of unrelated donor bone marrow transplantation: HLA-C mismatching is associated with a strong adverse effect on transplantation outcome. Blood 2004;**104**: 1923-30.
3. Petersdorf EW, Anasetti C, Martin PJ et al. Limits of HLA mismatching in unrelated hematopoietic cell transplantation. Blood 2004;**104**: 2976-80.
4. Sasazuki T, Juji T, Morishima Y et al. Effect of matching of class I HLA alleles on clinical outcome after transplantation of hematopoietic stem cells from an unrelated donor. New England J of Medicine 1998;**339**: 1177-85.
5. Ballen K, Spitzer T. The great debate: haploidentical or cord blood transplant. Bone marrow transplantation 2010;**46**: 323-9.
6. Ballen KK, Koreth J, Chen YB et al. Selection of optimal alternative graft source: mismatched unrelated donor, umbilical cord blood, or haploidentical transplant. Blood 2012;**119**: 1972-80.
7. Bashey A, Solomon S. T-cell replete haploidentical donor transplantation using post-transplant CY: an emerging standard-of-care option for patients who lack an HLA-identical sibling donor. Bone marrow transplantation 2014.
8. *Bone Marrow Donors Worldwide, www.bmdw.org [monograph on the internet]*. Available from: www.bmdw.org
9. *Europdonor, www.europdonor.nl [monograph on the internet]*.
10. Lown R, Marsh S, Switzer G et al. Ethnicity, length of time on the register and sex predict donor availability at the confirmatory typing stage. Bone marrow transplantation 2014;**49**: 525-31.
11. Barker JN, Byam CE, Kernan NA et al. Availability of cord blood extends allogeneic hematopoietic stem cell transplant access to racial and ethnic minorities. Biology of Blood and Marrow Transplantation 2010;**16**: 1541-8.
12. Broxmeyer HE, Douglas GW, Hangoc G et al. Human umbilical cord blood as a potential source of transplantable hematopoietic stem/progenitor cells. Proceedings of the National Academy of Sciences 1989;**86**: 3828-32.
13. Gluckman E, Broxmeyer H, Auerbach AD et al. Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling. N Engl J Med 1989;**321**: 1174-8.
14. Gluckman E, Rocha V, Boyer-Chammard A et al. Outcome of cord-blood transplantation from related and unrelated donors. New England Journal of Medicine 1997;**337**: 373-81.
15. Rocha V, Cornish J, Sievers EL et al. Comparison of outcomes of unrelated bone marrow and umbilical cord blood transplants in children with acute leukemia. Blood 2001;**97**: 2962-71.
16. Rocha V, Labopin M, Sanz G et al. Transplants of umbilical-cord blood or bone marrow from unrelated donors in adults with acute leukemia. New England Journal of Medicine 2004;**351**: 2276-85.
17. Bender JG, Unverzagt K, Walker DE et al. Phenotypic Analysis and Characterization of CD34+ Cells from Normal Human Bone Marrow, Cord Blood, Peripheral Blood, and Mobilized Peripheral Blood from Patients Undergoing Autologous Stem Cell Transplantation. Clinical immunology and immunopathology 1994;**70**: 10-8.
18. Eapen M, Rubinstein P, Zhang M-J et al. Outcomes of transplantation of unrelated donor umbilical cord blood and bone marrow in children with acute leukaemia: a comparison study. The Lancet 2007;**369**: 1947-54.
19. Komanduri KV, John LSS, de Lima M et al. Delayed immune reconstitution after cord blood transplantation is characterized by impaired thymopoiesis and late memory T-cell skewing. Blood 2007;**110**: 4543-51.
20. Niehues T, Rocha V, Filipovich AH et al. Factors affecting lymphocyte subset reconstitution after either related or unrelated cord blood transplantation in children–a Eurocord analysis. British journal of haematology 2001;**114**: 42-8.
21. Danby R, Rocha V. Current Strategies to Improve Engraftment in Cord Blood Transplantation. J Stem Cell Res Ther 2014;**4**: 2.
22. Rocha V, Broxmeyer HE. New approaches for improving engraftment after cord blood transplantation. Biology of Blood and Marrow Transplantation 2010;**16**: S126-S32.
23. Ballen KK, Gluckman E, Broxmeyer HE. Umbilical cord blood transplantation: the first 25 years and beyond. Blood 2013;**122**: 491-8.
24. Blommestein H, Verelst S, Huijgens P et al. Real-world costs of autologous and allogeneic stem cell transplantations for haematological diseases: a multicentre study. Annals of hematology 2012;**91**: 1945-52.

25. Barker JN, Scaradavou A, Stevens CE. Combined effect of total nucleated cell dose and HLA match on transplantation outcome in 1061 cord blood recipients with hematologic malignancies. Blood 2010;**115**: 1843-9.
26. Peffault de Latour R, Purtill D et al. Influence of nucleated cell dose on overall survival of unrelated cord blood transplantation for patients with severe acquired aplastic anemia: a study by eurocord and the aplastic anemia working party of the European group for blood and marrow transplantation. Biology of Blood and Marrow Transpl 2011;**17**: 78-85.
27. Wagner JE, Barker JN, DeFor TE et al. Transplantation of unrelated donor umbilical cord blood in 102 patients with malignant and nonmalignant diseases: influence of CD34 cell dose and HLA disparity on treatment-related mortality and survival. Blood 2002;**100**: 1611-8.
28. Eapen M, Klein JP, Sanz GF et al. Effect of donor–recipient HLA matching at HLA A, B, C, and DRB1 on outcomes after umbilical-cord blood transplantation for leukaemia and myelodysplastic syndrome: a retrospective analysis. The lancet oncology 2011;**12**: 1214-21.
29. van Rood JJ, Stevens CE, Smits J et al. Reexposure of cord blood to noninherited maternal HLA antigens improves transplant outcome in hematological malignancies. Proceedings of the National Academy of Sciences 2009;**106**: 19952-7.
30. van Rood JJ, Scaradavou A, Stevens CE. Indirect evidence that maternal microchimerism in cord blood mediates a graft-versus-leukemia effect in cord blood transplantation. Proceedings of the National Academy of Sciences 2012;**109**: 2509-14.
31. Solves P, Moraga R, Saucedo E et al. Comparison between two strategies for umbilical cord blood collection. Bone marrow transpl 2003;**31**: 269-73.
32. Surbek DV, Visca E, Steinmann C et al. Umbilical cord blood collection before placental delivery during cesarean delivery increases cord blood volume and nucleated cell number available for transplantation. American journal of obstetrics and gynecology 2000;**183**: 218-21.
33. Guttridge MG, Soh TG, Belfield H et al. Storage time affects umbilical cord blood viability. Transfusion 2014;**54**: 1278-85.
34. Louis I, Wagner E, Dieng MM et al. Impact of storage temperature and processing delays on cord blood quality: discrepancy between functional in vitro and in vivo assays. Transfusion 2012;**52**: 2401-5.
35. Barker JN, Byam C, Scaradavou A. How I treat: the selection and acquisition of unrelated cord blood grafts. Blood 2011;**117**: 2332-9.
36. Rubinstein P, Dobrila L, Rosenfield RE et al. Processing and cryopreservation of placental/umbilical cord blood for unrelated bone marrow reconstitution. Proceedings of the National Academy of Sciences 1995;**92**: 10119-22.
37. Lapierre V, Pellegrini N, Bardey I et al. Cord blood volume reduction using an automated system (Sepax) vs. a semi-automated system (Optipress II) and a manual method (hydroxyethyl starch sedimentation) for routine cord blood banking: a comparative study. Cytotherapy 2007;**9**: 165-9.
38. Berz D, McCormack EM, Winer ES et al. Cryopreservation of hematopoietic stem cells. American journal of hematology 2007;**82**: 463-72.
39. Laroche V, McKenna DH, Moroff G et al. Cell loss and recovery in umbilical cord blood processing: a comparison of postthaw and postwash samples. Transfusion 2005;**45**: 1909-16.
40. Nagamura-Inoue T, Shioya M, Sugo M et al. Wash-out of DMSO does not improve the speed of engraftment of cord blood transplantation: follow-up of 46 adult patients with units shipped from a single cord blood bank. Transfusion 2003;**43**: 1285-95.
41. Brunstein CG, Barker JN, Weisdorf DJ et al. Umbilical cord blood transplantation after nonmyeloablative conditioning: impact on transplantation outcomes in 110 adults with hematologic disease. Blood 2007;**110**: 3064-70.
42. Somers JA, Brand A, van Hensbergen Y et al. Double umbilical cord blood transplantation: A study of early engraftment kinetics in leukocyte subsets using HLA-specific monoclonal antibodies. Biology of Blood and Marrow Transplantation 2012.
43. Moretta A, Andriolo G, Lisini D et al. In vitro evaluation of graft-versus-graft alloreactivity as a tool to identify the predominant cord blood unit before double cord blood transplantation. Biology of Blood and Marrow Transplantation 2012;**18**: 1108-18.
44. Gutman JA, Turtle CJ, Manley TJ et al. Single-unit dominance after double-unit umbilical cord blood transplantation coincides with a specific CD8+ T-cell response against the nonengrafted unit. Blood 2010;**115**: 757-65.
45. Newell LF, Milano F, Nicoud IB et al. Early CD3 peripheral blood chimerism predicts the long-term engrafting unit following myeloablative double-cord blood transplantation. Biology of Blood and Marrow Transplantation 2012;**18**: 1243-9.
46. Brunstein CG, Gutman JA, Weisdorf DJ et al. Allogeneic hematopoietic cell transplantation for hematologic malignancy: relative risks and benefits of double umbilical cord blood. Blood 2010;**116**: 4693-9.

47. Jacobson CA, Turki AT, McDonough SM et al. Immune reconstitution after double umbilical cord blood stem cell transplantation: comparison with unrelated peripheral blood stem cell transplantation. Biology of blood and marrow transplantation 2012;**18**: 565-74.
48. Wagner Jr JE, Eapen M, Carter S et al. One-Unit versus Two-Unit Cord-Blood Transplantation for Hematologic Cancers. New England Journal of Medicine 2014;**371**: 1685-94.
49. Kelly S, Sola C, De Lima M, Shpall E. Ex vivo expansion of cord blood. Bone marrow transplantation 2009;**44**: 673-81.
50. Zhang CC, Kaba M, Iizuka S et al. Angiopoietin-like 5 and IGFBP2 stimulate ex vivo expansion of human cord blood hematopoietic stem cells as assayed by NOD/SCID transplantation. Blood 2008;**111**: 3415-23.
51. Sangeetha V, Kale VP, Limaye LS. Expansion of cord blood CD34 cells in presence of zVADfmk and zLLYfmk improved their in vitro functionality and in vivo engraftment in NOD/SCID mouse. PloS one 2009;**5**: e12221-e.
52. Peled T, Landau E, Mandel J et al. Linear polyamine copper chelator tetraethylenepentamine augments long-term ex vivo expansion of cord blood-derived CD34+ cells and increases their engraftment potential in NOD/SCID mice. Experimental hematology 2004;**32**: 547-55.
53. Delaney C, Varnum-Finney B, Aoyama K et al. Dose-dependent effects of the Notch ligand Delta1 on ex vivo differentiation and in vivo marrow repopulating ability of cord blood cells. Blood 2005;**106**: 2693-9.
54. Bray SJ. Notch signalling: a simple pathway becomes complex. Nature reviews Molecular cell biology 2006;**7**: 678-89.
55. Chaurasia P, Gajzer DC, Schaniel C et al. Epigenetic reprogramming induces the expansion of cord blood stem cells. The Journal of clinical investigation 2014;**124**: 2378-95.
56. Mahmud N, Petro B, Baluchamy S et al. Differential Effects of Epigenetic Modifiers on the Expansion and Maintenance of Human Cord Blood Stem/Progenitor Cells. Biology of Blood and Marrow Transplantation 2014;**20**: 480-9.
57. Fares I, Chagraoui J, Gareau Y et al. Pyrimidoindole derivatives are agonists of human hematopoietic stem cell self-renewal. Science 2014;**345**: 1509-12.
58. Robinson SN, Simmons PJ, Thomas MW et al. Ex vivo fucosylation improves human cord blood engraftment in NOD-SCID IL-2Rγ^{null} mice. Experimental hematology 2012;**40**: 445-56.
59. Cutler C, Multani P, Robbins D et al. Prostaglandin-modulated umbilical cord blood hematopoietic stem cell transplantation. Blood 2013;**122**: 3074-81.
60. Goessling W, Allen RS, Guan X et al. Prostaglandin E2 enhances human cord blood stem cell xenotransplants and shows long-term safety in preclinical nonhuman primate transplant models. Cell Stem Cell 2011;**8**: 445-58.
61. Hoggatt J, Singh P, Sampath J, Pelus LM. Prostaglandin E2 enhances hematopoietic stem cell homing, survival, and proliferation. Blood 2009;**113**: 5444-55.
62. Morrison SJ, Scadden DT. The bone marrow niche for haematopoietic stem cells. Nature 2014;**505**: 327-34.
63. Kunisaki Y, Bruns I, Scheiermann C et al. Arteriolar niches maintain haematopoietic stem cell quiescence. Nature 2013;**502**: 637-43.
64. Mendelson A, Frenette PS. Hematopoietic stem cell niche maintenance during homeostasis and regeneration. Nature medicine 2014;**20**: 833-46.
65. Robinson S, Ng J, Niu T et al. Superior ex vivo cord blood expansion following co-culture with bone marrow-derived mesenchymal stem cells. Bone marrow transplantation 2006;**37**: 359-66.
66. Corselli M, Chin CJ, Parekh C et al. Perivascular support of human hematopoietic stem/progenitor cells. Blood 2013;**121**: 2891-901.
67. Glettig DL, Kaplan DL. Extending human hematopoietic stem cell survival in vitro with adipocytes. BioResearch open access 2013;**2**: 179-85.
68. Spindler TJ, Tseng AW, Zhou X, Adams GB. Adipocytic Cells Augment the Support of Primitive Hematopoietic Cells In Vitro But Have No Effect in the Bone Marrow Niche Under Homeostatic Conditions. Stem cells and development 2013;**23**: 434-41.
69. Dumont N, Boyer L, Émond H et al. Medium conditioned with MSC-derived osteoblasts improves the expansion and engraftment properties of cord blood progenitors. Experimental hematology 2014.
70. Shpall EJ, Quinones R, Giller R et al. Transplantation of ex vivo expanded cord blood. Biology of Blood and Marrow Transplantation 2002;**8**: 368-76.
71. De Lima M, McMannis J, Gee A et al. Transplantation of ex vivo expanded cord blood cells using the copper chelator tetraethylenepentamine: a phase I/II clinical trial. Bone marrow transplantation 2008;**41**: 771-8.
72. de Lima M, McNiece I, Robinson SN et al. Cord-blood engraftment with ex vivo mesenchymal-cell coculture. New England Journal of Medicine 2012;**367**: 2305-15.

73. https://clinicaltrials.gov. Registry and results database of publicly and privately supported clinical studies of human participants conducted around the world.
74. Delaney C, Heimfeld S, Brashem-Stein C et al. Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution. Nature medicine 2010;**16**: 232-6.
75. Wagner JE, Brunstein C, McKenna D et al. Acceleration of Umbilical Cord Blood (UCB) Stem Engraftment: Results of a Phase I Clinical Trial with Stemregenin-1 (SR1) Expansion Culture. Biology of Blood and Marrow Transplant 2015;**21**: S48-S9.
76. Horwitz ME, Chao NJ, Rizzieri DA et al. Umbilical cord blood expansion with nicotinamide provides long-term multilineage engraftment. The Journal of clinical investigation 2014;**124**: 3121.
77. Gullo F, van der Garde M, Russo G et al. Computational modeling of the expansion of human cord blood CD133+ hematopoietic stem/progenitor cells with different cytokine combinations. Bioinformatics 2015;31: 2524-22.
78. Yoshida M, Tsuji K, Ebihara Y et al. Thrombopoietin alone stimulates the early proliferation and survival of human erythroid, myeloid and multipotential progenitors in serum-free culture. British journal of haematology 1997;**98**: 254-64.
79. Schipper L, Brand A, Reniers N et al. Effects of thrombopoietin on the proliferation and differentiation of primitive and mature haemopoietic progenitor cells in cord blood. British journal of haematology 1998;**101**: 425-35.
80. Schipper LF, Brand A, Reniers N et al. Differential maturation of megakaryocyte progenitor cells from cord blood and mobilized peripheral blood. Experimental hematology 2003;**31**: 324-30.
81. Somers JA, Braakman E, van der Holt B et al. Rapid induction of single donor chimerism after double umbilical cord blood transplantation preceded by reduced intensity conditioning: results of the HOVON 106 phase II study. haematologica 2014: haematol. 2014.106690.
82. Wallet HL, Sobh M, Morisset S et al. Double umbilical cord blood transplantation for hematological malignancies: a long-term analysis from the SFGM-TC registry. Experimental Hematology 2013;11: 924-33.
83. Bernstein SH, Nademanee AP, Vose JM et al. A multicenter study of platelet recovery and utilization in patients after myeloablative therapy and hematopoietic stem cell transplantation. Blood 1998;**91**: 3509-17.
84. Bolwell B, Pohlman B, Sobecks R et al. Prognostic importance of the platelet count 100 days post allogeneic bone marrow transplant. Bone marrow transplantation 2004;**33**: 419-23.
85. van Hensbergen Y, Schipper LF, Brand A et al. Ex vivo culture of human CD34+ cord blood cells with thrombopoietin (TPO) accelerates platelet engraftment in a NOD/SCID mouse model. Experimental hematology 2006;**34**: 943-50.
86. Schipper LF, Brand A, Fibbe WE, Van Hensbergen Y. Functional Characterization of TPO-Expanded CD34+ Cord Blood Cells Identifies CD34–CD61– Cells as Platelet-Producing Cells Early After Transplantation in NOD/SCID Mice and rCD34+ Cells as CAFC Colony-Forming Cells. Stem Cells 2012;**30**: 988-96.
87. Noort WA, Kruisselbrink AB, Kruger M et al. Mesenchymal stem cells promote engraftment of human umbilical cord blood–derived CD34+ cells in NOD/SCID mice. Experimental hematology 2002;**30**: 870-8.
88. de Carvalho Bittencourt M, Perruche S et al. Intravenous injection of apoptotic leukocytes enhances bone marrow engraftment across major histocompatibility barriers. Blood 2001;**98**: 224-30.
89. Van Besien K, Liu H, Jain N et al. Umbilical cord blood transplantation supported by third-party donor cells: rationale, results, and applications. Biology of Blood and Marrow Transplantation 2013;**19**: 682-91.
90. Sakaguchi S, Yamaguchi T, Nomura T et al. Regulatory T cells and immune tolerance. Cell 2008;**133**: 775-87.
91. Di Ianni M, Falzetti F, Carotti A et al. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. Blood 2011;**117**: 3921-8.
92. Caplan AI. Mesenchymal stem cells. Journal of orthopaedic research 1991;**9**: 641-50.
93. Friedenstein AJ, Gorskaja J, Kulagina N. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Experimental hematology 1976;**4**: 267-74.
94. Pittenger MF, Mackay AM, Beck SC et al. Multilineage potential of adult human mesenchymal stem cells. science 1999;**284**: 143-7.
95. Bernardo ME, Fibbe WE. Mesenchymal stromal cells: sensors and switchers of inflammation. Cell Stem Cell 2013;**13**: 392-402.
96. Kebriaei P, Isola L, Bahceci E et al. Adult human mesenchymal stem cells added to corticosteroid therapy for the treatment of acute graft-versus-host disease. Biology of Blood and Marrow Transplantation 2009;**15**: 804-11.
97. Le Blanc K, Frassoni F, Ball L et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. The Lancet 2008;**371**: 1579-86.

98. Lucchini G, Introna M, Dander E et al. Platelet-lysate-expanded mesenchymal stromal cells as a salvage therapy for severe resistant graft-versus-host disease in a pediatric population. Biology of blood and marrow transplantation 2010;**16**: 1293-301.
99. Pérez-Simon JA, Lopez-Villar O, Andreu EJ et al. Mesenchymal stem cells expanded in vitro with human serum for the treatment of acute and chronic graft-versus-host disease: results of a phase I/II clinical trial. haematologica 2011;**96**: 1072-6.
100. Prasad VK, Lucas KG, Kleiner GI et al. Efficacy and safety of ex vivo cultured adult human mesenchymal stem cells (Prochymal™) in pediatric patients with severe refractory acute graft-versus-host disease in a compassionate use study. Biology of Blood and Marrow Transplantation 2011;**17**: 534-41.
101. Ringden O, Le Blanc K. Mesenchymal stem cells for treatment of acute and chronic graft-versus-host disease, tissue toxicity and hemorrhages. Best practice & research Clinical haematology 2011;**24**: 65-72.
102. Baron F, Lechanteur C, Willems E et al. Cotransplantation of mesenchymal stem cells might prevent death from graft-versus-host disease (GVHD) without abrogating graft-versus-tumor effects after HLA-mismatched allogeneic transplantation following nonmyeloablative conditioning. Biology of blood and marrow transplantation 2010;**16**: 838-47.
103. Kuzmina LA, Petinati NA, Parovichnikova EN et al. Multipotent mesenchymal stromal cells for the prophylaxis of acute graft-versus-host disease - a phase II study. Stem cells international 2011;**2012**.
104. Friedman R, Betancur M, Boissel L et al. Umbilical cord mesenchymal stem cells: adjuvants for human cell transplantation. Biology of Blood and Marrow Transplantation 2007;**13**: 1477-86.
105. Hiwase SD, Dyson PG, To LB, Lewis ID. Cotransplantation of placental mesenchymal stromal cells enhances single and double cord blood engraftment in nonobese diabetic/severe combined immune deficient mice. Stem Cells 2009;**27**: 2293-300.
106. in 't Anker PS, Noort WA, Kruisselbrink AB et al. Nonexpanded primary lung and bone marrow–derived mesenchymal cells promote the engraftment of umbilical cord blood–derived CD34+ cells in NOD/SCID mice. Experimental hematology 2003;**31**: 881-9.
107. Kim D-W, Chung Y-J, Kim T-G et al. Cotransplantation of third-party mesenchymal stromal cells can alleviate single-donor predominance and increase engraftment from double cord transplantation. Blood 2004;**103**: 1941-8.
108. Masuda S, Ageyama N, Shibata H et al. Cotransplantation with MSCs improves engraftment of HSCs after autologous intra-bone marrow transplantation in nonhuman primates. Experimental hematology 2009;**37**: 1250-7. e1.
109. Watt SM, Gullo F, van der Garde M et al. The angiogenic properties of mesenchymal stem/stromal cells and their therapeutic potential. British medical bulletin 2013: ldt031.
110. Bonnet D, Bhatia M, Wang J et al. Cytokine treatment or accessory cells are required to initiate engraftment of purified primitive human hematopoietic cells transplanted at limiting doses into NOD/SCID mice. Bone marrow transplantation 1999;**23**: 203.
111. Schrepfer S, Deuse T, Reichenspurner H et al. Stem cell transplantation: the lung barrier. Transplantation proceedings: Elsevier, 2007:573-6.
112. Lazarus HM, Koc ON, Devine SM et al. Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. Biology of Blood and Marrow Transplantation 2005;**11**: 389-98.
113. Le Blanc K, Samuelsson H, Gustafsson B et al. Transplantation of mesenchymal stem cells to enhance engraftment of hematopoietic stem cells. Leukemia 2007;**21**: 1733-8.
114. Ball LM, Bernardo ME, Roelofs H et al. Cotransplantation of ex vivo–expanded mesenchymal stem cells accelerates lymphocyte recovery and may reduce the risk of graft failure in haploidentical hematopoietic stem-cell transplantation. Blood 2007;**110**: 2764-7.
115. Macmillan M, Blazar B, DeFor T, Wagner J. Transplantation of ex-vivo culture-expanded parental haploidentical mesenchymal stem cells to promote engraftment in pediatric recipients of unrelated donor umbilical cord blood: results of a phase I–II clinical trial. Bone marrow transplantation 2008;**43**: 447-54.
116. Lee S, Lee M, Yoo K et al. Co-transplantation of third-party umbilical cord blood-derived MSCs promotes engraftment in children undergoing unrelated umbilical cord blood transplantation. Bone marrow transpl 2013;**48**: 1040-5.
117. Wu K-H, Sheu J-N, Wu H-P et al. Cotransplantation of Umbilical Cord–Derived Mesenchymal Stem Cells Promote Hematopoietic Engraftment in Cord Blood Transplantation: A Pilot Study. Transplantation 2013;**95**: 773-7.
118. Chen Y-B, Aldridge J, Kim HT et al. Reduced-intensity conditioning stem cell transplantation: comparison of double umbilical cord blood and unrelated donor grafts. Biology of Blood and Marrow Transplantation 2012;**18**: 805-12.
119. Hwang W. Cord blood banking and transplantation. ISBT Science Series 2011;**6**: 324-7.

120. Nieda M, Nicol A, Denning-Kendall P et al. Endothelial cell precursors are normal components of human umbilical cord blood. British journal of haematology 1997;**98**: 775-7.
121. Erices A, Conget P, Minguell JJ. Mesenchymal progenitor cells in human umbilical cord blood. British journal of haematology 2000;**109**: 235-42.
122. Ingram DA, Mead LE, Tanaka H et al. Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. Blood 2004;**104**: 2752-60.
123. Lee OK, Kuo TK, Chen W-M et al. Isolation of multipotent mesenchymal stem cells from umbilical cord blood. Blood 2004;**103**: 1669-75.
124. Sanchez-Ramos JR. Neural cells derived from adult bone marrow and umbilical cord blood. Journal of neuroscience research 2002;**69**: 880-93.
125. Yoshida S, Ishikawa F, Kawano N et al. Human Cord Blood–Derived Cells Generate Insulin-Producing Cells In Vivo. Stem Cells 2005;**23**: 1409-16.
126. Bonanno G, Mariotti A, Procoli A et al. Human cord blood CD133+ cells immunoselected by a clinical-grade apparatus differentiate in vitro into endothelial-and cardiomyocyte-like cells. Transfusion 2007;**47**: 280-9.
127. Wu Y, Wang J, Scott PG, Tredget EE. Bone marrow-derived stem cells in wound healing: a review. Wound Repair and Regeneration 2007;**15**: S18-S26.
128. Au P, Daheron LM, Duda DG et al. Differential in vivo potential of endothelial progenitor cells from human umbilical cord blood and adult peripheral blood to form functional long-lasting vessels. Blood 2008;**111**: 1302-5.
129. Christopeit M, Schendel M, Föll J et al. Marked improvement of severe progressive systemic sclerosis after transplantation of mesenchymal stem cells from an allogeneic haploidentical-related donor mediated by ligation of CD137L. Leukemia 2007;**22**: 1062-4.
130. Duijvestein M, Vos ACW, Roelofs H et al. Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. Gut 2010;**59**: 1662-9.
131. Sun L, Wang D, Liang J et al. Umbilical cord mesenchymal stem cell transplantation in severe and refractory systemic lupus erythematosus. Arthritis & Rheumatism 2010;**62**: 2467-75.
132. Strunk D, Rohde E, Lanzer G, Linkesch W. Phenotypic characterization and preclinical production of human lineage-negative cells for regenerative stem cell therapy. Transfusion 2005;**45**: 315-26.
133. Mund JA, Estes ML, Yoder MC et al. Flow cytometric identification and functional characterization of immature and mature circulating endothelial cells. Arteriosclerosis, thrombosis, and vascular biology 2012;**32**: 1045-53.
134. Smythe J, Fox A, Fisher N et al. Measuring angiogenic cytokines, circulating endothelial cells, and endothelial progenitor cells in peripheral blood and cord blood: VEGF and CXCL12 correlate with the number of circulating endothelial progenitor cells in peripheral blood. Tissue Engineering Part C: Methods 2008;**14**: 59-67.
135. Dominici M, Le Blanc K, Mueller I et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;**8**: 315-7.
136. Martins A, Paiva A, Morgado J et al. Quantification and immunophenotypic characterization of bone marrow and umbilical cord blood mesenchymal stem cells by multicolor flow cytometry Transplantation proceedings: Elsevier, 2009:943-6.
137. Timmermans F, Van Hauwermeiren F, De Smedt M et al. Endothelial outgrowth cells are not derived from CD133+ cells or CD45+ hematopoietic precursors. Arteriosclerosis, thrombosis, and vascular biology 2007;**27**: 1572-9.
138. Marcus AJ, Woodbury D. Fetal stem cells from extra-embryonic tissues: do not discard. Journal of cellular and molecular medicine 2008;**12**: 730-42.
139. Pappa KI, Anagnou NP. Novel sources of fetal stem cells: where do they fit on the developmental continuum? 2009.
140. O'Donoghue K, Fisk NM. Fetal stem cells. Best Practice & Research Clinical Obstetrics & Gynaecology 2004;**18**: 853-75.
141. Secco M, Zucconi E, Vieira NM et al. Multipotent stem cells from umbilical cord: cord is richer than blood! Stem cells 2008;**26**: 146-50.
142. Sensebé L, Bourin P. Mesenchymal stem cells for therapeutic purposes. Transplantation 2009;**87**: S49-S53.
143. Zuk PA, Zhu M, Ashjian P et al. Human adipose tissue is a source of multipotent stem cells. Molecular biology of the cell 2002;**13**: 4279-95.
144. Guo Z, Li H, Li X et al. In Vitro Characteristics and In Vivo Immunosuppressive Activity of Compact Bone-Derived Murine Mesenchymal Progenitor Cells. Stem Cells 2006;**24**: 992-1000.
145. Scherjon SA, Kleijburg-van der Keur C, Noort WA et al. Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation. Blood 2003;**102**: 1548-9.
146. Brooke G, Rossetti T, Pelekanos R et al. Manufacturing of human placenta-derived mesenchymal stem cells for clinical trials. British journal of haematology 2009;**144**: 571-9.

147. Sarugaser R, Lickorish D, Baksh D et al. Human umbilical cord perivascular (HUCPV) cells: a source of mesenchymal progenitors. Stem cells 2005;**23**: 220-9.
148. Wexler SA, Donaldson C, Denning-Kendall P et al. Adult bone marrow is a rich source of human mesenchymal 'stem'cells but umbilical cord and mobilized adult blood are not. British journal of haem 2003;**121**: 368-74.
149. Bieback K, Kern S, Klüter H, Eichler H. Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. Stem cells 2004;**22**: 625-34.
150. Bruyn CD, Delforge A, Martiat P, Bron D. Ex vivo expansion of megakaryocyte progenitor cells: cord blood versus mobilized peripheral blood. Stem cells and development 2005;**14**: 415-24.

Chapter 1:

Direct comparison of Wharton Jelly and bone marrow derived mesenchymal stromal cells to enhance engraftment of cord blood CD34^{+} transplants.

Mark van der Garde, Melissa van Pel, Jose E. Millán Rivero,
Alice de Graaf-Dijkstra, Manon Slot, Yoshiko Kleinveld,
Suzanne M. Watt, Helene Roelofs and Jaap Jan Zwaginga

Stem Cells and Development Epub ahead of print

Abstract

Co-transplantation of $CD34^+$ hematopoietic stem and progenitor cells (HSPC) with mesenchymal stromal cells (MSC) enhances HSPC engraftment. For these applications, MSC are mostly obtained from bone marrow. However, MSC can also be isolated from the Wharton's jelly (WJ) of the human umbilical cord. This source, regarded to be a 'waste product', enables relatively low cost MSC acquisition without any burden to the donor. Here, we evaluated the ability of WJ MSC to enhance HSPC engraftment. First, we compared cultured human WJ MSC with human bone marrow-derived MSC (BM MSC) for *in vitro* marker expression, immunomodulatory capacity and differentiation into three mesenchymal lineages. Although we confirmed that WJ MSC have a more restricted differentiation capacity, both WJ MSC and BM MSC expressed similar levels of surface markers and exhibited similar immune inhibitory capacities. Most importantly, co-transplantation of either WJ MSC or BM MSC with CB $CD34^+$ cells into NOD-SCID mice showed similar enhanced recovery of human platelets and $CD45^+$ cells in the peripheral blood and a 3-fold higher engraftment in the BM, blood and spleen six weeks after transplantation when compared to transplantation of $CD34^+$ cells alone. Upon co-incubation, both MSC sources increased the expression of adhesion molecules on $CD34^+$ cells, although SDF-1-induced migration of $CD34^+$ cells remained unaltered. Interestingly, there was an increase in CFU-GEMM when CB $CD34^+$ cells were cultured on monolayers of WJ MSC in the presence of exogenous thrombopoietin, and an increase in BFU-E when BM MSC replaced WJ MSC in such cultures. Our results suggest that WJ MSC is likely to be a practical alternative for BM MSC to enhance CB $CD34^+$ cell engraftment.

Introduction

Cord blood (CB) is used as an alternative source for hematopoietic stem -and progenitor cell (HSPC) transplantation. [1-3] However, the successful outcome of CB transplantation is limited by the relatively low number of transplantable HSPC in these grafts, which results in delayed hematopoietic recovery post-transplant. [4] Double CB transplantation in this respect increases the number of transplantable HSPC, but the time to recovery of donor neutrophils and platelets in the peripheral blood post-transplant is still inferior to transplantation of bone marrow (BM) or mobilized peripheral blood (mPB) grafts. [5] One method to overcome this CB associated disadvantage is to enhance the engraftment of HSPC by co-transplantation of accessory cells such as mesenchymal stromal cells (MSC). [6]
MSC were first identified in BM as multipotent cells and characterized largely by *in vitro* attributes. [7] These included their ability to differentiate into mesodermal cells, such as adipocytes, chondrocytes and osteoblasts, their adherence to plastic and their expression of specific cell surface markers. [8] In addition, MSC have the capacity to modulate immune responses. [9] Interestingly, in animal models, co-transplantation of human CB-derived $CD34^+$ cells with human MSC was shown to improve hematopoietic engraftment. [10,11] Both local and systemic mechanisms may play a role in this latter process, for example, by the MSC promoting homing to the bone marrow or its vasculature or releasing pro-angiogenic, immunomodulatory or growth factors that promote engraftment. [9,12,13]
Although originally identified in cultures obtained from bone marrow aspirates, [14,15] MSC can also be isolated from other sources such as adipose tissue, [16] compact bone, [17] amniotic fluid, [18] cord blood,[19] the umbilical cord [20,21] or the placenta. [22]
MSC cultured from Wharton's Jelly (WJ MSC) of the umbilical cord display unique characteristics such as a greater expansion capacity and faster *in vitro* growth compared to BM MSC. [23,24] Moreover, WJ MSC have some logistical advantages over BM MSC. Notably, the umbilical cord is considered a waste product and WJ MSC can therefore be obtained from this source at relatively low cost and without burden to the donor. The WJ could therefore be a promising source for the clinical application of MSC. [25,26]
With this in mind, we set out to compare the effect of co-transplantation of human CB-derived $CD34^+$ cells with either BM or WJ MSC on hematopoietic engraftment in immune deficient NOD-SCID mice. Furthermore, we assessed whether co-transplantation of WJ MSC that were autologous to the CB $CD34^+$ cells affected this engraftment when compared to co-transplantation with allogeneic WJ MSC.

Materials and methods

Umbilical cord blood (CB) and umbilical cord collection. CB was drawn from the umbilical vein at birth at >36 weeks gestation after written informed consent from the mother at hospitals in the Netherlands according to Netcord–FACT standards and with ethical permission from the medical ethical board of the Leiden University Medical Center (LUMC), Leiden, The Netherlands. Blood was collected by gravity drainage into Macopharma collection bags containing 21 ml Citrate Phosphate Dextrose Adenine-1 (Macopharma, Utrecht, The Netherlands). The blood was stored at 4°C and processed within 48 hours of collection. Umbilical cords were collected concomitantly with the CB in a sterile container containing PBS with 1% (v/v) antibiotic/antimycotic mix (Life Technologies, Woerden, The Netherlands).

CD34$^+$ cell purification. Mononuclear cells were isolated from CB using a sterile Ficoll density gradient (1.077 g/cm^3, Pharmacy LUMC, Leiden, The Netherlands). The CD34$^+$ cell fraction was isolated from the mononuclear cell fraction by double CD34$^+$ cell selection using immunomagnetic beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The purity of the isolated CD34$^+$ cell fraction was verified by flow cytometry (Beckman Coulter, Woerden, The Netherlands) using CD45-FITC and CD34-PE antibodies (all Beckman Coulter, ISHAGE protocol [27]), and was higher than 90% for all CD34$^+$ cells used throughout the experiments. Cells were cryopreserved in IMDM with 10% (v/v) DMSO and 4% (w/v) human serum albumin (Pharmacy LUMC) and stored at -150˚C until use.

MSC isolation and culture. ***Umbilical cord-derived MSC:*** MSC were isolated with an explant method as described in DeBruyn *et al.* [28] Briefly, the cords were cut into 5 cm segments and then longitudinally and the vein and arteries tissue removed. The segments were placed on 10 cm culture dishes (Greiner, Alphen a/d Rijn, The Netherlands) with the inside of the cord, i.e. the Wharton Jelly, facing the bottom of the plate. MSC medium (DMEM supplemented with 15% (v/v) FBS and 1% (v/v) Antibiotic-Antimycotic solution, all Life Technologies) was added to the plate until the segments were submerged in medium. The culture plates were placed in a humidified incubator at 37˚C and 5% CO_2. Medium was refreshed every 3 days. After 10 days, the segments were removed and the MSC adhering to the plate were grown to confluence and passaged into culture flasks. Since we did not separate the (sub)amnion an the Wharton Jelly, we cannot exclude that a small part of the MSC derived from the (sub)amnion. However in line with the original description of this method by the De Bruyn et al [28] we decided to use the cells WJ MSC throughout the manuscript. ***BM-derived MSC***: BM was collected from patients undergoing knee or hip replacement surgery at the LUMC with informed consent of the donor and with ethical permission from the medical ethical board of the Leiden University Medical Center (LUMC). Mononuclear cells were isolated from the BM suspensions by gradient centrifugation with Ficoll (1.077 g/ml, pharmacy LUMC) and loaded into culture flasks containing DMEM with 10% (v/v) FBS and 1% (v/v) Penicillin and Streptomycin (all Life Technologies). After overnight culture in a humidified incubator at 37˚C and 5% CO_2, non-adhering cells were washed from the flask with PBS. Adherent cells were grown to confluence and passaged. After 3 passages, cells were cryopreserved in FBS with 10% (v/v) DMSO (pharmacy LUMC). The MSC that were used throughout this study were between passage 3 and 6.

Flow cytometry. Flow cytometry analysis for cell surface marker expression was performed with a Beckman Coulter FC500 or a BD FACSCalibur running CXP or CellQuest Pro software respectively. Isolated CD34$^+$ cells were analyzed for the expression of CD34 and CD45 (both from Beckman Coulter) and MSC were analyzed for the expression of CD105, CD90, CD80, CD73, CD45, CD34, CD31, HLA-ABC and HLA-DR (all from BD Biosciences).

Differentiation of UC and BM MSC into mesodermal lineages. The WJ MSC and BM MSC were analyzed for their ability to differentiate into adipocytes, chondrocytes and osteoblasts as described previously. [29,30] In brief, MSC were cultured in specific adipogenic, chondrogenic and osteogenic differentiation media. After 21 days, the osteogenic cultures were analyzed for the presence of osteoblasts by staining of calcium deposits with Alizarin red and alkaline phosphatase with fast blue. In the adipogenic cultures, lipid droplets were visualized with Oil red O staining and, in the chondrogenic cultures, cells were stained with Toluidine blue. We used an arbitrary scoring system that

assesses the degree of differentiation in the cultures. Cultures showing no differentiated cells were scored as 0, a few differentiated cells as 1, moderate differentiation as 2 and full differentiation as 3 (Figure 1).

Gene expression of adipogenic and osteogenic differentiation cultures. BM MSC and WJ MSC were cultured for 2 weeks in specific adipogenic and osteogenic differentiation media or normal MSC medium (control); The cells of the cultures were lysed and RNA was isolated using the DirectZol RNA miniprep kit (Zymo Research, Irvine, USA). cDNA was subsequently prepared using the High Capacity RNA to cDNA kit (Life Technologies, Grand Island, USA). Expression of osteogenic and adipogenic genes was analyzed using TaqMan Gene Expression assays (see below) and the ViiA 7 Real-Time PCR System (Life Technologies). Relative expression of the genes was calculated with the ΔΔCt method normalized to RPL13a.Osteogenic genes: Runt-related transcription factor 2 **(**RUNX2), Osterix, (OSX), Osteocalcin (OC), Bone Morphogenetic Protein 2 (BMP2). Adipogenic genes: Peroxisome Proliferator-Activated Receptor-gamma (PPARg), Fatty Acid Binding Protein 4 (FABP4), Perilipin (PLIN)

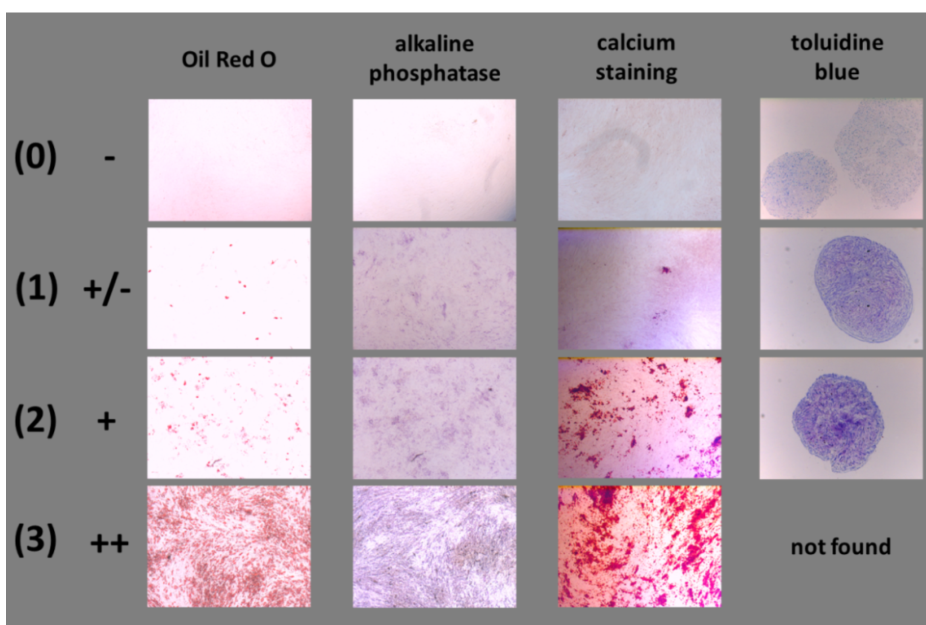


Figure 1: *Scoring system of the level of differentiation of WJ MSC or BM MSC into 3 mesodermal lineages. The amount of staining was assessed for the all different donors after differentiation cultures and compared to reference stains to determine the level of staining. 0=no differentiated cells, 1= <20% differentiated cells d, 2= <60% differentiated cells, 3= >60% differentiated cells.*

Immune inhibition of adult PBMC and CB MNC by WJ MSC and BM MSC. To analyze the effect of MSC on the proliferation of mononuclear cells obtained from adult peripheral blood (PBMC), $1x10^5$ PBMC were cultured for 5 days in 24 well plates with αCD3αCD28 beads (Life Technologies) alone or in combination with different concentrations of BM -or WJ-derived MSC in a fully humidified incubator at 37°C and 5% CO_2. Cell proliferation was measured by ^{3}H-thymidine incorporation.

Co-culture of MSC and CB CD34$^+$ cells with TPO. MSC obtained from BM and WJ were thawed and plated into a 24 well plate at $1.25x10^5$ cells/well and grown overnight in MSC medium (DMEM with 10% (v/v) FCS). After 24h, the MSC were irradiated (10 Gy) and the cells were washed twice with PBS. CB CD34$^+$ cells were added to the wells at 10^5 cells/well and cultured in expansion medium with Nplate (50ng/ml, TPO analog, Amgen, Breda, the Netherlands) as described previously. [31] After 10 days of culture, the hematopoietic cells were harvested by collecting all non adherent cells by aspirating the supernatant, washing the plates with PBS and spinning down the collected cell suspension. The cells were counted and analyzed for the expression of CD34-PE, CD61-PE-Cy7 and CD45-FITC (all Beckman Coulter) by flow cytometry. The hematopoietic stem and progenitor cells (HSPC) that were cultured were subsequently analyzed for their capacity to generate myeloid colonies in Methocult (Stemcell Technologies, Vancouver, Canada) as described previously. [32]

Transwell migration experiments. Four different cell suspensions were prepared. These were identical to the *in vivo* experiment described below. After 30 minutes of incubation of CB CD34$^+$ cells with MSC, part of the cell suspension was analyzed for the expression of CD34, CD11a, CD11b, CD184, CD49e and CD49d (antibodies all from Beckman Coulter) using flow cytometry. The remaining cells were used for migration experiments in transwell plates (Costar, (VWR) Amsterdam, The Netherlands), 6.5 mm diameter with 5 µm pore filters. The lower compartment of the well and the filter were coated with 2 ng/ml fibronectin (Sigma, St Louis, USA) for 15 minutes at 37°C. The lower compartments of the plates were loaded with IMDM and 100ng/ml SDF-1α (R&D Systems, Abingdon, UK). All cells were placed in the upper compartment of the plate and incubated for 5 hours at 37ºC and 5% CO_2 in a humidified incubator. After incubation, the numbers of CD34$^+$ cells that were harvested from the lower compartment were counted to determine the proportion of cells that migrated.

Transplantation in NOD-SCID mice. Female 5-6-week old NOD-SCID mice (Charles River, l'Arbresle, France) were kept in micro-isolator cages in laminar flow racks in the LUMC animal facilities. The animal ethical committee of the LUMC approved all animal experiments. NOD SCID mice received 3.5 Gy total body irradiation 24 hours before transplantation. Mice were transplanted with $1x10^5$ CB-derived CD34$^+$ cells alone or in combination with $1x10^6$ MSC through tail vein injections. Peripheral blood was collected from the tail vein at weekly intervals starting 3 weeks after transplantation. Blood collection and human platelet measurements were performed as described previously [32]. Briefly, human platelets were stained with a non-cross reactive mouse-anti-human CD41-PE and mouse anti human CD45-PC7 (both Beckman Coulter). Erythrocytes were lysed with IOTest3 lysing solution (Beckman Coulter) for 10 min. at room temperature. Flow-Count™ fluorospheres (Beckman Coulter) were added to the cells to enable analysis of the absolute number of circulating human platelets. Analysis was performed by flow cytometry (FC500, Beckman Coulter) using CXP software. Six weeks after transplantation, mice were sacrificed and bone marrow cells were obtained by flushing femurs with IMDM.

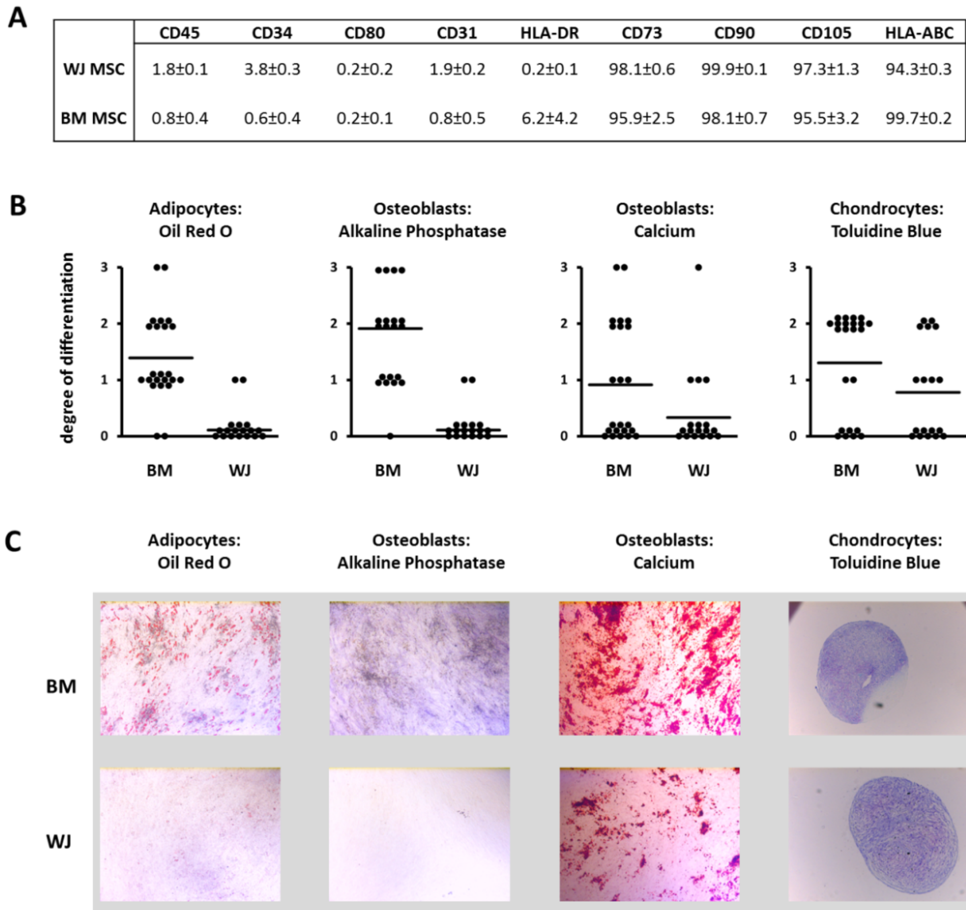
Next, human cell engraftment and multilineage chimerism were analyzed by flow cytometry using goat-anti-mouse-CD45-PE (LCA, Ly-5, 30-F11, Pharmingen, Erebodegem, Belgium), mouse-anti-human CD45-FITC, CD33-FITC, CD34-PE, CD19-PE (all from Beckman Coulter) and the appropriate isotype controls. Erythrocytes were lysed with IO Test3 Lysing solution (Beckman Coulter). Analysis was performed by flow cytometry (FC500, Beckman Coulter) using CXP software.

Statistics. All statistics were done using IBM SPSS Statistics (version 20, www.ibm.com/SPSS_Statistics*).* Results are presented as mean ± S.E.M. To test for statistical significance, a Mann-Whitney test or one way ANOVA was used. Results were considered to be significant if the p-value was equal to or less than 0.05.

Results

WJ-derived MSC have similar marker expression but limited differentiation potential compared to BM-derived MSC

Following isolation and subsequent expansion, the phenotype of the WJ MSC was determined and compared to BM MSC (Figure 2A). Similar to BM MSC, WJ MSC expressed HLA-ABC, CD73, CD90 and CD105 and lacked expression of the pan hematopoietic marker CD45 and the endothelial/hematopoietic marker CD31. A small population of BM MSC expressed HLA-DR (BM MSC 6.2±4.2% vs WJ MSC 0.2±0.1%); while a small subset of WJ MSC expressed CD34 (WJ MSC 3.8±0.3% vs. BM MSC 0.6±0.4%).
We next analyzed the ability of BM MSC and WJ MSC to differentiate into adipocytes, chondrocytes and osteoblasts. The majority of BM-derived MSC isolates had the capacity to differentiate into chondrocytes and adipocytes and half of the BM MSC isolates differentiated into osteoblasts. In contrast, more than 25% of the WJ MSC isolates (4 out of 18) showed adipocytes generation and less than 15 % of the WJ MSC isolates (2 out of 18) showed osteoblast differentiation (Figure 2B and C). Moreover, in these respective 4 and 2 cultures adipocyte and osteoblast differentiation was sporadic (grade 1). Chondrogenic differentiation occurred in half of the WJ isolates (9 out of 18). Thus, although BM MSC and WJ MSC are phenotypically similar, WJ MSC are limited with respect to their capacity to differentiate into mature mesodermal cell types as shown by their lower degree of differentiation compared to BM MSC.
For one MSC donor of each type of MSC, these functional differences were also investigated by the comparison of differentiation specific gene expression. As shown before, no osteoblast or adipocyte specific staining was observed in the WJ MSC culture, whereas positive staining for both cell types could be found in the BM MSC cultures (Figure 3A). The expression of the adipocyte related genes peroxisome proliferator-activated receptor-γ (PPARG), fatty acid binding protein 4 (FABP4) and perilipin (PLIN) was clearly upregulated in the BM MSC cultures, while WJ MSC only showed upregulation of PPARG (Figure 3B). For osteoblast related genes this method showed to be unreliable since osteogenic differentiated BM MSC did not show any increase in osteogenic gene expression, despite the presence of osteogenic specific staining (Figure 3A and 3C).



	CD45	CD34	CD80	CD31	HLA-DR	CD73	CD90	CD105	HLA-ABC
WJ MSC	1.8±0.1	3.8±0.3	0.2±0.2	1.9±0.2	0.2±0.1	98.1±0.6	99.9±0.1	97.3±1.3	94.3±0.3
BM MSC	0.8±0.4	0.6±0.4	0.2±0.1	0.8±0.5	6.2±4.2	95.9±2.5	98.1±0.7	95.5±3.2	99.7±0.2

***Figure 2: Characterization of the Wharton's Jelly MSC**. **A:** Expression of cell surface markers by WJ MSC and BM MSC. The percentage of cells (±SEM; n=10) that express the respective markers is shown. **B:** Ability of WJ MSC (n=18 different isolates) and BM MSC (n=23 different isolates) to differentiate into adipocytes (Oil Red O), osteoblasts (alkaline phosphatase and calcium deposition) and chondrocytes (toluidine blue staining). 0=no differentiated cells, 1= <20% differentiated cells d, 2= <60% differentiated cells, 3= >60% differentiated cells (see also Figure S1). The bar represents the mean of all experiments **C:** Representative images of differentiation cultures after incubation with cell differentiation specific stains.*

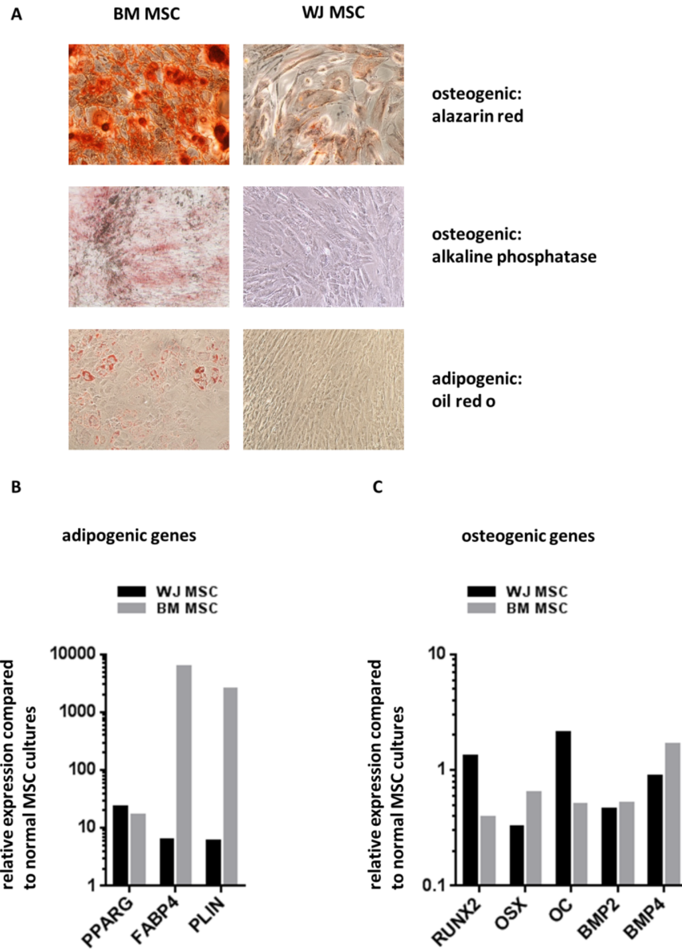


Figure 3: differentiation cultures of BM and WJ MSC, A: *Staining of the differentiation cultures for cell specific markers/deposits of osteoblasts (alizarin red and alkaline phosphatase) and adipocytes (oil red O)****, B:*** *Relative expression of adipogenic genes by BM MSC and WJ MSC after adipogenic differentiation cultures. Shown is the difference in up or down regulation of the genes by MSC in differentiation cultures compared to cultures in normal MSC medium (2^ΔΔCt, normalized to RPL13a).****, C:*** *Relative expression of osteogenic genes by BM MSC and WJ MSC after osteogenic differentiation cultures. Shown is the difference in up or down regulation of the genes by MSC in differentiation cultures compared to cultures in normal MSC medium (2^ΔΔCt, normalized to RPL13a).*

WJ MSC and BM MSC inhibit T cell proliferation of PBMC stimulated with αCD3αCD28 beads

Next, we compared *in vitro* immunomodulatory properties of WJ MSC and BM MSC in co-cultures with un-stimulated and αCD3αCD28-stimulated PBMC. In this setting, MSC were not immunogenic themselves, since co-incubation with MSC did not lead to proliferation of unstimulated PBMC. Moreover, WJ MSC and BM MSC inhibited proliferation of stimulated PBMC and this reduction was MSC dose dependent (Figure 4, $p<0.0001$ for all ratios of MSC and PBMC compared to stimulated PBMC alone). Interestingly, co-culture of BM MSC and PBMC at a 1:1 ratio resulted in decreased inhibition of proliferation (43±6%) as compared to a 1:2 ratio of BM MSC (70±4%, $p<0.001$). Additionally, at this 1:1 ratio, WJ MSC were more inhibitory than BM-MSC (Figure 4; 68±4% vs. 43±6% inhibition respectively, $p<0.00005$).

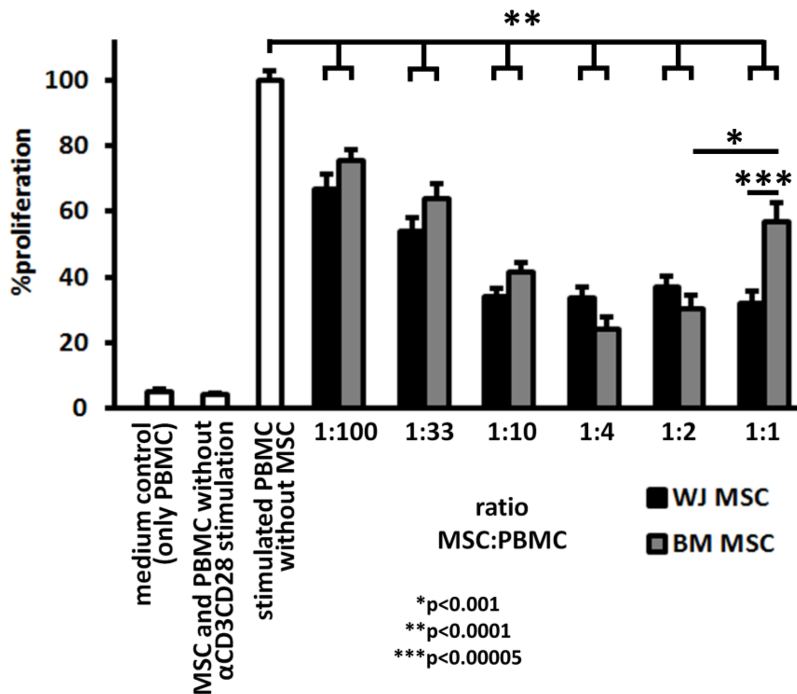
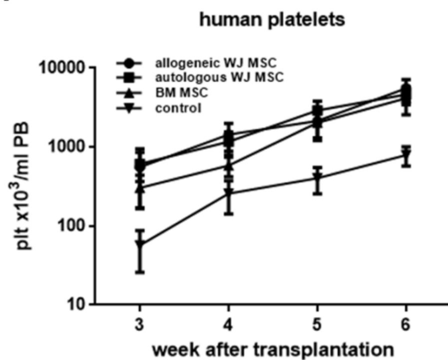
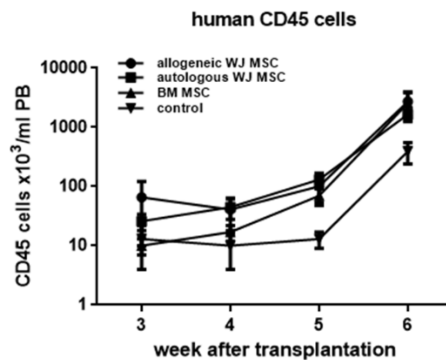


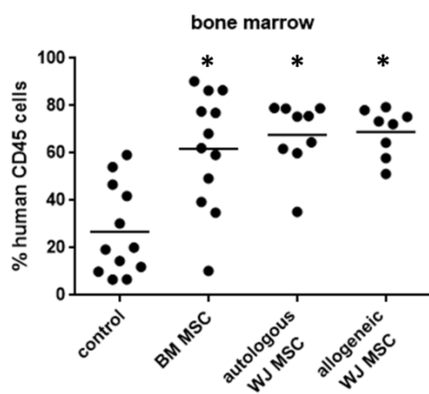
Figure 4: WJ and BM MSC inhibit T cell proliferation. *BM or WJ MSC were mixed with $1x10^5$ PBMC at different ratios and stimulated with αCD3αCD28 beads for 5 days. Proliferation of the PBMC was measured by (^3H) thymidine incorporation on day 5 and compared to a control without MSC. MSC obtained from both sources significantly inhibited the proliferation of the PBMC in a dose dependent manner (n=8, *p<0.0001 for all ratios; at a 1:1 ratio of MSC:PBMC comparing WJ MSC with BM MSC **p<0.00005).*

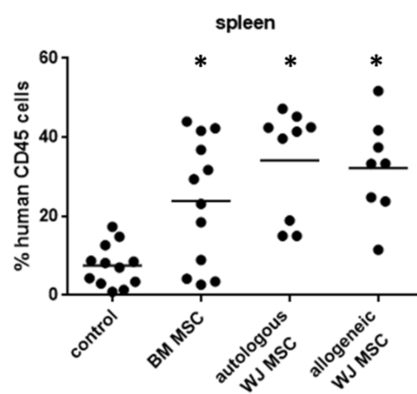
Co-transplantation of MSC enhances the engraftment of CB-derived $CD34^+$ cells in NOD SCID mice irrespective of the donor source

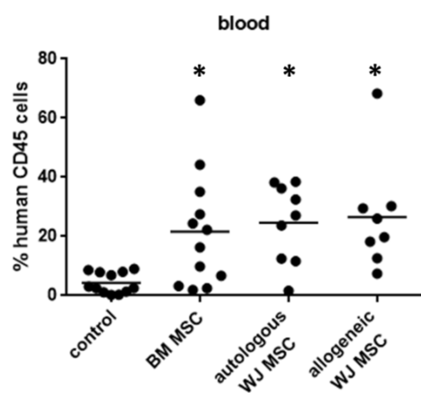
To evaluate and compare the effects of the different MSC on HSC engraftment *in vivo*, we co-transplanted human CB-derived $CD34^+$ cells and human MSC into sublethally irradiated NOD SCID mice (n=3 experiments with 3 different donors). Additionally, autologous WJ MSC (i.e. MSC generated from the umbilical cord of the $CD34^+$ cell donor), and allogeneic WJ MSC (MSC generated from the umbilical cord of another donor) were compared with co-transplantation of allogeneic BM derived MSC and transplantation of $CD34^+$ cells alone. Starting from 3 weeks after transplantation until the mice were sacrificed at week 6, we analyzed the peripheral blood (PB) of the mice for the presence of human platelets (Figure 5A) and human $CD45^+$ cells at weekly intervals (Figure 5B). All recipient mice had detectable levels of human platelets in their peripheral blood as early as week 3. Co-transplantation of MSC from all sources resulted in higher levels of circulating platelets compared to transplantation of $CD34^+$ cells alone ($CD34^+$ cells alone: 57±31 plt/µl PB vs. with BM MSC: 304±135 plt/µl PB, $p=0.073$, with autologous WJ MSC: 610±244 plt/µl PB, $p<0.05$ or with allogeneic MSC: 556±390 plt/µl PB, $p<0.05$). At 6 weeks after transplantation, platelet levels were on average 5-fold higher in the $CD34^+$ and MSC co-transplanted groups when compared to the platelet levels in recipients of $CD34^+$ cells alone ($CD34^+$ cells alone: 790±216 plt/µl PB vs. BM MSC and $CD34^+$ cells: 4146±1586 plt/µl PB, $p<0.005$, autologous WJ MSC and $CD34^+$ cells: 4649±1203 plt/µl PB, $p<0.005$ and allogeneic WJ MSC and $CD34^+$ cells: 5546±1654 plt/µl PB, $p<0.05$). Similarly, co-transplantation of WJ MSC significantly increased human $CD45^+$ cells in the PB from week 4 onwards as compared to transplantation with $CD34^+$ cells alone ($40.6±22.7x10^3$ and $45.1±17x10^3$ versus $10.1±5.7x10^3$ $CD45^+$ cells/ml for $CD34^+$ cells with autologous and allogeneic WJ MSCs vs $CD34^+$ cells alone respectively, $p<0.05$). Slower recovery of circulating human leukocytes was observed with co-transplanted BM MSC as compared to co-transplanted WJ MSC. Co-transplantation of BM MSC increased circulating $CD45^+$ cells 5 weeks after transplantation reaching levels similar to co-transplanting WJ MSC at this time point ($69.7±21.9x10^3$ versus $12.6±3.9x10^3$ $CD45^+$ cells/ml for $CD34^+$ cells with BM MSC or for $CD34^+$ transplantation only, $p<0.05$). Six weeks after transplantation, the mice were sacrificed and the bone marrow was analyzed for the presence of human hematopoietic cells. Co-transplantation of MSC obtained from both WJ and BM increased the frequency of human $CD45^+$ cells in the bone marrow by at least 2-fold as compared to transplantation of $CD34^+$ cells alone (Figure 5C, $CD34^+$ cells alone: 26.7±5.5% human $CD45^+$ cells vs. $CD34^+$ cells with BM MSC 61.8±7.1%, $p<0.0005$, with autologous WJ MSC: 67.7±4.8%, $p<0.0001$ or with allogeneic WJ MSC 68.9±3.6%, $p<0.0001$). A similar pattern was observed for human $CD45^+$ cell chimerism in spleen and peripheral blood. Co-transplantation of MSC and $CD34^+$ cells increased chimerism in the spleen by at least 3-fold compared to transplantation of $CD34^+$ cells alone (Figure 5D, $CD34^+$ cells alone: 7.5±1.5% vs. co-transplantation with BM MSC: 23.9±4.6%, $p<0.05$, with autologous WJ MSC: 34.1±4.5%, $p<0.005$ or with allogeneic WJ MSC: 32.2±4.3%, $p<0.001$) and in the blood at least 5-fold (Figure 5E, $CD34^+$ cells alone: 4.2±1.0% human $CD45^+$ cells vs. co-transplantation with BM MSC: 21.7±5.6%, $p<0.05$, with autologous WJ MSC: 24.7±4.5% or with allogeneic WJ MSC: 26.5±6.6%, $p<0.005$). BM cells harvested from the femurs of the mice expressed similar percentages of the common myeloid marker CD33, the lymphoid markers CD19 and CD3 and the stem/progenitor cell marker CD34 irrespective of the co-transplantation of MSC (Figure 5F). In our model, autologous and allogeneic WJ MSCs enhanced total human $CD45^+$ cell reconstitution to a similar extent.

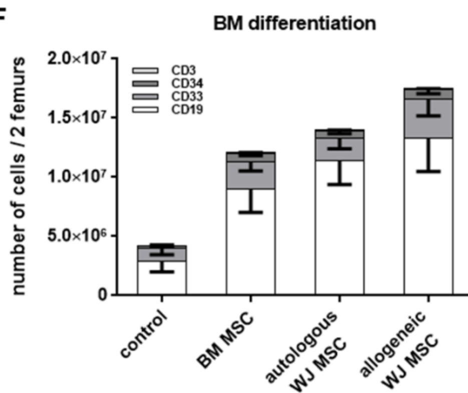

A
human platelets
allogeneic WJ MSC
autologous WJ MSC
BM MSC
control
plt x10³/ml PB
10000
1000
100
10
3
4
5
6
week after transplantation


B
human CD45 cells
allogeneic WJ MSC
autologous WJ MSC
BM MSC
control
CD45 cells x10³/ml PB
10000
1000
100
10
1
3
4
5
6
week after transplantation


C
bone marrow
% human CD45 cells
100
80
60
40
20
0
*
control
BM MSC
autologous WJ MSC
allogeneic WJ MSC


D
spleen
% human CD45 cells
60
40
20
0
*
control
BM MSC
autologous WJ MSC
allogeneic WJ MSC


E
blood
% human CD45 cells
80
60
40
20
0
*
control
BM MSC
autologous WJ MSC
allogeneic WJ MSC


F
BM differentiation
CD3
CD34
CD33
CD19
number of cells / 2 femurs
2.0×10⁷
1.5×10⁷
1.0×10⁷
5.0×10⁶
0
control
BM MSC
autologous WJ MSC
allogeneic WJ MSC

Co-culture of WJ MSC and CB CD34+ cells with TPO enhances CFU-GEM formation, while BM MSC enhances BFU-E formation

Several functional characteristics of MSC might play a role in their observed ability to facilitate engraftment. MSC have been shown previously to support the growth of human CB CD34+ cells. [33-37] Furthermore, we have shown that transplantation of TPO expanded CB CD34+ enhanced early platelet repopulation while retaining long term hematopoietic engraftment capacity in NOD-SCID mice. [31,32] Combining these 2 potential engraftment enhancing strategies, we compared the capacity of human WJ MSC and BM MSC to support differentiation and expansion of human CB-derived CD34+ cells in the presence of exogenous TPO. To this end, CD34+ cells were cultured for 10 days on monolayers of MSC obtained from different sources in the presence of exogenous TPO and analyzed for expansion of total MNC and CFU formation of the cultured CD34+ cells.

Cultures of CD34+ cells on either BM MSC or WJ MSC monolayers did not enhance the TPO induced expansion of total nucleated cells (Figure 6A, WJ MSC 4.8±0.9 fold expansion and BM MSC 4.1±1.5 fold expansion vs. no stromal support 4.0±0.8 fold expansion) over this time period. Additionally, the ratio between the 3 main cell subpopulations that are typically formed when CB CD34+ are cultured with TPO, namely residual CD34+ cells (rCD34+), CD34-- CD61- cells (Lin-) and CD34-61+ cells (CD61+), [38] were similar between cultures without stromal support and those on MSC mono-layers from different sources (Figure 6B). To investigate the differentiation potential of the remaining hematopoietic stem and progenitor cells (HSPC) in the TPO induced cultures, we next analyzed their colony-forming capacity in CFU assays. To this end, HSPC were first separated from the MSC after co-culture and subsequently cultured in semi-solid cultures in the presence of cytokines. Interestingly, HSPC derived from cultures on BM MSC monolayers exhibited an increased capacity to form BFU-E colonies (Figure 6C, BM MSC 42.8±10.2 BFU-E/1000 rCD34+ cells compared to no MSC 14.5±6.1 BFU-E/1000 rCD34+ cells, $p<0.05$). HSPC cultured in the presence of WJ MSC gave rise to higher numbers of CFU-GEMM (35.8±9.9 colonies/1000 CD34+ cells vs. 20.6±6.3/1000 CD34+ without stromal support; Figure 6C, right panel, n=5 experiments, $p<0.05$).

***Figure 5: Co-transplantation of MSC and CD34+ cells enhances peripheral blood recovery and bone marrow engraftment in NOD SCID mice** , **A:** Human platelet recovery in the PB of the mice after transplantation. **B:** Human CD45+ cell recovery in the PB of the mice after transplantation. **C:** Percentage of human CD45+ cells in the BM of the mice 6 weeks after transplantation **D:** Percentage of human CD45+ cells in the spleen of the mice 6 weeks after transplantation. **E:** Percentage of human CD45+ cells in the blood of the mice 6 weeks after transplantation. Bars represent the mean of all mice **F:** Lineage differentiation of the human CD45+ cells in the BM 6 weeks after transplantation. The total number of human CD45+ cells in the femurs off all mice was analyzed for the expression of lymphoid markers CD19 and CD3, myeloid marker CD33 and stem/progenitor cell marker CD34. (n=3 experiments with 3 different CB donors/MSC isolates)*

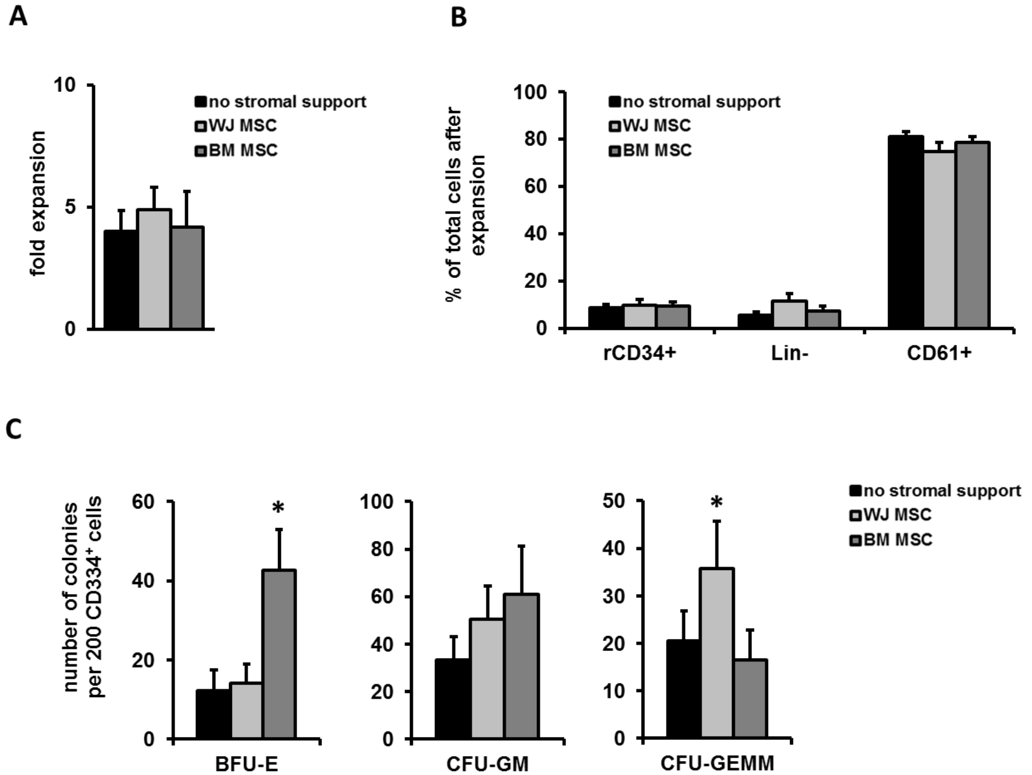


Figure 6: Co-culture of BM or WJ MSC with CB CD34*⁺ *cells supports the expansion of CB CD34*⁺ *cells in the presence of TPO. *CB CD34⁺ cells obtained from different donors (n=5) were cultured with TPO for 10 days in the presence or absence of MSC obtained from different sources. Next, the composition of the expanded cells was analyzed using flow cytometry and the capacity to form myeloid colonies was analyzed with CFU assays.* ***A:*** *Fold expansion (depicted as the total number of hematopoietic nucleated cells after culture divided by the number of input cells) in the absence or presence of stromal cells* ***B:*** *Percentage of the 3 major populations of the total hematopoietic cells observed after expansion in the absence or presence of stromal support from MSC from different sources; rCD34⁺ = residual CD34 cells, Lin⁻ = CD34⁻CD61⁻Lineage⁻ cells, and CD61⁺ = CD34⁻CD61⁺ cells.* ***C:*** *Colony-forming capacity of TPO expanded CD34⁺ cells after culture in the absence or presence of stromal support. (*p<0.05).*

Incubation of CB-derived CD34+ cells with MSC does not significantly alter their migration towards CXCL12, but increases the expression of adhesion markers

Hematopoietic stem cell homing to the marrow is the primary step for their engraftment and relies on their adhesive and migratory capacities. We investigated whether MSC change the migratory characteristics of CB $CD34^+$ cells towards CXCL12 in transwell migration studies. To this end, CB $CD34^+$ cells and MSC were incubated together for 30 minutes to mimic the time that $CD34^+$ cells and MSC are in the same tube prior to infusion. Aliquots of the cell suspensions were placed in transwell plates while others were analyzed in parallel for adhesion marker expression. Additionally, the effect of the presence of either autologous or allogeneic WJ MSC on the migration of $CD34^+$ cells was tested (Figure 7A). Autologous WJ MSC tended to increase $CD34^+$ migration when compared to the migration of $CD34^+$ cells alone; this difference however, was not significant (11.3±5.2% vs 6.8±2.2% for $CD34^+$ cell migration with or without autologous WJ MSC). Next, we investigated the expression of several adhesion markers known to be involved in homing to the BM. [39] In this respect, we analyzed the expression of CD11a, CD11b, CD49d, CD49e and the CXCL12 receptor CD184 (CXCR4) on $CD34^+$ cells after 30 minutes incubation with MSC (Figure 7B). Regardless of the MSC source, incubation of CB $CD34^+$ cells with MSC seemed to induce a general increase in marker expression. However, even between MSC from the same source, expression levels varied considerably. Therefore, only CD49d and CD49e expression was significantly increased after incubation with BM MSC and allogeneic WJ MSC respectively ($p<0.05$).

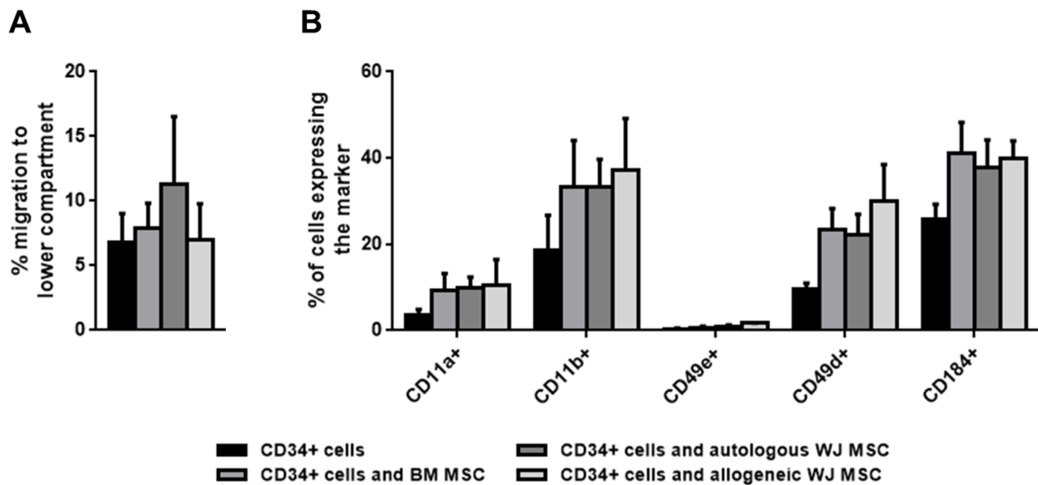


Figure 7: In vitro homing characteristics of and adhesion molecule expression of $CD34^+$ in the presence of MSC obtained from different sources. A: *Migration of $CD34^+$ cells towards SDF-1α in the presence of MSC obtained from WJ or BM.* ***B:*** *Expression of adhesion markers and CXCR4 on CB-derived $CD34^+$ cells after incubation with WJ MSC or BM MSC.*

Discussion

In the present study, we compared the effect of human MSC obtained from WJ and BM on the engraftment of CB-derived $CD34^+$ cells in an immune-deficient murine transplant model. The recovery of human platelets and $CD45^+$ cells in the peripheral blood of these mice was significantly enhanced by co-transpantation of either WJ or BM MSC from 3 weeks onwards. At 6 weeks post-transplantation, the percentage of human $CD45^+$ cells in the BM, spleen and peripheral blood was at least 3-fold higher when MSC were co-transplanted with $CD34^+$ cells compared to transplantation of $CD34^+$ cells alone. MSC obtained from BM and WJ were comparable in their capacity to enhance the engraftment of CB-derived $CD34^+$ cells.
Although the mechanism is so far not determined, MSC induced $CD34^+$ cell engraftment has been suggested to be associated with their immune-modulatory capacity. MSC from different tissues including WJ are known to modulate immunological responses. [9,40] At high levels of IFN-γ and TNF-α, MSC have an anti-inflammatory effect for which secreted indoleamine 2,3-dioxygenase (IDO) is one of the proposed mediators. [41,42] Conversely, in steady state conditions at low levels of IFN-γ and TNF-α, allogeneic MSC are able to stimulate an immunological response. [9] The absence of inflammatory signals directly after transplantation could therefore not only diminish the immunosuppressive effect of the co-transplanted MSC but may even have an immune activating effect. The immunosuppressive properties of MSC, however, are most likely to mediate a beneficial effect on the outcome of HCT e.g. by exhibiting a prophylactic effect on the occurrence or severity of Graft versus Host Disease or host mediated graft rejection. [43,44] In agreement, a study in which mononuclear cells (MNC) obtained from two CB units were transplanted demonstrated that either removal of the immune competent cells from the graft or MSC co-transplantation alleviated single CB donor dominance and improved overall engraftment. [10] In our *in vitro* experiments MSC from the BM and WJ were equally potent in inhibiting the proliferation of αCD3αCD28 stimulated PBMC. However, as we use immune deficient NOD SCID mice for our *in vivo* transplantation experiments and transplants without any immune competent cells, these immune modulating qualities do not seem to be instrumental for the similar engraftment enhancing effect of both MSC sources since. Alloimmunization between the recipient and the transplanted cells or between the $CD34^+$ cell purified HSC graft and the co-transplanted allogeneic MSC are therefore not likely to occur. In agreement, no difference in the engraftment enhancing capacity of autologous and allogeneic WJ MSC was found. Since immune related components cannot be assessed in our model, the marked increase of engraftment must therefore be caused by other MSC-derived factors.
Alternatively, MSC may play a direct role in regenerating the bone marrow niche, first by homing to the marrow and differentiating into stromal tissues and second by inducing the proliferation and differentiation of HSC. Concerning the first option, our studies show that the differentiation capacity of WJ MSC is variable and limited and thus unlikely to be the cause for their engraftment stimulating effect. Corroborated by a study from Bosch et al., [45] this also makes WJ MSC less interesting candidates for therapeutic bone or cartilage regeneration. Concerning the second option, WJ MSC have been shown to support the growth of CB-derived $CD34^+$ cells *ex vivo.* [33-37] As we previously showed that TPO expanded CB $CD34^+$ cells contributed to both improved platelet recovery and BM engraftment, [31] we combined these 2 different mechanisms in an *in vitro* experiment and investigated whether MSC could further enhance TPO-induced effects on CD34+ cultures. In this regard additional presence of MSC did not change TPO induced expansion and neither was the composition of the formed subpopulations changed. CFU cultures of the

expanded cells did however show that the MSC have a different effect on the types of cells that are formed after expansion. Cultures of HSPC on BM MSC monolayers exhibited an increased capacity to form BFU-E colonies while HSPC cultured in the presence of WJ MSC gave rise to higher numbers of CFU-GEMM. These observations might be of conceptual importance since CFU-GEMM are correlated with the presence of more primitive stem cells. [46] Hence, WJ MSC in this respect might have a better potential to preserve the more immature $CD34^+$ cells in culture with TPO than BM MSC. Other studies have shown that the co-culture of MSC, including WJ MSC, can enhance the fold expansion of both total nucleated cells and CD34+ cells. [33,35,37] These studies used a cocktail of cytokines including SCF, Flt3L and TPO in their culture protocol. Adding SCF and Flt3L to the expansion medium can significantly enhance the fold expansion of the cells, [47] and the absence of these cytokines could therefore explain the lack of TNC and CD34+ cell expansion in our experiments.
However, for both the MSC induced marrow niche regenerating mechanisms to become relevant, homing of MSC with the HSPCs to the bone marrow is necessary. This has so far not been convincingly shown. [11,48,49] Although co-localization of MSC and $CD34^+$ cells in the pelvis has been reported [49], [48] MSC are more often detected in various organs, but not in the BM [11] and entrapment of MSC in the lungs has been described as a possible explanation for the lack of homing of the MSC. [49]
MSC-induced homing of $CD34^+$ cells to the HSC niche might be another explanation for increased engraftment by local (e.g. by cell to cell contact in the marrow) or systemic (e.g. by paracrine factors production) support. Our *in vitro* studies, with brief exposure of $CD34^+$ cells to MSC could not consistently show enhanced migration of $CD34^+$ cells towards CXCL12. However, in the presence of MSC, CD34+ cells upregulate surface markers that are associated with their homing to or retention in the BM marrow (e.g. CD11a, CD11b, CD184, CD49e and CD49d). [39,50,51]
In conclusion, our data support the use of human WJ MSC as an alternative source to enhance the engraftment of human CB-derived $CD34^+$ cells. Although MSC induced homing of HPSC to the BM, seems an interesting explanation from our studies, for a real life estimate of MSC role in engraftment support other mechanisms also need to be considered. Engraftment in this regard is likely dependent on (interacting) factors like the immune status of the recipient and hematopoietic immune (activating) cells in the transplants. However, the immune-deficient recipient mice and the CD34+ isolated stem cell transplant that were used in our studies, makes MSC induced immunomodulation an unlikely explanation for the observed enhanced engraftment *in vivo*. Our *in vitro* experiments, however, do suggest that WJ MSC may serve as an alternative source to BM MSC for immunomodulatory applications. A general advantage of WJ MSC is that the umbilical cord can be regarded as a waste product, and that WJ MSC can therefore be obtained at relatively low cost without harm or risk for the donor.
Since co-transplantation of allogeneic MSC did not show particular disadvantages, [52-54] and while co-transplantation of autologous MSC with donor hematopoietic stem cells might even inhibit engraftment, [55] our study does not support the paired use and banking of CB $CD34^+$ cells and WJ MSC of the same umbilical cord. However, concurrent collection of the umbilical cord with CB collection enables sharing the logistics, tissue typing and virological testing for two products from only one donor with additional saving of costs and effort to obtain MSC from BM. This new CB cord strategy would therefore create a relative cheap, off the shelf MSC product that can be provided by tissue banks to hospitals. The value of such an approach however will eventually depend on the therapeutic efficacy of MSC and more specifically WJ MSC.

References

1. Ballen KK, Gluckman E, Broxmeyer HE. Umbilical cord blood transplantation: the first 25 years and beyond. Blood 2013;**122**: 491-8.
2. Ballen KK, Koreth J, Chen YB et al. Selection of optimal alternative graft source: mismatched unrelated donor, umbilical cord blood, or haploidentical transplant. Blood 2012;**119**: 1972-80.
3. Fuchs E, O'Donnell PV, Brunstein CG. Alternative transplant donor sources: is there any consensus? Curr Opin Oncol 2013;**25**: 173-9.
4. Eapen M, Rocha V, Sanz G et al. Effect of graft source on unrelated donor haemopoietic stem-cell transplantation in adults with acute leukaemia: a retrospective analysis. The lancet oncology 2010;**11**: 653-60.
5. Brunstein CG, Gutman JA, Weisdorf DJ et al. Allogeneic hematopoietic cell transplantation for hematologic malignancy: relative risks and benefits of double umbilical cord blood. Blood 2010;**116**: 4693-9.
6. Danby R, Rocha V. Current Strategies to Improve Engraftment in Cord Blood Transplantation. J Stem Cell Res Ther 2014;**4**: 2.
7. Pittenger MF, Mackay AM, Beck SC et al. Multilineage potential of adult human mesenchymal stem cells. science 1999;**284**: 143-7.
8. Dominici M, Le Blanc K, Mueller I et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;**8**: 315-7.
9. Bernardo ME, Fibbe WE. Mesenchymal stromal cells: sensors and switchers of inflammation. Cell Stem Cell 2013;**13**: 392-402.
10. Kim D-W, Chung Y-J, Kim T-G et al. Cotransplantation of third-party mesenchymal stromal cells can alleviate single-donor predominance and increase engraftment from double cord transplantation. Blood 2004;**103**: 1941-8.
11. Noort WA, Kruisselbrink AB, Kruger M et al. Mesenchymal stem cells promote engraftment of human umbilical cord blood–derived CD34+ cells in NOD/SCID mice. Experimental hematology 2002;**30**: 870-8.
12. Bonnet D, Bhatia M, Wang J et al. Cytokine treatment or accessory cells are required to initiate engraftment of purified primitive human hematopoietic cells transplanted at limiting doses into NOD/SCID mice. Bone marrow transplantation 1999;**23**: 203.
13. Watt SM, Gullo F, van der Garde M et al. The angiogenic properties of mesenchymal stem/stromal cells and their therapeutic potential. British medical bulletin 2013: 1-29.
14. Caplan AI. Mesenchymal stem cells. Journal of orthopaedic research 1991;**9**: 641-50.
15. Friedenstein AJ, Gorskaja J, Kulagina N. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Experimental hematology 1976;**4**: 267-74.
16. Zuk PA, Zhu M, Ashjian P et al. Human adipose tissue is a source of multipotent stem cells. Molecular biology of the cell 2002;**13**: 4279-95.
17. Guo Z, Li H, Li X et al. In Vitro Characteristics and In Vivo Immunosuppressive Activity of Compact Bone-Derived Murine Mesenchymal Progenitor Cells. Stem Cells 2006;**24**: 992-1000.
18. Scherjon SA, Kleijburg-van der Keur C, Noort WA et al. Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation. Blood 2003;**102**: 1548-9.
19. Erices A, Conget P, Minguell JJ. Mesenchymal progenitor cells in human umbilical cord blood. British journal of haematology 2000;**109**: 235-42.
20. Sarugaser R, Lickorish D, Baksh D et al. Human umbilical cord perivascular (HUCPV) cells: a source of mesenchymal progenitors. Stem cells 2005;**23**: 220-9.
21. Seshareddy K, Troyer D, Weiss ML. Method to Isolate Mesenchymal-Like Cells from Wharton's Jelly of Umbilical Cord. Methods in cell biology 2008;**86**: 101-19.
22. Brooke G, Rossetti T, Pelekanos R et al. Manufacturing of human placenta-derived mesenchymal stem cells for clinical trials. British journal of haematology 2009;**144**: 571-9.
23. Fong C-Y, Richards M, Manasi N et al. Comparative growth behaviour and characterization of stem cells from human Wharton's jelly. Reproductive biomedicine online 2007;**15**: 708-18.
24. Troyer DL, Weiss ML. Concise Review: Wharton's Jelly-Derived Cells Are a Primitive Stromal Cell Population. Stem cells 2008;**26**: 591-9.
25. Bongso A, Fong C-Y. The therapeutic potential, challenges and future clinical directions of stem cells from the Wharton's jelly of the human umbilical cord. Stem Cell Reviews and Reports 2013;**9**: 226-40.
26. El Omar R, Beroud J, Stoltz J-F et al. Umbilical cord mesenchymal stem cells: the new gold standard for mesenchymal stem cell-based therapies? Tissue Engineering Part B: Reviews 2014;**20**: 523-44.
27. Sutherland DR, Anderson L, Keeney M et al. The ISHAGE guidelines for CD34+ cell determination by flow cytometry. Journal of hematotherapy 1996;**5**: 213-26.

28. De Bruyn C, Najar M, Raicevic G et al. A rapid, simple, and reproducible method for the isolation of mesenchymal stromal cells from Wharton's jelly without enzymatic treatment. Stem cells and development 2010;**20**: 547-57.
29. Duijvestein M, Wildenberg ME, Welling MM et al. Pretreatment with Interferon-γ Enhances the Therapeutic Activity of Mesenchymal Stromal Cells in Animal Models of Colitis. Stem Cells 2011;**29**: 1549-58.
30. Perez-Galarza J, Carlotti F, Rabelink MJ et al. Optimizing reporter constructs for in vivo bioluminescence imaging of IFN-γ stimulated mesenchymal stromal cells. Experimental hematology 2014;**42**: 793-803.
31. van der Garde M, van Hensbergen Y, Brand A et al. Thrombopoietin Treatment of One Graft in a Double Cord Blood Transplant Provides Early Platelet Recovery While Contributing to Long-Term Engraftment in NSG Mice. Stem cells and development 2015;**24**: 67-76.
32. van Hensbergen Y, Schipper LF, Brand A et al. Ex vivo culture of human CD34+ cord blood cells with thrombopoietin (TPO) accelerates platelet engraftment in a NOD/SCID mouse model. Experimental hematology 2006;**34**: 943-50.
33. Alakel N, Jing D, Muller K et al. Direct contact with mesenchymal stromal cells affects migratory behavior and gene expression profile of CD133+ hematopoietic stem cells during ex vivo expansion. Experimental hematology 2009;**37**: 504-13.
34. Bakhshi T, Zabriskie RC, Bodie S et al. Mesenchymal stem cells from the Wharton's jelly of umbilical cord segments provide stromal support for the maintenance of cord blood hematopoietic stem cells during long-term ex vivo culture. Transfusion 2008;**48**: 2638-44.
35. Fong C, Gauthaman K, Cheyyatraivendran S et al. Human umbilical cord Wharton's jelly stem cells and its conditioned medium support hematopoietic stem cell expansion ex vivo. Journal of Cellular Biochemistry 2012;**113**: 658-68.
36. Magin A, Körfer N, Partenheimer H et al. Primary cells as feeder cells for coculture expansion of human hematopoietic stem cells from umbilical cord blood-a comparative study. Stem cells and development 2009;**18**: 173-86.
37. Zhang Y, Chai C, Jiang X-S, Teoh S-H, Leong KW. Co-culture of umbilical cord blood CD34þ cells with human mesenchymal stem cells. Tissue engineering 2006;**12**: 2161-70.
38. Schipper LF, Brand A, Reniers N et al. Differential maturation of megakaryocyte progenitor cells from cord blood and mobilized peripheral blood. Experimental hematology 2003;**31**: 324-30.
39. Voermans C, Rood P, Hordijk P et al. Adhesion molecules involved in transendothelial migration of human hematopoietic progenitor cells. Stem Cells 2000;**18**: 435-43.
40. Weiss ML, Anderson C, Medicetty S et al. Immune Properties of Human Umbilical Cord Wharton's Jelly-Derived Cells. Stem cells 2008;**26**: 2865-74.
41. Tipnis S, Viswanathan C, Majumdar AS. Immunosuppressive properties of human umbilical cord-derived mesenchymal stem cells: role of B7-H1 and IDO. Immunology and Cell Biology 2010;**88**: 795-806.
42. Valencic E, Piscianz E, Andolina M et al. The immunosuppressive effect of Wharton's jelly stromal cells depends on the timing of their licensing and on lymphocyte activation. Cytotherapy 2010;**12**: 154-60.
43. Baron F, Lechanteur C, Willems E et al. Cotransplantation of mesenchymal stem cells might prevent death from graft-versus-host disease (GVHD) without abrogating graft-versus-tumor effects after HLA-mismatched allogeneic transplantation following nonmyeloablative conditioning. Biology of blood and marrow transplantation 2010;**16**: 838-47.
44. Kuzmina LA, Petinati NA, Parovichnikova EN et al. Multipotent mesenchymal stromal cells for the prophylaxis of acute graft-versus-host disease—a phase II study. Stem cells international 2012; 968213.
45. Bosch J, Houben AP, Radke TF et al. Distinct differentiation potential of "MSC" derived from cord blood and umbilical cord: are cord-derived cells true mesenchymal stromal cells? Stem cells and development 2011;**21**: 1977-88.
46. Broxmeyer HE, Douglas GW, Hangoc G et al. Human umbilical cord blood as a potential source of transplantable hematopoietic stem/progenitor cells. Proceedings of the National Academy of Sciences 1989;**86**: 3828-32.
47. Gullo F, van der Garde M, Russo G et al. Computational modeling of the expansion of human cord blood CD133+ hematopoietic stem/progenitor cells with different cytokine combinations. Bioinformatics 2015; **31**: 2514-22.
48. Hiwase SD, Dyson PG, To LB, Lewis ID. Cotransplantation of placental mesenchymal stromal cells enhances single and double cord blood engraftment in nonobese diabetic/severe combined immune deficient mice. Stem Cells 2009;**27**: 2293-300.
49. Schrepfer S, Deuse T, Reichenspurner H et al. Stem cell transplantation: the lung barrier Transplantation proceedings: Elsevier, 2007:573-6.

50. Coombe DR, Watt S, Parish C. Mac-1 (CD11b/CD18) and CD45 mediate the adhesion of hematopoietic progenitor cells to stromal cell elements via recognition of stromal heparan sulfate. Blood 1994;**84**: 739-52.
51. Lai CY, Yamazaki S, Okabe M et al. Stage-Specific Roles for Cxcr4 Signaling in Murine Hematopoietic Stem/Progenitor Cells in the Process of Bone Marrow Repopulation. Stem Cells 2014;**32**: 1929-42.
52. Bernardo M, Ball L, Cometa A et al. Co-infusion of ex vivo-expanded, parental MSCs prevents life-threatening acute GVHD, but does not reduce the risk of graft failure in pediatric patients undergoing allogeneic umbilical cord blood transplantation. Bone marrow transplantation 2011;**46**: 200-7.
53. Le Blanc K, Frassoni F, Ball L et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. The Lancet 2008;**371**: 1579-86.
54. Macmillan M, Blazar B, DeFor T, Wagner J. Transplantation of ex-vivo culture-expanded parental haploidentical mesenchymal stem cells to promote engraftment in pediatric recipients of unrelated donor umbilical cord blood: results of a phase I–II clinical trial. Bone marrow transplantation 2008;**43**: 447-54.
55. Nauta AJ, Westerhuis G, Kruisselbrink AB et al. Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a nonmyeloablative setting. Blood 2006;**108**: 2114-20.

Chapter 2:

Thrombopoietin treatment of one graft in a double cord blood transplant provides early platelet recovery while contributing to long term engraftment in NSG mice

Mark van der Garde, Yvette van Hensbergen,
Anneke Brand, Manon C. Slot, Alice de Graaf-Dijkstra,
Arend Mulder, Suzanne M. Watt and Jaap Jan Zwaginga

Stem Cells and Development 2014, 24: 67-76.

Abstract

Human cord blood (CB) hematopoietic stem (HSC) cell transplants demonstrate delayed early neutrophil and platelet recovery and delayed longer term immune reconstitution compared to bone marrow and mobilized peripheral blood transplants. Despite advances in enhancing early neutrophil engraftment, platelet recovery after CB transplantation is not significantly altered when compared to contemporaneous controls. Recent studies have identified a platelet biased murine HSC subset, maintained by thrombopoietin (TPO) which has enhanced capacity for short and long term platelet reconstitution, can self-renew, and can give rise to myeloid and lymphoid biased HSCs. In previous studies, we have shown that transplantation of human CB $CD34^+$ cells pre-cultured in TPO as a single graft accelerates early platelet recovery as well as yielding long term repopulation in immune deficient mice. Here, using a double CB murine transplant model, we investigated whether TPO cultured human CB $CD34^+$ cells have a competitive advantage or disadvantage over untreated human CB $CD34^+$ cells in terms of i) short and longer term platelet recovery and ii) longer term hematological recovery. Our studies demonstrate that the TPO treated graft shows accelerated early platelet recovery without impairing the platelet engraftment of untreated $CD34^+$ cells. Notably, this was followed by a dominant contribution to platelet production via the untreated $CD34^+$ cell graft over the intermediate to longer term. Furthermore, although the contribution of the TPO treated graft to long term hematological engraftment was reduced, the TPO treated and untreated grafts both contributed significantly to long term chimerism *in vivo*.

Introduction

Cord blood (CB) transplantation is used as an alternative for bone marrow or mobilized peripheral blood grafts, particularly when no HLA matched related or unrelated donor can be found. [1-4] However, in contrast to the other cell sources, un-manipulated CB hematopoietic stem and progenitor cells (HSPCs) demonstrate defective CXCL12-mediated homing and adhesion to endothelium and delayed hematological engraftment and reconstitution. [5-7] Higher HSPC dosages, measured in terms of CFU content and $\geq 1.8 \times 10^5$ viable $CD34^+$ cells transplanted per kg recipient body weight in this respect, seem needed to improve the median time to and probability of neutrophil and platelet engraftment, factors essential for reducing transplant related morbidity and mortality. [8-17] The restricted number of $CD34^+$ HSPCs in one cord blood unit, however, is often a hurdle to treat adults. To overcome this limitation, adults and large children are generally considered for double CB (dCB) transplantation, [18-21] with both improved Non Relapse Mortality (NRM) and increased relapse-free survival (RFS) reported using this dCB approach. [18-21] The relapse mediated advantages of dCB transplants are still partly offset by NRM as a result of increased infectious complications and bleeding caused by delayed neutrophil and platelet recovery when compared to matched related or unrelated mobilized peripheral blood and bone marrow grafts. [18] One solution to this delayed hematological engraftment is to expand the numbers of HSPCs in the graft leading to long term repopulating cells most preferably in combination with expansion of neutrophil and platelet progenitors to enable early hematological repopulation as well. Unfortunately most ex vivo manipulations seem to be associated with the loss of long term repopulation and/or the skewing of progenitors towards neutrophil differentiation.

Although substantially higher $CD34^+$ cell numbers have been generated by *ex vivo* culture with cytokines and small molecules or the homing/engraftment of these cells has been enhanced with small molecules in the attempts reported so far, [22-32] the co-transplantation of an un-manipulated CB unit with an ex vivo expanded CB unit or part of a unit has been regarded as a sensible precaution as the long term repopulating HSCs often originate from the non-expanded CB unit. Importantly, the first cautious attempts to expand one of the grafts prior to dCB transplantation have significantly reduced the time to neutrophil recovery, [24,26-28,30] although platelet recovery has remained substantially delayed when compared to current rather than historical transplant outcomes. [33]

Thrombopoietin (TPO) is known to be critical for both HSC maintenance and platelet production. [34-40] Sanjuan-Pla et al. [41] have recently identified, in the mouse, a TPO-dependent platelet biased HSC expressing Sca-1, c-kit, CD150 and vWF which exists at the apex of the hematopoietic hierarchy and which not only generates platelets over the short and longer term but also can give rise to both myeloid and lymphoid biased HSCs. This corresponding TPO dependent platelet biased subset has not been identified in the human, principally because in the mouse a vWF-eGFP reporter is used. However, previous studies from our group and others have demonstrated that culturing human CB $CD34^+$ cells for 7-9 days with thrombopoietin (TPO) can lead to improved early platelet recovery in immune-deficient mice when compared to transplantation with untreated CB $CD34^+$ cells in the single CB transplant setting, without apparently compromising longer term hematological reconstitution. [42-44] Since thrombocytopenia is a common complication of both single and double CB HSC transplants, it has been proposed from these studies that TPO treatment ex vivo of one or part of one CB unit in a dCB transplant setting might be especially useful in preventing delays in platelet recovery post-transplant and hence in improving overall patient survival. [1,45] Although purified and ex vivo manipulated $CD34^+$ are most likely to be combined with an un-manipulated CB unit in the transplant setting,

the influence of ex vivo manipulation on hematological reconstitution should be compared first for a fixed number of CD34+ cells in surrogate models of dCB transplantation ahead of assessing other immunologic and matching dependent effects of the graft on repopulation and final chimerism.
In this paper, we therefore used a dCB transplant model in NSG mice and isolated $CD34^+$ cells from three different CB donor pairs to first determine if TPO treated $CD34^+$ HSPCs from one CB unit when combined with untreated $CD34^+$ cells from another CB unit could both improve and sustain platelet recovery when compared with a dCB transplant of untreated $CD34^+$ cells of both donors. Secondly, we assessed if untreated CB $CD34^+$ cells from one donor had a competitive advantage or disadvantage when co-transplanted with the same number of TPO-treated vs untreated CB $CD34^+$ cells from a second donor in terms of short or longer term platelet recovery and longer term hematological reconstitution. Interestingly, the TPO cultured $CD34^+$ cells were responsible for improved early platelet recovery and contributed substantially to longer term platelet recovery in the dCB transplant setting, although the dominant contribution to platelet recovery in the intermediate to longer term arose from the untreated $CD34^+$ cells. Furthermore, although there was less contribution from the TPO-treated $CD34^+$ cell graft, long term hematological cell reconstitution was dependent on both the TPO treated and the untreated grafts. Finally, the hematopoietic potential of the untreated CB cells units was not negatively influenced by the co-transplantation of TPO treated cells.

Materials and Methods

Umbilical cord blood (CB) collection. CB was drawn from the umbilical vein at birth at >36 weeks gestation after written informed consent from the mother at peripheral hospitals in the Netherlands according to Netcord–FACT standards and with ethical permission from the medical ethical board of the Leiden University Medical Center (LUMC), Leiden, The Netherlands. Blood was collected by gravity drainage into Macopharma collection bags containing 21 ml Citrate Phosphate Dextrose Adenine-1 (Macopharma, Utrecht, The Netherlands). The blood was stored at 4°C and processed within 48 hours of collection.

$CD34^+$ cell purification. Mononuclear cells were isolated from CB using a sterile Ficoll density gradient (1.077 g/cm^3, Pharmacy LUMC). The $CD34^+$ cell fraction was isolated from the mononuclear cell fraction by double $CD34^+$ cell selection using immunomagnetic beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The purity of the isolated $CD34^+$ cell fraction was verified by flow cytometry (Beckman Coulter, Woerden, The Netherlands) using CD45-FITC and CD34-PE antibodies (Beckman Coulter) and the ISHAGE protocol.[46] Cells were cryopreserved in 10% (v/v) DMSO in 4% (w/v) human serum albumin; (Pharmacy LUMC) in IMDM and stored at -150˚C until use.

Cultures. $CD34^+$ cells were cultured in 24 well sterile tissue culture plates, at a concentration of $1x10^5$ cells/ml, in Iscove's modified Dulbecco's medium (IMDM) (Gibco, Breda, The Netherlands) supplemented with 20% (v/v) AB heparinized plasma (Sanquin Blood Supply Foundation, Rotterdam, The Netherlands), 0.5 mg/ml human transferrin saturated with $FeCl_3.H_20$ (Sigma, Zwijndrecht, The Netherlands), 0.34% (v/v) human serum albumin (20% (w/v) stock; Cealb® CLB, Amsterdam, The Netherlands), 1% (v/v) penicillin/streptomycin (PenStrep; Bio-Whittaker, Verviers, Belgium), 0.05 mM β-mercapto-ethanol (Sigma), and 50 ng/ml mpl-ligand (Nplate) or thrombopoietin (TPO, a kind gift from KIRIN Brewery Ltd., Tokyo, Japan). At day 7, the medium was refreshed by

demi-dilution, with medium containing 50 ng/ml TPO or Nplate. At day 9, the cells were split into two new wells and diluted 1:1 with medium without TPO and harvested on day 10. The total viable cell number was counted using flow cytometry and the degree of expansion calculated. The composition of the cultured cells was analysed by flow cytometry using mouse-anti-human CD45, CD61, CD34, CD3 and CD56 antibodies (all from Beckman Coulter). It should be noted that previous comparisons of TPO with Nplate in parallel cultures of $CD34^+$ cells from 5 donors gave similar expansion rates and percentages of the predominant populations during culture. However, because of accessibility to reagents, TPO was changed to Nplate in the last experiment. Cells were analysed for HLA epitope expression before and after expansion with custom-made, in house human HLA epitope specific monoclonal antibodies. [47-49]

Transplantation in NSG mice. Female 5-6 week old NSG mice (Charles River, l'Arbresle, France) were kept in micro-isolator cages in laminar flow racks in the LUMC animal facilities. The animal ethical committee of the LUMC approved all animal experiments. NSG mice received 2.85 Gy total body irradiation 24 hours before transplantation. Mice were transplanted i.v. with cells (Fig. 1) using 4 mice per group. Three dCB transplantation experiments were performed with one CB donor pair for each experiment, each experiment consisting of three groups of four mice within each group. Mice received one of the following combinations of cells in their transplants (Fig. 1): Group 1) the first donor CB TPO treated $CD34^+$ cells (X1) and the second donor CB untreated $CD34^+$ cells (N2); Group 2) the second donor CB TPO treated $CD34^+$ cells (X2) and the first donor CB untreated $CD34^+$ cells (N1); and Group 3) both CB donor cells infused as untreated $CD34^+$ cells (N1 and N2). For the analysis of the data, all mice that received one TPO treated graft and one untreated graft (groups 1 and 2) were compared with the mice that received two untreated grafts (group 3). The number of cells that each of the mice received are described in the Supplementary data.

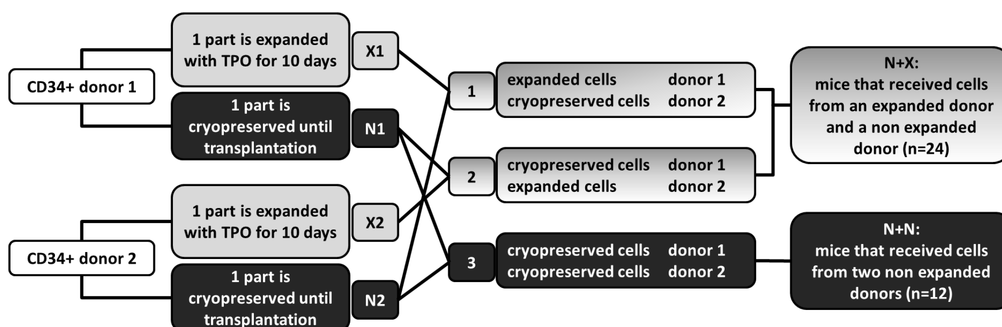


Figure 1: Schematic representation of the experimental strategy. *Isolated $CD34^+$ cells from 6 human CB donors were divided into 3 donor pairs. The cells from each donor were then split into two parts and cryopreserved. One part was cultured with TPO for 9 days, the other part was thawed before transplantation. After TPO based culture, cells were combined with untreated cells from the other donor of the donor pair creating 6 groups with TPO treated cells and untreated cells (N+X group), and the untreated cells of both donors of the donor pairs were combined creating 3 groups with untreated cells from two donors. Cells from each group were transplanted in 4 NSG mice.*

Analysis of peripheral blood after transplantation. Blood was collected from the tail vein twice weekly during the first 3 weeks after transplantation and once weekly thereafter. Blood collection and human platelet measurements were performed as described previously. [49-51] Briefly, human platelets were stained with non-cross reactive mouse-anti-human CD41-PE and human CD45 cells were stained with mouse anti human CD45-PC7 (both Beckman Coulter). To determine donor origin HLA epitope specific monoclonal antibodies conjugated with Alexa 488 or PE-Dy647 were used. [47,49] Erythrocytes were lysed with IOTest3 lysing solution (Beckman Coulter) for 10 min. at room temperature. All cells were fixed with a 0.1% (w/v) formaldehyde solution (Pharmacy LUMC). Flow-Count™ fluorospheres (Beckman Coulter) were added to the cells to enable the measurement of the absolute number of circulating human platelets. Analysis was performed by flow cytometry (FC500, Beckman Coulter) using CXP software.

Analysis of the bone marrow, spleen and blood after 20 weeks. Twenty weeks after transplantation, mice were sacrificed and the bone marrow recovered from the femurs by flushing with IMDM containing 1% (v/v) PenStrep (Life Technologies) and 10U/ml Na-heparin (Pharmacy LUMC). Single cell suspensions were prepared from the spleen that were washed with IMDM with 1% (v/v) PenStrep (Life Technologies) and 10U/ml Na-heparin (Pharmacy LUMC) and the blood was collected via cardiac puncture. Evaluation of human cell engraftment and the relative lineage distribution of the engrafted human cells in the bone marrow, spleen and blood were performed with flow cytometry analysis. Cells were labelled with rat-anti-mouse-CD45-PE (BD Biosciences, Breda, The Netherlands), mouse-anti-human CD45-FITC, CD33-PE, CD34-PE, CD19-PE and CD3-ECD (all from Beckman Coulter), and the appropriate isotype controls. Subsequently, erythrocytes were lysed with IO Test3 lysing solution (Beckman Coulter) for 10 mins. at room temperature and fixed with a 0.1% (w/v) formaldehyde solution. Chimerism was analysed with HLA specific antibodies. [47-49] Analysis was performed by flow cytometry (FC500) using CXP software (Beckman Coulter).

Hematopoietic progenitor cell assays. To investigate whether bone marrow cells harvested 20 weeks after transplantation contained human hematopoietic progenitor cells (HPC), 3 different *in vitro* assays were used; HALO cultures, myeloid CFU cultures and cobblestone area forming cell (CAFC) assays. The HALO assay (HemoGenix, Colorado Springs, USA) was used to enumerate the number of colony forming cells. In short, $1.25x10^4$ human $CD45^+$ cells from the bone marrow of each mouse were suspended in HALO master mix (containing EPO, GM-CSF, IL-2, IL-3, Il-6, IL-7, SCF, TPO and Flt3-L) and incubated in a 96 well plate for 6 days at 37˚C and 5% CO_2 in a humidified incubator. After 6 days, the cells were harvested from the wells and lysed, after which the ATP content was measured with a luciferase/luciferin assay using a standard ATP curve to determine the ATP concentration. Methocult CFU assays (H4434 Classic, Stemcell, Vancouver, Canada) were used to determine the capacity to differentiate into different myeloid lineages. Here, $2x10^4$ cells were suspended in classic Methocult medium and cultured for 2 weeks at 37˚C and 5% CO_2 in a humidified incubator after which different differentiated colonies were counted with an inverted microscope. CAFC assays were used to determine whether the cells were primitive hematopoietic progenitor cells [50,52]. In short, $6.4x10^4$ cells from 1 representative mouse of each group for each experiment were seeded onto an irradiated NIH3T3 monolayer, growing on a collagen coated 24 well plate in CAFC medium containing 3% (v/v) FCS, 3% (v/v) horse serum, glutamine, PenStrep, hydrocortisone and β-mercaptoethanol. Five wells/mouse were seeded with bone marrow cells. Cells were

cultured for 5 weeks at 37˚C and 5% CO_2 in a humidified incubator and colonies were counted at week 4 and week 5 with an inverted microscope.

Statistics. All statistics were done using IBM SPSS Statistics 20. Unless stated otherwise, significance was determined with unpaired two tailed Student's T tests or Mann Whitney tests. Results were considered to be significant if the p-value was equal or smaller than 0.05.

Results

Culturing CD34+ cells with thrombopoietin

The CD34+ cell purity before TPO culture or transplant was 92-98%. After culturing the human CB CD34+ cells with TPO, the expression of CD61 (*ITGB3,* GPIIIa or beta3 integrin) which forms part of the GPIIb/IIIa (CD41/CD61) complex that binds to vWF, fibronectin, finrinogen and vitronectin [36,43,50] was measured. Three broad cell populations were detected: 1) total CD34+ cells (both CD34+CD61+ and CD34+CD61-), 2) CD34-CD61- cells and 3) CD34-CD61+ cells. As observed previously,[43,44,50] TPO induced differentiation from CB CD34+ cells first to the double negative CD34-CD61- population and then to a strongly expanded CD34-CD61+ population (Figure 2). With the relatively short TPO exposure, we aimed to and succeeded in generating approximately as many CD34-CD61- cells as the CD34+ starting population. This double negative population has been shown to be responsible for the earlier platelet recovery in *in vivo* models [50]. With the expected significant inter-experimental and inter-donor variation, TPO exposure, however, also led to a variable and sometimes higher reduction in absolute CD34+ cell numbers for some donors. However, both CD34+CD61- and CD34+CD61+ cells remained present in the graft (Figure 2 and Table 1).

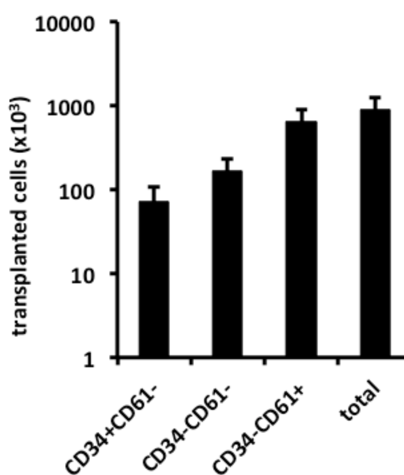


Figure 2: Number of cells and composition of the TPO treated graft of the 6 different donor grafts transplanted into the mice that received a TPO treated graft and an untreated graft. *The transplanted cell number was based on equivalent CD34+ cell numbers in the original graft before culturing with TPO and in the untreated CB unit. Thus, the total number of cells in the dCB transplant was variable between all donors that received one TPO treated graft. Although all transplants included a fixed number of CD34+ cells from the untreated donor graft, the cell numbers that were culture derived from the same number of CD34+ cells of the other donor graft varied between $2.6x10^5$ and $24.0x10^5$ cells. Moreover, the composition of the grafts was also variable, although all grafts contained high numbers of expanded megakaryocytic cells, ranging from $2.0x10^5$ to $17.3x10^5$ cells and relatively low numbers of CD34-CD61+ cells.*

Table 1: Graft compositions after TPO expansion of $CD34^+$ cells from the 6 different donors used in the 3 different transplantation experiments. *Shown are the results of each population after CD34 and CD61 analysis with flow cytometry; (graft 1,2 graft 3,4 and graft 5, 6 were respectively combined in the different experiments). Nr=number of.*

CD34+CD61-	***nr CD34+ d0***	***nr CD34+ d10***	***fold expansion from CD34+***
graft 1	188,520	78,732	0.42
graft 2	195,260	249,080	1.28
graft 3	92,420	12,164	0.13
graft 4	96,890	50,922	0.53
graft 5	97,450	31,715	0.33
graft 6	93,200	9,846	0.11
CD34+CD61+	***nr CD34+ d0***	***nr CD34+CD61+ d10***	***fold expansion from CD34+***
graft 1	188,520	12,636	0.07
graft 2	195,260	91,010	0.47
graft 3	92,420	4,871	0.05
graft 4	96,890	7,424	0.08
graft 5	97,450	14,030	0.14
graft 6	93,200	35,373	0.38
CD34-CD61-	***TNC d0***	***nr CD34-CD61- d10***	***fold expansion from TNC***
graft 1	200,000	377,136	1.89
graft 2	200,000	318,535	1.59
graft 3	100,000	34,629	0.35
graft 4	100,000	82,276	0.82
graft 5	100,000	141,126	1.41
graft 6	100,000	64,592	0.65
CD34-CD61+	***TNC d0***	***nr CD34-CD61+ d10***	***fold expansion from TNC***
graft 1	200,000	503,496	2.52
graft 2	200,000	1,736,375	8.68
graft 3	100,000	203,337	2.03
graft 4	100,000	135,378	1.35
graft 5	100,000	992,129	9.92
graft 6	100,000	287,190	2.87

Total platelet and leukocyte recovery in the peripheral blood (PB) of NSG mice receiving TPO-treated and untreated CD34$^+$ cell grafts

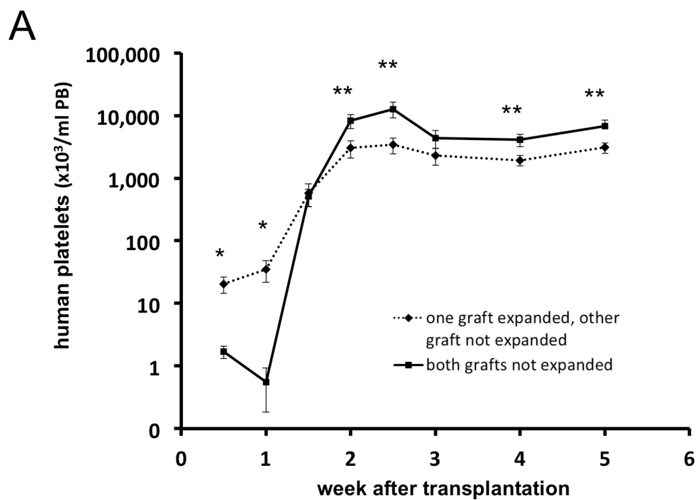
At day 4 and onwards after transplantation of TPO treated and/or untreated CB cells into NSG mice, human platelets were observed in the peripheral blood (PB). A significantly higher number and more rapid increase of human platelets in the PB of mice was seen when mice were transplanted with CD34$^+$ cells where those from one donor were first cultured with TPO (Figure 3A). Ten days after transplantation, however, a rapid increase in platelet numbers was observed in mice that received two untreated and uncultured grafts (N+N group). With a notable transient peak at 2.5 weeks post transplantation, the platelet concentration in the N+N mice even exceeded the numbers found in mice that received one TPO treated graft together with one untreated graft (N+X group) for up to 6 weeks. From that time on, the differences, although still present between the two groups, were no longer significant with platelet numbers gradually increasing in both groups up to 18 weeks after transplantation (Figure 3B).

The human CD45$^+$ cell concentrations in PB (signifying total human leukocytes) for the different groups are shown in Figure 4, with all mice showing a gradual increase in PB human CD45$^+$ cells, which began to plateau for the N+N group at 6 weeks post-transplant and for the N+X group at week 12 post-transplant (Figure 3C). A trend towards a slightly higher total human CD45$^+$ cell recovery was observed in mice from the N+N group between 2 to10 weeks post-transplant, but the difference from the N+X group was only statistically significant at week 6 after transplantation ($p \leq 0.05$).

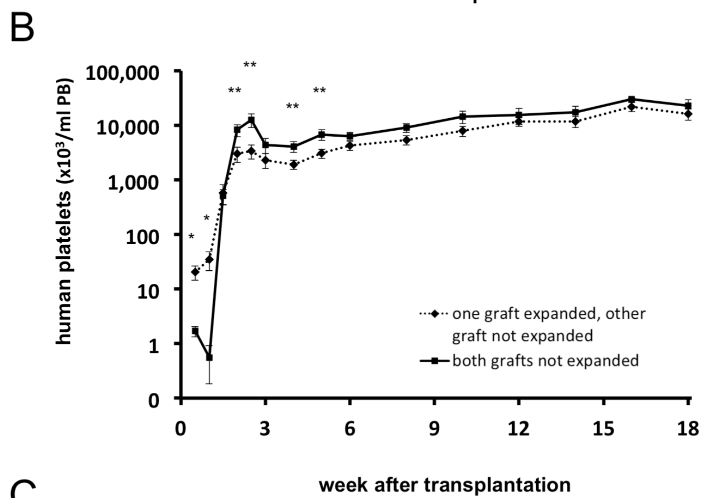
Overall long term engraftment in bone marrow, spleen and peripheral blood comparing the TPO treated and untreated grafts with the double untreated grafts

Twenty weeks after transplantation, the mice were sacrificed and their blood, bone marrow and spleens showed that 72-89% of the CD45$^+$ cell population was derived from human as opposed to murine leukocytes (Table 2). Additionally, both myeloid and lymphoid lineages were detected. In the lymphoid lineage, CD19$^+$ B cells far outweighed the concentration of CD3$^+$ T cells (Table 2). The percentage of human CD34$^+$ cells based on the total human CD45$^+$ cell fraction in the bone marrow was greater than 0.5% in all mice at 20 weeks, with the variation observed between the 3 experiments being 2.91±0.42 %, 6.39±1.16 % and 0.80±1.11 % for experiments 1, 2 and 3 respectively. Between the N+N and N+X groups, no significant differences in the percentages of lineage specific and CD34$^+$ cells in bone marrow or spleens were observed (Table 2).

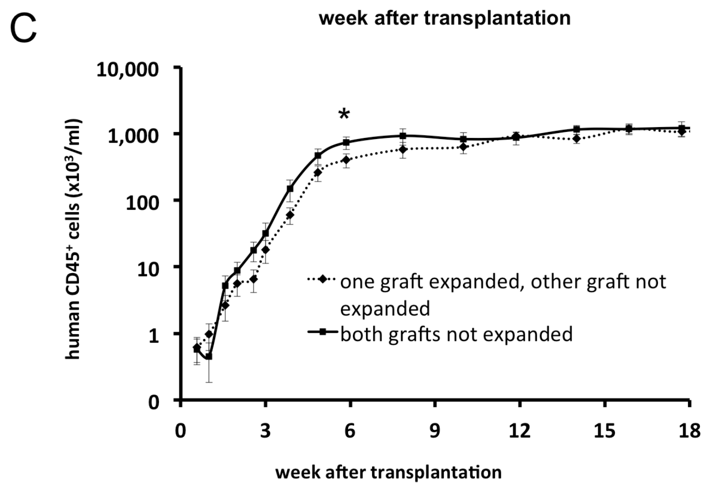
A



B



C



	BM	
	one unit TPO treated other unit untreated	***Both units untreated***
CD45	88.7 (±2.2)	86.1 (±2.8)
CD34	3.7 (±0.7)	2.7 (±0.8)
CD19	57.2 (±4.1)	52.2 (±7.0)
CD33	21.7 (±2.8)	20.8 (±4.5)
CD3	1.1 (±0.3)	1.8 (±0.4)
	Spleen	
	one unit TPO treated other unit untreated	***Both units untreated***
CD45	71.9 (±3.2)	77.9 (±4.9)
CD34	n.d.	n.d.
CD19	31.8 (±6.0)	46.1 (±9.5)
CD33	5.9 (±2.2)	2.7 (±0.8)
CD3	6.1 (±1.7)	7.4 (±1.5)
	Blood	
	one unit TPO treated other unit untreated	***Both units untreated***
CD45	78.7 (±2.0)	73.9 (±3.7)
CD34	n.d.	n.d.
CD19	n.d.	n.d.
CD33	n.d.	n.d.
CD3	3.0 (±1.1)	6.9 (±3.1)

Table 2. Mean Percentage (±SEM) of CD45+, CD34+, CD19+, and CD3+ cells of the total human CD45+ cells in the bone marrow (BM) and spleen of the mice that received a TPO cultured graft and an untreated graft and the mice that received two untreated grafts. *The number of CD45+ cells in the different hematopoietic organs was approximately similar for the N+N and the N+X group. There were differences between the percentages of the different lineages, but these were not significant.*

Fig. 3: Mean human platelet or leukocyte concentration (per µl PB, ±SEM) in the peripheral blood (PB) of the mice after transplantation. *(♦, dashed line) one of the CB donor grafts was TPO cultured (X), (■, straight line) both CB donor grafts were untreated (N).* ***A:*** *first 5 weeks after transplantation, **p≤0.05, *p≤0.02.* ***B:*** *week 1-18 after transplantation. Cells from the N+X group had earlier platelet recovery in the first week after transplantation. In contrast, cells from the N+N group had higher platelet numbers 2 weeks after transplantation after which the platelet numbers of both groups both gradually increase towards a plateau.* ***C:****). Leukocyte recovery was slightly faster for the N+N group, but only significantly higher 6 weeks after transplantation, *p≤0.05*

In vitro assays of the engrafted bone marrow cells

Three assays were carried out to assess the human progenitor cell potential of the bone marrow (BM) cells 20 weeks post-transplant. We verified that colonies were not mouse derived by performing similar cultures of the BM cell suspension from mice not receiving human dCB transplants and none of these yielded colonies. First, using the HALO assay where the mean concentration of ATP produced is a measure of proliferating cells, no significant difference was observed between human cells derived from the bone marrow from the N+X or N+N groups of mice (Figure 4A). Secondly, to study whether the engrafted human cells were capable of differentiating into different myeloid lineages, CFU assays were performed with the same marrow samples. In both the N+N and the N+X group, lineage forming potential was shown (Figure 4B) by the generation of colonies of all different myeloid lineages: Burst Forming Unit Erythroid (BFU-E) colonies (mean=9.1±3.7 colonies for the mice from the N+X group and 9.7±5.1 for the N+N mice, the Granulocyte/Macrophage colonies (CFU-GM, mean=63.2±10.9 for the mice of the N+X group and 94.0±39.9 for the mice of the N+N group) and the Granulocyte/ Erythrocyte/ Macrophage/ Megakaryocyte colonies (CFU-GEMM, 8.8±3.2 for the mice of the N+X group graft and 13.0±9.2 for the mice of the N+N group). Although bone marrow from mice of the N+N group on average formed more colonies of all different myeloid lineages, none of these differences was significant (p=0.7 for the total number

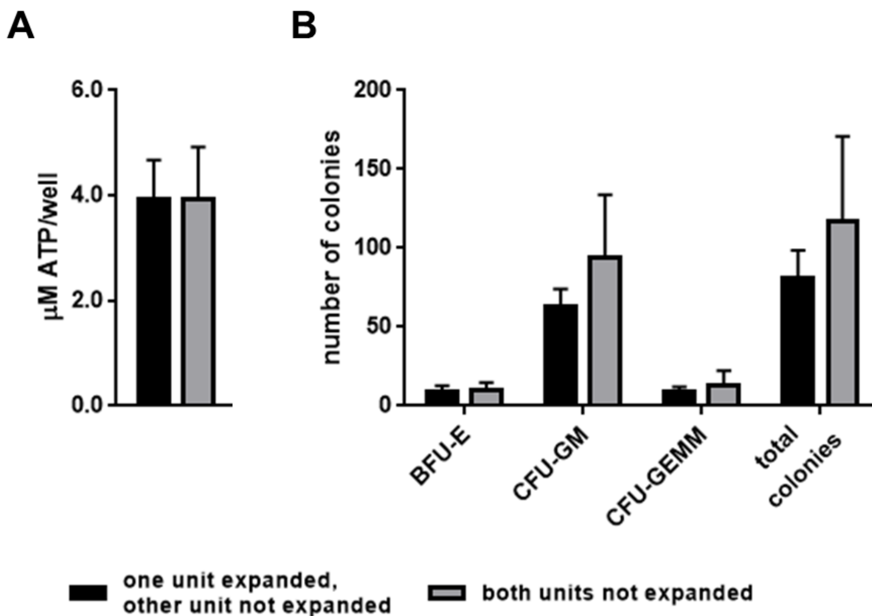


Figure 4, A: Mean (±S.E.M.) ATP levels produced by expanded cells harvested from the bone marrow of the mice 20 weeks after transplantation. *Shown is the mean ATP concentration/well of all mice of the groups that received one TPO treated graft and an untreated graft (black bar) and the mice that received two untreated grafts (white bar). There was no difference in ATP production after HALO culture between the N+X and the N+N group.* ***B: Mean number of different myeloid colonies formed by the engrafted bone marrow cells of a mouse of each representative group of each experiment after 2 weeks of culture in Methocult.*** *Cells from the N+N group produced more colonies than cells from the N+X group, but this difference was not significant.*

Finally, we used the *in vitro* CAFC assay to show that the harvested bone marrow from the transplanted mice still contained primitive cells, since the CAFC assay is used as a surrogate assay for predicting long term engraftment potential [52]. Although cells from a representative mouse of each group were capable of generating CAFC colonies in at least 2 of the five wells (results not shown), less CAFC colonies could be grown from the bone marrow cells of the mice that received mixed transplants. These culture results of all bone marrow cells were followed up by *in vivo* analysis of donor specific cell counts both in the PB and in the marrow.

Platelet and leukocyte recovery in the peripheral blood comparing the TPO treated and the untreated donor grafts

To analyze how much the TPO treated and the untreated donor grafts contribute to platelet production, PB samples were stained with present on one and not the other co-transplanted graft. Since the HLA staining of platelet is not absolute, we could analyze the donor contribution in two donor pairs only (n=12 mice). Figure 5A show that the platelets produced in the first week after transplantation were indeed derived from the $CD34^+$ cells that had been TPO treated. In contrast, when the graft was not TPO treated, platelet production commenced after week one only. However, from two weeks, the untreated $CD34^+$ cells produced higher platelet numbers and this progressed throughout the remainder of the experiment. Total $CD45^+$ leukocyte production from the TPO treated $CD34^+$ cells was significantly impaired. Although detectable $CD45^+$ leukocyte levels were observed after 1.5 weeks from both the TPO cultured unit and the untreated unit, the increase in leukocytes was more rapid when the unit from a non TPO cultured graft and the total leukocyte production from this untreated graft was higher throughout the experiment (Figure 5B). Leukocyte production from the TPO cultured graft, however, remained present and constant although at a lower level than leukocytes from the untreated graft.

Bone marrow chimerism in mice co-transplanted with one TPO treated and one untreated graft

In agreement with the results in peripheral blood, human hematopoiesis (i.e. human $CD45^+$ cells) in the bone marrow of mice from the N+X group 20 weeks after transplantation on average demonstrated increased human hematopoiesis from the untreated graft compared to the TPO treated graft (66±4% vs. 34±4% mean$\pm$SEM). Assuming that the untreated donor grafts would theoretically contribute equally to engraftment (i.e. the contribution of each graft is 50%), the skewing in favor of the untreated donor graft was highly significant ($p \leq 0.001$). This skewed chimerism in the bone marrow for human $CD45^+$ cells (Table 3) was also observed for $CD34^+$ cells ($p \leq 0.01$) and B lymphoid cells ($CD19^+$, $p \leq 0.01$), while the dominance was less clear for T cells ($CD3^+$, $p=0.555$) and myeloid cells ($CD33^+$, $p=0.314$). Indeed, in the N+N group, chimerism as expected was equal between the two donors. These combined results indicate that both the untreated graft and the TPO cultured graft can provide long term reconstitution but that the initial difference in stem cell numbers between the TPO cultured and untreated grafts is reflected in their eventual chimerism.

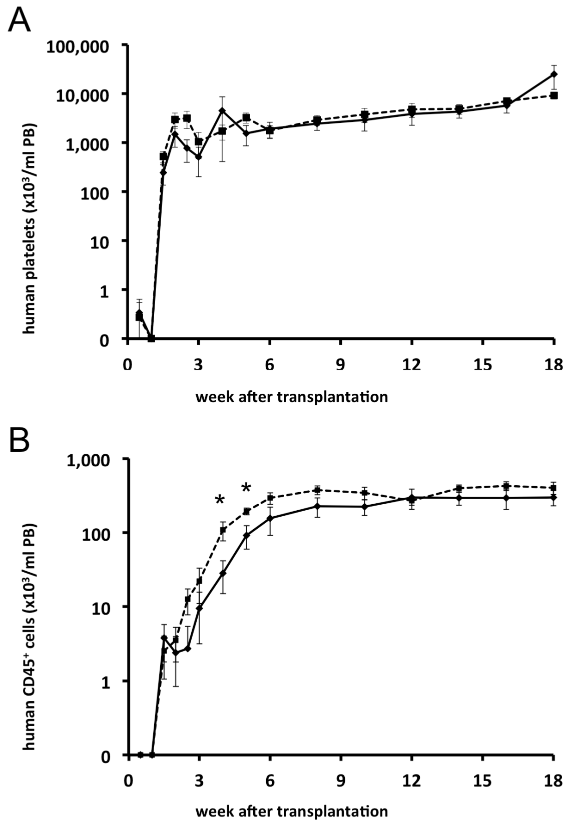


Figure 5: Mean (±SEM) platelet (A) and leukocyte (B) concentrations in the PB of one of the donor grafts in experiments 1 and 2 when the donor graft was untreated (♦, straight line) or TPO cultured (■, dashed line). *Cells from the TPO cultured donor graft had earlier platelet recovery in the first week after transplantation. In contrast, cells from the N+N group had higher platelet numbers from 2 weeks after transplantation up to the end of the experiment. Leukocyte numbers were higher for the untreated donor graft throughout the whole experiment.*

Fraction	*TPO treated unit*	*Untreated unit*
CD45	34.2 (±4.3)	65.8 (±4.3)
CD34	35.0 (±4.9)	65.0 (±4.9)
CD19	35.7 (±4.8)	64.3 (±4.8)
CD33	43.8 (±6.0)	56.2 (±6.0)
CD3	45.2 (±8.0)	54.4 (±8.0)

Table 3. Mean (±SEM) contribution to the BM cells of the TPO cultured graft and the untreated graft in the mice receiving cells from a TPO treated graft and an untreated graft (N+X Group). *On average dominance came from the untreated graft*

Platelet and leukocyte recovery in the peripheral blood and long term bone marrow, spleen and blood chimerism mediated by the untreated donor graft when it is transplanted with a TPO treated or a untreated donor graft

Using the same donor specific HLA antibodies used for the analysis of the contribution of each donor graft in the N+X group, we next studied if the engraftment efficacy of the untreated $CD34^+$ cells from one donor could be changed by the presence of TPO treated or of untreated cells of another donor. During the experiment, human platelet numbers originating from the untreated donor $CD34^+$ cells were not significantly different regardless of whether they were co-transplanted with TPO cultured cells or untreated $CD34^+$ from another donor (Figure 6A, n=16). On the contrary, a temporarily higher number of total human $CD45^+$ leukocytes originated from the untreated graft when it was co-transplanted with a TPO cultured graft. This difference became clear at week 2 and was significant at week 4 and 5 after transplantation (Figure 6B, $p \leq 0.05$ for both). The longer term human $CD45^+$ repopulation in the PB was not significantly changed by the manipulation status of the other donor's graft.

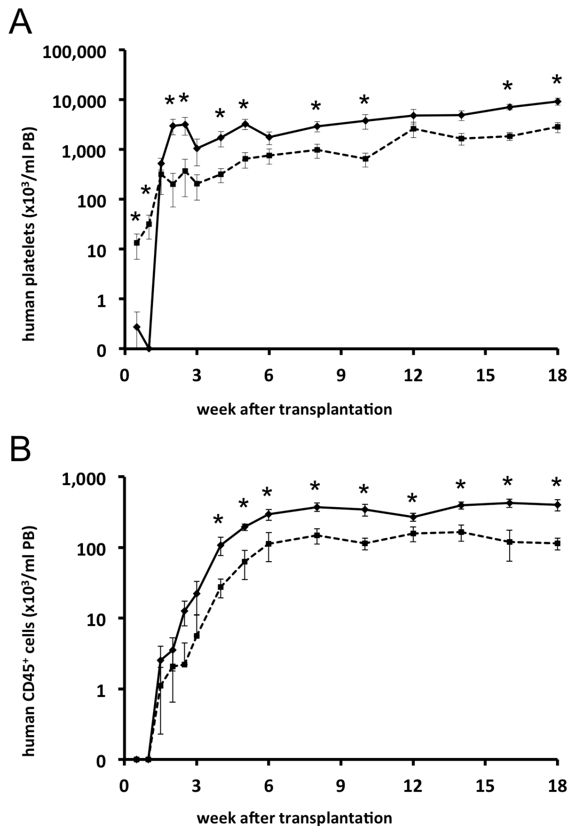


Figure 6: Human platelet (A) and leukocyte (B) concentrations of one of the donors in experiments 1 and 2 when an untreated graft was co-transplanted with either another untreated graft (♦, straight line) or a TPO cultured graft (■, dashed line). **p≤0.05. Platelet production was approximately equal throughout the experiment, regardless of co-transplantation with TPO treated or untreated cells. Leukocyte production was slightly faster when untreated cells were co-transplanted with TPO treated cells as compared to co-transplantation with untreated cells*

Discussion

Thrombocytopenia is a common complication of allogeneic HSC transplantation, with platelet recovery being particularly delayed after CB HSC transplants[1,2,4,53]. Transplant patients may also present with secondary failure of platelet recovery[54]. Thrombocytopenia at day +100 and secondary failure of platelet recovery post allogeneic HSC transplant have been associated with poorer survival. [45,54] Current expansion protocols for one CB unit in a dCB transplant setting still do not show substantial improvements in platelet recovery when transplant outcomes are compared with current rather than with historical outcome data [27,28,30]. Furthermore, given that their administration can induce reticulin formation in the bone marrow and possibly bone marrow fibrosis and that TPO in the myeloablative ransplant setting failed to improve platelet recovery, the use of thrombopoietin receptor agonists to promote platelet recovery post-transplant still requires additional safety and efficacy studies. [35]Interestingly, platelet primed or biased HSCs have been identified in mice. [41,55,56] Their phenotypic profiles have not yet been analyzed in a single study from one group and further subset analysis is needed. thrombopoietin receptor agonists to promote platelet recovery post-transplant still requires additional safety and efficacy studies. [35] However, current combined results from three groups suggest that they are c-kit^{hi+} and CD41^{+}, dependent on TPO, capable of generating long term myeloid and lymphoid biased HSCs as well as generating in vitro CD41^{+}CD61^{+} megakaryocytes, and are responsible for early and longer term platelet recovery. [41,55,56] The therapeutic importance of such specialized HSC subsets in human HSC transplantation remains to be defined. In this paper, we show that TPO treated human CD34^{+} grafts from one CB donor can successfully be combined with untreated CD34^{+} cells from another donor to a) improve short term platelet engraftment from the TPO treated cell graft and to b) generate long term hematological reconstitution in an immune deficient mouse model of dCB transplantation, while c) the untreated CD34^{+} HSCs that are co-transplanted as a safety net are not hampered in their engraftment potential by the CD34^{+} cell manipulation with TPO in the other cord blood graft. These observations are important in two respects. The first is because delayed platelet engraftment in the weeks following dCB transplantation remains a significant problem,[1,2,4,53] which has not yet been substantially addressed in the clinical transplant setting. The second is that, if a platelet primed HSC exists in the human as it does in the mouse, we now have developed a validated *in vivo* dCB transplant model with which to identify and study the efficacy of the putative human TPO dependent platelet biased HSC, and to define its lineage relationships in the context of other identified human HSPC subsets and following their interactions with specific factors, agonists or antagonists. [57,58]
The studies presented here build on our previous short term (6 week) engraftment studies with single CB transplants in immune deficient mice, where we have shown that TPO treatment can improve early platelet recovery and that this recovery is mostly dependent on the TPO mediated generation of CD34^{-}CD61^{-} cells. [44,50] Importantly in our current studies, by analyzing engraftment using donor specific HLA antibodies in the dCB transplant setting, we could further demonstrate that this improved *in vivo* early platelet recovery (around day 7) was indeed originating from TPO treated CD34^{+} cell graft, while the untreated CD34^{+} cells formed platelets at later time points (significantly from around week 2) and dominated over the TPO cultured cells.
We limited the number of variables in our experiments because we wanted to specifically investigate if residual CD34^{+} HSPCs after TPO mediated culture could contribute to long term hematological reconstitution and if the TPO treated graft could influence the engraftment of untreated CD34^{+} HSPCs in a double cord blood setting. When we co-

transplanted untreated CD34+ cells from one donor graft with TPO-treated or untreated CD34+ cells from a second donor graft, we could show, by using donor specific HLA antibodies, that TPO treated CD34+ cells generated fewer CD45+ cells in the peripheral blood of NSG mice than the same graft which had not been cultured in TPO when co-transplanted with a non expanded graft. This could be attributed to an absolute loss of a proportion of CD34+ HSPCs or platelet biased HSCs, during the TPO based culture because of their redirection towards platelet production. Thus, although its contribution was lower when compared to grafting untreated CB CD34+ cells, we could show that the TPO cultured CD34+ graft provided a stable and substantial contribution to longer term platelet recovery and hematological reconstitution in *in vivo* dCB transplants, indicating that a significant proportion of platelet biased HSCs might also be maintained in short term TPO mediated cultures, as shown before. [50] This finding was corroborated by our CAFC and CFU assays, which are reported to correlate with hematopoietic stem cell engraftment. In bone marrow of all mice, the long term culture initiating (CAFC) cells were maintained but again fewer of these cells were found in bone marrow from mice that received one TPO cultured CD34+ graft. Similarly, bone marrow cells from mice receiving two non treated CB CD34+ grafts formed on average a greater number of myeloid colonies than those from mice receiving one untreated and one TPO cultured CB graft; however, in both groups all types of colonies were present. Our combined results therefore show a consistent , clearly lower number of HSPCs in the bone marrow of combined untreated and TPO-cultured graft (N+X) transplanted mice, which is in agreement with the decreased CD34+ HSPC and possibly platelet biased HSC content in these combined grafts. Notwithstanding this, there was no difference in the combined hematopoietic repopulation quality between the N+X and N+N group. While the percentage of human chimerism in the bone marrow was higher than 80% in both groups reflecting the use of an optimal transplanted CD34+ cell dose in our experiments, a smaller number of transplanted untreated CD34+ cells in one graft might not be able to compensate for the TPO induced CD34+ HSPC loss in the cultured graft and might thus eventually compromise the combined engraftment in mice. This requires further study using defined subsets of HSPCs. Complementing these studies, we found that the untreated CD34+ cells produced more CD45+ leukocytes when co-transplanted with a TPO cultured graft than when co-transplanted with a graft of uncultured CD34+ cells. This stimulatory effect needs to be confirmed and further investigated. Importantly however, the hematopoietic repopulation capacity of un-manipulated CD34+ cells as safety back up for manipulated CB transplantations is not hampered by co-transplantation of TPO manipulated CD34+ cells.

In conclusion, we have developed an *in vivo* transplant model with which to study the relative contribution to short and longer term platelet recovery and longer term hematological reconstitution of and competition between human CB grafts from different donors or lineage biased HSC subsets from these grafts. Furthermore, our studies allow us to also analyze the effects of exogenous factors on expanding the content of or enhancing the differentiation of such cell subsets prior to transplant. Such approaches will undoubtedly provide us with strategies to enhance both short and longer term platelet recovery as well as to manipulate the HSC and HSPC subset content of such grafts for improved outcomes following clinical CB HSC transplantation.

References

1. Ballen KK, Gluckman E, Broxmeyer HE. Umbilical cord blood transplantation: the first 25 years and beyond. Blood 2013;**122**: 491-8.
2. Ballen KK, Koreth J, Chen YB et al. Selection of optimal alternative graft source: mismatched unrelated donor, umbilical cord blood, or haploidentical transplant. Blood 2012;**119**: 1972-80.
3. Fuchs E, O'Donnell PV, Brunstein CG. Alternative transplant donor sources: is there any consensus? Curr Opin Oncol 2013;**25**: 173-9.
4. Watt SM. Article 04238. In: Caplan M, ed. Umbilical Cord Blood Banking. Reference Module in Biomedical Sciences. Kidlington, UK: Elsevier Press, 2014.
5. Xia L, McDaniel JM, Yago T et al. Surface fucosylation of human cord blood cells augments binding to P-selectin and E-selectin and enhances engraftment in bone marrow. Blood 2004;**104**: 3091-6.
6. Hoggatt J, Singh P, Sampath J, Pelus LM. Prostaglandin E2 enhances hematopoietic stem cell homing, survival, and proliferation. Blood 2009;**113**: 5444-55.
7. Hidalgo A, Frenette PS. Enforced fucosylation of neonatal CD34+ cells generates selectin ligands 2005.
8. Avery S, Shi W, Lubin M, Gonzales AM et al. Influence of infused cell dose and HLA match on engraftment after double-unit cord blood allografts. Blood 2011;**117**: 3277-85.
9. Barker JN, Scaradavou A, Stevens CE. Combined effect of total nucleated cell dose and HLA match on transplantation outcome in 1061 cord blood recipients with hematologic malignancies. Blood 2010;**115**: 1843-9.
10. Lemarie C, Esterni B, Calmels B et al. CD34+ progenitors are reproducibly recovered in thawed umbilical grafts, and positively influence haematopoietic reconstitution after transplantation. Bone marrow transplantation 2007;**39**: 453-60.
11. Migliaccio AR, Adamson JW, Stevens CE et al. Cell dose and speed of engraftment in placental/umbilical cord blood transplantation: graft progenitor cell content is a better predictor than nucleated cell quantity. Blood 2000;**96**: 2717-22.
12. Page KM, Mendizabal A, Betz-Stablein B et al. Optimizing donor selection for public cord blood banking: influence of maternal, infant, and collection characteristics on cord blood unit quality. Transfusion 2014;**54:** 340-55.
13. Page KM, Zhang L, Mendizabal A et al. Total colony-forming units are a strong, independent predictor of neutrophil and platelet engraftment after unrelated umbilical cord blood transplantation: a single-center analysis of 435 cord blood transplants. Biology of Blood and Marrow Transplantation 2011;**17**: 1362-74.
14. Page KM, Zhang L, Mendizabal A et al. The Cord Blood Apgar: a novel scoring system to optimize selection of banked cord blood grafts for transplantation (CME). Transfusion 2012;**52**: 272-83.
15. Scaradavou A, Smith KM, Hawke R et al. Cord Blood Units with Low CD34+ Cell Viability Have a Low Probability of Engraftment after Double Unit Transplantation. Biology of Blood and Marrow Transplantation 2010;**16**: 500-8.
16. Terakura S, Azuma E, Murata M et al. Hematopoietic engraftment in recipients of unrelated donor umbilical cord blood is affected by the CD34+ and CD8+ cell doses. Biology of Blood and Marrow Transplantation 2007;**13**: 822-30.
17. Wagner JE, Barker JN, DeFor TE et al. Transplantation of unrelated donor umbilical cord blood in 102 patients with malignant and nonmalignant diseases: influence of CD34 cell dose and HLA disparity on treatment-related mortality and survival. Blood 2002;**100**: 1611-8.
18. Brunstein CG, Gutman JA, Weisdorf DJ et al. Allogeneic hematopoietic cell transplantation for hematologic malignancy: relative risks and benefits of double umbilical cord blood. Blood 2010;**116**: 4693-9.
19. Oran B. Can double-cord transplants provide a more potent graft-vs-leukemia effect? Best Practice & Research Clinical Haematology 2012;**25**: 479-82.
20. Scaradavou A, Brunstein CG, Eapen M et al. Double unit grafts successfully extend the application of umbilical cord blood transplantation in adults with acute leukemia. Blood 2013;**121**: 752-8.
21. Verneris MR, Brunstein CG, Barker J et al. Relapse risk after umbilical cord blood transplantation: enhanced graft-versus-leukemia effect in recipients of 2 units. Blood 2009;**114**: 4293-9.
22. Broxmeyer HE, Hoggatt J, O'Leary HA et al. Dipeptidylpeptidase 4 negatively regulates colony-stimulating factor activity and stress hematopoiesis. Nature medicine 2012;**18**: 1786-96.
23. Christopherson KW, Hangoc G, Mantel CR, Broxmeyer HE. Modulation of hematopoietic stem cell homing and engraftment by CD26. Science 2004;**305**: 1000-3.
24. Cutler C, Multani P, Robbins D et al. Prostaglandin-modulated umbilical cord blood hematopoietic stem cell transplantation. Blood 2013;**122**: 3074-81.
25. Dahlberg A, Delaney C, Bernstein ID. Ex vivo expansion of human hematopoietic stem and progenitor cells. Blood 2011;**117**: 6083-90.

26. Danby R, Rocha V. Improving engraftment and immune reconstitution in umbilical cord blood transplantation. Frontiers in immunology 2014;**5**.
27. de Lima M, McNiece I, Robinson SN et al. Cord-blood engraftment with ex vivo mesenchymal-cell coculture. New England Journal of Medicine 2012;**367**: 2305-15.
28. Delaney C, Heimfeld S, Brashem-Stein C et al. Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution. Nature medicine 2010;**16**: 232-6.
29. Fares I, Chagraoui J, Gareau Y et al. Pyrimidoindole derivatives are agonists of human hematopoietic stem cell self-renewal. Science 2014;**345**: 1509-12.
30. Horwitz ME, Chao NJ, Rizzieri DA et al. Umbilical cord blood expansion with nicotinamide provides long-term multilineage engraftment. The Journal of clinical investigation 2014;**124**: 3121.
31. Kelly S, Sola C, De Lima M, Shpall E. Ex vivo expansion of cord blood. Bone marrow transplantation 2009;**44**: 673-81.
32. Ramirez P, Wagner J, DeFor T et al. CXCR4 expression in CD34+ cells and unit predominance after double umbilical cord blood transplantation. Leukemia 2013;27: 1181-1183.
33. Guttridge MG, Soh TG, Belfield H et al. Storage time affects umbilical cord blood viability. Transfusion 2014;**54**: 1278-85.
34. de Sauvage FJ, Carver-Moore K, Luoh S-M et al. Physiological regulation of early and late stages of megakaryocytopoiesis by thrombopoietin. The Journal of experimental medicine 1996;**183**: 651-6.
35. Kuter DJ. The biology of thrombopoietin and thrombopoietin receptor agonists. International journal of hematology 2013;**98**: 10-23.
36. Ng AP, Kauppi M, Metcalf D et al. Characterization of thrombopoietin (TPO)-responsive progenitor cells in adult mouse bone marrow with in vivo megakaryocyte and erythroid potential. Proceedings of the National Academy of Sciences 2012;**109**: 2364-9.
37. Ng AP, Kauppi M, Metcalf D et al. Mpl expression on megakaryocytes and platelets is dispensable for thrombopoiesis but essential to prevent myeloproliferation. Proceedings of the National Academy of Sciences 2014;**111**: 5884-9.
38. Qian H, Buza-Vidas N, Hyland CD et al. Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells. Cell stem cell 2007;**1**: 671-84.
39. Yagi M, Ritchie KA, Sitnicka E et al. Sustained ex vivo expansion of hematopoietic stem cells mediated by thrombopoietin. Proceedings of the National Academy of Sciences 1999;**96**: 8126-31.
40. Yoshihara H, Arai F, Hosokawa K et al. Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. Cell stem cell 2007;**1**: 685-97.
41. Sanjuan-Pla A, Macaulay IC, Jensen CT et al. Platelet-biased stem cells reside at the apex of the haematopoietic stem-cell hierarchy. Nature 2013;**502**: 232-6.
42. Mattia G, Milazzo L, Vulcano F et al. Long–term platelet production assessed in NOD/SCID mice injected with cord blood CD34+ cells, thrombopoietin–amplified in clinical grade serum–free culture. Experimental hematology 2008;**36**: 244-52.
43. Schipper LF, Brand A, Reniers N et al. Differential maturation of megakaryocyte progenitor cells from cord blood and mobilized peripheral blood. Experimental hematology 2003;**31**: 324-30.
44. van Hensbergen Y, Schipper LF, Brand A et al. Ex vivo culture of human CD34+ cord blood cells with thrombopoietin (TPO) accelerates platelet engraftment in a NOD/SCID mouse model. Experimental hematology 2006;**34**: 943-50.
45. Bolwell B, Pohlman B, Sobecks R et al. Prognostic importance of the platelet count 100 days post allogeneic bone marrow transplant. Bone marrow transplantation 2004;**33**: 419-23.
46. Sutherland DR, Anderson L, Keeney M et al. The ISHAGE guidelines for CD34+ cell determination by flow cytometry. Journal of hematotherapy 1996;**5**: 213-26.
47. Mulder A, Kardol MJ, Arn JS et al. Human monoclonal HLA antibodies reveal interspecies crossreactive swine MHC class I epitopes relevant for xenotransplantation. Molecular immunology 2010;**47**: 809-15.
48. Somers JA, Brand A, van Hensbergen Y et al. Double umbilical cord blood transplantation: A study of early engraftment kinetics in leukocyte subsets using HLA-specific monoclonal antibodies. Biology of Blood and Marrow Transplantation 2012.
49. van Hensbergen Y, Mulder A, Cornelissen JJ, Brand A. Validation of human monoclonal HLA Class I antibodies to evaluate the kinetics of donor chimerism in different cell subsets after double-cord-blood transplantation in the NOD/SCID model. Transfusion 2013;**53**: 104-14.
50. Schipper LF, Brand A, Fibbe WE, Van Hensbergen Y. Functional Characterization of TPO-Expanded CD34+ Cord Blood Cells Identifies CD34− CD61− Cells as Platelet-Producing Cells Early After Transplantation in NOD/SCID Mice and rCD34+ Cells as CAFC Colony-Forming Cells. Stem Cells 2012;**30**: 988-96.
51. Schipper LF, Van Hensbergen Y, Fibbe WE, Brand A. A sensitive quantitative single-platform flow cytometry protocol to measure human platelets in mouse peripheral blood. Transfusion 2007;**47**: 2305-14.

52. Breems D, Blokland E, Neben S, Ploemacher R. Frequency analysis of human primitive haematopoietic stem cell subsets using a cobblestone area forming cell assay. Leukemia 1994;**8**: 1095.
53. Wallet HL, Sobh M, Morisset S et al. Double umbilical cord blood transplantation for hematological malignancies: a long-term analysis from the SFGM-TC registry. Experimental Hematology 2013;41: 924-33.
54. Bruno B, Gooley T, Sullivan KM et al. Secondary failure of platelet recovery after hematopoietic stem cell transplantation. Biology of Blood and Marrow Transplantation 2001;**7**: 154-62.
55. Gekas C, Graf T. CD41 expression marks myeloid-biased adult hematopoietic stem cells and increases with age. Blood 2013;**121**: 4463-72.
56. Shin JY, Hu W, Naramura M, Park CY. High c-Kit expression identifies hematopoietic stem cells with impaired self-renewal and megakaryocytic bias. The Journal of experimental medicine 2014;**211**: 217-31.
57. Görgens A, Radtke S, Möllmann M et al. Revision of the human hematopoietic tree: granulocyte subtypes derive from distinct hematopoietic lineages. Cell reports 2013;**3**: 1539-52.
58. Laurenti E, Doulatov S, Zandi S et al. The transcriptional architecture of early human hematopoiesis identifies multilevel control of lymphoid commitment. Nature immunology 2013;**14**: 756-63.

Chapter 3:

Cryopreservation of cord blood CD34$^+$ cells before or after thrombopoietin expansion differentially affects early platelet recovery in NOD SCID mice

Yvette van Hensbergen*, Mark van der Garde*,
Anneke Brand, Manon C. Slot, Alice de Graaf-Dijkstra,
Suzanne Watt and Jaap Jan Zwaginga

***equally contributing first authors**

Transfusion 2015, 55: 1772–1781

Abstract

BACKGROUND: Expansion of human cord blood CD34+ cells with thrombopoietin (TPO) can accelerate delayed platelet recovery following transplantation into immunodeficient mice. Clinical implementation, however, will depend on practical and effective protocols. The best timing of TPO- expansion in relation to cryopreservation in this respect is unknown.

STUDY DESIGN AND METHODS: In this study, we evaluated whether the order of cryopreservation and TPO expansion affected the expansion rate and numbers of clonogenic hematopoietic progenitor cells in vitro or platelet and longer term hematopoietic repopulation in NOD SCID mice in vivo.

RESULTS: Our results demonstrate higher expansion rates and the generation of higher numbers of multi-lineage and megakaryocytic progenitors (CFU-GEMM and CFU-Mk) in vitro when freshly isolated cord blood CD34+ cells are first cultured with TPO and then cryopreserved and thawed as compared to TPO expansion post CD34+ cell cryopreservation. In contrast, the cells produced with the latter strategy showed higher expression of CD62L and a superior SDF-1α mediated migration. This might play a role in an also observed superior early platelet recovery after transplantation of these cells into NOD SCID mice. The hematopoietic engraftment in the bone marrow six weeks after transplantation was not different between the two strategies.

CONCLUSION: Although TPO expansion before cryopreservation would yield higher nucleated cell and clonogenic myeloid and megakaryocyte cell numbers and enable earlier availability, cord blood TPO expansion after cryopreservation is likely to be clinically more effective, despite the lower number of cells obtained after expansion. Moreover, the latter strategy is logistically more feasible.

Introduction

Allogeneic cord blood (CB) transplantation is used as an alternative for bone marrow or mobilized peripheral blood grafts when no suitable HLA matched related or unrelated donor is available.[1,2] CB, however, contains a limited number of hematopoietic stem/progenitor cells and is, compared to other graft sources, associated with longer intervals to reach safe neutrophil and platelet levels. Double CB transplantation can increase the cell content, but is associated with poorer platelet recovery and a higher risk of grade II and IV acute and extensive chronic graft versus host disease.[3] Moreover, delayed immune reconstitution is observed after both single and double CB transplants.[1-3]
One strategy to overcome this problem is to expand (the whole or part of) a single CB unit in such a way that long term hematological reconstitution is preserved while early neutrophil and platelet engraftment is enhanced. Another strategy is to combine an expanded CB graft to bridge the engraftment delay with an un-manipulated CB graft to safeguard long-term hematological reconstitution.[4] In this regard, substantial improvements in early neutrophil recovery have been demonstrated in clinical trials that combined post cryopreservation expanded $CD34^+$ cells from a single CB unit with an unmanipulated CB unit.[5-7]
We have previously developed a 10 day thrombopoietin (TPO) protocol for expansion/ differentiation of human CB $CD34^+$ cells which has shown to improve early platelet recovery in immunodeficient mice when compared to transplantation with unexpanded, cryopreserved CB $CD34^+$ cells. With this protocol, the engraftment capacity in the bone marrow was maintained.[8-11] However, engraftment and longer-term hematological reconstitution is influenced not only by hematopoietic stem and progenitor cell numbers, but also by the viability and functionality of these cells.[12] The time and the storage temperature prior to cryopreservation of CB units are well recognized as important determinants affecting in vitro characteristics such as post thaw viability, recovery and hematological engraftment post-transplant in human recipients.[13-16] The effect of cryopreservation on the outcome of ex vivo expansion is however indistinct and mainly compared by in vitro yields and viability parameters and less often in clinical studies.[17-20]
Although adequate proliferation and differentiation ability of $CD34^+$ cells in culture with TPO has been observed after thawing of CB $CD34^+$ cells,[9,11] several studies have reported a decrease in various colony formation assays after cryopreservation and thawing of expanded megakaryocyte progenitor cells[14,21]. Moreover, this resulted in a high cell death and a dramatic loss of post-thaw CFU-GEMM and CFU-Mk progenitors.[19]
Since the effect of cryopreservation on ex vivo expansion and subsequent transplantation of UCB $CD34^+$ cells is equivocal, expansion protocols require validation.
In this study, we evaluated and compared whether the order of expansion and cryopreservation, i.e. expansion before or after cryopreservation, affects the expansion capacity and differentiation ability in vitro, and the platelet recovery and engraftment capacity in NOD SCID mice. To investigate this, we compared isolated CB $CD34^+$ cells from the same UCB unit and expanded these cells in parallel pre- and post-cryopreservation. To achieve this, one half of the freshly isolated $CD34^+$ cells was immediately TPO expanded and subsequently cryopreserved and thawed (Expanded/Cryopreserved/Thawed: ECT pathway) while the other half was cryopreserved directly after isolation and expanded after thawing (Cryopreserved/ Thawed/Expanded: CTE pathway). In vitro characteristics - fold expansion, differentiation and migratory capacity - and in vivo characteristics - platelet recovery and 6-week hematological engraftment capacity in a NOD SCID mouse model - were compared between the two pathways.

Materials and Methods

Thrombopoietin expansion of cord blood CD34$^+$ cells. Umbilical cord blood (CB) (>36 weeks gestation) was obtained after written informed consent from the mother collected into 25 ml Citrate Phosphate Dextrose Adenine-1 and stored for a maximum of 48 hours at 4°C until processed. The protocol was approved by the LUMC Medical Ethical Committee. Mononuclear cells were isolated on Ficoll (<1.077 g/cm^3, Pharmacy LUMC, Leiden, The Netherlands). CD34$^+$ cells were then isolated by CD34$^+$ magnetic cell sorting (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The purity of the isolated CD34$^+$ cell fraction was verified by flow cytometry with CD45-FITC and CD34-PE (all from Beckman Coulter, Mijdrecht, The Netherlands).[7-9] 5x10^4 CD34$^+$ cells/ml were cultured in 24 well tissue culture plates in IMDM medium (Gibco, Breda, The Netherlands) supplemented with 20% (v/v) AB heparinized plasma (Sanquin Blood Bank, Rotterdam, The Netherlands), 0.5 mg/ml human transferrin saturated with $FeCl_3.H_20$ (Sigma, Zwijndrecht, The Netherlands), 0.34% (v/v) human serum albumin (Cealb® Sanquin, Amsterdam, The Netherlands), 1% (v/v) penicillin/ streptomycin (Bio-Whittaker, Verviers, Belgium), 0.05 mM β-mercapto-ethanol (Sigma, St Louis, MISS., USA) for 10 days at 37°C and 5% CO_2 in a humidified atmosphere with 50 ng/ml TPO (a kind gift from KIRIN Brewery Ltd., Japan), and media containing 50 ng/ml TPO refreshed at day 7 by semi-dilution. At day 9, the cells were split into two new wells and diluted 1:1 in medium without TPO and harvested at day 10.[8,9] The total cell expansion was calculated and the composition of the cultured cells analyzed by flow cytometry using mouse-anti-human CD45, CD61 and CD34 antibodies.[8,9,11] For the migration experiments, the cells were expanded with 50 ng/ml Nplate (Amgen, Thousand Oaks, USA), a fusion protein homolog of TPO, of which fold expansion and characteristics of expanded cells were similar in paired experiments with CB CD34$^+$ cells from 6 different donors.[10]

Cryopreservation and thawing of the cells Before cryopreservation, the fresh or cultured CD34$^+$ cells were resuspended in IMDM with 2% (v/v) human serum albumin (HSA Cealb Sanquin), stored on ice for at least 15 minutes and diluted 1:1 with ice-cold cryopreservation medium (60% (v/v) IMDM medium (Gibco) supplemented with 20% (v/v) HSA Cealb®) and 20% (v/v) DMSO (Pharmacy LUMC)). The cells were cryopreserved in a controlled rate freezer at a 1°C-reduction per minute to –80°C before being stored in liquid nitrogen. Thawing was performed quickly in a 37°C water bath and cells were resuspended gently on ice in IMDM medium containing 2.5% HSA (v/v), 10U/ml heparin (Pharmacy LUMC) and 1% (v/v) penicillin/streptomycin. Cell viability was assessed with 7AAD.

Transplantation in NOD SCID mice. Female 5-6 week old NOD SCID mice (Charles River, France) were kept in micro-isolator cages in laminar flow racks in the LUMC animal facilities. Mice were given autoclaved acidified water containing 0.07 mg/ml Polymixin-B (Bufa B.V., Uitgeest, The Netherlands), 0.09 mg/ml ciproxin (Bayer B.V., Mijdrecht, The Netherlands) and 0.1 mg/ml Fungizone (Bristol-Myers-Squibb, Woerden, The Netherlands). All animal experiments were approved by the LUMC animal ethical committee. NOD SCID mice were sub lethally irradiated (3.5Gy) up to 24 hours before i.v. infusion with the expanded cells. Blood collection via tail incision was performed twice weekly during the first 3 weeks after transplantation and once weekly thereafter. The human platelets concentration in the blood was analyzed with flow cytometry as described before[22], using mouse-anti-human CD41-PE and Flow-Count fluorspheres™ (all from Beckman Coulter). Six weeks after transplantation, mice were sacrificed, and their

bone marrow was analyzed for human cell engraftment and the relative lineage distribution with flow cytometry analysis using goat-anti-mouse-CD45-PE (LCA, Ly-5, 30-F11, Pharmingen, Alphen a/d Rijn, The Netherlands), mouse-anti-human CD45-FITC, CD33-FITC, CD34-PE, CD19-PE and CD3-ECD (all from Beckman Coulter) or appropriate isotype controls.

Clonogenic assays. To determine the clonogenic capacity of the cells, progenitor cell (CFU-GEMM) and megakaryocyte colony forming unit (CFU-Mk) assays were performed. The CFU-GEMM assay was performed as described by the manufacturer. In short, 2.7×10^4 BM-WBC (bone marrow-white blood cells), 0.32×10^4 $CD34^+$ or TPO expanded cells were added to Methocult™ (StemCell Technologies Inc.) in a final volume of 1.5 ml. 1.1 ml of the Methocult™ cell suspension was dispensed in 35 mm dishes (Greiner, Alphen a/d Rijn, The Netherlands) and cultured for 14 days at 37°C, 5% CO_2 in a humidified atmosphere (>95%). Each sample was tested in duplicate. After 14 days, the number of granulocyte, erythroid and monocyte colonies in the culture dishes was counted manually. The CFU-Mk assay was performed in MegaCult™-C medium with cytokines (StemCell Technologies Inc.). The assay was performed as described by the manufacturer. In short, 2×10^5 BM-WBC cells or 0.25×10^4 $CD34^+$ or TPO expanded cells per 3.3 ml were cultured in a chamber slide (StemCell Technologies Inc.) for 10 days at 37°C, 5% CO_2 in a humidified atmosphere (>95%). After 10 days, the number of megakaryocyte colonies in each well of the chamber slide was counted manually.

Expression of adhesion markers and migration of the cells cryopreserved before and after expansion. Expression of adhesion markers (CD11a-b, CD49d-e, CD62L) and the stem cell homing marker CD184 (CXCR4) on the three subpopulations was determined by flow cytometry in combination with CD61-PC7 and CD34-PC5 antibodies (all Beckman Coulter). Migration experiments were performed in transwell plates (Costar, Amsterdam, the Netherlands), with 5 μm pore filters. Before adding the cell suspensions, the lower compartment of the well and the bottom of the filter were coated for 15 minutes at 37°C with 2 ng/ml fibronectin (Sigma). 1×10^5 TPO expanded cells were added to the upper compartment. The lower chamber contained media with or without human rCXCL12 (SDF-1α, 100 ng/ml; Sigma). Plates were incubated for 5 hours at 37ºC and 5% CO_2 in a humidified incubator, before harvesting the migrated cells from the lower chamber. Before and after incubation, all cells were analyzed by flow cytometry for the expression of CD45, CD34 and CD61 and cell number calculated in the presence of flow count beads (all from Beckman Coulter). The percentage of migrating cells was calculated by dividing the number of cells from the lower compartment by the number of cells that were added to the upper compartment.

Statistics. All statistics were done using IBM SPSS Statistics (version 20, www.ibm.com/SPSS_*Statistics).* Results are presented as mean ± S.E.M. For the comparison of fold expansion, clonogenic potential platelet recovery, bone marrow engraftment and migration potential, significance was determined with Student's two tailed t test. Correlation between fold expansion and clonogenic potential was done with Spearman's Rank Correlation Coefficient. Differences between the different subpopulations after expansion were determined with a one way ANOVA. Results were considered to be significant if the p-value was equal to or less than 0.05.

Results

Thrombopoietin expansion of $CD34^+$ cells pre- and post-cryopreservation

$CD34^+$ cells isolated from fresh CB were split in two fractions. One part of the cells was directly expanded with TPO for 10 days and cryopreserved thereafter (pathway ECT, Expansion/ Cryopreservation/Thawing). The other half was cryopreserved directly after isolation and expanded with TPO after thawing (pathway CTE, Cryopreservation/Thawing/ Expansion) (Figure 1). Overall, the results demonstrate that the fold total nucleated cell expansion was significantly higher ($p \leq 0.05$) when the $CD34^+$ cells were TPO expanded before cryopreservation (Table 1, Figure 2, ECT = 17.8±4.3 fold, n=10, versus CTE =10.0±2.2 fold, n=10). After TPO expansion, three major populations of cells ($CD34^+CD61^-$, $CD34^-CD61^-Lineage^-$ and $CD34^-CD61^+$) are typically found.

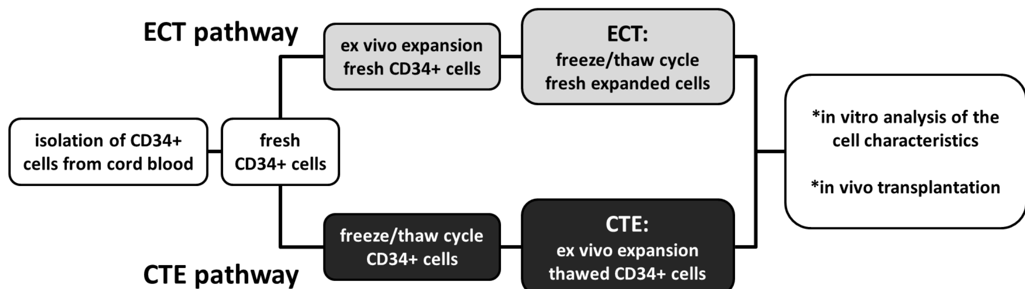


Figure 1: Flow chart of the experimental design. *Human $CD34^+$ cells were isolated from fresh CB and split in two fractions. The $CD34^+$ cells were either directly expanded with TPO for 10 days and cryopreserved (ECT pathway), or the $CD34^+$ cells were cryopreserved directly after isolation and TPO expanded after thawing (CTE pathway).*

We previously showed that $CD34^+CD61^-$ cells (hereafter called residual CD34+ cells, $rCD34^+$), correlate in the mouse with sustained engraftment, while $CD34^-CD61^-Lineage^-$ (hereafter called Lin- cells) accelerated platelet recovery. Remarkably, $CD34^-CD61^+$ cells (hereafter called CD61+ cells) appear not to contribute to platelet production nor to hematopoietic engraftment.[8] Post-thaw viability of the TPO expanded cells in the ECT pathway after cryopreservation and thawing was 83.8±5.7%, 90.5±16.4% and 79.9±2.3% for the rCD34, Lin- and $CD61^+$ populations respectively. Notably, we did not observe major differences in viability between cells that were derived from the either the ECT or CTE pathway (for 5 donors; Table 1). The CTE pathway yielded less Lin- cells (16.6±3.8 vs ECT 21.7±1.4%; p=0.590) and more $rCD34^+$ cells (CTE 14.0±3.1% vs ECT 11.2±2.1%; p=0.590) (Table 1), although neither of these shifts was significant.

Table 1: Subset distribution of TPO expanded cells *In vitro characteristics of the cells after completion of the ECT or CTE pathway. Values are mean (± SEM, *p≤0.05).*

pathway		***ECT***	***CTE***
fold expansion		17.8 (±4.3)	10.0 (±2.2)*
proportion subpopulation	**rCD34+**	11.2 (±2.1)	14.0 (±3.1)
(% of total cells)	**Lin-**	21.7 (±1.4)	16.6 (±3.8)
	CD61+	61.4 (±1.9)	63.8 (±4.3)
post thaw viability	**rCD34+**	83.8(±5.7)	74.2 (±3.5)
(% of viable cells)	**Lin-**	90.5 (±16.4)	n.a
	CD61+	79.9 (±2.3)	n.a.
total nr of CFU-GEMM/1000 cultured cells		2272 (±522)*	1444 (±300)*
total nr of CFU-MK/1000 cultured cells		196 (±34)*	107 (±28)*

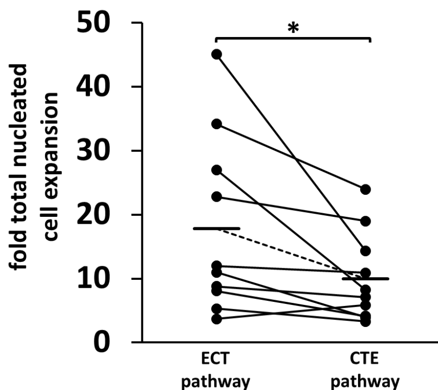


Figure 2: Fold total nucleated cell expansion comparing the ECT and CTE strategies. *Human CB CD34⁺ cells were isolated and ex vivo expanded for 10 days with TPO directly (ECT) or after cryopreservation (CTE) (n=10). Fold expansion after 10 days. Although variation in the fold expansion was observed between donors, expansion CD34⁺ cells via the ECT pathway resulted in a higher number of cells than expansion via the CTE pathway (*p≤0.05; paired Student's two tailed T test).*

Clonogenic progenitor content of TPO expanded cells

Next, the TPO expanded cells from the ECT and the CTE pathways were examined for their CFU-GEMM and CFU-Mk content. For each 1,000 $CD34^+$ cells plated, the number of CFU-GEMM after TPO expansion was higher in the CTE pathway than in the ECT pathway (300±43 vs. 241±46 per 10^3 $CD34^+$ cells plated respectively, mean±SEM, n=5; $p \leq 0.05$; Figure 3A), while the number of CFU-Mk was higher for the ECT compared to the CTE pathway (26.9±6.2 vs. 21.3±5.1 per 10^3 $CD34^+$ cells plated respectively, mean±SEM, n=6; $p \leq 0.05$; Figure 3B). When the absolute numbers of clonogenic cells were calculated on the basis of CD34+ cells present in the final products, CFU-GEMM and CFU-Mk numbers were on average 1.6 and 1.8-fold higher respectively in the ECT protocol as compared to the CTE protocol (ECT vs. CTE: 2,272±522 vs. 1,444±300 CFU-GEMM and 196±34 vs. 107±28 CFU-Mk; n=5 and n=6 independent experiments respectively; Figures 3C and 3D). As indicated in Figure 3, there was a significant and strong positive correlation between the absolute numbers of CFU-GEMM and CFU-Mk in the TPO expanded products and the fold

nucleated cell expansion regardless of whether the CTE and ECT protocols were used (Figure 4A and 4B, Spearman's rank correlation r=0.721 and 0.806 respectively and p≤0.05 and p≤0.01 respectively). The number of GFU-GEMM and CFU-Mk were strongly correlated, irrespective of the number of colonies that were formed (Figure 4C, r=0.927, p=0001).

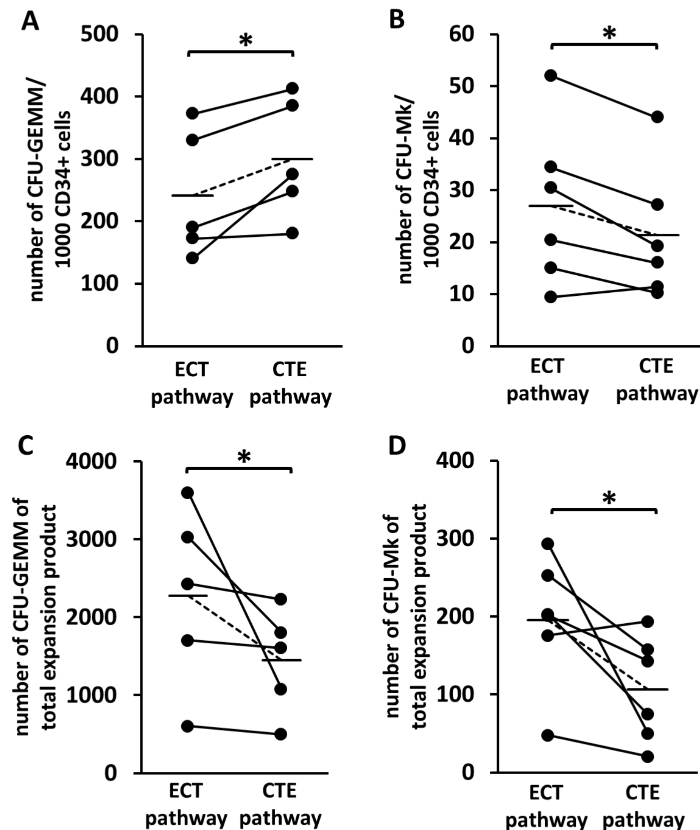


Figure 3: CFU-GEMM and CFU-Mk cultures from cells generated by the ECT vs. the CTE strategy. *Fresh human CB CD34⁺ cells were TPO expanded and subsequently cryopreserved and then thawed (ECT), or were first cryopreserved and then expanded with TPO after thawing (CTE). Shown is the number of CFU-GEMM* ***(A)*** *and CFU-Mk* ***(B)*** *grown from 1000 CD34⁺ cells. Cells expanded with the CTE pathway showed lower CFU-GEMM numbers than when grown from the ECT pathway. In contrast, CD34⁺ cells generated via this pathway showed higher CFU-Mk numbers than CD34⁺ cells derived from the CTE pathway (*p≤0.05). When calculated as the absolute number of colonies formed from the total expansion product, the ECT pathway generated more CFU-GEMM* ***(C)*** *and CFU-Mk* ***(D)****.*

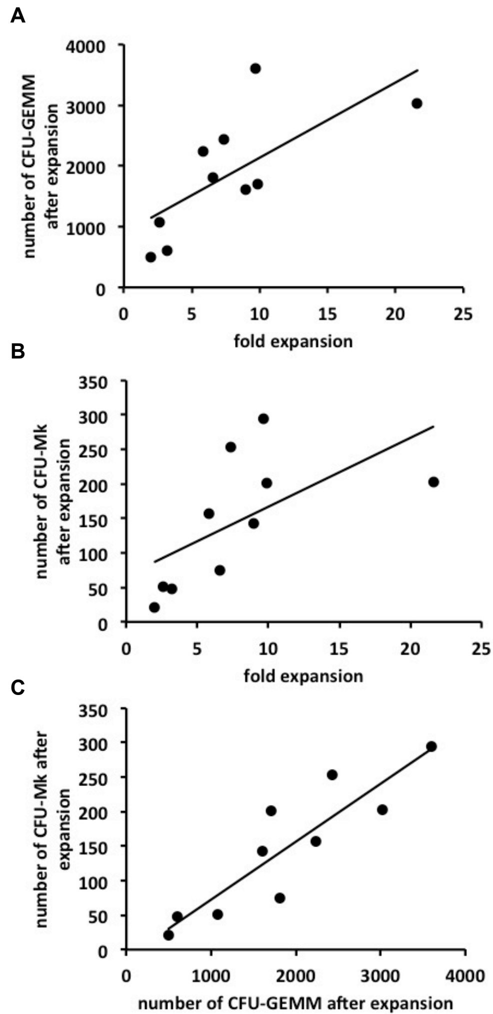


Figure 4. Correlation between fold expansion and formation of CFU-GEMM and CFU-Mk colonies from cells generated by the ECT vs. the CTE strategy. *The absolute number of CFU-GEMM **(A)** and CFU-Mk **(B)** in the total expansion product correlated significantly with the fold expansion in the presence of TPO (Spearman rank correlation 0.721 and 0.806, p-values $\leq$0.05 and $\leq$0.01 respectively). **(C)** The absolute number of CFU-Mk correlated with the absolute number of CFU-GEMM in the total TPO expansion product (Spearman rank correlation coefficient 0.927, p$\leq$0.0001).*

Platelet recovery in the peripheral blood and engraftment in the bone marrow, blood and spleen of NOD SCID mice

The $CD34^+$ cells of three different CB units were expanded via the ECT and CTE pathway and subsequently transplanted into NOD SCID mice (n=3 mice per group). Since the ECT pathway yields higher expansion numbers than the CTE pathway, the absolute cell numbers transplanted in NOD SCID mice were higher for the ECT protocol than for the CTE protocol (on average $12.5\pm6.0x10^6$ cells/mouse for the ECT pathway vs. $5.5\pm3.1x10^6$cells/mouse for the CTE pathway). At day 5 after transplantation, human platelets could be measured in all mice, however, the human platelet concentration was higher in mice that received cells expanded with the CTE pathway (Figure 5A, $p\leq0.05$). Platelet recovery was measured in the peripheral blood from day 5 until 6 weeks after transplantation. From day 12 onwards, the kinetics and extent of platelet recovery were comparable and not statistically different between the CTE and ECT pathway, although transplantation of cells from the ECT pathway generally resulted in lower peripheral blood platelet numbers (Figure 5B). At 6 weeks after transplantation mice were sacrificed to evaluate engraftment of human cells in the bone marrow, peripheral blood and spleen. The percentage of human $CD45^+$ cells within the total $CD45^+$ population (mouse plus human $CD45^+$ cells) tended to be higher in the mice that received cells prepared via the CTE pathway, but this difference was not significant (Figure 5C) Moreover, the lineage differentiation of the human cells in the bone marrow, as determined by the percentage of $CD34^+$, $CD33^+$ and $CD19^+$ cells, was similar (Figure 5D).

Adhesion marker expression and migratory capacity of cells expanded via the ECT or the CTE pathway

Because of the difference in initial platelet recovery shortly after transplantation between the ECT and CTE strategies, the expression of adhesion and homing markers (CD11a/b, CD49d/e, CD62L, CD184/CXCR4) and the migratory capacity of the subpopulations of the expanded cells were measured for both the ECT and CTE expansion methods. A marked increase in mean fluorescence intensity of the CD62L expression was found in the $rCD34^+$ and Lin- population after expansion via the CTE pathway (Table 2) ($rCD34^+$: 3.2-fold difference, p=0.011 and Lin-: 3.9-fold difference, p=0.002).

Figure 5: Engraftment of human cells expanded with the ECT or CTE pathway in an NOD SCID model. *CB $CD34^+$ cells were cryopreserved and expanded according to either the ECT pathway or the CTE pathway and subsequently transplanted in NOD SCID mice (n=9 mice/pathway).* ***(A):*** *Human platelet recovery, defined as the concentration of human platelets/µl PB, for each individual mouse at day 5 after transplantation, showing a significant advantage on platelet recovery in the mice that received cell expanded via the CTE pathway, $p\leq0.05$* ***(B):*** *Mean human platelet concentration of all mice of each group throughout the experiment (*$p\leq0.05$)* ***(C):*** *Percentage of human cell CD45+chimerism in bone marrow, spleen and PB six weeks after transplantation, measured as a percentage of total murine and human $CD45^+$ cells. Mice receiving CTE expanded cells had on average higher percentages of human cells in all organs tested, although not significant.* ***(D):*** *Similar average distribution of different hematopoietic subsets was observed in the human $CD45^+$ marrow cells*

Moreover, the relative percentage of CD62L co-expression was significantly higher in these sub-populations when cells were cultured via the CTE pathway as compared to the ECT pathway (Figure 6A, rCD34$^+$: 91.9±2.4% vs. 58.3±18.3%, 1.6-fold difference; p=0.011; Lin-: 44.9±3.6% vs. 10.3±4.3%, 4.4-fold difference; p=0.00016). In migration experiments, the CD62L$^+$ cells migrated significantly better than CD62L$^-$ cells, which was unrelated to the expansion pathway (Figure 6B). Interestingly however, cells of the CTE pathway migrated better towards an SDF-1a gradient (the CXCR-4 ligand) than cells from the ECT pathway (Figure 6B; $p \leq 0.05$), despite the observation that CD184 (CXCR4) expression for both pathways was equal (Table 2).

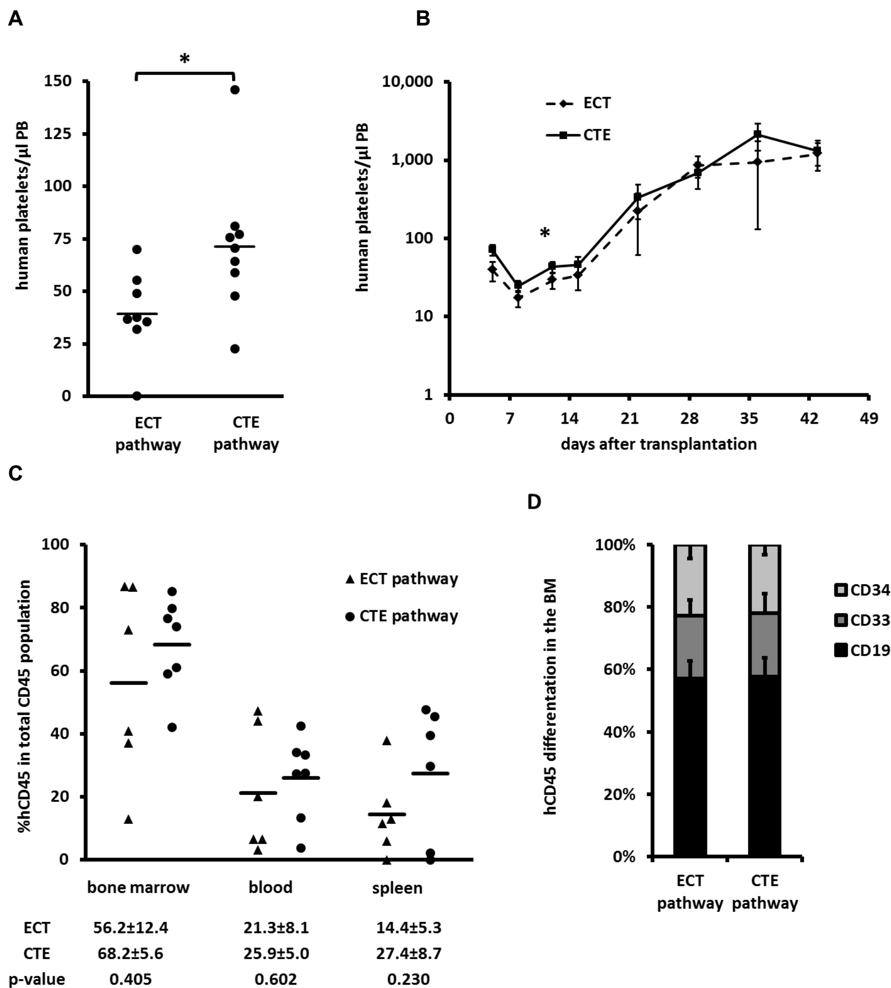


Table 2: Difference in mean fluorescence intensity (±SD) of adhesion markers on TPO expanded cells after expansion by the two different methods. *$CD34^+$ cells were cultured following the ECT pathway (TPO expansion of fresh $CD34^+$ cells and cryopreservation of TPO expanded cells) or the CTE pathway (TPO expansion of cryopreserved $CD34^+$ cells) and adhesion and migration marker expression was examined on the sub-populations obtained after thawing of the TPO expanded cells (ECT) of directly after TPO expansion (CTE). *p<0.05, §p<0.01*

	subpopulation					
	CD34+		*Lin-*		*CD61+*	
	ECT	***CTE***	***ECT***	***CTE***	***ECT***	***CTE***
CD11a	7.4 (±1.5)	6.9 (±2.1)	7.4 (±4.4)	5.8 (±1.3)	2.2 (±0.6)	1.7 (±0.5)
CD11b	0.4 (±0.0)	0.6 (±0.3)	0.6 (±0.2)	0.8 (±0.3)	0.8 (±0.2)	0.8 (±0.1)
CD49d	4.1 (±0.5)	4.5 (±0.5)	5.4 (±1.2)	5.5 (±0.8)	4.7 (±0.7)	4.5 (±0.4)
CD49e	1.0 (±0.2)	0.8 (±0.3)	1.2 (±0.1)	1.0 (±0.4)	4.0 (±0.9)	4.1 (±0.2)
CD62L	3.0 (±0.6)	9.4 (±0.6)*	0.7 (±0.1)	2.9 (±0.1)§	1.1 (±0.4)	1.1 (±0.3)
CD184	0.7 (±0.0)	0.6 (±0.2)	2.0 (±1.5)	2.6 (±1.3)	14.2 (±8.1)	11.6 (±4.2)

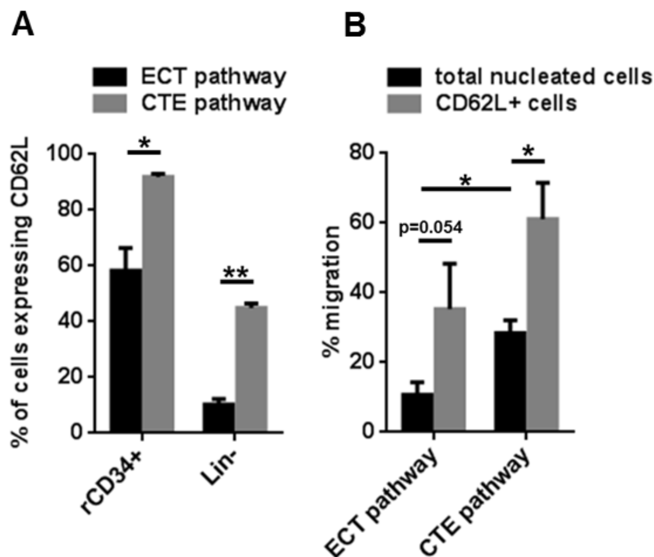


Figure 6: CD62L expression of the expanded cells (A) and percentage of cells that migrated towards an SDF-1α gradient in a trans-well plate (B). A: *Percentage of cells that express CD62L of the cell population responsible for bone marrow engraftment (rCD34+) and the population responsible for early platelet engraftment (Lin-) after expansion via the ECT pathway (black bars) or the CTE pathway (grey bars). Higher CD62L expression was found for both populations after expansion with the CTE pathway. (**p≤0.01, *p<0.05).* ***B:*** *Cells expressing CD62L pathway showed better migration towards the SDF-1α gradient regardless of the expansion pathway and cells expanded with the CTE pathway showed better migration that cells expanded with the ECT pathway.*

Discussion

Expansion of CB CD34+ cells with TPO accelerates platelet recovery in vivo without hampering bone marrow engraftment at 6 weeks in immunodeficient mice.[8-11,23]. Anticipating clinical application, pre- or post-cryopreservation TPO expansion of hematopoietic stem/progenitor cells might influence subsequent in vivo platelet recovery and hematological reconstitution, an issue that has not been investigated so far. We compared the effect of TPO-expansion pre- versus post-cryopreservation of CB CD34+ cells in vitro and in the NOD SCID mice model.
The results show that TNC (total nucleated cell) expansion with TPO is higher when freshly isolated CB CD34+ cells are cultured for 10 days and subsequently cryopreserved (ECT), as compared to CD34+ cells from the same donor expanded after cryopreservation and thawing (CTE). Although the absolute number of rCD34+ cells on average was higher after the CTE protocol, the Lin- cell subset, which has been shown to correlate best with early platelet recovery in vivo in NOD SCID mice[8] was more abundant using the ECT protocol. Surprisingly, and in contrast to the higher expansion and the higher putative platelet precursor numbers observed after the ECT protocol, mice that received CTE cells had a significantly higher number of platelets early after transplantation. This was not at the expense of bone marrow engraftment, which tended also to be higher with the CTE; albeit engraftment as well as the lineage differentiation of the engrafted human cells in the mouse bone marrow at six weeks after transplantation in this respect was not significantly different between the two strategies. As mentioned, compromised viability after thawing of ECT cells is an unlikely explanation for the results as viability of all subsets, including the Lin- cell recovery, exceeded 75%.
However, CD34+ cells are heterogeneous[24] and more extensive studies are required to identify phenotypically the rCD34+ cells after TPO expansion from the non-TPO exposed CD34+ cells with respect to their sensitivity to cryopreservation.
An alternative explanation for the discrepancy between the quantitative in vitro and qualitative in vivo results between the two protocols might be found in the capacity of the cells to home to and engraft in specific bone marrow niches. Migration towards an SDF-1a gradient is a surrogate marker for this process. Additionally, recent studies by Niswander et al.[25,26] demonstrate an additive effect of SDF-1 after TPO treatment in vivo and implicate the SDF-1α in the movement of platelet progenitor cells to the bone marrow vasculature, where they can then promote a resulting increase in circulating platelet numbers. Indeed, we observed that a higher percentage of cells from the CTE pathway migrated towards SDF-1α. The higher CD62L expression, an important migration and homing receptor [27-29] on both rCD34+ and Lin- cells from the CTE pathway, may play a role in this. Indeed, CD62L expression has been correlated to speed of hematopoietic recovery,[30] and in particular with platelet recovery.[31] In this regard, the lower migration towards an SDF-1α gradient of the CD62L- population we observed seems compatible with previous reports. The lower number of CD62L expressing cells derived from the ECT pathway might in this regard explain why fewer cells from the ECT pathway are able to migrate towards or within the bone marrow. However, admittedly CD62L expression is unlikely to be the only explanation for the difference in the in vivo results between the ECT and the CTE pathway. Migration of CD62L+ cells derived from the CTE pathway, whether rCD34+ or Lin- was higher when compared to cells derived from the ECT pathway, indicating that other factors play a role in the migration as well. Important in this respect, no significant differences could be found in the expression of the SDF-1α receptor, CXCR4 (CD184), which was used as the chemo-attractant in these experiments,

although there was higher expression of CXCR4 on the $CD34^-CD61^-$ platelet progenitors than on the $CD34^+$ progenitors post TPO expansion.
In terms of CB transplant logistics, expansion of fresh $CD34^+$ cells followed by cryopreservation would be virtually impossible from a feasibility point of view with respect to the low volume to be cryopreserved, the high costs for the purification and the low efficiency as only a small proportion of UCB (~1%) are used for allogeneic transplantation. Expansion of cryopreserved CB units that are selected for an unrelated allogeneic transplantation therefore is much more practical in the clinical setting. From our comparison in which we started with equal $CD34^+$ cell numbers, we have shown that 10 days of TPO expansion both before and post cryopreservation did lead to similar improved platelet recovery and to similar longer term hematological recoveries.
In conclusion, our study shows that CB $CD34^+$ cells can be TPO-expanded both pre- and post-cryopreservation. The choice between cytokine expansion of CB before or after cryopreservation is essentially a trade-off between different advantages and disadvantages with respect to feasibility, costs and available UCB products, and whether the cells are to be used for specific autologous or allogeneic transplants. Pre-cryopreservation expansion allows quality control analyses on the expansion, as well as immediate availability of an expanded graft together with higher levels of expansion. For unrelated allografts, pre-cryopreservation expansion however introduces a logistical stalemate because it is not possible to predict which CB units will be required and which ones should be expanded before cryopreservation. Post-cryopreservation expansion allows a careful choice for this purpose of the presently banked units. In the case of TPO mediated expansion, better early platelet recovery and similar long term engraftment for unrelated allografts remains. The 10 day culture delay before such a transplant is available in this respect seems a minor disadvantage. We therefore conclude that from a logistical, financial and clinical standpoint, expansion is better performed after cryopreservation for CB allotransplants and that the expansion protocol needs to be clearly assessed if the CB unit is expanded ex vivo and cryopreserved ahead of its use in transplantation.

References

1. Ballen KK, Gluckman E, Broxmeyer HE. Umbilical cord blood transplantation: the first 25 years and beyond. Blood 2013;**122**: 491-8.
2. Watt SM. Article 04238. In: Caplan M, ed. Umbilical Cord Blood Banking. Reference Module in Biomedical Sciences. Kidlington, UK: Elsevier Press, 2014.
3. Wagner Jr JE, Eapen M, Carter S et al. One-Unit versus Two-Unit Cord-Blood Transplantation for Hematologic Cancers. New England Journal of Medicine 2014;**371**: 1685-94.
4. Danby R, Rocha V. Current Strategies to Improve Engraftment in Cord Blood Transplantation. J Stem Cell Res Ther 2014;**4**: 2.
5. de Lima M, McNiece I, Robinson SN et al. Cord-blood engraftment with ex vivo mesenchymal-cell coculture. New England Journal of Medicine 2012;**367**: 2305-15.
6. Delaney C, Heimfeld S, Brashem-Stein C et al. Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution. Nature medicine 2010;**16**: 232-6.
7. Horwitz ME, Chao NJ, Rizzieri DA, Long GD et al. Umbilical cord blood expansion with nicotinamide provides long-term multilineage engraftment. The Journal of clinical investigation 2014;**124**: 3121.
8. Schipper LF, Brand A, Fibbe WE, Van Hensbergen Y. Functional Characterization of TPO-Expanded CD34+ Cord Blood Cells Identifies CD34− CD61− Cells as Platelet-Producing Cells Early After Transplantation in NOD/SCID Mice and rCD34+ Cells as CAFC Colony-Forming Cells. Stem Cells 2012;**30**: 988-96.
9. Schipper LF, Brand A, Reniers N et al. Differential maturation of megakaryocyte progenitor cells from cord blood and mobilized peripheral blood. Experimental hematology 2003;**31**: 324-30.
10. van der Garde M, van Hensbergen Y, Brand A et al. Thrombopoietin treatment of one graft in a double cord blood transplant provides early platelet recovery while contributing to long term engraftment in NSG mice. Stem cells and development 2014;**24**: 67-76.
11. van Hensbergen Y, Schipper LF, Brand A et al. Ex vivo culture of human CD34+ cord blood cells with thrombopoietin (TPO) accelerates platelet engraftment in a NOD/SCID mouse model. Experimental hematology 2006;**34**: 943-50.
12. Purtill D, Smith K, Devlin S et al. Dominant unit CD34+ cell dose predicts engraftment after double-unit cord blood transplantation and is influenced by cord blood bank practice. Blood 2014.
13. Guttridge MG, Soh TG, Belfield H et al. Storage time affects umbilical cord blood viability. Transfusion 2013.
14. Kurtz J, Seetharaman S, Greco N, Moroff G. Assessment of cord blood hematopoietic cell parameters before and after cryopreservation. Transfusion 2007;**47**: 1578-87.
15. Page KM, Mendizabal A, Betz-Stablein B et al. Optimizing donor selection for public cord blood banking: influence of maternal, infant, and collection characteristics on cord blood unit quality. Transfusion 2013.
16. Scaradavou A, Smith KM, Hawke R et al. Cord Blood Units with Low CD34+ Cell Viability Have a Low Probability of Engraftment after Double Unit Transplantation. Biology of Blood and Marrow Transplantation 2010;**16**: 500-8.
17. Almici C, Carlo-Stella C, Wagner J et al. Clonogenic capacity and ex vivo expansion potential of umbilical cord blood progenitor cells are not impaired by cryopreservation. Bone marrow transplantation 1997;**19**: 1079-84.
18. Lazzari L, Lucchi S, Montemurro T et al. Evaluation of the effect of cryopreservation on ex vivo expansion of hematopoietic progenitors from cord blood. Bone marrow transplantation 2001;**28**: 693-8.
19. Pick M, Eldor A, Grisaru D et al. Ex vivo expansion of megakaryocyte progenitors from cryopreserved umbilical cord blood: a potential source of megakaryocytes for transplantation. Experimental hematology 2002;**30**: 1079-87.
20. Rice AM, Wood JA, Milross CG et al. Prior cryopreservation of ex vivo-expanded cord blood cells is not detrimental to engraftment as measured in the nod-scid mouse model. Journal of hematotherapy & stem cell research 2001;**10**: 157-65.
21. Nicol A, Nieda M, Donaldson C et al. Analysis of cord blood CD34+ cells purified after cryopreservation. Experimental hematology 1995;**23**: 1589-94.
22. Schipper LF, Van Hensbergen Y, Fibbe WE, Brand A. A sensitive quantitative single-platform flow cytometry protocol to measure human platelets in mouse peripheral blood. Transfusion 2007;**47**: 2305-14.
23. Mattia G, Milazzo L, Vulcano F et al. Long–term platelet production assessed in NOD/SCID mice injected with cord blood CD34+ cells, thrombopoietin–amplified in clinical grade serum–free culture. Experimental hematology 2008;**36**: 244-52.
24. Notta F, Doulatov S, Laurenti E et al. Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. Science 2011;**333**: 218-21.

25. Niswander LM, Fegan KH, Kingsley PD et al. SDF-1 dynamically mediates megakaryocyte niche occupancy and thrombopoiesis at steady-state and following radiation injury. Blood 2014: blood-2014-01-547638.
26. Thon JN. SDF-1 directs megakaryocyte relocation. Blood 2014;**124**: 161-3.
27. Hattori Y, Kato H, Nitta M, Takamoto S. Decrease of L-selectin expression on human CD34+ cells on freeze-thawing and rapid recovery with short-term incubation. Experimental hematology 2001;**29**: 114-22.
28. Voermans C, Kooi ML, Rodenhuis S et al. In vitro migratory capacity of CD34+ cells is related to hematopoietic recovery after autologous stem cell transplantation. Blood 2001;**97**: 799-804.
29. Voermans C, Rood P, Hordijk P et al. Adhesion molecules involved in transendothelial migration of human hematopoietic progenitor cells. Stem Cells 2000;**18**: 435-43.
30. Watanabe T, Dave B, Heimann D et al. Cell adhesion molecule expression on CD34+ cells in grafts and time to myeloid and platelet recovery after autologous stem cell transplantation. Experimental hematology 1998;**26**: 10-8.
31. Dercksen MW, Gerritsen WR, Rodenhuis S et al. Expression of adhesion molecules on CD34+ cells: CD34+ L-selectin+ cells predict a rapid platelet recovery after peripheral blood stem cell transplantation. Blood 1995;**85**: 3313-9.

Chapter 4:

No synergistic effect of co-transplantation of MSC and ex vivo TPO expanded CD34+ cord blood cells on platelet recovery and bone marrow engraftment in NOD SCID mice

Mark van der Garde, Anneke Brand, Manon C Slot,
Alice de Graaf-Dijkstra,Jaap Jan Zwaginga
and Yvette van Hensbergen

Stem Cells and Development
2015, 24: 1448-1456

Abstract

After cord blood transplantation, early platelet recovery in immune deficient mice is obtained by expansion of cord blood (CB) CD34+ cells with TPO as single growth factor. Moreover, improvement of hematopoietic engraftment has been shown by co-transplantation of Mesenchymal Stem Cells (MSC). We investigated whether a combination of both approaches would further enhance the outcome of CB transplantation in NOD SCID mice. NOD SCID mice were transplanted with either CB CD34+ cells, CD34+ cells with MSC, TPO expanded CD34+ cells or TPO expanded CD34+ cells with MSC. We analyzed human platelet recovery in the peripheral blood (PB) from day 4 after transplantation onwards and human bone marrow (BM) engraftment at week 6. The different transplants were assessed in vitro for their migration capacity and expression of CXCR4. TPO expansion improved the early platelet recovery in the PB of the mice. Co-transplantation of MSC with CD34+ cells improved BM engraftment and platelet levels in the PB 6 weeks after transplantation. Combining TPO expansion and MSC co-transplantation however, neither resulted in a more efficient early platelet recovery nor in better BM engraftment and even very low or absent BM engraftment occurred. In vitro, MSC boosted the migration of CD34+ cells, suggesting a possible mechanism for the increase in engraftment. Our results show that co-transplantation of MSC with TPO expanded CD34+ cells at most combines, but does not increase the separate advantages of these different strategies. A combination of both strategies even adds a risk of non engraftment.

Introduction

Cord blood (CB) is an alternative hematopoietic graft source for almost 20% of the patients for whom no HLA matched donor can be found [1-4]. However, CB contains relatively low numbers of hematopoietic stem cells (HSCs), which translates into delayed neutrophil recovery and slow and impaired platelet engraftment when compared to transplantation with bone marrow (BM) or G-CSF mobilized peripheral blood stem cell (PBSC) grafts [5-7]. Several strategies to overcome this are under investigation such as the selection of CB units containing large cell numbers, double CB transplantation, the ex vivo manipulation of CB cells and co-transplantation of accessory cells, such as MSC [8]. Ex vivo culture of CB cells , depending on culture conditions and growth factors, often alters the functionality of the cord blood cells and/or the composition of the cord blood graft [9,10]. In this respect, ex vivo culture with TPO as single growth factor accelerates platelet recovery in the PB of mice, without impairment of engraftment in the BM [11-13]. These platelets are derived from TPO induced lineage negative (CD34-CD61-Lin-) cells preceding megakaryocyte formation [12].
Mesenchymal stem cells (MSC) are also investigated to boost engraftment. These multipotent stromal cells are characterized by three characteristics, 1) plastic adhesion in culture, 2) the expression of a set of distinct markers and 3) the ability to differentiate into three mesodermal lineages [14]. MSC can be isolated from both adult [15,16] and fetal tissues [17,18]. MSC have anti-proliferative, immunosuppressive and anti-inflammatory effects [19] and are currently evaluated in clinical studies for the treatment of immune mediated disorders such as Crohn's disease [20], systemic lupus [21] and systemic sclerosis [22]. In hematopoietic stem cell transplantation (HST), post-transplant infusions of MSC [23-28] as well as co-transplantation of MSC [29,30] are explored for the treatment and prophylaxis of graft versus host disease and/or graft rejection. In animal models, co-transplantation of MSC, improves engraftment after HST [18,31-35]. Clinical studies have so far shown variable results when MSC co-transplantation was compared to neutrophil and/or platelet recovery of historical controls [36,37]. While improved neutrophil recovery is reported more consistently [38,39], the median time to platelet engraftment remained delayed, compared to unrelated BM or PBSC transplants [40].
Combining TPO expansion of CB CD34+ cells and MSC co-transplantation could enhance post CB transplant PB platelet recovery as well as BM engraftment. In this study, we therefore compared the engraftment potential of both approaches in an NOD SCID mouse model.

Materials and methods

$CD34^+$ cell purification. Umbilical cord blood (CB) was collected with written consent from the mother according to Netcord–FACT standards and with ethical permission from the medical ethical board of the Leiden University Medical Center (LUMC). Mononuclear cells were isolated from CB using a ficoll density gradient. The $CD34^+$ cell fraction was isolated using magnetic $CD34^+$ isolation beads (Miltenyi Biotec, Bergisch Gladbach, Germany).. The purity of the isolated $CD34^+$ cell fraction was verified by flow cytometry (Beckman Coulter, Woerden, The Netherlands) with CD45-FITC and CD34-PE (Beckman Coulter). The percentage of $CD34^+/CD45^+$ cells in the isolated fraction was 91±3%.

Expansion of the $CD34^+$ cells. $CD34^+$ cells were cultured at 37°C and 5% CO_2 in a humidified atmosphere in IMDM medium (Gibco, Breda, The Netherlands) supplemented with 20% (v/v) AB heparin plasma (Sanquin Blood Supply Foundation, Rotterdam, The

Netherlands), 0.5 mg/ml human transferrin saturated with $FeCl_3.H_20$ (Sigma, Zwijndrecht, The Netherlands), 0.34% (v/v) human serum albumin 20% (Cealb® CLB, Amsterdam, The Netherlands), 1% (v/v) penicillin/streptomycin (Bio-Whittaker, Verviers, Belgium), 0.05 mM β-mercapto-ethanol (Sigma), and 50 ng/ml mpl-ligand (TPO, kind gift from KIRIN Brewery Ltd., Japan). The cells were plated in 24 well sterile TC plates, at a concentration of $5x10^4$ cells/ml. At day 7, the medium was refreshed by semi-dilution, with medium containing 50 ng/ml TPO. At day 9 the cells were split into two new wells and 1:1 diluted with medium without TPO. At day 10, the cells were harvested and the total cell expansion was calculated and subsequently the composition of the cultured cells was analyzed by flow cytometry using mouse-anti-human CD45, CD61, CD34, CD14 and CD15 antibodies (all Beckman Coulter).

Culture of mesenchymal stem cells. Mesenchymal stem cells were obtained from fetal lung tissue as previously described [18]. The cells were cultured in M199 supplemented with 10% FCS, 1% pen/strep, 20 μg/ml EGF and 8 U/ml heparin, in gelatin coated tissue culture flasks at 37°C and 5% CO_2 in a humidified atmosphere.

Transplantation in NOD/scid mice. Female, 5-6 weeks old, NOD SCID mice (Charles River, France) were kept the animal facilities of the LUMC. The animal ethical committee of the LUMC approved all animal experiments. The mice were irradiated sub lethally (3.5 Gy) 24 hours before i.v. transplantation with the different transplants: 1) $2*10^5$ $CD34^+$ cells (hereafter referred to as CD34), 2) $2*10^5$ $CD34^+$ cells 10 days expanded with TPO (hereafter referred to as CD34-E), 3) $2x10^5$ $CD34^+$ cells + $1x10^6$ FL MSC (hereafter referred to as CD34/MSC) and 4) $2x10^5$ $CD34^+$ cells 10 days expanded with TPO + $1x10^6$ FL MSC (hereafter referred to as CD34-E/MSC). A schematic representation of the experiment is shown in figure 1.
Blood collection via tail vein incision was performed twice weekly during the first 3 weeks after transplantation and once weekly thereafter. Blood collection and human platelets measurements were performed as described previously [41]. Briefly, human platelets were stained with a non-cross reactive mouse-anti-human CD41-PE (Beckman Coulter) and erythrocytes were lysed with IQTest3 Lysing solution (Beckman Coulter). Flow-Count™ fluorospheres (Beckman Coulter) were added to enable the measurement of the absolute number of circulating human platelets. The detection limit was $1x10^3$ platelets/ml of PB. Analysis was performed with flow cytometry (EPICS® XL-MCL, Beckman Coulter) running system II software.
Six weeks after transplantation, mice were sacrificed and the bone marrow was obtained from the femur. Cells were resuspended in IMDM and cells were labeled with goat-anti-mouse-CD45-PE (LCA, Ly-5, 30-F11, Pharmingen), mouse-anti-human CD45-FITC (Beckman Coulter), and the appropriate isotype controls. Subsequently erythrocytes were lysed with IO Test 3 Lysing solution according to the manufactures procedures (Beckman Coulter). Analysis was performed with flow-cytometry (EPICS® XL-MCL, Beckman Coulter) running system II software.

Migration experiments. Four different cell suspensions were prepared identical to the in vivo experiment. After 30 minutes of incubation cells were analyzed for the expression of CD45, CD34, CD61, CXCR4, CD49d and CD49e with flow cytometry and placed in the upper compartment of a trans-well plate [Costar, Amsterdam, the Netherlands], with a 100 ng/ml SDF gradient in the lower compartment, both containing IMDM. (Gibco). Plates were incubated for 5 hours at 37ºC and 5% CO_2 in a humidified incubator. After incubation, cells were harvested from both compartments. After incubation all cells were

analyzed for the expression of CD45, CD34, and CD61 (all Beckman Coulter) to calculate the number of cells that have migrated.

Statistical Analysis. All statistics were done with SPSS, version 20. All results are presented as mean±SEM. To compare groups, a student's T test (normally distributed) or Mann Whitney test (not normally distributed) was used. Differences were considered significant when $p<0.05$. If multiple groups were compared, an ANOVA or a Holm's sequential Bonferroni adjustment was applied.

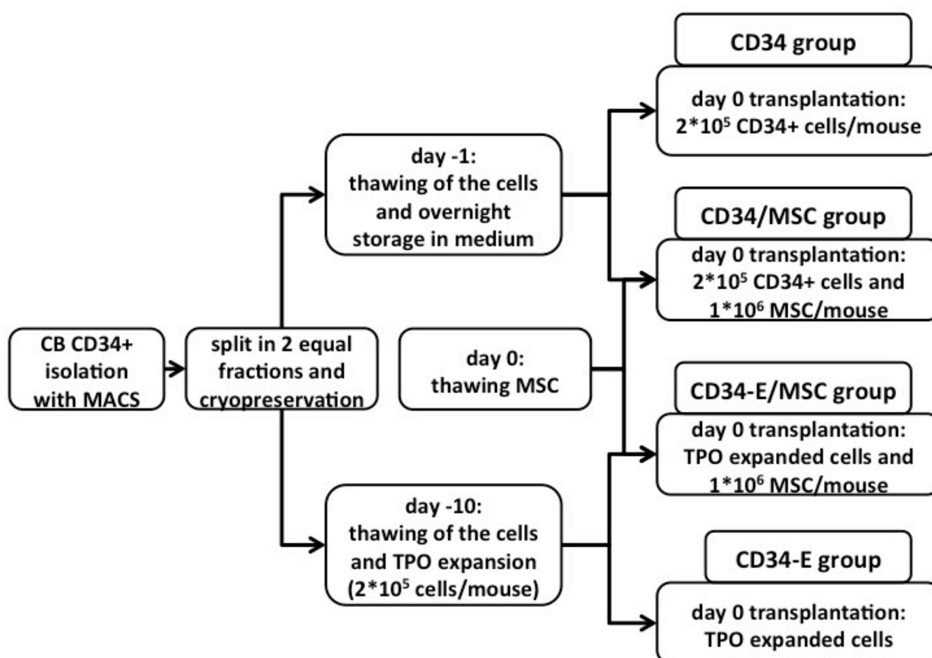


Figure 1: *flow chart of the transplantation experiment. Four different types of transplants were prepared (cell numbers/mouse):* ***CD34****: $2*10^5$ un-manipulated CD34+ cells (control group)* ***CD34/MSC****: $2*10^5$ un-manipulated CD34+ cells with $1*10^6$ fetal lung MSC* ***CD34-E/MSC****: the total expansion product of $2*10^5$ CD34+ cells expanded with TPO with $1*10^6$ fetal lung MSC* ***CD34-E****: the total expansion product of $2*10^5$ CD34+ cells expanded with TPO*

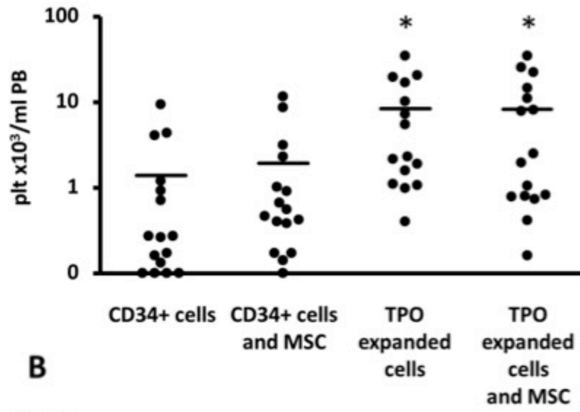
Results

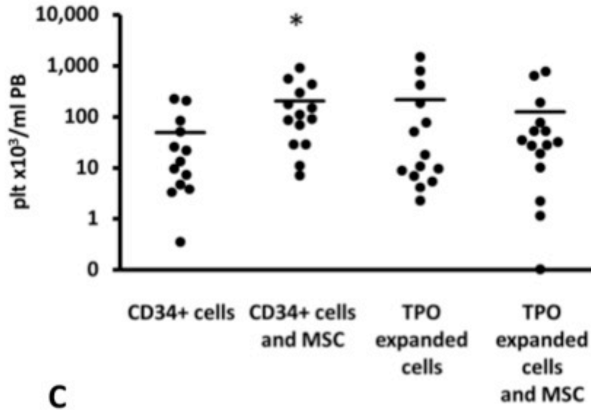
Platelet recovery in the peripheral blood of NOD-SCID mice

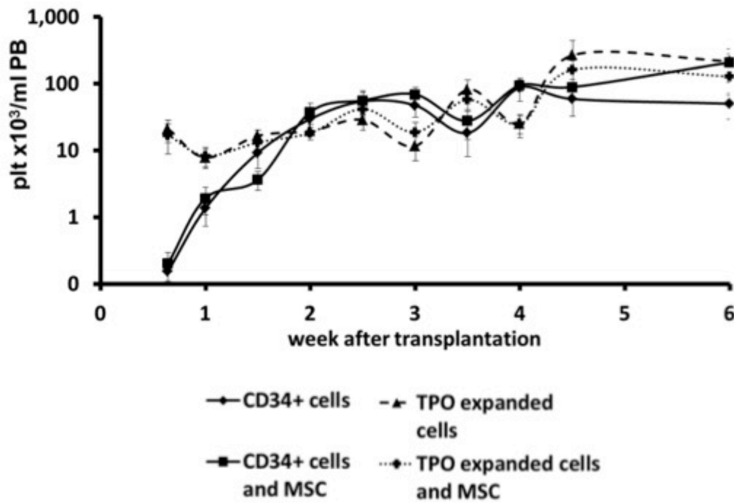
To study the different strategies to overcome delayed and reduced engraftment of CB cells, mice were transplanted with either unmanipulated CD34+ cells (CD34 group), TPO expanded CD34+ cells (CD34-E group), unmanipulated CD34+ cells with MSC (CD34/MSC group) or TPO expanded CD34+ cells with MSC (CD34-E/MSC group) while platelet recovery as well as bone marrow engraftment were studied.

Similar to earlier studies [11-13,42], in the first week after transplantation, mice that received TPO expanded cells (CD34-E and CD34-E/MSC groups) showed accelerated early platelet (plt) recovery in the PB when compared to mice transplanted with only $CD34^+$ cells (CD34 group) (figure 2A and 2C), as shown by significantly higher platelet concentrations in the PB of the mice 8 days after transplantation (CD34-E group:7.8±2.5x10^3 plt/ml PB, CD34-E/MSC group: 8.3±2.7 x10^3 plt/ml, compared to the control group, CD34: 1.4±0.6x10^3 plt/ml, $p<0.005$ for both groups). The TPO induced increased PB platelet concentration was present for two weeks after transplantation while after week 2, the mean PB platelet levels in all groups slowly increased. Co-transplantation of MSC in contrast, did not significantly increase early platelet repopulation as compared to CD34+ cells or TPO expanded CD34+ cells respectively (CD34/MSC: 1.9±0.8x10^3 plt/ml compared to CD34: 1.4±0.6x10^3 plt/ml and CD34-E/MSC: 8.3±2.7x10^3 plt/ml compared to CD34-E: 7.8±2.5x10^3 plt/ml). Compared to unmanipulated CD34+ grafts, all mice that received a manipulated graft and/ or additional MSCs showed higher platelet concentrations six weeks after transplantation (mean platelet concentration ±SEM in PB: CD34/MSC: 207.4±68.2x10^3, CD34-E: 216.2±112.2x10^3 and CD34-E/MSC: 127.0±60x10^3 plt/ml) as compared to the mice that received unmanipulated CD34+ cells (CD34: 49.8±21.0x10^3 plt/ml PB). However, only for the CD34/MSC group this difference was significant ($p<0.01$). The mean increase of long term platelet engraftment in the CD34-E group was due to a wide SD not significant because a number of mice engrafted exceptionally well, thereby increasing the mean value of the group. The median values of these groups, 18.0x10^3 plt/ml PB for CD34-E versus 17.4x10^3 plt/ml PB for CD34, were similar. Vice versa, the (non-significant) lower mean platelet concentration of the group that was transplanted with CD34-E/MSC was the result of a number of mice with very low or non-engraftment.

***Figure 2**. **A:** platelet concentration in the peripheral blood (PB) of each of the mice 8 days after transplantation. Bars represent the mean platelet concentration of each group. On average, both groups that were transplanted with TPO expanded cells had significantly higher concentrations of platelets in the PB (*p<0.02). **B:** platelet concentration of each of the mice 40 days after transplantation. Bars represent the mean platelet concentration of each group. On average, all groups had higher concentrations of platelets in the PB than the control group, but this difference was only significant for the group that was transplanted with CD34+ cells and MSC (*p<0.05). **C:** kinetics of platelet recovery in the mice throughout the experiment. Shown are the mean±SEM values of the four different transplants.*


A
100
10
1
0
plt x10³/ml PB
*
*
CD34+ cells
CD34+ cells and MSC
TPO expanded cells
TPO expanded cells and MSC

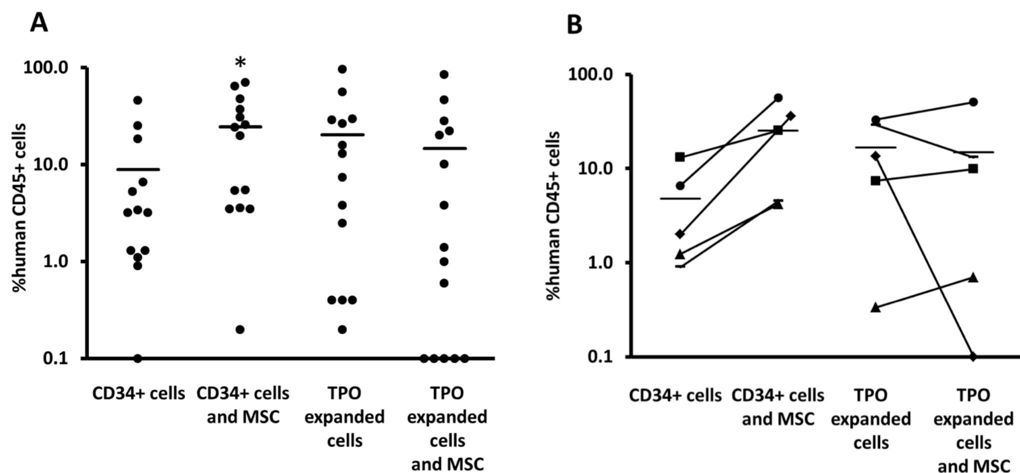

B
10,000
1,000
100
10
1
0
plt x10³/ml PB
*
CD34+ cells
CD34+ cells and MSC
TPO expanded cells
TPO expanded cells and MSC


C
1,000
100
10
1
0
plt x10³/ml PB
0
1
2
3
4
5
6
week after transplantation
CD34+ cells
TPO expanded cells
CD34+ cells and MSC
TPO expanded cells and MSC

Human engraftment in the bone marrow

The percentage of human CD45 cells in the bone marrow 6 weeks after transplantation of the mice is shown in figure 3A. Co-transplantation of MSC increased the engraftment when compared to transplantation of CD34+ cells alone with higher percentages of human cells in the BM (CD34/MSC: 24.5±6.3% vs. CD34: 8.9±3.7%; p<0.05). In line with the 6 weeks PB platelet counts, TPO expanded cells also induced engraftment, but again this was not significant (CD34-E: 20.2±7.4%; p=0.402 compared to the CD34 group).

Interestingly, whereas co-transplantation of MSC with CD34+ cells significantly improved the BM engraftment of the mice at week 6, co-transplantation of MSC with TPO expanded CD34+ cells did not enhance BM engraftment and seemed even less favorable when compared to transplantation of TPO expanded cells alone (%human CD45 cells in the BM for CD34-E/MSC: 14.7±6.2% vs. 20.2±7.4% for CD34-E). Again, as seen with the low platelet counts at week 6, low or non-engraftment in a subset of mice did account for this difference. To study if this finding was related to the quality of a particular CB unit, we compared the mean BM engraftment of mice that received cells from the same cord blood unit (figure 3B). Again, co-transplantation of MSC with CD34+ cells increased the engraftment (on average 7.1±-2.1 fold) for all of the five different cord blood units that were used in this study. Co-transplantation of MSC with TPO expanded cells, however, again showed very low engraftment for one of the cord blood units and no engraftment for a second unit (0-0.1% of human CD45+ cells in the bone marrow). Whereas co-transplantation of MSC with TPO expanded cells of these two specific units impaired engraftment, co-transplantation of MSC with the unmanipulated CD34+ cells of these two units increased the engraftment 5 and 17 fold respectively. The latter was in line with the average increase in engraftment (7.1±2.1 fold) when MSC are co-transplanted with unmanipulated CD34+ cells, whilst they decreased the engraftment (1.1±0.4 fold) when co-transplanted with TPO expanded cells.



Co-transplantation of MSC with TPO expanded cells on different time points

TPO expanded grafts contain a higher cell number as compared to unmanipulated CD34+ cells. Thus, mice transplanted with CD34-E grafts receive more cells. Co-transplantation of MSC with these high cell numbers might result in e.g. obstruction of the lung circulation and impairment of the expected positive influence of MSC on marrow engraftment contributing to the non-engraftments we observed in some cases. To prevent this possible blocking of circulatory beds by MSC, we performed a pilot experiment in which we infused MSC 4 hours after TPO expanded cells. . As shown in figure 4, this later timing of MSC co-transplantation did not significantly alter the level of platelets in the PB 6 weeks after transplantation (Fig 4A, 125.8±58.2 plt/ml PB for MSC transplantation concomitantly with the hematopoietic cell transplantation vs. 134.4±62.6 x10^3 plt/ml PB for MSC transplantation 4 hours after hematopoietic cell transplantation, p=0.873). Moreover, the percentage of human CD45+ cells in the bone marrow was not significantly changed when MSC were directly infused with CD34+ cells or after 4 hours (Fig 4B, 22.2±6.4% vs 16.1±8.3%, p=0.522).

Figure 3. A: *Percentage of human CD45 cells as a percentage of the total CD45 cells in the BM of the mice 6 weeks after transplantation. Bars represent mean values of each group. All groups had higher mean percentages of human CD45 in their BM than the control group, but this was only significant for the group that received CD34+ cells and MSC (p<0.05).* ***B:*** *The mean percentage of human CD45+ cells of the mice that were transplanted with cells from the same cord blood unit were calculated and compared for each cell type (CD34+ cells or TPO expanded cells) when transplanted with or without MSC, showing an increase or a decrease in engraftment when MSC were added to the CD34+ cells or the TPO expanded cells of this cord blood. Co-transplantation of MSC with CD34+ cells increased the engraftment of the cells of all of the cord blood units. Co-transplantation of MSC with TPO expanded only increased the engraftment of the cells of three cord blood units. The average fold increase in engraftment by the co-transplantation of MSC with CD34+ cells was 7.1±2.1, The average fold increase in engraftment by the co-transplantation of MSC with TPO expanded cells was 1.1±0.4.*

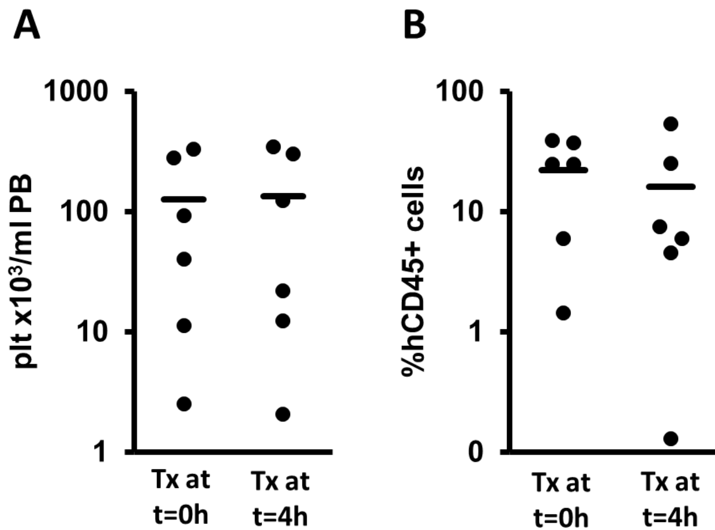


*Figure 4: Engraftment results of mice transplanted with TPO expanded cells and co-transplanted with MSC at the time of hematopoietic cell transplantation (Tx at t=0h) or 4 hours after transplantation (Tx at t=4h). Bars represent mean values of each group. No differences in the platelet engraftment (**A**) in the peripheral blood or the human CD45+ cell engraftment (**B**) in the BM were detected when the MSC were transplanted on different time points.*

Migration properties and expression of migration related molecules of the transplanted cells

SDF-1α is the main chemo-attractant for hematopoietic cells to home to the bone marrow and migration capacity of cells towards SDF-1α is therefore a crucial step in engraftment [43-46]. To investigate if TPO expansion and/or MSC affect the migratory capacity of the cells, we analyzed the migration capacity of the grafts towards a 100 ng/ml SDF-1α gradient in Transwell plates (figure 5A). Addition of MSC improved the migration of the CD34+ cells by 3 fold (21.6±4.8% with MSC vs. 6.7±2.4% for CD34+ cells alone, $p<0.05$). Also TPO expansion improved the migration of the remaining CD34+ cells in the transplant 8-9 fold (48.7±8.9% $p<0.005$). Addition of MSC did not further enhance the already increased migration of residual CD34+ cells in the TPO expanded CB (57.2±10.5%).

Because TPO expansion generated CD34-CD61-Lin- cells that establish PB platelet recovery, we also investigated the effect of MSC on migration of this sub-population [12]. Figure 5B in this respect shows that, similarly as with residual CD34+ cells, co-transplantation of MSC does not alter the migration of CD34-CD61- cells (37.0±4.1% for TPO expanded transplants, 33.2±2.1% for TPO expanded cells with MSC, $p=0.818$).

The expression of CXCR4, the receptor for SDF1α, on (residual) CD34+ (figure 5C) or CD34-CD61-Lin- cells (figure 5D) was analyzed with flow cytometry (figure 5C and 5D) but was not significantly altered by their incubation with MSC nor by TPO expansion alone or in combination with MSC. However, the expression of CD49d and CD49e was upregulated in TPO expanded CD34+ cells as opposed to non expanded CD34+ cells and CD34+ cells that were incubated with MSC.

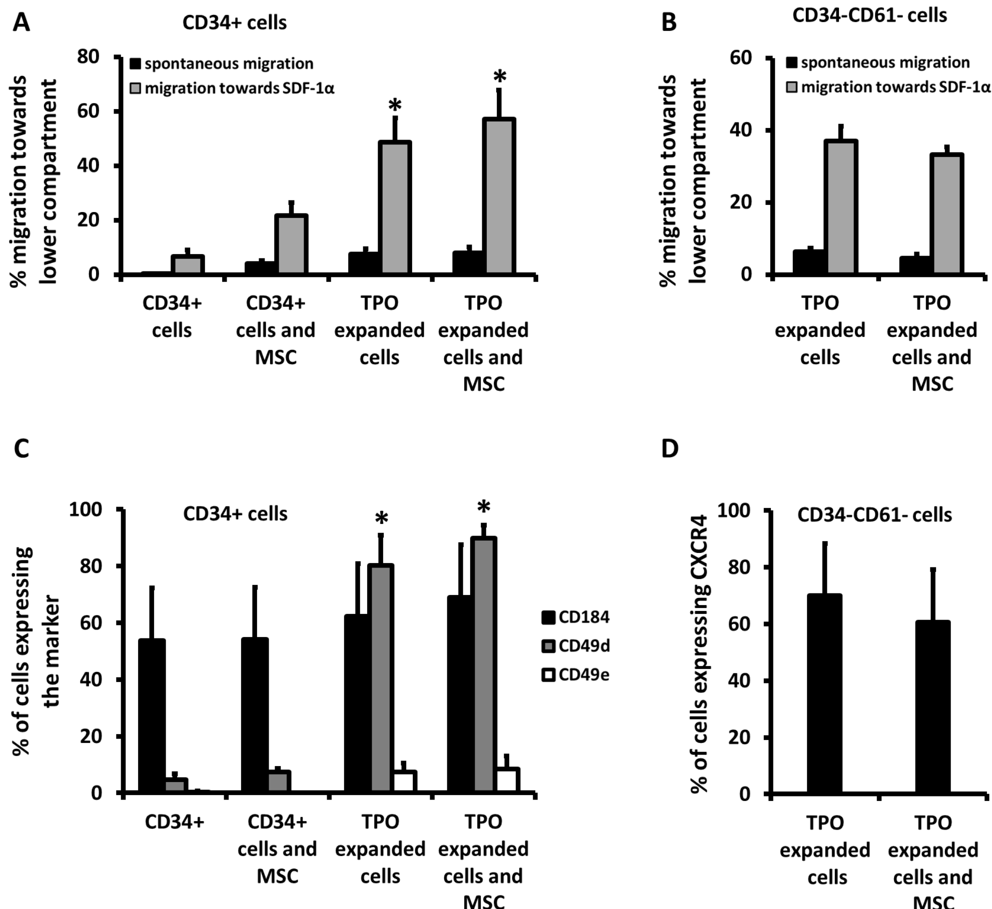


Figure 5. A: *Percentage of CD34+ cells that have migrated through a trans-well system towards the lower compartment of the plate containing medium with 100ng/ml SDF-1α gradient (black bars) or medium alone (gray bars, spontaneous migration). CD34+ cells are either un-manipulated cells (control group and group that was transplanted with CD34+ cells and MSC) or the CD34+ subpopulation of the cells after expansion with TPO. Both addition of MSC and TPO expansion improved the migration of the CD34+ cells significantly. Addition of MSC to TPO expanded cells did not improve the migration of the subpopulation (*p<0.05).* ***B:*** *Percentage of cells of the CD34-CD61-Lin- subpopulation found after TPO expansion of CD34+ cells that have migrated through a trans-well system towards the lower compartment of the plate containing medium with 100ng/ml SDF-1α gradient (black bars) or medium alone (gray bars, spontaneous migration). Addition of MSC to TPO expanded cells did not improve the migration of the subpopulation.* ***C:*** *Percentage of CD34+ cells expressing CXCR4, CD49d or CD49e. There was no difference in the expression of CXCR4 between the different transplants suggesting that the differences found in the transwell migration are not due a difference in the expression of the receptor for SDF-1α. TPO expanded cells did express higher percentages of CD49d and to a lesser extend CD49e (*p<0.0001).**D:*** *Percentage of cells of the CD34-CD61- subpopulation found after TPO expansion of CD34+ cells expressing CXCR4. No differences in the expression of the receptor of SDF-1α were found*

Discussion

Several studies have shown that co-transplantation of MSC improves the bone marrow engraftment in animal models [18,31-35], but the effect on the speed of platelet recovery has not been investigated. In this study, we observed that MSC co-transplantation with either unmanipulated CD34+ cells or with TPO expanded CD34+ cells had no effect on the recovery of platelets in the peripheral blood within 2 weeks after transplantation. The early platelet recovery seen after transplantation of CB cells expanded with TPO originates from CD34-CD61-Lin- cells. This population that is (partly) committed to the megakaryocyte lineage is unique for CB and not observed among TPO expanded adult stem cell sources and this maturation pattern may contribute to the delayed and slow platelet recovery observed after CB transplantation. [12,47]. The absence of this Lin-neg population in unmanipulated CD34+ CB explains the lack of improvement of early platelet recovery when MSC are co-transplanted with uncultured CB CD34+ cells. Also, in line with earlier studies in NOD SCID mice showing that MSC co-transplantation improved BM engraftment and repopulation for CD34+ CB cells [12,13,18,31-34], we observed that MSC boosted both PB platelet levels after 6 weeks and BM engraftment.
The mechanism behind the improved engraftment by MSC is still unknown. We found enhanced SDF-1α migration capacity of CD34+ cells by MSC in vitro, which was not related to a change in the expression of CXCR4. This suggests that MSC induced improvement of engraftment might be partly attributed to this increased migration capacity. However, the interaction between MSC and stem cells on homing is complex [48,49] and conclusive proof for this or other mechanisms is still lacking. Homing of MSC to the marrow was studied as determinant for the observed engraftment potentiating effect and these studies have shown conflicting results. Both Noort et al. and Kim et al. did not find MSC in the BM of the mice after transplantation [18,34]. Noort at al analyzed the presence of MSC in multiple organs with RT PCR and did not find any MSC in the BM, spleen, liver or thymus, but only sequestration of the MSC in the a lung. This 'lung barrier' was corroborated by Schrepfer et al [50], who IV injected labeled MSC and found high bioluminescence in the lungs with in vivo imaging and ex vivo analysis in contrast to only trace signals from other organs such as the spleen, the tibia and the liver. Moreover, efforts from Noort et al to bypass the lung barrier by intra-cardiac injection did not result in the detection of MSC in the BM either. Only Hiwase et al. suggested that MSC and HSC can migrate in conjunction to the marrow [32]. Other mechanisms, induced by MSC secreted cytokines and growth factors might also play a role. MSC are known to support and maintain blood vessels [51] and might contribute to marrow regeneration by inducing vascularization. Even MSC mediated immunomodulation enhancing allogeneic tolerance has to be considered [36].
The aim of our study was to investigate whether co-transplantation with MSC had a synergistic effect on accelerated platelet recovery and/or improved BM engraftment of TPO expanded CB. We observed that MSC did not potentiate short term platelet engraftment of TPO expanded CD34+ cells. This could be associated with an unchanged migration pattern of CD34-CD61-Lin- cells towards an SDF-1α gradient in vitro since MSC do not influence the homing capacity of the TPO generated CD34-CD61-Lin- cells responsible for early platelet repopulation. However, a causal role on the lack of synergy in platelet recovery between the two populations is elusive. Furthermore the increase in BM engraftment, seen with the co-transplantation of MSC with unmanipulated CD34+ cells is not seen when MSC are co-transplanted with TPO expanded cells. Strikingly, in some situations the combination seemed to decrease the 6 week PB platelet numbers as well as human CD45+ BM engraftment. This could largely be attributed to the fact that some mice treated with the combination of TPO expansion and MSC virtually showed non-

engraftment (only 0-0.1% of human CD45 cells in the bone marrow). If these 'non' or 'very low' engrafters were not taken into account when calculating the mean engraftment percentage of this group, the level of human CD45+ cells in the BM rose from 14.7% to 22.0%, which is similar to mice transplanted with only TPO expanded cells (20.2%). A synergy of the two approaches which each separately have shown to improve platelet recovery or BM engraftment in NOD/SCID mice was clearly absent. Most importantly, adding MSC to TPO expanded cells may result in engraftment failure.

These non or very low engraftment cases in the combined approach could hypothetically be caused by a phenomenon called the lung barrier. Because of their larger size, MSC might become trapped in the lung. In addition, MSC can adhere to hematopoietic stem cell, further impairing their homing to the marrow [50]. The high cell numbers in the TPO expanded transplants in combination with the MSC may be critical to develop this complication. Although we did observe acute deaths in both groups that were co-transplanted with MSC, possibly caused by pulmonary embolism, non engrafters were only found in the group with TPO expanded cells combined with MSC. To see whether the simultaneous presence of high numbers of cells transplanted after TPO expansion together with MSC influenced the engraftment, we performed additional experiments in which we transplanted the MSC 4 hours after the transplantation of the TPO expanded cells. Although no differences in the engraftment of both platelets in the PB and human CD45 cells in the BM after 6 weeks were discerned, one mouse again showed hardly any BM engraftment after 6 weeks. Interestingly, this mouse was given MSC 4 hours after infusion of TPO expanded cells. Thus, entrapment of MSC adhered to TPO expanded progenitor cells in the lung does not seem to be an explanation for non-engraftment.

TPO expanded CD34+ cells migrate better *in vitro* than fresh CD34+ cells or fresch CD34+ cells with MSC. Whether this improved *in vitro* migration also translates into better *in vivo* homing is not definite. A previously conducted homing study with ^{99m}Tc-tropolone-labeled cells [13] which showed a similar proportion (approximately 0.5%) of the fresh CD34+ or TPO expanded CD34+ cells that were transplanted homed towards the femur. In these experiments fresh CD34+ cells or their expanded equivalent were transplanted, i.e. higher numbers of cells were transplanted in the TPO expanded group. The absolute number of homing cells is therefore higher in the group that was transplanted with TPO expanded cells. However, the experiment did not discern the proportion of each of the three major subpopulations found after TPO expansion that homed to the BM. Although more cells homed to the BM in the mice that received TPO expanded cells and the largest population of these cells (CD61+) migrated less in vitro than the two other populations (CD34+ and CD34-CD61- cells (48.7±8.9% and 37.0±4.1% respectively vs. 15.4±2.1% for CD61+ cells), we cannot with certainty conclude that TPO expanded CD34+ cells home better to the BM than fresh CD34+ cells. Despite the higher migration rate of TPO expanded cells in vitro, the lack of improvement in engraftment suggests that other characteristics such as TPO induced changes to their immature characteristics, their stemness and thus long term engraftment potential, are more important in this respect.

In this paper we additionally focused on the added engraftment effect by MSC co-transplantation with TPO expanded cells. It is likely that MSC do not improve the homing capacity of TPO expanded CD34+ cells since they do not affect migration in vitro and transplantation of MSC four hours after the transplantation of CD34+ cells does not alter the engraftment of the cells.

Superior migration of TPO expanded cells over the fibronectin coated transwell plates despite a lack of difference in CXCR4 expression might be explained by their higher expression of adhesion molecules for which fibronectin is a ligand, such as CD49d and CD49e [52], Blocking of CD49d and CD49e with antibodies reduces the migration of CD34+

cells in fibronectin coated plates [53]. In the migration experiments, short term incubation with MSC did not lead to a change in the expression of adhesion markers on CD34+ cells. The increased migration and enhanced engraftment of non expanded CD34+ cells in the presence of MSC can therefore not be explained by a change in adhesion molecule expression. Strikingly, expansion with CD34+ cells, either with MSC or TPO might change the longer term the engraftment capacity of the cells. This was suggested by studies showing that after transplantation of one CB unit expanded by culture ex vivo with MSC or TPO and one unmanipulated unit, the unmanipulated CB graft establishes long-term engraftment [42,54].

In conclusion, co-transplantation of MSC can improve engraftment after 6 weeks while TPO expansion improves early platelet recovery. MSC co-transplantation combined with TPO expansion at best combines but gives no synergy on either of these effects. However, the combination of high cell numbers introduces the risk of non- engraftment. More precise characterization of these non engrafting events will be essential to combine these approaches.

References

1. Ballen KK, Gluckman E, Broxmeyer HE. Umbilical cord blood transplantation: the first 25 years and beyond. Blood 2013.
2. Ballen KK, Koreth J, Chen YB et al. Selection of optimal alternative graft source: mismatched unrelated donor, umbilical cord blood, or haploidentical transplant. Blood 2012;**119**: 1972-80.
3. Fuchs E, O'Donnell PV, Brunstein CG. Alternative transplant donor sources: is there any consensus? Curr Opin Oncol 2013;**25**: 173-9.
4. Watt SM. Article 04238. In: Caplan M, ed. Umbilical Cord Blood Banking. Reference Module in Biomedical Sciences. Kidlington, UK: Elsevier Press, 2014.
5. Brunstein CG, Gutman JA, Weisdorf DJ et al. Allogeneic hematopoietic cell transplantation for hematologic malignancy: relative risks and benefits of double umbilical cord blood. Blood 2010;**116**: 4693-9.
6. Eapen M, Rubinstein P, Zhang M-J et al. Outcomes of transplantation of unrelated donor umbilical cord blood and bone marrow in children with acute leukaemia: a comparison study. The Lancet 2007;**369**: 1947-54.
7. Rocha V, Labopin M, Sanz G et al. Transplants of umbilical-cord blood or bone marrow from unrelated donors in adults with acute leukemia. New England Journal of Medicine 2004;**351**: 2276-85.
8. Danby R, Rocha V. Current Strategies to Improve Engraftment in Cord Blood Transplantation. J Stem Cell Res Ther 2014;**4**: 2.
9. Kelly S, Sola C, De Lima M, Shpall E. Ex vivo expansion of cord blood. Bone marrow transplantation 2009;**44**: 673-81.
10. Tung S, Parmar S, Robinson S et al. Ex vivo expansion of umbilical cord blood for transplantation. Best Practice & Research Clinical Haematology 2010;**23**: 245-57.
11. Mattia G, Milazzo L, Vulcano F et al. Long–term platelet production assessed in NOD/SCID mice injected with cord blood CD34+ cells, thrombopoietin–amplified in clinical grade serum–free culture. Experimental hematology 2008;**36**: 244-52.
12. Schipper LF, Brand A, Fibbe WE, Van Hensbergen Y. Functional Characterization of TPO-Expanded CD34+ Cord Blood Cells Identifies CD34− CD61− Cells as Platelet-Producing Cells Early After Transplantation in NOD/SCID Mice and rCD34+ Cells as CAFC Colony-Forming Cells. Stem Cells 2012;**30**: 988-96.
13. van Hensbergen Y, Schipper LF, Brand A et al. Ex vivo culture of human CD34+ cord blood cells with thrombopoietin (TPO) accelerates platelet engraftment in a NOD/SCID mouse model. Experimental hematology 2006;**34**: 943-50.
14. Dominici M, Le Blanc K, Mueller I et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;**8**: 315-7.
15. Caplan AI. Mesenchymal stem cells. Journal of orthopaedic research 1991;**9**: 641-50.
16. Chamberlain G, Fox J, Ashton B, Middleton J. Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. Stem cells 2007;**25**: 2739-49.
17. Campagnoli C, Roberts IA, Kumar S et al. Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. Blood 2001;**98**: 2396-402.
18. Noort WA, Kruisselbrink AB, Kruger M et al. Mesenchymal stem cells promote engraftment of human umbilical cord blood–derived CD34+ cells in NOD/SCID mice. Experimental hematology 2002;**30**: 870-8.
19. Nauta AJ, Fibbe WE. Immunomodulatory properties of mesenchymal stromal cells. Blood 2007;**110**: 3499-506.
20. Duijvestein M, Vos ACW, Roelofs H et al. Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. Gut 2010;**59**: 1662-9.
21. Sun L, Wang D, Liang J et al. Umbilical cord mesenchymal stem cell transplantation in severe and refractory systemic lupus erythematosus. Arthritis & Rheumatism 2010;**62**: 2467-75.
22. Christopeit M, Schendel M, Föll J et al. Marked improvement of severe progressive systemic sclerosis after transplantation of mesenchymal stem cells from an allogeneic haploidentical-related donor mediated by ligation of CD137L. Leukemia 2007;**22**: 1062-4.
23. Kebriaei P, Isola L, Bahceci E et al. Adult human mesenchymal stem cells added to corticosteroid therapy for the treatment of acute graft-versus-host disease. Biology of Blood and Marrow Transplantation 2009;**15**: 804-11.
24. Le Blanc K, Frassoni F, Ball L et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. The Lancet 2008;**371**: 1579-86.
25. Lucchini G, Introna M, Dander E et al. Platelet-lysate-expanded mesenchymal stromal cells as a salvage therapy for severe resistant graft-versus-host disease in a pediatric population. Biology of blood and marrow transplantation 2010;**16**: 1293-301.

26. Pérez-Simon JA, Lopez-Villar O, Andreu EJ et al. Mesenchymal stem cells expanded in vitro with human serum for the treatment of acute and chronic graft-versus-host disease: results of a phase I/II clinical trial. haematologica 2011;**96**: 1072-6.
27. Prasad VK, Lucas KG, Kleiner GI et al. Efficacy and safety of ex vivo cultured adult human mesenchymal stem cells (Prochymal™) in pediatric patients with severe refractory acute graft-versus-host disease in a compassionate use study. Biology of Blood and Marrow Transplantation 2011;**17**: 534-41.
28. Ringden O, Le Blanc K. Mesenchymal stem cells for treatment of acute and chronic graft-versus-host disease, tissue toxicity and hemorrhages. Best practice & research Clinical haematology 2011;**24**: 65-72.
29. Baron F, Lechanteur C, Willems E et al. Cotransplantation of mesenchymal stem cells might prevent death from graft-versus-host disease (GVHD) without abrogating graft-versus-tumor effects after HLA-mismatched allogeneic transplantation following nonmyeloablative conditioning. Biology of blood and marrow transplantation 2010;**16**: 838-47.
30. Kuzmina LA, Petinati NA, Parovichnikova EN et al. Multipotent mesenchymal stromal cells for the prophylaxis of acute graft-versus-host disease—a phase II study. Stem cells international 2011;**2012**.
31. Friedman R, Betancur M, Boissel L et al. Umbilical cord mesenchymal stem cells: adjuvants for human cell transplantation. Biology of Blood and Marrow Transplantation 2007;**13**: 1477-86.
32. Hiwase SD, Dyson PG, To LB, Lewis ID. Cotransplantation of placental mesenchymal stromal cells enhances single and double cord blood engraftment in nonobese diabetic/severe combined immune deficient mice. Stem Cells 2009;**27**: 2293-300.
33. in 't Anker PS, Noort WA, Kruisselbrink AB et al. Nonexpanded primary lung and bone marrow–derived mesenchymal cells promote the engraftment of umbilical cord blood–derived CD34+ cells in NOD/SCID mice. Experimental hematology 2003;**31**: 881-9.
34. Kim D-W, Chung Y-J, Kim T-G, Kim Y-L, Oh I-H. Cotransplantation of third-party mesenchymal stromal cells can alleviate single-donor predominance and increase engraftment from double cord transplantation. Blood 2004;**103**: 1941-8.
35. Masuda S, Ageyama N, Shibata H et al. Cotransplantation with MSCs improves engraftment of HSCs after autologous intra-bone marrow transplantation in nonhuman primates. Experimental hematology 2009;**37**: 1250-7. e1.
36. Ball LM, Bernardo ME, Roelofs H et al. Cotransplantation of ex vivo–expanded mesenchymal stem cells accelerates lymphocyte recovery and may reduce the risk of graft failure in haploidentical hematopoietic stem-cell transplantation. Blood 2007;**110**: 2764-7.
37. Macmillan M, Blazar B, DeFor T, Wagner J. Transplantation of ex-vivo culture-expanded parental haploidentical mesenchymal stem cells to promote engraftment in pediatric recipients of unrelated donor umbilical cord blood: results of a phase I–II clinical trial. Bone marrow transplantation 2008;**43**: 447-54.
38. Lee S, Lee M, Yoo K et al. Co-transplantation of third-party umbilical cord blood-derived MSCs promotes engraftment in children undergoing unrelated umbilical cord blood transplantation. Bone marrow transplantation 2013;**48**: 1040-5.
39. Wu K-H, Sheu J-N, Wu H-P et al. Cotransplantation of Umbilical Cord–Derived Mesenchymal Stem Cells Promote Hematopoietic Engraftment in Cord Blood Transplantation: A Pilot Study. Transplantation 2013;**95**: 773-7.
40. Chen Y-B, Aldridge J, Kim HT et al. Reduced-intensity conditioning stem cell transplantation: comparison of double umbilical cord blood and unrelated donor grafts. Biology of Blood and Marrow Transplantation 2012;**18**: 805-12.
41. Schipper LF, Van Hensbergen Y, Fibbe WE, Brand A. A sensitive quantitative single-platform flow cytometry protocol to measure human platelets in mouse peripheral blood. Transfusion 2007;**47**: 2305-14.
42. van der Garde M, van Hensbergen Y, Brand A et al. Thrombopoietin treatment of one graft in a double cord blood transplant provides early platelet recovery while contributing to long term engraftment in NSG mice. Stem cells and development 2014.
43. Peled T, Shoham H, Aschengrau D et al. Nicotinamide, a SIRT1 inhibitor, inhibits differentiation and facilitates expansion of hematopoietic progenitor cells with enhanced bone marrow homing and engraftment. Experimental hematology 2012;**40**: 342-55. e1.
44. Quesenberry PJ, Becker PS. Stem cell homing: rolling, crawling, and nesting. Proceedings of the National Academy of Sciences 1998;**95**: 15155-7.
45. Vermeulen M, Le Pesteur F, Gagnerault M-C et al. Role of adhesion molecules in the homing and mobilization of murine hematopoietic stem and progenitor cells. Blood 1998;**92**: 894-900.
46. Voermans C, Rood P, Hordijk P et al. Adhesion molecules involved in transendothelial migration of human hematopoietic progenitor cells. Stem Cells 2000;**18**: 435-43.
47. Schipper LF, Brand A, Reniers N et al. Differential maturation of megakaryocyte progenitor cells from cord blood and mobilized peripheral blood. Experimental hematology 2003;**31**: 324-30.

48. Angelopoulou M, Novelli E, Grove JE et al. Cotransplantation of human mesenchymal stem cells enhances human myelopoiesis and megakaryocytopoiesis in NOD/SCID mice. Experimental hematology 2003;**31**: 413-20.
49. Kang SK, Shin IS, Ko MS et al. Journey of mesenchymal stem cells for homing: strategies to enhance efficacy and safety of stem cell therapy. Stem cells international 2012;**2012**.
50. Schrepfer S, Deuse T, Reichenspurner H et al. Stem cell transplantation: the lung barrier Transplantation proceedings: Elsevier, 2007:573-6.
51. Watt SM, Gullo F, van der Garde M et al. The angiogenic properties of mesenchymal stem/stromal cells and their therapeutic potential. British medical bulletin 2013: ldt031.
52. Turner ML, Masek LC, Hardy CL et al. Comparative adhesion of human haemopoietic cell lines to extracellular matrix components, bone marrow stromal and endothelial cultures. British journal of haematology 1998;**100**: 112-22.
53. Voermans C, Gerritsen WR, von dem Borne AE, van der Schoot CE. Increased migration of cord blood-derived CD34+ cells, as compared to bone marrow and mobilized peripheral blood CD34+ cells across uncoated or fibronectin-coated filters. Experimental hematology 1999;**27**: 1806-14.
54. de Lima M, McNiece I, Robinson SN et al. Cord-blood engraftment with ex vivo mesenchymal-cell coculture. New England Journal of Medicine 2012;**367**: 2305-15.

Chapter 5

Dual use of cord blood for both hematopoietic stem cell transplantation and other therapies

Mark van der Garde, Anneke Brand, Manon Slot, Ben Meijer and Yvette van Hensbergen

Transfusion
submitted

Abstract

BACKGROUND: Cord blood (CB) contains CD45 negative cells that have clinical potential cellular therapy. Separate banking of these CD45- cells could extend the use of CB beyond hematopoietic stem cell transplantation (HST). The 'dual' use of CB is only possible if removal of the CD45- cells does not compromise the hematopoietic quality of the graft. We analyzed the influence of CD45- cells on the hematopoietic properties of a CB graft.

STUDY DESIGN AND METHODS: CB mononuclear cells were separated into CD45+ and CD45- cells. Both fractions were analyzed for marker expression, the influence on immunological responses, the capacity to generate hematopoietic colonies and the influence on the engraftment of CB CD34+ in NOD SCID mice.

RESULTS: CD45- cells neither stimulated an immunological response of PBMC nor affected the response to CB CD45+ cells and were not able to form hematopoietic colonies. Co-transplantation of CD45- cells did not alter the engraftment of CB CD34+ cells in NOD SCID mice, whereas CD45+ cells improved the engraftment.

CONCLUSION: Removal of CD45- cells does not compromise the hematopoietic quality of cord blood *in vitro* and in the *in vivo* engraftment in NOD/SCID mice. This would allow for the separate banking of these cells and their use for other clinical applications.

Introduction

Cord blood (CB) is currently banked in public banks for the purpose of unrelated allogeneic hematopoietic stem cell transplantation (HST) for patients lacking a suitable related or unrelated donor. Besides the public banking of CB, private banks store donor-directed CB to provide engineered regenerative or immunomodulatory cells when needed in the future. Indeed in addition to hematopoietic stem cells (HSC), CB contains cell populations with non-hematological clinical potential such as mesenchymal stem/stromal cells (MSC) and endothelial cells (EC).[1,2] EC and MSC are used for regenerative cellular therapy such as vascular repair and wound healing, [3,4] or as therapy for immune related diseases. [5] In HST, MSC are used for the treatment or prophylaxis of graft versus host disease (GvHD)[6] and the co-transplantation of MSC and CB HSC is investigated for the enhancement of engraftment.[7]
Isolation of the non-hematopoietic cells from the hematopoietic fraction and separate cryopreservation of these fractions could expand the use of CB to both HST and other therapies. Before 'dual' banking can be considered it must be ensured that the removal of non-hematopoietic cells leaves the hematopoietic quality of the CB graft uncompromised.
Most culture protocols for the culture of EPC or MSC from CB involves the overnight culture of the whole mononuclear cell fraction (MNC), after which the non-adherent cells are removed. However, this does not allow for the dual use of the unit since the hematopoietic cells are discarded in this process. Separation of the two fractions must therefore be done on markers that identify both MSC and EPC or by depletion from the hematopoietic cells using the pan hematopoietic marker CD45, or a set of hematopoietic lineage markers. Endothelial cells can be identified by the expression of vascular endothelial growth factor receptor 2 (VEGF-R2) and VE Cadherin (CD144) and have been identified in fresh cord blood, but the frequency of these cells is very low and highly variable between donors. [8] MSC can be identified by a combination of distinct makers and properties set by the international society for cellular therapy (ISCT).[9] Besides the ability to adhere to plastic and to differentiate into 3 mesodermal linegaes, MSC should express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14, CD19 and HLA-DR. Efforts to identify MSC like cells in CB based on marker expression alone have shown cell types that express certain markers, but confirmation by culture experiments is still needed to confidently state that these cells are indeed MSC.[10] Both MSC and EPC are derived from the non-hematopoietic CD45 negative fraction of CB.[8,11] Separating the CD45 negative cells from the hematopoietic fraction might therefore preserve the possibility to culture both cell types.
In this study we have investigated the influence of CD45- cells on the engraftment of CB cells. For this purpose we have determined the proportion of CD45- cells among CB mononuclear cell suspensions and analyzed these cells for the expression of hematopoietic and non-hematopoietic markers. In addition, we isolated the CD45- cells from the CD45+ cells and analyzed their capacity to grow hematopoietic and non-hematopoietic cells *in vitro*. The influence of the CD45- fraction on the immunological responses of CB CD45+ cells and adult PBMC was analyzed with mixed leukocyte reactions. To investigate the influence of CD45- cells on transplantation *in vivo*, we co-transplanted CD45- or CD45+ cells with CB CD34+ cells and analyzed the engraftment in the bone marrow six weeks after transplantation.

Materials & methods

Collection and processing of umbilical cord blood. Umbilical cord blood (CB) was drawn from the umbilical vein from newborns and collected in Citrate Phosphate Dextrose Adenine-1 (Macopharma, Utrecht, The Netherlands) after written informed consent from the mother and approval by the ethical committee of the Leiden University Medical Center (LUMC). Mononuclear Cell suspensions (MNC) were prepared from whole cord blood by density gradient separation with Ficoll (1.077 g/ml, pharmacy LUMC). CD34+ or $CD45^+$ and $CD45^-$ cells were subsequently separated by Magnetic Activated Cell Sorting (MACS, Miltenyi, Bergisch Gladbach, Germany).

Phenotypic characterization. Before analysis all samples were lysed with IO3 test RBC lysis solution (Beckman Coulter, Woerden, the Netherlands). Samples were measured on a Beckman Coulter Cytomics FC500 flow cytometer. CD45 FITC, CD45 ECD and CD45-PC7 conjugated monoclonal antibodies (Beckman Coulter, Woerden, The Netherlands) were used to discriminate $CD45^-$ and $CD45^+$ cells. The fractions were further analyzed for the expression of CD2PE, CD3PE, CD4PE, CD8PE, CD14PE, CD15PE, CD19PE, CD33PE, CD56PE, CD34PE, CD61PC7, CD90PC5, CD31FITC, CD146FITC (Beckman Coulter), CD133 PE (Miltenyi), GpA PE (Dako, Glostrup, Denmark), HLA-ABC PE and HLA-DR PE (BD Biosciences, Breda, the Netherlands).

Mixed Lymphocyte Reactions. Selected HLA class I antigens of both CB cells and PBMC from healthy donors were determined by flow cytometry with custom made human allele specific monoclonal HLA class I antibodies. Subsequently, an HLA class I mismatched pair consisting of one CB donor and one healthy donor was selected based on HLA class I antigen disparity.[12] The response of PBMC to CB CD45+ cells and the immunomodulatory capacity of the CB CD45- cells was determined by culturing 10^5 PBMC with 10^5 irradiated CB $CD45^+$ cells either with and without different concentrations of CB CD45- cells ($2.5*10^4$, $5*10^4$ or $1*10^5$). Similarly, we analyzed the immunomodulatory effect of CB CD45- cells on the response of CB CD45+ cells against PBMC (10^5 CB CD45+ cells cultured with 10^5 irradiated PBMC with and without the addition of 0, $2.5*10^4$, $5*10^4$ or 10^5 CB $CD45^-$ cells). The cells were cultured for 5 days in medium (IMDM, Life Tech. with 10% human serum, Sanquin) at 37˚C and 5% CO_2, after which immune function was measured directly by quantification of intracellular ATP levels of T-cells with an ATP-luciferase assay.[13] CD3 cells were isolated with Dynal beads (Invitrogen, Breda, the Netherlands) and lysed. Luciferase (Life Tech.) was added to the well and luminesce was measured with a luminometer (Berthold technologies, Bad Wilbad, Germany). The ATP produced by the T cells of each of the conditions was averaged (4 wells/condition) and divided by the average ATP content of the medium control in order to obtain a relative stimulation index (SI) that was used to compare different experiments.

Colony formation experiments. MNC, CD45- and CD45+ fractions were analyzed for the capacity to grow hematopoietic colony forming units (CFU) and endothelial colonies. CFU assays were performed as described before.[14] For the culture of endothelial cells the isolated fractions were cultured in fibronectin coated flaks overnight in EGM-2 (Lonza, Verviers, Belgium) after which non-adherent cells were removed. Adherent cells were grown to confluence and analyzed for the expression of CD31 and CD146 with flow cytometry.

Analysis of the influence of engraftment of CB CD45$^-$ and CD45+ cells. All animal experiments were conducted with ethical approval of the Experimental Animal Committee of the LUMC. CB CD34$^+$ cells, CD45$^+$ and CD45$^-$ cells were isolated as described above. Sublethally irradiated NOD SCID mice were transplanted with either 10^5 CD34$^+$ cells alone, 10^5 CD34$^+$ cells+ 10^6 CD45$^+$ cells, or 10^5 CD34$^+$ cells+10^6 CD45- cells. After 6 weeks the mice were sacrificed and the femoral bone marrow, blood and spleen was harvested and analyzed for the presence of human and mouse CD45$^+$ cells (human CD45-PC7 (BC), murine CD45-PE (BD)) and human CD34$^+$, CD33$^+$, CD19$^+$ cells (CD34PC5, CD19PE, CD33FITC (BC)).

Statistics. Statistics were done with SPSS version 20 using a Mann Whitney test. Differences were considered to be significant when $p<0.05$.

Results

Composition of the CD45- fraction

From eight CB units the MNC were analyzed for the expression of hematopoietic, stem cell and non-hematopoietic markers. The suspensions contained on average 29±3% CD45- cells of which the majority expressed red cell marker glycophorin A (GpA) or platelet/megakaryocyte marker CD61 (Table 1, 27.9±8.5% and 46.9±11.2% respectively). Cells expressing stem cell and/or non-hematopoietic markers such as CD34, CD133, CD90, CD146, CD31 and CD271 were only found in small quantities (Table 1). Approximately 1% of the CD45- cells expressed lineage markers other than GpA or CD61. Expression of HLA Class I was similar between the CD45- and CD45+ fractions (31.6±5.9% vs 35.0±5.8% respectively), but only few CD45- cells expressed HLA-DR (2.3±1.1%) compared to CD45+ cells (36.0±2.1%).

Table 1: *Expression levels of hematopoietic, non-hematopoietic and immunological markers by cells of the CD45+ fraction and the CD45- fraction after density gradient separation of whole CB and MACS isolation of the fractions. The majority of CD45- cells were GpA or CD61 positive. Part of the CD45- cells expressed HLA class I but HLA class II expression was low compared to CD45+ cells. Expression of non-hematopoietic and stem cells markers was only found in small quantities.*

percentage in CD45neg or CD45pos fraction	*type of marker*	**CD45 negative fraction (28.54±3.08)**	**CD45 postive fraction (71.46±3.08)**
CD61+	***hematopoietic***	46.92 (±11.24)	26.02 (±5.30)
GPA		27.93 (±8.52)	4.17 (±0.80)
Lineage markers		0.96 (±0.41)	91.58 (±1.76)
CD38		0.03 (±0.01)	10.75 (±1.95)
CD80	***immunological***	0.07 (±0.02)	0.07 (±0.03)
HLA-ABC		31.64 (±5.94)	34.95 (±5.77)
HLA-DR		2.34 (±1.16)	35.98 (±2.12)
CD34	***stem cell***	0.47 (±0.14)	0.93 (±0.25)
CD90	***stem cell / mesenchymal***	9.78 (±4.16)	4.58 (±1.84)
CD271	***mesenchyhmal***	0.14 (±0.02)	0.29 (±0.08)
CD146	***endothelial***	0.11 (±0.05)	0.08 (±0.02)
CD31	***hematopoietic / endothelial***	3.78 (±2.03)	22.87 (±3.76)

The immunomodulatory capacity of CB CD45- cells on CD45+ cells. Mixed lymphocyte reactions were performed to determine the immunomodulatory capacity of $CD45^-$ cells. $CD45^-$ cells did not induce a proliferative response when compared to CD45+ cells (Figure 1A, mean SI 1.0±0.1 vs. 2.1±0.5, $p<0.05$). The addition of different concentrations of $CD45^-$ cells did not alter the proliferative response of PBMC stimulated by mismatched CB CD45+ cells ($p>0.9$). . The responder capacity of CD45+ CB cells upon stimulation with PBMC was low as compared to adult cells, but we did not observe an immunomodulatory effect of CB CD45- cells on the response of CB CD45+ cells to mismatched PBMC (Figure 1)

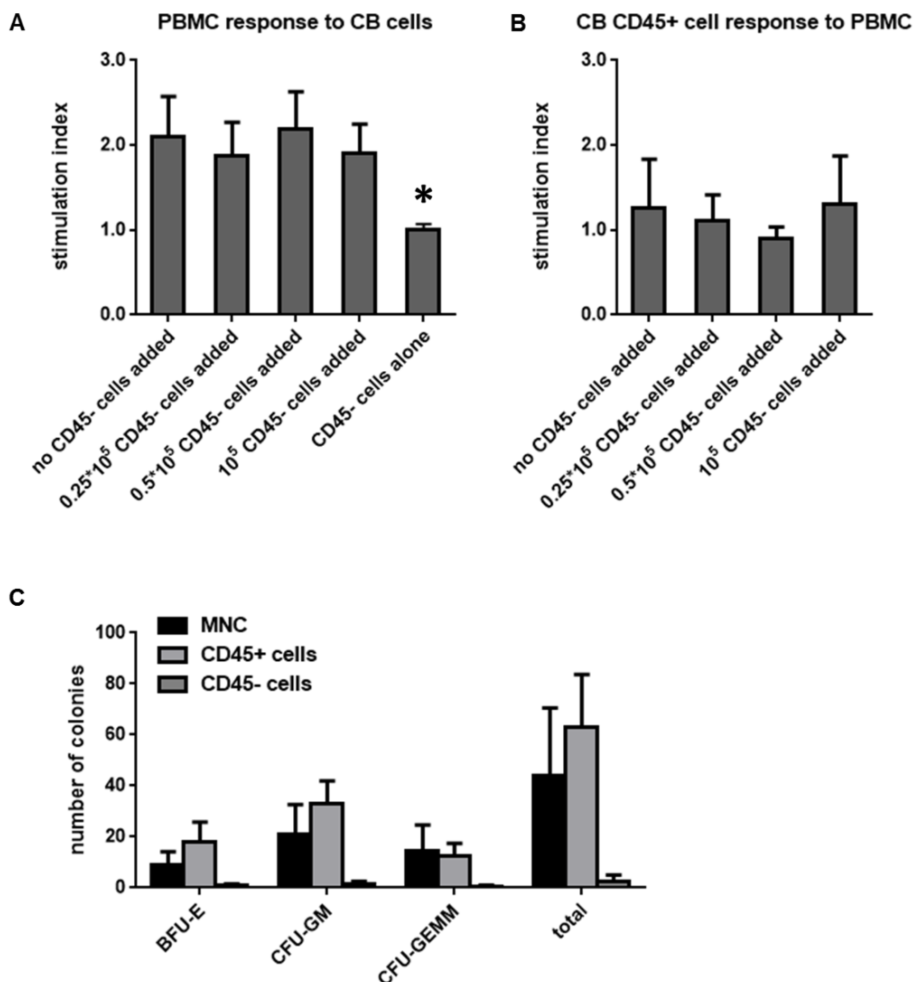
CD45- cells cannot generate hematopoietic colonies but are able to form endothelial cells. To investigate the capacity of CD45- cells to grow hematopoietic colonies, MNC, CD45+ and CD45- cells from 4 different donors were cultured in Methocult for 2 weeks (Figure 1C). CD45- cells from only one donor were able to grow a few colonies (3 BFU-E, 5 CFU-GM and 2 CFU-GEMM), but this number was markedly lower than the number of colonies grown from the CD45+ cells from this donor (27 BFU-E, 56 CFU-GM and 27 CFU-GEMM). From 7 donors the capacity to grow endothelial cells (EC) from CD45+ and CD45- cells was investigated. All 7 CD45- fractions were able to grow EC as confirmed by the expression of CD31 and CD146. In contrast, EC could only be grown from one of the CD45+ fractions (data not shown).

The influence of $CD45^-$ cells on the engraftment of CB $CD34^+$ cells in NOD-SCID mice. A total of 36 mice were transplanted with cells from 4 CB units. The mice were separated into three groups (3 mice/group), which were transplanted with $CD34^+$ cells alone, $CD34^+$ cells + $CD45^+$, or $CD34^+$ cells + $CD45^-$ cells. Figure 2A-C shows the percentage of human CD45 cells (hCD45) in the bone marrow (BM), blood and spleen of the mice 6 weeks after transplantation. The mean percentage of hCD45 in the BM was higher in the group that received CD34+ cells + $CD45^+$ cells (33.6±9.2% human CD45+ cells) as compared to mice transplanted with CD34+ cells alone (23.2±7.9%) or mice that received CD34+ cells + $CD45^-$ cells (20.1±6.2%). There were no differences found in the distribution of human CD19, CD33 and CD34 cells in the BM among the different groups (figure 2D).

***Figure 1: In vitro characteristics of the CD45- cells. A: Influence of CD45- on the response of PBMC to CD45+ cells**. 10^5 irradiated CB CD45+ cells were added to PBMC and cultured for 5 days. To analyze the influence of CD45- cells, different numbers of CD45- cells were added to the CD45+ cells and compared with the response of PBMC to CD45+ cells alone. T cell response was measured by quantification of intracellular ATP levels using an ATP/luciferase assay. The stimulation index is calculated by dividing the ATP produced by the T cells in the experimental conditions by the ATP produced by the T cells in the medium controls. Addition of CD45- cells to CD45+ cells did not alter the immunological response of PBMC and addition of CD45- did not elicit any response (*$p<0.05$). These results suggest that CD45- cells do not influence the immunological properties of a cord blood graft in vitro. **B: Influence of CD45- cells on the response of CB CD45+ to PBMC.** 10^5 irradiated PBMC were added to CB CD45+ cells and cultured for 5 days. To analyze the influence of CD45- cells, different numbers of irradiated CD45- cells were added to the PBMC cells and compared with the response of CB CD45+ cells to PBMC alone. T cell proliferation was measured with a Luciferase/Luciferin assay, the stimulation index is calculated by dividing the ATP produced in autologous control conditions by the ATP produced by the experimental conditions. Addition of CD45- cells to PBMC cells did not alter the immunological response of CB CD45+ cells.**C: Number of myeloid colonies cultured from MNC, purified CD45+ cells or purified CD45- cells.** Part of the MNC fraction was separated into CD45+ and CD45- cells. All fractions were analyzed for their capacity to generate colony forming units (CFU) in a standard Methocult culture. Shown is the mean±SEM number of CFU formed by each fraction of 4 separate experiments. Cells from the CD45- fraction were scarcely able to generate colonies and CD45+ cells generated more CFU than equal numbers of MNC (consisting of CD45+ and CD45- cells). These results suggest that CD45- cells do not significantly contribute to the formation of hematopoietic cells in vitro.*

Discussion

The majority of the CD45- fraction of CB consists of GpA+ cells and of CD61+ cells. GpA expressing cells partly consist of NRBC resistant to ammonia based red blood cell (RBC) lysis buffer and the remainder of the GpA+ cells consists of larger RBC that are not properly lysed. The number of NRBC in the CB HST grafts has been correlated to engraftment, but this was explained by a correlation between the number of NRBC and the number of HSC and not causal of direct support of NRBC to engraftment.[15] The CD61+ cells in the CD45- fraction of CB are likely mature megakaryocytes that have lost CD45+ expression or large platelet complexes. Although these cells do not have a nucleus, they do appear in the MNC fraction when measured with flow cytometry since their forward and sideward scatter fluorescence is similar to that of mononuclear cells. CD61+ cells do not contribute to early platelet recovery after transplantation as shown previously.[16]



\

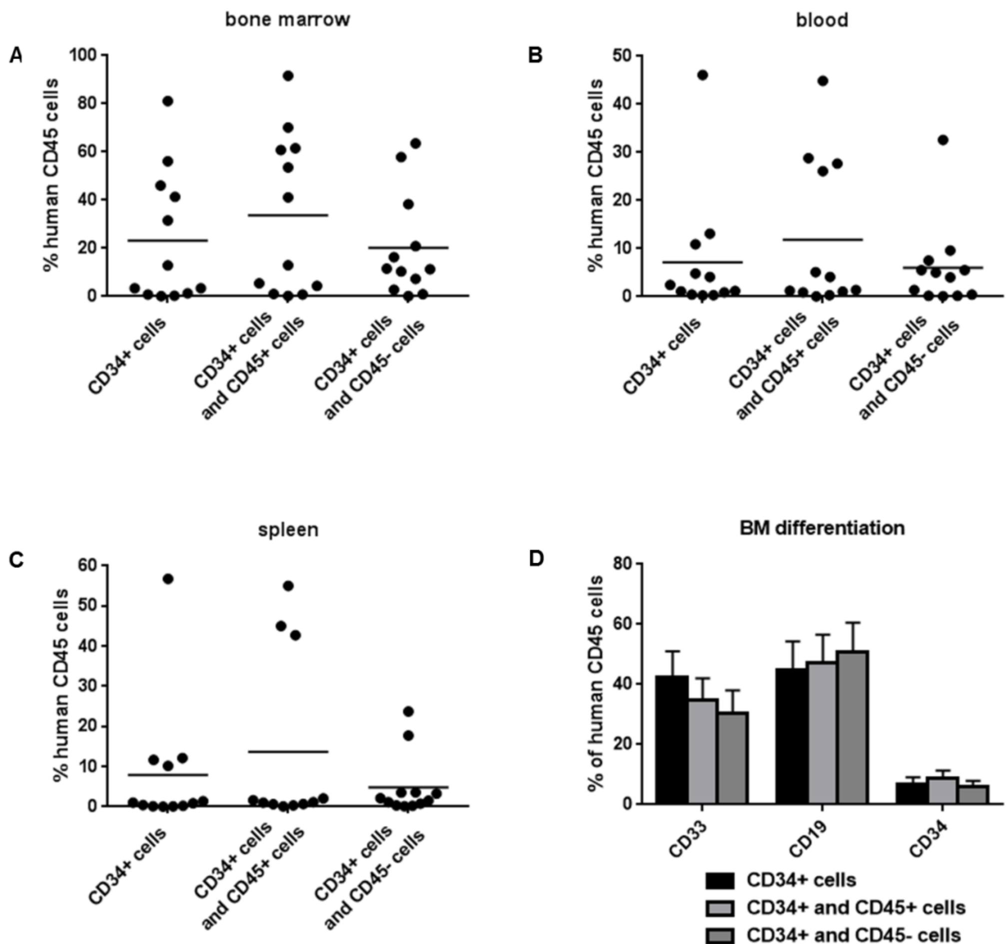


Figure 2: Influence of CD45+ and CD45- cells on the engraftment of CB CD34+ cells. *CD34+, CD45- and CD45+ cells we isolated from the MNC fraction of CB and transplanted into sublethally irradiated NOD SCID mice.* ***A, B, C:*** *Percentage of human CD45 cells of the total number of CD45 cells in the bone marrow, blood and spleen of NOD-SCID mice 6 weeks after transplantation. Only the co-transplantation of CD45+ cells increased the mean percentage of human CD45+ cells in the BM of the mice.* ***D:*** *Percentage of CD19, CD33 and CD34 of the human CD45 cells in the bone marrow of NOD-SCID mice, 6 weeks after transplantation. No significant differences were found in the distribution of the different hematopoietic cells in the bone marrow. These results suggest that CD45- cells do not influence the engraftment capacity of CB CD34+ cells.*

The expression of HLA class I antigen in the CD45- fraction can probably be explained by these mature megakaryocytes and platelets in the CD45- fraction that have lost CD45 expression and by NRBC expressing HLA.[17] The immune cells in a CB graft provide a graft versus leukemia (GvL) and can cause graft versus host disease (GvHD) and both of these effects could be altered by the CD45- cells in the graft. However, as expected by the absence of class II bearing cells, isolated CD45- cells do not elicit a T cell response of adult PBMC and addition of CD45- cells did not alter the response of PBMC to CB CD45+ cells.

Furthermore, the addition of CD45$^-$ cells to isolated CD45$^+$ cells did not alter the (already weak) responder capacity against the PBMC. The number of myeloid colonies that can be formed in culture is a predictor of neutrophil and platelet engraftment after CB HST.[18] In this study, the CD45- cells of only one of five donors was able to form a small number of hematopoietic colonies, and this number was 10 fold lower that the number of colonies formed by the CD45+ cells of the same donor. All other CD45- fractions did not show any growth of hematopoietic qualities, suggesting that the colonies formed by this particular donor might be caused by contamination with CD45+ cells. This suggests that all CD45- cells do not have hematopoietic progenitor potential. Furthermore, only CD45- cells were able to form endothelial colonies, showing that the non-hematopoietic potential of CB is preserved after isolation of the CD45- fraction.
In mouse models, co-transplantation endothelial cells and MSC have shown to improve the engraftment of CB CD34+ cells.[19,20] Removal of the progenitors of these cells might negatively influence engraftment. However, in such studies, the cultured cells and transfused cell numbers that are used are much higher than in un-cultured CB. Co-transplantation of isolated CD45- fraction did not enhance engraftment of CB CD34$^+$ cells, whereas co-transplantation of CD45+ cells increased engraftment, showing that removal of the CD45- fraction does not impair the engraftment of CD34+ cells. No differences were seen in the percentage of myeloid, lymphoid or stem cells in the bone marrow, regardless of whether the CD34+ cells were transplanted with CD45+ or CD45- cells or without additional cells, suggesting that removal of CD45- cells does not skew the differentiation of cells towards a specific lineage.
In conclusion, CD45- cells do not seem to influence the immunological properties of a graft and they are not needed for proper engraftment after transplantation in the NOD SCID model. This would allow for removal of the CD45- fraction of a CB unit and the use of this fraction for other purposes. The dual use of CB could expand the applicability of CB and thereby may (in the future) compensate for the costs of the collection and storage of CB HSC. Currently only 1% of the stored units have been used for transplantation. Dual banking could stimulate collection and storage in parallel with private banks, leaving the hematopoietic part for unrelated HST and the non-hematopoietic cells for future (autologous) regenerative therapy.

References

1. Ingram DA, Mead LE, Tanaka H et al. Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. Blood 2004;**104**: 2752-60.
2. Lee OK, Kuo TK, Chen W-M et al. Isolation of multipotent mesenchymal stem cells from umbilical cord blood. Blood 2004;**103**: 1669-75.
3. Au P, Daheron LM, Duda DG et al. Differential in vivo potential of endothelial progenitor cells from human umbilical cord blood and adult peripheral blood to form functional long-lasting vessels. Blood 2008;**111**: 1302-5.
4. Wu Y, Wang J, Scott PG, Tredget EE. Bone marrow-derived stem cells in wound healing: a review. Wound Repair and Regeneration 2007;**15**: S18-S26.
5. Sun L, Wang D, Liang J et al. Umbilical cord mesenchymal stem cell transplantation in severe and refractory systemic lupus erythematosus. Arthritis & Rheumatism 2010;**62**: 2467-75.
6. Ringden O, Le Blanc K. Mesenchymal stem cells for treatment of acute and chronic graft-versus-host disease, tissue toxicity and hemorrhages. Best practice & research Clinical haematology 2011;**24**: 65-72.
7. Le Blanc K, Samuelsson H, Gustafsson B et al. Transplantation of mesenchymal stem cells to enhance engraftment of hematopoietic stem cells. Leukemia 2007;**21**: 1733-8.
8. Smythe J, Fox A, Fisher N et al. Measuring angiogenic cytokines, circulating endothelial cells, and endothelial progenitor cells in peripheral blood and cord blood: VEGF and CXCL12 correlate with the number of circulating endothelial progenitor cells in peripheral blood. Tissue Engineering Part C: Methods 2008;**14**: 59-67.
9. Dominici M, Le Blanc K, Mueller I et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;**8**: 315-7.
10. Martins A, Paiva A, Morgado J et al. Quantification and immunophenotypic characterization of bone marrow and umbilical cord blood mesenchymal stem cells by multicolor flow cytometry. Transplantation proceedings 2009;**41**: 943-6.
11. Timmermans F, Van Hauwermeiren F, De Smedt M et al. Endothelial outgrowth cells are not derived from CD133+ cells or CD45+ hematopoietic precursors. Arteriosclerosis, thrombosis, and vascular biology 2007;**27**: 1572-9.
12. van Hensbergen Y, Mulder A, Cornelissen JJ, Brand A. Validation of human monoclonal HLA Class I antibodies to evaluate the kinetics of donor chimerism in different cell subsets after double-cord-blood transplantation in the NOD/SCID model. Transfusion 2013;**53**: 104-14.
13. Petty RD, Sutherland LA, Hunter EM, Cree IA. Comparison of MTT and ATP-based assays for the measurement of viable cell number. Journal of bioluminescence and chemiluminescence 1995;**10**: 29-34.
14. van der Garde M, van Hensbergen Y, Brand A et al. Thrombopoietin Treatment of One Graft in a Double Cord Blood Transplant Provides Early Platelet Recovery While Contributing to Long-Term Engraftment in NSG Mice. Stem cells and development 2014;**24**: 67-76.
15. Stevens CE, Gladstone J, Taylor PE et al. Placental/umbilical cord blood for unrelated-donor bone marrow reconstitution: relevance of nucleated red blood cells. Blood 2002;**100**: 2662-4.
16. Schipper LF, Brand A, Fibbe WE, Van Hensbergen Y. Functional Characterization of TPO-Expanded CD34+ Cord Blood Cells Identifies CD34− CD61− Cells as Platelet-Producing Cells Early After Transplantation in NOD/SCID Mice and rCD34+ Cells as CAFC Colony-Forming Cells. Stem Cells 2012;**30**: 988-96.
17. Robinson J, Sieff C, Delia D et al. Expression of cell-surface HLA-DR, HLA-ABC and glycophorin during erythroid differentiation. Nature 1981;**289**: 68-71.
18. Page KM, Zhang L, Mendizabal A et al. Total colony-forming units are a strong, independent predictor of neutrophil and platelet engraftment after unrelated umbilical cord blood transplantation: a single-center analysis of 435 cord blood transplants. Biology of Blood and Marrow Transplantation 2011;**17**: 1362-74.
19. Noort WA, Kruisselbrink AB, Kruger M et al. Mesenchymal stem cells promote engraftment of human umbilical cord blood–derived CD34+ cells in NOD/SCID mice. Experimental hematology 2002;**30**: 870-8.
20. Zeng L, Chen C, Song G et al. Infusion of endothelial progenitor cells accelerates hematopoietic and immune reconstitution, and ameliorates the graft-versus-host disease after hematopoietic stem cell transplantation. Cell biochemistry and biophysics 2012;**64**: 213-22.

General Discussion

25 Years of cord blood transplantation

Since the first cord blood transplantation (CBT) in 1989, over 30,000 CBT have been performed worldwide. Especially in the last 15 years there has been a considerable increase in cord blood transplantation (CBT). Given the minimum dose of cells per kg of recipient bodyweight that is needed for a successful outcome, CBT of a single cord blood unit was initially limited to children. The number of CBT performed in patients older than 16 years has steadily increased and in 2011 accounted for 54% of all CBT in the USA, although it must be noted that this only accounted for 6% off all allogeneic HST in adults. [1] According to the Center for International Blood and Marrow Transplant Research (CIBMTR), this increase can mostly be attributed to the steady increase of the number of banked CB units which makes it easier to find two (partially) matched units for a particular patient for double CBT (dCBT). The increase in CBT is reflected by the fact that research focusing on the improvement of CBT is still active and currently there are 418 ongoing clinical trials involving CBT. [2]

Despite the major contribution of CBT over the last 25 years, several problems need to be improved to make cord blood the best choice for unrelated donor HST. The most important problem in this respect is the higher chance of graft failure and delayed engraftment, which are both related to the low number of HSPC in a cord blood graft. Another problem is the relatively high cost; CBT is on average more expensive than matched unrelated bone marrow or peripheral blood stem cell (PBSC) HST. [3] The main cost drivers in this respect are laboratory costs such as tests, imaging, injections and radiation) and the number of inpatient days, both accounting for approximately 30% of the total cost. The time that the patient has to stay in hospital is directly related to delayed engraftment seen after CBT. Another aspect of cost is the high acquisition price of a cord blood unit, especially when the patient is transplanted with two cord blood units. [4] Because of these problems in recent years the increase in the number of CBT has stagnated (figure 1). A more efficient and or alternative use of banked CBs would be extremely important to reduce and compensate logistic costs while faster engraftment of a CB graft would reduce morbidity and mortality and lower the related patient treatment costs.

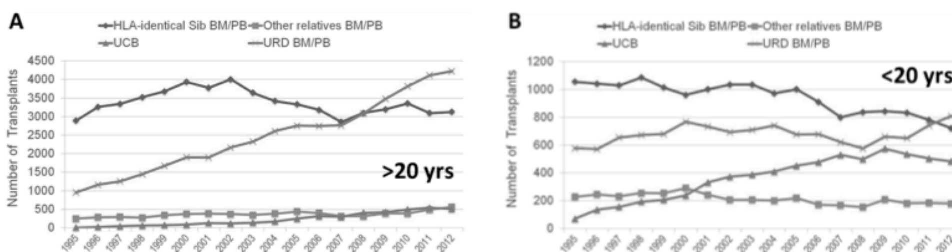


Figure 1: Number of allogeneic transplantations per donor source in centers that are registered with the CIBMRT for patients older than 20 years (A) and younger than 20 years (B). Figure adapted from the CIBMTR summary slides 2014 [5] (UCB: umbilical cord blood, BM: bone marrow, PB: mobilized peripheral blood stem cells, URD: unrelated donor)

This thesis focuses firstly on possibilities to improve the engraftment after CBT by the co-transplantation of mesenchymal stem cells (MSC) **(chapter 1)**, the in vitro expansion of CB **(chapters 2&3)** or a combination of both expansion and MSC co-transplantation **(chapter 4)**. Secondly, by investigating the use of CB for other cellular therapies without compromising the potential for HST a reduction of the cost for a CB product is **(chapter 5)**.

Co-transplantation of mesenchymal stem cells and CB to enhance the engraftment of HSC: practical considerations

For large-scale application of co-transplantation of MSC for HST, there is a need for an, of the shelf, low cost source of MSC. In this thesis we studied the engraftment enhancing effect three different sources of MSC, fetal lung **(chapter 4)**, adult bone marrow and the Wharton's Jelly of the umbilical cord **(chapter 1)**. We initially used fetal lung MSC since this source of MSC was previously used and shown to significantly improve the engraftment of CB CD34+ cells. [6] However, these MSC are harvested from aborted fetal tissues and besides ethical opposition to the use of such tissues, it is doubtful that the limited availability of this source is compatible with clinical applications on a large scale.
In **chapter 1** we show that the engraftment enhancing capacity of MSC harvested from the Wharton's Jelly (WJ) of the umbilical cord is similar to that of BM derived MSC. WJ in this respect is a more attractive source for the clinical application of MSC for several reasons: 1) The umbilical cord is considered to be waste material and is therefore practical free of ethical constraints. In contrast, MSC, harvest from the bone marrow and adipose tissue as other possible sources both require invasive and more or less painful procedures, i.e. aspiration of BM from the pelvis and liposuction respectively. Most ethical review boards are therefore reluctant to harvest MSC from these sources from non-related donors on a large scale. 2) If the umbilical cord containing the WJ is collected concurrently with the CB, the costs of HLA typing and infection screening of the donor, but also part of the collection logistics can be shared between both products, logically resulting in a reduction of the cost of both CB and a WJ MSC product. 3) Finally, WJ MSC are of fetal origin and therefore have a lower chance of viral transmission such as Epstein Barr virus and cytomegalovirus. A disadvantage, however, is that cord collections are far from sterile while BM or adipose aspiration can be done in a closed aseptic manner. Indeed, when we developed the protocol for the isolation of WJ MSC **(chapter 1)**, we were initially confronted with a variety of infections ranging from opportunistic bacteria to fungal infections. These infections could be virtually prevented by implementing a stringent disinfection protocol in which both ends of the umbilical cord are clamped to prevent influx of collection medium into the cord, followed by sterilization of the outside of the cord with first iodine and then 70% ethanol. We have chosen an 'explant' method to isolate the MSC instead of digestion of the cord tissue with enzymes [7,8] because it is less laborious and cost intensive than digestion of the tissue with enzymes. This method, despite challenges, has shown that isolation and culture of WJ MSC can be performed with GMP compliance. [7,8] Moreover, since the engraftment enhancing effect of WJ MSC is similar to that of BM MSC WJ MSC could be a suitable alternative for BM MSC and probably more convenient for large scale clinical application.

Co-transplantation of mesenchymal stem cells and CB to enhance the engraftment of HSC: scientific considerations

A large conceptual problem for all clinical applications of MSC, including the enhancement of HST engraftment, is the variability of the MSC between cultures. Although all harvested and cultured cells fulfill the minimal criteria defining them as MSC, [9] these products of the

cultures comprise a heterogeneous collection of cells, showing large variations with respect to more detailed immuno-phenotypes, proliferative capacity, differentiation capacity and the ability to secrete cytokines. [10] Moreover, with still inconclusive knowledge on the mechanism(s) by which MSC exert their effects, we can only expect to find associations between general in vitro tests and the actual in vivo results. These associations between the in vitro capacities on the one hand and the in vivo effects on the other hand however, would be of great importance to develop in vitro quality control tests as release criteria for an effective therapeutic functionality. In **chapters 1 and 4** we therefore analyzed different in vitro characteristics of MSC to see whether these characteristics could be correlated to the engraftment enhancing effect of MSC.

In theory, the engraftment enhancing effect of MSC can be achieved via 1) local mechanisms, e.g. repair of the stromal tissues in the BM or secretion of factors that assist in proliferation and/or differentiation of the HSC in the BM, or 2) systemic mechanisms, e.g. through an immunological effect on or assistance in the homing of the HSC to the BM.

The BM niche is likely to be the key target of MSC therapy. This niche consists of stromal cells such as pericytes, osteoblasts, osteoclasts and adipocytes that are important for the maintenance and proliferation of HSC. [11] This tissue is damaged by chemotherapy or irradiation of the recipient of HST and repair of the stromal components of marrow could be established by the homing of the MSC to the BM and their subsequent differentiation. Interestingly, analysis of the in vitro capacity to differentiate into osteoblast, adipocytes and chondrocytes **(chapter 1)** showed that WJ MSC are limited and variable in this respect compared to BM MSC. Since MSC from both sources were equally potent in enhancing the engraftment of CB CD34+ cells, it is unlikely that differentiation into stromal tissues of the recipient is responsible for the engraftment enhancing effect in our model. Moreover, and of considerable importance, homing of intravenously injected MSC to the BM has so far not been convincingly shown. [6,12]

Another mechanism for the engraftment enhancement effect could be related to the immuno-modulatory capacity of MSC. [12,13] The in vitro experiments in **chapter 1** show that the capacity of MSC to suppress an immunological response is similar between MSC derived from WJ or BM. Notwithstanding this possibility, the 3 fold engraftment enhancement mediated by MSCs of both sources is not likely due to their immune modulating capacities since transplantation was performed with purified stem cells in immune deficient NOD SCID mice and our model was therefore devoid of immunologically active cells.

Finally, the engraftment enhancing effect of MSC could be attributed to the ability of MSC to assist HSC homing to the BM. In **chapter 4** we show that MSC can improve the migration of non expanded CD34+ cells in transwell plates with an SDF-1α gradient. SDF-1α is main chemoattractant of the BM, and expression of its receptor CXCR4 is associated with the homing of HSC to the BM. [14]

Interestingly, this increased migration was not related to the expression of CXCR4, the receptor for SDF-1α, by the CD34+ cells. This could be explained by the in vitro experimental conditions. The plates are coated with fibronectin, a ligand for adhesion receptors CD49d and CD49e. [15] Earlier studies have shown that migration of CD34+ cells on fibronectin coated plates is increased compared to non-coated plates, and blocking of the adhesion receptors CD49d and CD49e diminishes this increase. [16] Indeed, we could show that incubation of the CD34+ cells with fetal lung MSC did upregulate the expression of CD49d and CD49e, which could explain the increased migration described in **chapter 5**. On the other hand, in **chapter 2** CD34+ migration was not increased by BM or WJ MSC despite a similar upregulation in CD49d and CD49e as was observed for fetal lung MSC.

These inconsistent results leave doubt that enhancement of migration after incubation with BM or WJ MSC is the main cause for enhancement of in vivo engraftment.
Lastly, the number of passages of the cultured MSC may play a role. A study that looked at the long term outcomes of MSC treatment for GvHD and hemorrhagic cystitis showed that in the GvHD patients the one year survival rate was only 21% in the patients that were transplanted with MSC that were cultured for 3 or more passages, as opposed to 75% in the patients that were treated with MSC from passage 1 or 2 [17].
Currently there are seven clinical trials involving the co-transplantation of mesenchymal stem cells listed on the website of the U.S. National Institutes of Health. [2] Whether these small scale studies that mostly focus on the safety of MSC co-transplantation [18-23] will give definite answers on engraftment mechanisms is doubtful and more detailed and larger in vivo studies on the mechanism behind engraftment enhancement are therefore needed to conclusively elucidate this mechanism.

Co-transplantation of mesenchymal stem cells and CB to enhance the engraftment of HSC: safety concerns

There are several safety concerns with respect to the clinical application of MSC. [24] In order to obtain a sufficient number of MSC for transplantation, primary MSC are cultured for several passages. During this culture it is possible that the cells acquire DNA mutations and chromosomal aberrations which can lead to malignant transformation of the cells after transplantation. Fortunately, so far such malignant transformations have not been observed in vivo or in vitro. [25] Another concern is ectopic tissue formation of the transplanted cells, but a study that analyzed tissue samples from 31 patients that were infused with MSC found no signs of this formation. Furthermore, the detection of DNA derived from the MSC donor correlated negatively with the time after infusion, i.e. the detectable levels of DNA coming from the MSC decreased with time, showing that the transplanted MSC gradually disappear [26].
Another safety concern associated with the co-transplantation of MSC and HSC apply to possibly unwanted immunomodulatory capacities of the MSC. Although MSC can, next to their engraftment enhancing effect, prevent GvHD, their immunomodulatory capacity can also be detrimental for the outcome of transplantation. In this respect, co-transplanted MSC might theoretically suppress the graft versus leukemia (GvL) effect and consequently increase the chance of relapse. A pilot study from 2008 showed that in a group of patients that was co-transplanted with MSC 6 out of 10 patients relapsed compared to only 3 out of 15 patients in the control group. [27] However, another study that compared historical controls with patients that were co-transplanted with MSC did not find such an increase in relapse incidence, but did find a MSC associated reduction in GvHD. [28]
Finally, the immunosuppressive effect of MSC could lead to a higher infection rate in patients that are co-transplanted with MSC. One of the mechanisms by which MSC suppress immune responses is the induction of indoleamine 2,3-dioxygenase (IDO), which catalyzes the conversion from tryptophan to kynurenine. [29] Kynurenine decreases T-cell cytotoxicity, [30] which can make the patient more vulnerable to infections such as CMV and EBV and opportunistic fungal infections. The previously mentioned study on the long term follow up of MSC treated patients found a high incidence of fungal infections in patients with acute steroid resistant GvHD that responded well to the therapy. [17] The long term effect of immune suppression by MSC also seems to play a role in the survival rate of these patients as they also show a high mortality rate due to infections two years after treatment.
Notwithstanding the so far excellent safety record of MSC treatment, larger randomized studies with longer follow up are needed to elucidate not only how MSCs exert their

beneficial effects in vivo but also, vice versa, if they can induce relapse, infection or other longer term unwanted side effects. These studies should take the conditioning regime, the specific disease and the GvHD course and treatment as parameters into account.

Clinical application of CB expansion with TPO: practical considerations

As mentioned in the introduction of this thesis, the clinical application of the transplantation of TPO expanded cells would be most suitable for patients undergoing unexpanded CB HST that are refractory to platelet transfusions. Although we show in **chapter 2** that the engrafted TPO expanded cells exhibit the potential for long term engraftment, recovery of CD45+ cells in the blood is delayed. Furthermore, the long term engraftment capacity is decreased as compared to non expanded CB cells and transplantation of a single graft of TPO expanded CD34+ cells is therefore not advisable. Transplantation of TPO expanded cells in combination with a non expanded unit to safeguard long term engraftment therefore seems to be the best alternative. Important in this respect is that the TPO expanded cells responsible for short term platelet recovery did not reduce or affect the long-term engraftment of non-expanded CD34+ cells nor influence the platelet recovery of these cells **(chapter 2)**.

For further translation of the in vivo experiments with immune deficient mice into clinical use, we still need to monitor the functionality of platelets derived from the TPO expanded cells that have engrafted; platelets derived from non-expanded CD34+ cells that engrafted in NOD SCID mice have shown this functionality in in vitro assays [31], GMP compliant upscaling of our expansion protocol to a therapeutic transplant size would be the next step towards clinical implementation. What is already convenient of the current protocol is the use of the clinical grade Nplate (romiplostim), an analog of TPO, which is registered to treat thrombocytopenic patients [32] and the fact that the expansion medium does not contain animal components such as fetal bovine serum.

Combining TPO expansion and the co-transplantation of MSC for the improvement of CB transplantation.

Transplantation of expanded cells and the co-transplantation of MSC both seem potent strategies for the enhancement of CB engraftment. Combining these strategies could be a way to synergistically improve the outcome of CB transplantation. In **chapter 4** we report on TPO- expanded CB CD34+ cells, shown to accelerate platelet recovery in the peripheral blood (PB) [33-36] with co-transplantation of MSC, shown to improve bone marrow engraftment. While both strategies themselves provided the benefits of each of the separate strategies, combining both methods did not yield any synergistic effect and even led to non engraftment events. Graft failure of CBT is believed to be caused by the low number of stem cells and/or HLA disparity between the donor and the the recipient. Both these mechanisms however are not likely to play a role in the non-engraftment events we observed in our in vivo experiments since identical grafts (and cell numbers) without MSC were able to reconstitute the bone marrow and immune cells are lacking in our model. We excluded that the non engraftment events in **chapter 4** might be due to the high amounts of infused cells when the TPO expanded cells and MSC were combined and possibly concomitant lung entrapment of hematopoietic stem cells, since transplantation of the MSC four hours after the transplantation of the TPO expanded cells still resulted in a mouse without engraftment. Without any synergistic effect of the co-transplantation of MSC and TPO expanded cells on either platelet recovery or engraftment we feel that this safety aspect does not make this combined approach a viable option for further implementation.

Expansion of long-term engraftment capacity of CB HSC in one of two transplanted cords: general considerations

Since several expansion protocols are known to increase the graft size to cell numbers that should ensure successful engraftment, [37-39] it is still considered to be a risk to transplant only one expanded unit into an adult patient. So far all clinical trials using expanded cells have therefore combined the expanded graft with a non expanded graft [37-40] to ensure the long term engraftment capacity of the transplant. Expansion of HSC inevitably leads to a certain degree of differentiation of the long term repopulating stem cell pool whereby the immaturity and self renewal capacity is lost leading to reduction in long term repopulation capacity.

Preservation of the long term repopulating capacity cannot be assessed properly with in vitro models while animal models also have intrinsic limitations. Clinical studies that have used expanded cells in combination with a unit of non expanded cells, showed that long term engraftment is usually skewed towards or eventually totally dependent on the non-expanded unit [38,40]. However, because of this double transplant approach it remains difficult to assess whether expanded cells indeed lose their long term engraftment ability or that non-expanded cells eventually out-compete the expanded cells in this respect. In light of this possibility, in **chapter 2** we specifically studied graft dependent blood recovery and engraftment in double graft transplantations of a TPO expanded and a non expanded CB graft in NOD SCID mice. By the use of HLA allele specific antibodies, the progeny of the different grafts could be distinguished in the blood, BM and spleen. Although the PB of the mice contained platelets that originated from the TPO expanded graft up to 20 weeks after transplantation, thus showing that TPO expansion does not deplete the capacity of the CB cells to differentiate into megakaryocytes and platelets, the chimerism of the cells in the blood, BM and spleen at 20 weeks was skewed towards the non expanded graft. This suggests that long term engraftment of the TPO expanded cells occurs but was less efficient than that of the non- expanded cells.

Two mechanisms by which the non-expanded unit becomes dominant should be considered. So far, by looking at clinical experience with double CB transplantation of two non expanded units, immunological processes and the viability and cell number of the individual units seem to be the most important factors in the establishment of dominance.

The number of specific immune competent T cells such as CD3 (CD4+CCR7+) cells in either graft seems to be associated to the establishment of dominance of that particular CB unit. [41-43] The remaining number of these types of cells in an expanded graft in this respect will depend on the type of/nature of expansion protocol. If the cells are expanded for the mononuclear fraction of the cord blood unit, the immune competent cells are likely to be affected by the culture conditions while in isolated and expanded CD133+ and CD34+ cells the immune competent cells are completely removed.

In the latter approach, the non expanded cells will clearly have the immunological advantage in a graft vs graft response leading to eventual dominance of this unit. In our in vivo model that was devoid of immune competent cells, the observed dominance of the non expanded cells should be attributable to another mechanism and possibly dependent on the number of (long term repopulating) stem cells that was higher in the non-expanded units.

The immune competence of grafts is not only important for engraftment and dominance in case of double transplant strategies, it is even more important if one realizes that the (minor) HLA mismatch between the graft and the patient is critical for the GvL effect. GvL is essential to prevent disease relapse and in essence required for total donor chimerism and eventual complete disease remission. [44] Expanded and non-expanded CB contain only low numbers of effective immunocompetent T cells but nevertheless show a similar

relapse rate as seen with matched unrelated donor grafts. However, in the case of relapse, additional blood from a CBT donor cannot be harvested for donor lymphocyte infusions (DLI). [45] A solution for this might be the expansion of T cells from the CD34 negative fraction after isolation of the stem cells prior to expansion. [46]

As mentioned above the main challenge is the expansion of the true long term repopulating hematopoietic stem. [47] The CD34+ cell population in CB is a heterogeneous population containing only a limited number of immature progenitors (long repopulating HSC) with the ability of complete self renewal and mostly consists of more mature progenitors (short term repopulating HSC) and committed myeloid or lymphoid progenitors. [48,49]

Earlier expansion protocols used a combination of cytokines that led to a high degree of differentiation and which coincides with the maturation of the most immature cells and thus loss of the long term repopulating capacity of the graft. Although a single long term repopulating cell is capable of eventual complete reconstitution of the bone marrow, [50] the speed to attain this is most critical for limiting morbidity and the mortality of the patients. More recent expansion protocols focus on the preservation of the true long term repopulating stem cell (LT HSC) by adding small molecules to the expansion medium. An example of one of these small molecules is Stemregenin 1 (SR1), an arylhydrocarbon receptor (AHR) antagonist. AHR is an important regulator of hematopoietic stem cells [51] and culture with SR1 has shown to increase the number of LT HSC in vitro and in immune deficient mouse models. [52,53] Because of the promising experimental results, a phase 1 trial has started to evaluate the clinical efficacy of expansion with SR1. [54] Other examples of small molecules that can increase the expansion of LT HSC are transcriptional such as UM171, a pyrimidoindole derivative, [55] and valproic acid, and histone deacetylase inhibitor. [56] However, as mentioned before, long term repopulation is difficult to assess in vitro or in animal models, and larger scale clinical trials are needed to investigate the value of these expansion strategies.

CB engraftment stimulating protocols: logistic considerations.

In **chapter 3** we show that the engraftment capacity of CB CD34+ cells expanded with TPO before and after cryopreservation is similar. However, as discussed in this chapter, expansion before cryopreservation is logistically largely impractical since it is not known which cord blood unit is going to be needed for transplantation and expansion of all collected units would be an extremely costly exercise. However, expansion from a cryopreserved unit is neither an easy option since it needs to be performed in a GMP certified cell culture facility and not all transplantation centers have such a facility available. A solution for this would be to centralize the expansion of the CB unit upon request at the CB banks.

In general, the engraftment enhancing strategies for CB transplantation that were investigated in this thesis have shown their merit in in vivo models, but before these strategies can be applied clinically, several practical and scientific issues have to be addressed. The most complicating issue in this respect is that before actual use in patients becomes possible, that the manufacturing of the involved cellular products, either ex vivo culture of HSC or the culture of accessory cells such as MSC or regulatory T cells, not only require GMP grade facilities, but also highly qualified personnel and rigorous compliance to laboratory protocols. Indeed, the various manipulations needed for these cell therapies make them so called advanced therapy medicinal products (ATMP) falling under medicinal regulations and new approval for each change in the manufacturing protocol has to be applied for.

Because of all these practical and regulatory hurdles, it would probably be advisable to prepare the grafts for these still experimental cell therapies in the specialized transplantation centers or blood bank facilities that are not only equipped to process cellular products under GMP conditions but also to facilitate validated GCP grade assays including immune and engraftment monitoring. Only under such conditions fast advance of knowledge is ensured and can reverse translation of outcomes lead to optimization of these therapies.

The use of CB for other purposes than HST

CB also contains non-hematopoietic cells that might be useful for regenerative or immunomodulatory cell therapies. The best known examples of these cells are endothelial progenitor cells (EPC) and MSC. The number of endothelial progenitor cells and MSC in CB is however very low e.g. for 2.99±2.45 endothelial progenitor cells per 10^8 mononuclear cells (MNC), [57] or 0-2.3 MSC per 10^8 MNC, [58] and the culture of these cells has proven to be difficult. [58] What factors influence the number of non-hematopoietic cells in CB is unknown, although the gestational age of the cord blood seems to play a role. [59] CB that was collected after 24 to 28 weeks of gestation seems to contain more MSC, whereas CB from weeks 33-36 predominantly contains EPC.

The regenerative potential of the non hematopoietic cells from CB is, with the exception of MSC immunotherapy, mostly useful in an autologous setting since therapies such as wound repair and bone or cartilage grafting requires the transplantation of live tissue that can be rejected by the host if the cells are of an allogeneic origin. Publically stored CB is exclusively banked for the purpose of allogeneic HST. Because only a few percent of the stored CB units is actually used for this purpose, CB banking is a costly activity. The number of privately banked CB units is almost double the number of publically banked units and virtually not used. [60]

Private CB banking initiatives successfully advertise autologous stored CB as superior treatment of (future) hematological malignancies of the child itself or one of its family members. The theoretical chance of needing the CB for the advertised purpose is only 1 in 2700 banked units which are estimated to be of potential benefit for the donor itself or its family. [61] Finding a way to utilize a single CB unit for both autologous (non-hematopoietic) and allogeneic (HSCT) purposes could have considerable advantages, as the 'dual' use of banked CB could be partly paid for by additional interest and financial support of the donors.

To explore this 'dual banking' strategy we investigated in **chapter 5** if the hematopoietic cells in CB could be separated from the non-hematopoietic cells based on the expression of the pan hematopoietic marker CD45. We specifically investigated whether removal of the CD45 negative cells would affect the in vitro and in vivo hematopoietic potential of the graft. The ability to form hematopoietic colonies in culture is correlated to the platelet and neutrophil engraftment capacity of a CB unit. [62] Removal of the CD45-negative cells neither affected the number of hematopoietic colonies that were formed by CD45-postive cells in a standard methocult assay. In agreement the CD45- negative cells showed no hematopoietic colony forming capacity. In mixed lymphocyte cultures, the CD45- cells were not immunogenic themselves and did not influence the PBMC response to CD45-postive cells or vice versa. We therefore hypothesize that CD45-negative cell removal will not likely influence the immunological properties of the remaining graft, which is important for both the incidence of GvHD and the GvL effect. Although our NOD-SCID transplant model does not test immunologic mechanisms, co-transplantation of CD45-negative cells with CD34+ cells did not change engraftment in NOD SCID mice of CD34+ cells alone. In contrast, co-transplantation of CD45-postive with CD34+ cells clearly

showed the added engraftment enhancing potential of the CD45+ population. The possible non-hematopoietic use of the remaining CD45- cells after CD45+ immuno-separation was shown by the fact that endothelial cell colonies could be cultured from the isolated CD45- cells but not from the CD45+ cells.
Our results, although preliminary, suggest that CD45-negative cells from CB might be used separately without altering the HST capacity of the CD45-postive cells. However, it must be noted that our transplant model does not allow for the testing of immunological mechanisms. Our findings should therefore be confirmed in models that better reflect the human setting in this respect. There are several practical and logistic obstacles for this 'dual-use' banking initiative. Firstly, the time that cells are needed for either autologous or allogeneic use will usually not coincide. This will make pre-cryopreservation separation and separate storage more preferable with respect to the quality of the cells, but this will increase the cost of banking. Again, the problem with this strategy is that it is not known beforehand which specific cell type of which particular donor will be needed in the future and culturing and banking a variety of different cell types would increase the cost for processing and banking of the CB unit. Therefore, post cryopreservation separation and culture is more practical and likely to be the only option that can be considered. However, although endothelial progenitor cells can be cultured from cord blood that was stored for over 20 years, [63] the culture of MSC from frozen CB is very difficult. [58] Loss of cells by the freeze thaw procedures combined with the already low and variable number of non-hematopoietic cells in CB will therefore limit the number of successful cell cultures from cryopreserved CB.
For MSC, considered as one of the most promising cell types for cell therapies that can also be used in an allogeneic setting, it is important to realize that they can be more easily obtained from the Wharton's Jelly of the UC itself and private CB banks have started to offer the storage of these cells. In this respect it must be noted that WJ MSC show clear aberrances from the typical MSC phenotype and its therapeutic use can therefore not be extrapolated from findings obtained with other MSC sources. In **chapter 1** we found a limited and variable differentiation capacity into osteoblast, adipocytes and chondrocytes which was previously shown by earlier studies. [64] However, and perhaps more important, WJ MSC were shown to possess important in vitro immunomodulatory and in vivo engraftment enhancing capacities that are similar to BM MSC, which are much more difficult to obtain.
In conclusion, new use of cord and cord blood cells seem possible. Adapted cord and cord blood banking strategies and manipulation strategies (most likely post cryopreservation) are however, needed for this new use and the cost for this is considerate and should, depending on the development of the progress in cellular therapies be weighed carefully. This new use not only involves creating greater HST efficacy of cord blood but also non-hematopoietic like regenerative and more specific immunomodulatory indications. Isolation of the CD45-postive cell from CB for the use in HSCT, in this regard could make the remaining CD45-negative cells a new asset for these purposes. The logistics and methodology to optimize such new therapeutic vistas need consistent bench to bed side synergies between scientists and clinicians, but also dedicated grants supporting this vision and international and multicenter collaboration.

CB transplantation or haploidentical transplantation: the choice of the right graft source

Despite many scientific and clinical advances, the increase in CB transplantation seems to have stagnated in the last years (Figure 1). This stagnation might be due to center specific preferences in using other unrelated donor transplants such as haploidentical grafts from

a child or parent of the patient [65] or the use of mismatched (un)related donor grafts. [66] Another reason is that high incidence of graft versus host disease (GvHD), which was historically a big problem with haploidentical graft transplantation [67,68], is more manageable. Besides T-cell depletion as a main preventive measure, large progress has been made in the understanding, treatment [9] and prevention of GvHD, such as the post-transplant administration of methotrexate [10,11]. Additionally, experimental therapies are now being investigated such as the treatment with MSC infusions both prophylactically [28,69] or when first line treatment for GvHD with corticoids fails. [70-75] Alternatively, co-transplantation of regulatory T cells has shown promising results in prevention of GvHD. [76,77] This improved treatment and prevention of GvHD has revived the interest in haploidentical HST.

Haploidentical grafts also have other advantages over CB grafts. [78-80] One of these advantages favoring haploidentical HST is the low acquisition cost. Secondly, a haploidentical donor graft is usually quickly available since nearly every patient has a haploidentical sibling, child or parent. In contrast, for CB, despite the increasing number of banked units, the variability of the cell dose in the banked units still limits the choice of sufficiently large grafts. Thirdly, the immune reconstitution after haploidentical transplantation is still faster compared to CB transplantation and reduces the risk of infection, the largest cause of non-relapse mortality, especially in the first three months after transplantation [81]. A last and often mentioned advantage of non-CB grafts is the possibility of post-transplant donor lymphocyte infusions (DLI) to prevent or treat relapse. Whether this applies to haploidentical grafts is however debatable since haploidentical DLI –although possible- carries again a very high risk of GVHD and is therefore very rarely applied. Conversely the strong T cell depletion needed to control GVHD in the case of haploidentical GvHD is offset by a higher relapse rate, and T cell replete grafts [82] or post-transplant DLI [83] in these patients again increases the risk of GvHD. Hence, development of more practical relapse treatment solutions for both CB and haploidentical transplantations is therefore needed and crucial for choice of the most suitable graft source. In this respect, DLI from CB in the near future is likely to become possible by – although costly - expansion of T cells from the CD34 negative fraction of the graft. [46]

Finally, it is good to realize that most comparisons between different graft sources are based on different studies performed in separate centers with retrospective outcome analyses. Moreover, variability in patient groups and diseases treated in these studies make these comparisons even more complicated. While certain graft sources might be better in the treatment of a certain group of patients or of a certain disease, the experience of a center with a certain transplant platform/approach is probably one of the most critical determinants to optimize the disease and patient related outcome.

Prospective randomized and multicenter studies not only covering morbidity and mortality but also treatment costs and quality of life issues are therefore needed to correctly evaluate the pros and cons of each graft source. [84,85] The continuing research on the enhancement of CB transplantation, such as HSC and lymphocyte expansion, however, is accelerating and could improve the outcome for CB recipients in such a way that this might shift the balance for graft choice towards CB.

References

1. Pasquini M, Wang Z. Current use and outcome of hematopoietic stem cell transplantation: CIBMTR Summary Slides 2013. Available at: http://www.cibmtr.org 2013.
2. https://clinicaltrials.gov. Registry and results database of publicly and privately supported clinical studies of human participants conducted around the world. 2015.
3. Blommestein H, Verelst S, Huijgens P et al. Real-world costs of autologous and allogeneic stem cell transplantations for haematological diseases: a multicentre study. Annals of hematology 2012;**91**: 1945-52.
4. Labopin M, Ruggeri A, Gorin NC et al. Cost-effectiveness and clinical outcomes of double versus single cord blood transplantation in adults with acute leukemia in France. haematologica 2014;**99**: 535-40.
5. Pasquini M, Wang Z. Current use and outcome of hematopoietic stem cell transplantation: CIBMTR Summary Slides 2014. Available at: http://www.cibmtr.org 2014.
6. Noort WA, Kruisselbrink AB, Kruger M et al. Mesenchymal stem cells promote engraftment of human umbilical cord blood–derived CD34+ cells in NOD/SCID mice. Experimental hematology 2002;**30**: 870-8.
7. Capelli C, Gotti E, Morigi M et al. Minimally manipulated whole human umbilical cord is a rich source of clinical-grade human mesenchymal stromal cells expanded in human platelet lysate. Cytotherapy 2011;**13**: 786-801.
8. Hartmann I, Hollweck T, Haffner S et al. Umbilical cord tissue-derived mesenchymal stem cells grow best under GMP-compliant culture conditions and maintain their phenotypic and functional properties. Journal of immunological methods 2010;**363**: 80-9.
9. Dominici M, Le Blanc K, Mueller I et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;**8**: 315-7.
10. Pevsner-Fischer M, Levin S, Zipori D. The origins of mesenchymal stromal cell heterogeneity. Stem Cell Reviews and Reports 2011;**7**: 560-8.
11. Morrison SJ, Scadden DT. The bone marrow niche for haematopoietic stem cells. Nature 2014;**505**: 327-34.
12. Kim D-W, Chung Y-J, Kim T-G et al. Cotransplantation of third-party mesenchymal stromal cells can alleviate single-donor predominance and increase engraftment from double cord transplantation. Blood 2004;**103**: 1941-8.
13. Hiwase SD, Dyson PG, To LB, Lewis ID. Cotransplantation of placental mesenchymal stromal cells enhances single and double cord blood engraftment in nonobese diabetic/severe combined immune deficient mice. Stem Cells 2009;**27**: 2293-300.
14. Peled A, Petit I, Kollet O et al. Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. Science 1999;**283**: 845-8.
15. Turner ML, Masek LC, Hardy CL et al. Comparative adhesion of human haemopoietic cell lines to extracellular matrix components, bone marrow stromal and endothelial cultures. British journal of haematology 1998;**100**: 112-22.
16. Voermans C, Gerritsen WR, von dem Borne AE, van der Schoot CE. Increased migration of cord blood-derived CD34+ cells, as compared to bone marrow and mobilized peripheral blood CD34+ cells across uncoated or fibronectin-coated filters. Experimental hematology 1999;**27**: 1806-14.
17. von Bahr L, Sundberg B, Lönnies L et al. Long-term complications, immunologic effects, and role of passage for outcome in mesenchymal stromal cell therapy. Biology of Blood and Marrow Transplantation 2012;**18**: 557-64.
18. Ball LM, Bernardo ME, Roelofs H et al. Cotransplantation of ex vivo–expanded mesenchymal stem cells accelerates lymphocyte recovery and may reduce the risk of graft failure in haploidentical hematopoietic stem-cell transplantation. Blood 2007;**110**: 2764-7.
19. Lazarus HM, Koc ON, Devine SM et al. Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. Biology of Blood and Marrow Transplantation 2005;**11**: 389-98.
20. Le Blanc K, Samuelsson H, Gustafsson B et al. Transplantation of mesenchymal stem cells to enhance engraftment of hematopoietic stem cells. Leukemia 2007;**21**: 1733-8.
21. Lee S, Lee M, Yoo K et al. Co-transplantation of third-party umbilical cord blood-derived MSCs promotes engraftment in children undergoing unrelated umbilical cord blood transplantation. Bone marrow transplantation 2013;**48**: 1040-5.
22. Macmillan M, Blazar B, DeFor T, Wagner J. Transplantation of ex-vivo culture-expanded parental haploidentical mesenchymal stem cells to promote engraftment in pediatric recipients of unrelated donor umbilical cord blood: results of a phase I–II clinical trial. Bone marrow transplantation 2008;**43**: 447-54.

23. Wu K-H, Sheu J-N, Wu H-P et al. Cotransplantation of Umbilical Cord–Derived Mesenchymal Stem Cells Promote Hematopoietic Engraftment in Cord Blood Transplantation: A Pilot Study. Transplantation 2013;**95**: 773-7.
24. Wang Y, Han Z-b, Song Y-p, Han ZC. Safety of mesenchymal stem cells for clinical application. Stem cells international 2012;**2012**.
25. Tarte K, Gaillard J, Lataillade J-J et al. Clinical-grade production of human mesenchymal stromal cells: occurrence of aneuploidy without transformation. Blood 2010;**115**: 1549-53.
26. Von Bahr L, Batsis I, Moll G, et al. Analysis of tissues following mesenchymal stromal cell therapy in humans indicates limited long-term engraftment and no ectopic tissue formation. Stem Cells 2012;**30**: 1575-8.
27. Ning H, Yang F, Jiang M et al. The correlation between cotransplantation of mesenchymal stem cells and higher recurrence rate in hematologic malignancy patients: outcome of a pilot clinical study. Leukemia 2008;**22**: 593-9.
28. Baron F, Lechanteur C, Willems E et al. Cotransplantation of mesenchymal stem cells might prevent death from graft-versus-host disease (GVHD) without abrogating graft-versus-tumor effects after HLA-mismatched allogeneic transplantation following nonmyeloablative conditioning. Biology of blood and marrow transplantation 2010;**16**: 838-47.
29. Meisel R, Zibert A, Laryea M et al. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2, 3-dioxygenase–mediated tryptophan degradation. Blood 2004;**103**: 4619-21.
30. Terness P, Bauer TM, Röse L et al. Inhibition of Allogeneic T Cell Proliferation by Indoleamine 2, 3-Dioxygenase–expressing Dendritic Cells Mediation of Suppression by Tryptophan Metabolites. The Journal of experimental medicine 2002;**196**: 447-57.
31. Salles II, Thijs T, Brunaud C et al. Human platelets produced in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice upon transplantation of human cord blood CD34+ cells are functionally active in an ex vivo flow model of thrombosis. Blood 2009;**114**: 5044-51.
32. Kuter DJ, Rummel M, Boccia R et al. Romiplostim or standard of care in patients with immune thrombocytopenia. New England Journal of Medicine 2010;**363**: 1889-99.
33. *Bone Marrow Donors Worldwide, www.bmdw.org [monograph on the internet]*. Available from: www.bmdw.org
34. Schipper LF, Brand A, Fibbe WE, Van Hensbergen Y. Functional Characterization of TPO-Expanded CD34+ Cord Blood Cells Identifies CD34− CD61− Cells as Platelet-Producing Cells Early After Transplantation in NOD/SCID Mice and rCD34+ Cells as CAFC Colony-Forming Cells. Stem Cells 2012;**30**: 988-96.
35. van der Garde M, van Hensbergen Y, Brand A et al. Thrombopoietin Treatment of One Graft in a Double Cord Blood Transplant Provides Early Platelet Recovery While Contributing to Long-Term Engraftment in NSG Mice. Stem cells and development 2014.
36. van Hensbergen Y, Schipper LF, Brand A et al. Ex vivo culture of human CD34+ cord blood cells with thrombopoietin (TPO) accelerates platelet engraftment in a NOD/SCID mouse model. Experimental hematology 2006;**34**: 943-50.
37. de Lima M, McNiece I, Robinson SN et al. Cord-blood engraftment with ex vivo mesenchymal-cell coculture. New England Journal of Medicine 2012;**367**: 2305-15.
38. Delaney C, Heimfeld S, Brashem-Stein C et al. Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution. Nature medicine 2010;**16**: 232-6.
39. Horwitz ME, Chao NJ, Rizzieri DA et al. Umbilical cord blood expansion with nicotinamide provides long-term multilineage engraftment. The Journal of clinical investigation 2014;**124**: 3121.
40. De Lima M, McMannis J, Gee A et al. Transplantation of ex vivo expanded cord blood cells using the copper chelator tetraethylenepentamine: a phase I/II clinical trial. Bone marrow transplantation 2008;**41**: 771-8.
41. Gutman JA, Turtle CJ, Manley TJ et al. Single-unit dominance after double-unit umbilical cord blood transplantation coincides with a specific CD8+ T-cell response against the nonengrafted unit. Blood 2010;**115**: 757-65.
42. Newell LF, Milano F, Nicoud IB et al. Early CD3 peripheral blood chimerism predicts the long-term engrafting unit following myeloablative double-cord blood transplantation. Biology of Blood and Marrow Transplantation 2012;**18**: 1243-9.
43. Somers JA, Brand A, van Hensbergen Y et al. Double umbilical cord blood transplantation: A study of early engraftment kinetics in leukocyte subsets using HLA-specific monoclonal antibodies. Biology of Blood and Marrow Transplantation 2012.
44. Horowitz MM, Gale RP, Sondel PM et al. Graft-versus-leukemia reactions after bone marrow transplantation. Blood 1990;**75**: 555-62.
45. Roddie C, Peggs KS. Donor lymphocyte infusion following allogeneic hematopoietic stem cell transplantation. Expert opinion on biological therapy 2011;**11**: 473-87.

46. Berglund S, Gertow J, Uhlin M, Mattsson J. Expanded umbilical cord blood T cells used as donor lymphocyte infusions after umbilical cord blood transplantation. Cytotherapy 2014;**16**: 1528-36.
47. Norkin M, Lazarus H, Wingard J. Umbilical cord blood graft enhancement strategies: has the time come to move these into the clinic&quest. Bone marrow transplantation 2013;**48**: 884-9.
48. Görgens A, Radtke S, Möllmann M et al. Revision of the human hematopoietic tree: granulocyte subtypes derive from distinct hematopoietic lineages. Cell reports 2013;**3**: 1539-52.
49. Doulatov S, Notta F, Laurenti E, Dick JE. Hematopoiesis: a human perspective. Cell stem cell 2012;**10**: 120-36.
50. Notta F, Doulatov S, Laurenti E et al. Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. Science 2011;**333**: 218-21.
51. Singh KP, Casado FL, Opanashuk LA, Gasiewicz TA. The aryl hydrocarbon receptor has a normal function in the regulation of hematopoietic and other stem/progenitor cell populations. Biochemical pharmacology 2009;**77**: 577-87.
52. Boitano AE, Wang J, Romeo R et al. Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells. Science 2010;**329**: 1345-8.
53. Smith BW, Rozelle SS, Leung A et al. The aryl hydrocarbon receptor directs hematopoietic progenitor cell expansion and differentiation. Blood 2013;**122**: 376-85.
54. Wagner JE, Brunstein C, McKenna D et al. Acceleration of Umbilical Cord Blood (UCB) Stem Engraftment: Results of a Phase I Clinical Trial with Stemregenin-1 (SR1) Expansion Culture. Biology of Blood and Marrow Transplant 2015;**21**: S48-S9.
55. Fares I, Chagraoui J, Gareau Y et al. Pyrimidoindole derivatives are agonists of human hematopoietic stem cell self-renewal. Science 2014;**345**: 1509-12.
56. Chaurasia P, Gajzer DC, Schaniel C et al. Epigenetic reprogramming induces the expansion of cord blood stem cells. The Journal of clinical investigation 2014;**124**: 0-.
57. Smythe J, Fox A, Fisher N et al. Measuring angiogenic cytokines, circulating endothelial cells, and endothelial progenitor cells in peripheral blood and cord blood: VEGF and CXCL12 correlate with the number of circulating endothelial progenitor cells in peripheral blood. Tissue Engineering Part C: Methods 2008;**14**: 59-67.
58. Bieback K, Kern S, Klüter H, Eichler H. Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. Stem cells 2004;**22**: 625-34.
59. Javed MJ, Mead LE, Prater D et al. Endothelial colony forming cells and mesenchymal stem cells are enriched at different gestational ages in human umbilical cord blood. Pediatric Research 2008;**64**: 68-73.
60. Butler MG, Menitove JE. Umbilical cord blood banking: an update. Journal of assisted reproduction and genetics 2011;**28**: 669-76.
61. Hwang W. Cord blood banking and transplantation. ISBT Science Series 2011;**6**: 324-7.
62. Page KM, Zhang L, Mendizabal A et al. Total colony-forming units are a strong, independent predictor of neutrophil and platelet engraftment after unrelated umbilical cord blood transplantation: a single-center analysis of 435 cord blood transplants. Biology of Blood and Marrow Transplantation 2011;**17**: 1362-74.
63. Broxmeyer HE, Lee M-R, Hangoc G et al. Hematopoietic stem/progenitor cells, generation of induced pluripotent stem cells, and isolation of endothelial progenitors from 21-to 23.5-year cryopreserved cord blood. Blood 2011;**117**: 4773-7.
64. Bosch J, Houben AP, Radke TF et al. Distinct differentiation potential of “MSC” derived from cord blood and umbilical cord: are cord-derived cells true mesenchymal stromal cells? Stem cells and development 2011;**21**: 1977-88.
65. Fuchs EJ, Huang X-j, Miller JS. HLA-haploidentical stem cell transplantation for hematologic malignancies. Biology of Blood and Marrow Transplantation 2010;**16**: S57-S63.
66. Lee SJ, Klein J, Haagenson M et al. High-resolution donor-recipient HLA matching contributes to the success of unrelated donor marrow transplantation. Blood 2007;**110**: 4576-83.
67. Beatty PG, Clift RA, Mickelson EM et al. Marrow transplantation from related donors other than HLA-identical siblings. New England Journal of Medicine 1985;**313**: 765-71.
68. Szydlo R, Goldman JM, Klein JP et al. Results of allogeneic bone marrow transplants for leukemia using donors other than HLA-identical siblings. Journal of Clinical Oncology 1997;**15**: 1767-77.
69. Kuzmina LA, Petinati NA, Parovichnikova EN et al. Multipotent mesenchymal stromal cells for the prophylaxis of acute graft-versus-host disease—a phase II study. Stem cells international 2011;**2012**.
70. Kebriaei P, Isola L, Bahceci E et al. Adult human mesenchymal stem cells added to corticosteroid therapy for the treatment of acute graft-versus-host disease. Biology of Blood and Marrow Transplantation 2009;**15**: 804-11.
71. Le Blanc K, Frassoni F, Ball L et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. The Lancet 2008;**371**: 1579-86.

72. Lucchini G, Introna M, Dander E et al. Platelet-lysate-expanded mesenchymal stromal cells as a salvage therapy for severe resistant graft-versus-host disease in a pediatric population. Biology of blood and marrow transplantation 2010;**16**: 1293-301.
73. Pérez-Simon JA, Lopez-Villar O, Andreu EJ et al. Mesenchymal stem cells expanded in vitro with human serum for the treatment of acute and chronic graft-versus-host disease: results of a phase I/II clinical trial. haematologica 2011;**96**: 1072-6.
74. Prasad VK, Lucas KG, Kleiner GI et al. Efficacy and safety of ex vivo cultured adult human mesenchymal stem cells (Prochymal™) in pediatric patients with severe refractory acute graft-versus-host disease in a compassionate use study. Biology of Blood and Marrow Transplantation 2011;**17**: 534-41.
75. Ringden O, Le Blanc K. Mesenchymal stem cells for treatment of acute and chronic graft-versus-host disease, tissue toxicity and hemorrhages. Best practice & research Clinical haematology 2011;**24**: 65-72.
76. Di Ianni M, Falzetti F, Carotti A et al. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. Blood 2011;**117**: 3921-8.
77. Brunstein CG, Miller JS, Cao Q et al. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. Blood 2011;**117**: 1061-70.
78. Ballen K, Spitzer T. The great debate: haploidentical or cord blood transplant. Bone marrow transplantation 2010;**46**: 323-9.
79. Ballen KK, Koreth J, Chen YB et al. Selection of optimal alternative graft source: mismatched unrelated donor, umbilical cord blood, or haploidentical transplant. Blood 2012;**119**: 1972-80.
80. Bashey A, Solomon S. T-cell replete haploidentical donor transplantation using post-transplant CY: an emerging standard-of-care option for patients who lack an HLA-identical sibling donor. Bone marrow transplantation 2014.
81. Jacobson CA, Turki AT, McDonough SM et al. Immune reconstitution after double umbilical cord blood stem cell transplantation: comparison with unrelated peripheral blood stem cell transplantation. Biology of blood and marrow transplantation 2012;**18**: 565-74.
82. Solomon SR, Sizemore CA, Sanacore M et al. Haploidentical transplantation using T cell replete peripheral blood stem cells and myeloablative conditioning in patients with high-risk hematologic malignancies who lack conventional donors is well tolerated and produces excellent relapse-free survival: results of a prospective phase II trial. Biology of Blood and Marrow Transplantation 2012;**18**: 1859-66.
83. Dodero A, Carniti C, Raganato A et al. Haploidentical stem cell transplantation after a reduced-intensity conditioning regimen for the treatment of advanced hematologic malignancies: posttransplantation CD8-depleted donor lymphocyte infusions contribute to improve T-cell recovery. Blood 2009;**113**: 4771-9.
84. Fuchs EJ, O'Donnel P, Brunstein CG. A Multi-Center, Phase III, Randomized Trial of Reduced Intensity(RIC) Conditioning and Transplantation of Double Unrelated Umbilical Cord Blood (dUCB) versus HLA-Haploidentical Related Bone Marrow (Haplo) for Patients with Hematologic Malignancies. The Blood and Marrow Transplant Clinical Trials Network 2012;**BTN CTN Protocol 1101**.
85. Roth JA, Bensink ME, O'Donnell PV et al. Design of a cost-effectiveness analysis alongside a randomized trial of transplantation using umbilical cord blood versus HLA-haploidentical related bone marrow in advanced hematologic cancer. Journal of comparative effectiveness research 2014;**3**: 135-44.

Summary

Umbilical cord blood (CB) is an alternative for mobilized peripheral blood stem cell or bone marrow grafts when a patient needs allogeneic hematopoietic stem cell transplantation (HST). However, CB HST is associated with increased incidence of graft failure and delayed engraftment and immune reconstitution compared to other graft sources, which is caused by the relatively low numbers of stem cells in a cord blood unit. There are a number of strategies that can be used to increase the number of stem cells in a cord blood graft, including double CB transplantation, ex vivo expansion of CB and the co-transplantation of accessory cells such as mesenchymal stem cells (MSC). For this thesis we investigated different aspects of the application of these strategies.
The co-transplantation of MSC has shown promising results in animal models. It enhances the engraftment of the stem cells leading to higher numbers of cells in the hematopoietic organs of the recipient. The majority of MSC used in the clinic are derived from the bone marrow of healthy donors. The harvest of these MSC is a costly procedure and involves a certain risk for the donor, and is therefore ethically constrained.
We compared in vitro characteristics and the engraftment enhancing capacity of BM MSC with MSC from the Wharton Jelly of the umbilical cord (WJ MSC). These MSC have several advantages over BM MSC such as a low collection costs and a relatively low chance of transmissible infections. Furthermore, being a waste product, WJ MSC are free of ethical constraints. Although less capable of differentiating into mesodermal lineages, WJ MSC have a similar capacity to inhibit immunological responses in vitro and to enhance the engraftment of CB CD34+ cells in vivo. WJ MSC would therefore be a good alternative for the use of BM MSC from healthy donors.
Ex vivo expansion of CB has successfully been applied in several phase I/II clinical trials. Our lab has previously developed a protocol for the expansion of CB CD34+ cells, using only TPO, which can improve platelet recovery in the peripheral blood. Expanded cells with this capacity would be especially helpful for patients that are refractory to platelet transfusions, since these patient have an increased risk of dying from hemorrhage after HST. For clinical application of these expanded cells, co-transplantation of non expanded cells is necessary to safeguard the long term reconstitution of all blood cells. We investigated the transplantation of expanded cells and non expanded CB cells and did not find any difference with respect to recovery of CD45+ cells in the peripheral blood of the mice or BM and spleen engraftment when compared to the transplantation of unmanipulated cells from two CB units. Platelet recovery was significantly improved in the first weeks after transplantation, which suggests that this strategy would be favorable over the transplantation of two unmanipulated units. We further investigated if the order of cryopreservation and expansion, i.e. before or after the banking of the CB unit, would influence the outcome of transplantation. Although the number of cells that are formed after expansion are higher when the CB cells are expanded before the cells are cryopreserved, we did not find any significant differences in the engraftment capacity compared to cells that were expanded after cryopreservation of the CB cells. The latter even proved to be more efficient in the recovery of platelets in the peripheral blood of the mice. This suggests that our expansion protocol can be applied to all CB units that are currently stored in CB banks.
Lastly we tried to combine the co-transplantation of MSC with the transplantation of TPO expanded cells. Each strategy by itself can either improve overall engraftment or early platelet recovery in the peripheral blood. A combination of both strategies could therefore further improve the outcome of CB transplantation. However, we found that, although each single strategy improved the outcome of transplantation in the way we expected, the combination of strategies did neither further improve early platelet recovery nor engraftment. Furthermore, we encountered events of non engraftment in several mice,

which suggest that the combination of MSC and expanded cells can lead to unwanted adverse events. We could not determine the cause of these adverse events and therefore concluded that this strategy cannot be translated into a clinical protocol until the cause leading to these events is elucidated.

Recommendations

WJ MSC are a promising source of MSC for clinical application. We therefore recommend that the protocol for the collection of the umbilical cord and processing of the cells should be translated into a GMP grade protocol.

Transplantation of TPO expanded cells in combination with unmanipulated cells seems to be safe with respect to the early recovery of platelets and the long repopulation of the BM. The expansion protocol could therefore be translated into a GMP grade protocol and simultaneously scaled up from culturing small quantities of cells to whole CB units. A phase I clinical trial with TPO expanded cells in combination with unmanipulated CB cells would be an option if there is demand from clinicians that have a sufficient number of patients that would benefit from the transplantation of these cells.

Samenvatting

Navelstrengbloed (NB) is een alternatief voor gemobiliseerd perifeer bloed of beenmerg grafts voor patiënten die een allogene hematopoietische stamceltransplantatie (HST) nodig hebben. NB HST wordt echter geassocieerd met een verhoogde incidentie van graft failure en vertraagde engraftment en immuun-reconstitutie in vergelijking met andere bronnen voor HST grafts. Dit wordt veroorzaakt door het relatief lage aantal stamcellen in een NB eenheid. Er zijn een aantal strategieën die het aantal stamcellen in een NB eenheid kunnen vergroten, waaronder transplantatie van 2 NB eenheden, ex vivo expansie van NB en de co-transplantatie van accessoire cellen zoals mesenchymale stamcellen (MSC). Voor dit proefschrift hebben wij verschillende aspecten van deze strategieën onderzocht.
De co-transplantatie van MSC heeft in diermodellen veelbelovende resultaten laten zien. Het verbeterd de engraftment van de hematopoietische stamcellen, wat leidt tot hogere aantallen cellen in de hematopoietische organen van de ontvanger. De meerderheid van de MSC die in de kliniek gebruikt worden komen van het beenmerg van gezonde donoren. Het oogsten van deze MSC is een kostbare procedure en draagt een zeker risico met zich mee voor de donor en is daarom ethisch gezien niet wenselijk. We hebben daarom de in vitro eigenschappen en de engraftment vergrotende capaciteit van BM MSC vergeleken met MSC afkomstig van een andere bron: de Wharton's Jelly van de navelstreng. Deze MSC hebben een aantal voordelen vergeleken met BM MSC, zoals lage kosten voor collectie en een relatief lage kans op overdraagbare infecties. Er zijn bovendien, aangezien WJ MSC geoogst worden van een afval product, geen ethische bezwaren voor het gebruik van deze cellen. Hoewel deze cellen minder capaciteit hebben om te differentiëren in mesodermale cellijnen, hebben WJ MSC een vergelijkbare capaciteit om in vitro immunologische reacties te remmen en de engraftment van NB CD34+ cellen in vivo te vergroten. WJ MSC zouden daarom een goed alternatief zijn voor het gebruik van BM MSC van gezonde donoren.
De ex vivo expansie van NB is succesvol toegepast in verschillende fase I/II klinische studies. Ons lab heeft een protocol ontwikkeld voor de expansie van NB CD34+ cellen, alleen gebruik makend van TPO, dat het herstel van plaatjes in het perifere bloed (PB) kan verbeteren. Geëxpandeerde cellen met deze capaciteit zouden in het bijzonder geschikt zijn voor patiënten die refractoir zijn voor plaatjestransfusies aangezien deze patiënten een verhoogd risico hebben om te overleiden van bloedingen na een HST. Voor de klinische applicatie van deze geëxpandeerde cellen is het noodzakelijk om niet geëxpandeerde cellen mee te transplanteren om de lange termijn reconstitutie van alle bloedcellen veilig te stellen. We hebben de transplantatie van geëxpandeerde en niet geëxpandeerde onderzocht en vonden geen verschil met betrekking tot het herstel van CD45+ cellen in het PB van de muizen of de engraftment in het beenmerg en milt als we dit vergeleken met de transplantatie van cellen van twee niet gemanipuleerde NB eenheden. Het herstel van plaatjes was significant verbeterd in de eerste week na de transplantatie, wat suggesteerd dat deze strategie favorable is vergeleken met de transplantatie van twee niet gemanipuleerde NB eenheden. Vervolgens hebben we onderzocht of de volgorde van cryopreservatie en expansie, i.e. voor of na het opslaan van de NB eenheid, de uitkomst van de transplantatie beïnvloed. Hoewel het aantal cellen dat gevormd wordt na expansie hoger is als de NB cellen geëxpandeerd worden voor de cellen gecryopreserveerd worden, vonden we geen significante verschillen in de engraftment capaciteit vergeleken met cellen die geëxpandeerd werden na cryopreservatie. Expansie post cryopreservatie leidde zelfs tot een bet plaatjesherstel in het perifere bloed van de muizen. Dit duidt erop dat ons expansieprotocol toegepast kan worden op alle NB eenheden die momenteel opgeslagen zijn in de NB banken.
Tot slot hebben we geprobeerd de co-transplantatie van MSC en de expansie van TPO te combineren. Elke afzonderlijke strategie kan ofwel de engraftment, ofwel het

plaatjesherstel in het perifere bloed verbeteren. Een combinatie van deze strategieën zou dus de uitkomst van een NB transplantatie nog verder kunnen verbeteren. We vonder echter dat, hoewel elke afzonderlijke strategie de uitkomst van de transplantatie naar verwachting verbeterde, een combinatie van beide strategieën noch het plaatsjes herstel noch de engraftment verder kon verbeteren. Bovendien observeerden we zelfs gevallen van non engraftment in meerdere muizen, wat erop duidt dat de combinatie van MSC en geëxpandeerde cellen tot ongewenste neveneffecten kan leiden. We konden niet vaststellen wat de oorzaak van de neveneffecten was en hebben daarom geconcludeerd dat deze strategie niet in een klinisch protocol vertaald kan worden totdat de oorzaak van deze neveneffect is verhelderd.

Aanbevelingen

WJ MSC zijn een veelbelovende bron voor het gebruik van MSC in klinische applicaties. We bevelen daarom aan dat het protocol voor de collectie van de navelstreng en het verwerken van de cellen vertaald moet worden in een GPM geaccrediteerd protocol.

Transplantatie van TPO geëxpandeerde cellen in combinatie met niet geëxpandeerde cellen lijkt veilig te zin m.b.t. het versnelde herstel van plaatjes in het perifere bloed en de lange termijn repopulatie van het beenmerg. Het expansie-protocol zou daarom vertaald moeten worden in een GMP protocol en gelijktijdig opgeschaald moeten worden van het kweken van kleine aantallen cellen tot gehele NB eenheden. Een fase 1 klinische studie met TPO geëxpandeerde cellen in combinatie met niet gemanipuleerde NB cellen zou een optie zijn als er een vraag is vanuit clinici dat een voldoende aantal patiënten voordeel zou hebben bij de transplantatie van deze cellen.

Acknowledgements

Als eerste zou ik natuurlijk mijn dank uit willen laten gaan naar de mensen die mij de mogelijkheid hebben gegeven voor het volgen van mijn promotietraject: Yvette van Hensbergen, Anneke Brand en Jaap Jan Zwaginga. Het tot stand komen van deze thesis niet mogelijk zijn geweest zonder de hulp van iedereen in het Sanquin Leiden lab, en met name Manon, Alice, Jos, Yoshiko en John.

Verder zou ik graag de volgende mensen willen bedanken:

Heleen, Mellisa en Jose, voor hun hulp bij het WJ MSC project, Professor Suzanne Watt, alle medewerkers van de navelstrangbloedbank (NBB), alleen al voor de eindeloze voorraad navelstrengbloed maar ook voor hun bereidheid om altijd mee te denken, de medewerkers van de stamcelbank van het LUMC voor het beschikbaar stellen van BM MSC, Riane Jahan-Willems (NBB) en de mederwerkers van de afdeling verloskunde voor het opzetten van de collectie van navelstrengen in het Rijnland Ziekenhuis en medewerkers van de afdeling verloskunde van het LUMC.

Als laatste wil ik uiteraard veel dank betuigen aan mijn ouders en broer, die me bij gestaan hebben in goede en wat slechtere tijden en zonder wiens steun ik dit nooit bereikt zou hebben.

Publication List

Mark van der Garde, Yvette van Hensbergen, Anneke Brand, Manon C Slot, Alice de Graaf-Dijkstra, Arend Mulder, Suzanne M Watt and Jaap Jan Zwaginga, *Thrombopoietin Treatment of One Graft in a Double Cord Blood Transplant Provides Early Platelet Recovery While Contributing to Long-Term Engraftment in NSG Mice.* **Stem cells and development 2015: (24) 67-76**

Yvette van Hensbergen*, **Mark van der Garde***, Anneke Brand, Manon C. Slot, Alice de Graaf-Dijkstra, Suzanne M Watt and Jaap Jan Zwaginga, ***equally contributing first authors**. *Cryopreservation of cord blood CD34$^+$ cells before or after thrombopoietin expansion differentially affects early platelet recovery in NOD SCID mice.* **Transfusion 2015: (55) 1772-1781**

Mark van der Garde, Anneke Brand, Manon C Slot, Alice de Graaf-Dijkstra, Jaap Jan Zwaginga and Yvette van Hensbergen. *No synergistic effect of co-transplantation of MSC and ex vivo TPO expanded CD34+ cord blood cells on platelet recovery and bone marrow engraftment in NOD SCID mice.* **Stem Cells and Development 2015: (24) 1448-1456**

Mark van der Garde, Melissa van Pel, Jose E. Millán Rivero, Alice de Graaf-Dijkstra, Manon Slot, Yoshiko Kleinveld, Suzanne Watt, Helene Roelofs and Jaap Jan Zwaginga. *Direct comparison of Wharton Jelly and bone marrow derived mesenchymal stromal cells to enhance engraftment of cord blood CD34$^+$ transplants.* **Stem Cells and Development 2015: published online ahead of print**

Suzanne M Watt SM, Francesca Gullo, **Mark van der Garde**, Daniel Markeson, Rosalba Camicia, Cheen P Khoo and Jaap Jan Zwaginga. *The angiogenic properties of mesenchymal stem/stromal cells and their therapeutic potential.* **British medical bulletin 2013: (1) 1-29**

Sarah JM Hale, Ashley BH Hale, Youyi Zhang, Dominic Sweeney, Nita Fisher, **Mark van der Garde**, Rita Grabowska, Emma Pepperell, Keith Channon, Enca Martin-Rendon and Suzanne M. Watt. *CXCR2 modulates bone marrow vascular repair and haematopoietic recovery post-transplant.* **British Journal of Haematology 2015: (169) 552-564**

Francesca Gullo*, **Mark van der Garde***, Giulia Russo, Marzio Pennisi, Santo Motta, Francesco Pappalardo and Suzanne Watt, ***equally contributing first authors.** *Computational modeling of the expansion of human cord blood CD133+ hematopoietic stem/progenitor cells with different cytokine combinations.* **Bioinformatics 2015: 31: 2514-2522**

List of acronyms

AB	antibody
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
BFU-E	burst forming unit erythrocyte
BM	bone marrow
CAFC	cobblestone area forming cell
CB	cord blood
CBT	cord blood transplantation
CFU	colony forming unit
CFU-GM	CFU granulocyte macrophage
CFU-GEMM	CFU **g**ranulocyte erythrocyte monocyte megakaryocyte
CFU-Mk	CFU megakaryocyte
CTE	cryopreserved/thawed/expanded
dCB	double cord blood
DLL-1	delta like ligand 1
DMSO	dimethyl sulfoxide
ECT	expanded/cryopreserved/thawed
FCS	fetal calf serum
Flt3L	Flt3 ligand
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	good manufactoring practice
GvHD	graft versus host disease
GvL	graft versus leukemia
HLA	human leukocyte antigen
HSC	hematopoietic stem cell
HSPC	hematopoietic stem and progenitor cells
HST	hematopoietic stem cell transplantation
IL-X	interleukin-X
IMDM	Iscove's modified Dulbecco's medium
Lin-	lineage negative
LUMC	Leiden University Medical Center
MDS	myelodysplastic syndrom
MNC	mononuclear cells
MMUD	mismatched unrelated donor
MSC	mesenchymal stem/stromal cell
MUD	matched unrelated donor
NBB	navelstrengbloedbank
NOD SCID	non obese diabetic severe combined immuno deficient
NRM	non relapse mortality
PB	peripheral blood
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PBSC	peripheral blood stem cells
rCD34+	residual CD34+ cells (after TPO expansion)
RFS	relapse free survival
SCF	stem cell factor
SEM	standard error of mean
TEPA	tetraethylenepentamine
TNC	total nucleated cell count
TPO	thrombopoietin
T-reg	regulatory T cell
UC	umbilical cord
UCB	umbilical cord blood
WBC	white blood cell
WJ	Wharton Jelly