Improving Umbilical Cord Blood Stem Cell Engraftment by Ex Vivo Expansion of Hematopoietic Stem and Progenitor Cells

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Improving Umbilical Cord Blood Stem Cell Engraftment by Ex Vivo Expansion of Hematopoietic Stem and Progenitor Cells

Het verbeteren van klinische uitkomsten na stamceltransplantatie met navelstrengbloed door het vermeerderen van hematologische stam- en voorlopercellen

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Chapter 1

General introduction and outline of the thesis

1. HEMATOPOIESIS AND HEMATOPOIETIC STEM CELLS

Hematopoiesis (from Greek: αἷμα/aíma is "blood" and ποιεῖν/poieín means "to make") is defined as the production of all types of blood cells, including formation, development and differentiation of those cells. This process starts prenatally, where it progresses in wave-like stages (**figure 1**). The first and second wave take place in the yolk sac (day 16 and day 19). During the third wave, hematopoietic stem cells (HSC) originate in the dorsal aorta (aorta-gonad-mesonephros region, AGM) at approximately 4 weeks of gestation [1]. Subsequently, HSC home to the placenta (and are present in the umbilical cord blood (UCB) as well [2]) and the fetal liver, which is the primary source of erythrocytes from the 9th week of gestation. From there, HSC further expand and eventually seed the bone marrow, which becomes the predominant site of hematopoiesis at the end of the second trimester. At birth, HSC can still be detected in the umbilical cord blood. After birth, hematopoietic activity in the liver ceases and the bone marrow becomes the only site for hematopoiesis [3, 4].

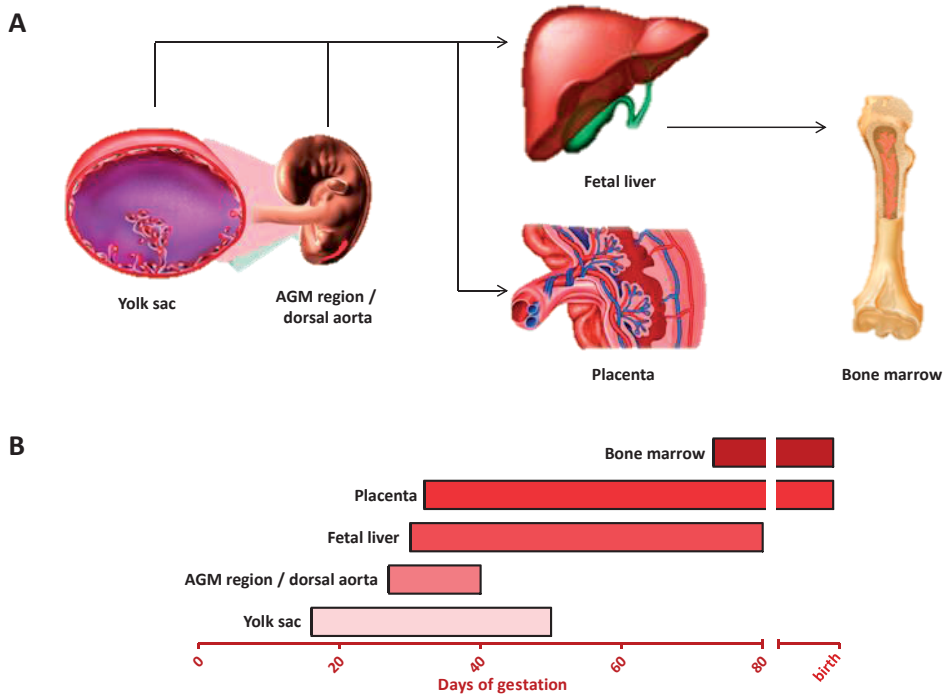


Figure 1. Hematopoietic development and migration during human embryogenesis
A. Migration of hematopoiesis
B. Hematopoietic development in the human embryo in time.
Adapted from [5] and [6]

In adults, all mature blood cells are derived from a small population of HSC that reside in the bone marrow. HSC are self-renewing and have the ability to differentiate into progenitors of all different hematopoietic lineages and eventually generate mature blood cells, including erythrocytes, platelets, and leukocytes. Within the bone marrow, HSC reside in anatomically defined regions, the so-called niche. The bone marrow niche consists of niche cells (e.g. osteoblasts, adipocytes and mesenchymal stromal cells), extracellular matrix and soluble factors such as cytokines, chemokines and signaling molecules derived from the extracellular matrix [7]. Fate decisions of HSC, balancing self-renewal, proliferation and differentiation, are controlled in the bone marrow niche by signaling molecules, such as bone morphogenetic proteins and hematopoietic cytokines, direct cell-cell contact of HSC with the niche cells and interaction with the extracellular matrix [8, 9]. In addition, other factors like the circadian rhythm and oxygen level regulate HSC maintenance by influencing the niche cells and the production of cytokines [10, 11]. It is hypothesized that the bone marrow comprises two distinct niches: an endosteal niche housing the quiescent HSC with self-renewing capacity and a perivascular niche where more actively proliferating HSC reside [12-14].

In contrast to more mature blood cells, HSC are morphologically indistinguishable from immature hematopoietic progenitor cells. In order to identify HSC flow cytometry, gene expression profiling, *in vitro* functional assays and (re)transplantation into immunodeficient mice have been used. HSC are characterized by the expression of a number of cell surface markers which can be used to purify and quantitate HSC. In humans, the marker profile that leads to the highest enrichment of HSC is: $CD34^+$, $CD38^-$, $CD45RA^-$, $CD90^+$ and $CD49f^+$ [15]. In mice, HSC are highly purified within the LSK (Lin^-, c-Kit^+, Sca-1^+), $CD34^-$, $CD150^+$, $CD48^-$, $CD41^-$, $flt3^-$ and $CD49b^{lo}$ population [16].

Transcription factors are able to modulate expression levels of their target genes and are thereby able to influence cell fate decisions. Several genes have been identified that appear to be active in HSC, including Scl1, Gata1, Gata2 and Runx1 [17] as well as associated transcription factors forming the gene regulatory network of HSC [18]. However, the ability of HSC to both self-renew and differentiate complicates capturing HSC function in one gene regulatory network. It is beyond the scope of this thesis to describe these proposed gene regulatory networks in further detail.

Multipotency, a key feature of HSC, can be demonstrated by colony forming unit (CFU) assays. Upon culture of human bone marrow in semisolid methylcellulose medium, colonies containing granulocytes, erythrocytes, monocytes and megakaryocytes (CFU-GEMM) are formed, indicating the presence of pluripotent hematopoietic progenitors [19]. Another technique identifying cells with HSC features is the long-term culture initiating cell (LTC-IC) assay. This assay, where cells are cultured for 5 to 8 weeks on a suitable feeder cell layer followed by replating in methylcellulose to score colonies, identifies primitive hematopoietic cells, based on their capacity to produce progenitors for at least

5 weeks [20]. However, the essential property of the ability of HSC to reconstitute long-term hematopoiesis cannot be measured by these assays. Despite these limitations, *in vitro* assays are still used in order to identify the presence of highly immature hematopoietic cells, which might be an indication of the presence of HSC.

An *in vivo* transplantation assay is required to address the essential feature of the HSC: being able to reconstitute long-term hematopoiesis. To demonstrate the presence of HSC, human cell populations comprising HSC are transplanted into immunodeficient mice, after which human engraftment can be observed in the murine bone marrow [21, 22]. The progeny of HSC contribute to long-term hematopoietic recovery, which can be observed after 3 to 4 months upon transplantation. Repopulation before that time point is derived from committed progenitors and multipotent short term repopulating HSC present in the graft. Additionally, bone marrow from engrafted mice can be retransplanted into secondary recipient mice, thereby showing the presence of the true self-renewing long term repopulating HSC [23]. Currently, these SCID repopulating cells (SRC) represent the cell population that is the closest to HSC.

2. HEMATOPOIETIC STEM CELL TRANSPLANTATION

2.1 Introduction

Following the observation that irradiation is especially harmful for bone marrow [24], research was initiated to explore whether transplanted bone marrow would protect individuals from otherwise lethal effects of irradiation, given at a toxic bone marrow dose. Injection of homologous bone marrow rescued mice [25], rats [26], hamsters [27] and rabbits [28] from lethal irradiation. The first successful bone marrow transplantation in humans was reported in 1957 by Thomas *et al*., who reported the clinical course of a patient with leukemia receiving bone marrow of its identical twin via intravenous infusion upon treatment with chemotherapy and radiation [29]. It took more than 10 years, until the first successful true-allogeneic, non-twin transplantations were performed, with the donors being siblings of the patients [30, 31]. Currently, allogeneic hematopoietic stem cell transplantation (allo-SCT) is the treatment of choice for many patients. In total, more than 400,000 allo-SCT have been performed worldwide [32]. Primarily, allo-SCT is performed in patients suffering from severe hematological diseases such as leukemia, myelodysplastic syndrome, lymphoma and aplastic anemia, but is used for many other disorders as well (**table 1**) [33]. The therapeutic effect depends on both intensive chemotherapy and irradiation and on eradication of residual leukemia cells of the recipient by donor immune cells present in the graft, an immune-mediated effect known as 'graft versus leukemia' (GVL).

Table 1. Indications for allo-SCT [33]

Malignancies	Other diseases
Acute myeloid leukemia	Aplastic anemia
Acute lymphoblastic leukemia	Paroxysmal nocturnal hemoglobinuria
Chronic myeloid leukemia	Fanconi's anemia
Myelodysplastic syndrome	Blackfan-Diamond anemia
Non-Hodgkin's lymphoma	Thalassemia major
Hodgkin's disease	Sickle cell anemia
Chronic lymphocytic leukemia	Severe combined immunodeficiency
Multiple myeloma	Wiskott-Aldrich syndrome
Juvenile chronic myeloid leukemia	Inborn errors of metabolism

2.2 Immunological principles of allogeneic hematopoietic stem cell transplantation

Two types of allogeneic immune reactivity should be distinguished. First, the so-called host versus graft (HVG) reaction, mediated by recipient T cells, which may result in rejection of the graft and thereby in graft failure. Rejection is primarily determined by differences in highly polymorphic human leukocyte antigens (HLA) between donor and recipient [34]. HLA antigens are essentially involved in the afferent and efferent phases of a T cell dependent immune response and therefore abundantly expressed on hematopoietic cells (both class I and II), but also on every nucleated cell (class I) [35, 36]. Major HLA-antigens continuously present foreign and autologous peptides to $CD4^+$ T cells (class II) or to $CD8^+$ T cells (class I) in order to direct an immune response towards infectious micro-organisms, but not to recipient tissues [37]. In addition to major HLA antigens, minor antigens have been identified, which refer to peptides derived from human proteins and tissues, that may differ between donor and recipient and may also be involved in alloreactivity after transplantation [38]. Furthermore, the degree of immune suppression given to the recipient also strongly affects the probability of rejection and whether or not the donor graft is depleted from T cells [39]. Apart from host versus graft reactions, a reverse type of alloreactivity can occur, exerted by donor T cells present in the donor graft recognizing recipient tissues (graft versus host), again due to differences in HLA major and HLA minor antigens. Clinically, such alloreactivity is termed graft versus host disease (GVHD) with acute and chronic variants based upon the time of onset and clinical manifestations [40]. Acute GVHD may present itself as a maculopapular rash, persistent nausea and/or emesis, abdominal cramps with diarrhea and liver function abnormalities consistent with hepatitis [41, 42]. In contrast, patients with chronic GVHD demonstrate skin involvement resembling lichen planus or the cutaneous manifestations of scleroderma, dry oral mucosa with ulcerations and sclerosis of the gastrointestinal tract and liver function abnormalities consistent with chronic hepatitis [43]. Chronic GVHD can be

preceded by acute GVHD, either direct or after a disease-free interval, or it may occur de novo without clinically evident acute GVHD preceding. Although GVHD is a severe complication of allo-HSCT, that needs immunosuppressive treatment, the alloreactive T cells may also recognize and eradicate the underlying leukemia, an effect known as the GVL effect. This GVL effect plays a major role in reducing the risk of relapse following allo-HSCT [44]. Both GVHD and GVL are the result of graft cells recognizing recipient cells, based on differences in HLA antigens. However, it is still unclear whether the specificity of T cells responsible for GVHD differ from that of T cells mediating the GVL effect. There are several strategies to prevent GVH alloreactivity, including T cell depletion of the graft and the use of immunosuppressive agents such as cyclosporine, tacrolimus, sirolimus, mycophenolate and corticosteroids. However, these strategies also suppress GVL effects and may therefore be associated with an enhanced frequency of relapse [45]. Prevention of host versus graft alloreactivity is primarily based on matching for HLA-antigens between donor and recipient in conjunction with immunosuppressive therapy prior to transplantation, aimed at inhibition of the recipients immune response. In contrast to solid organ transplantation, allo-HSCT is generally followed by tolerance for donor hematopoietic cells by the recipient, resulting in a durable engrafted and functional hematopoietic system [46]. Tolerance results from both central and peripheral mechanisms, including central (thymic) deletion of newly developed, donor derived auto-reactive T cells and peripheral tolerance by the functional inhibition of auto(recipient)reactive donor T cells by regulatory T cells and inhibitory cytokines such as IL-10 [47]. In general, tolerance is established by 6 months after transplantation allowing then to stop further immunosuppressive therapy, provided that no graft versus host disease is present.

2.3 Conditioning regimens prior to hematopoietic stem cell transplantation

The aim of condition therapy prior to allo-SCT is threefold: reducing the disease burden, deterging the bone marrow and thereby making room for the donor cells and suppressing the recipients immune system and thereby allowing engraftment of the HSC [48]. However, the latter aim is most probably the most important, as the high rates of engraftment following non-myeloablative conditioning has shown that intensive immunosuppressive therapy is sufficient[49]. Several regimens are defined based on their intensity and toxicity. Three types of conditioning were defined by Bacigalupo *et al*. [50], namely, myeloablative (MA) conditioning, reduced intensity conditioning (RIC) and nonmyeloablative (NMA) conditioning. MA conditioning is defined as a combination of agents expected to produce profound pancytopenia and myeloablation within 1 to 3 weeks from administration; pancytopenia is long lasting, usually irreversible, and in most instances fatal, unless hematopoiesis is restored by hematopoietic stem cell infusion. MA regimens that are frequently applied include total body irradiation (TBI, 10-12 Gy) in combination with cyclophosphamide and the combination of busulfan and cyclophosphamide. NMA

regimens are those that will cause minimal cytopenia, do not require stem cell support, and mainly consist of immunosuppressive agents. A well-known example of the latter is the fludarabine plus TBI (2 Gy) schedule, as was developed by Storb *et al*. in Seattle [51], which results in engraftment and appears to be associated with a low rate of early mortality. RIC regimens are an intermediate category of regimens that fit neither the MA nor NMA definition. RIC regimens cause cytopenia, which may be prolonged and may require stem cell support, but it is possible that autologous recovery would occur eventually. An array of RIC regimen were developed the last decades, including disease specific regimens and more generally applicable regimens, but randomized comparative studies are lacking, leaving the question which regimen is to be preferred unanswered.

2.4 Hematologic and immunologic reconstitution following hematopoietic stem cell transplantation

Hematopoietic recovery following allo-SCT depends on many variables, but the quality of the donor graft in terms of stem cell numbers and whether the graft was manipulated prior to transplantation are the main denominators of hematopoietic recovery. In general, following allo-SCT from an HLA-identical sibling donor, neutrophil recovery and platelet recovery usually take, respectively, approximately 14 and 25 days [52]. Alternative donor stem cell grafts usually are associated with a more protracted recovery, which is most pronounced following umbilical cord blood stem cell transplantation (UCBT), which may take 3 to 4 weeks for neutrophil recovery and up to 3 months for platelet recovery, which is due to the low number of stem cells and the multiple HLA-mismatches between donor and recipient [53, 54]. Lymphoid recovery may take much longer than myeloid recovery, which is mainly due to insufficient thymopoiesis after allo-SCT in adult patients. While NK cells may rapidly recover, B cells usually take up to several months and T cell recovery may even be extended up to 12 months [55]. Full immune reconstitution requires the restoration of both non-hematopoietic and hematopoietic components. The non-hematopoietic components of the immune system (the skin and mucosa surfaces) are part of the innate (natural) immunity, which is present from birth and which is not adapted or developed during infections such as the adaptive part of the immune system, including B cells and T cells. Especially, $CD4^+$ T cell counts may be insufficient beyond one year post-transplantation, indicating the slow and retarded recovery of adaptive immune function [56]. For T cell recovery, both peripheral expansion of transplanted T cells and formation of newly developed T cell in the thymus (thymopoiesis) are pivotal. Impaired recovery of thymopoiesis is a major risk factor for retarded T cell recovery and subsequent susceptibility for opportunistic infections and treatment related mortality [57]. Thymopoiesis can be monitored by measuring the frequency of signal joint T cell receptor rearrangement excision circles (sjTRECs). SjTREC positive $CD3^+$ cells are T cells

newly formed by thymopoiesis. Measuring sjTREC positive T cells is however not routinely applied in clinical care.

Many variables may affect the timing of reconstitution, including the occurrence of GVHD, conditioning regimen, type of donor, source of hematopoietic progenitor cells, number of HLA-mismatches and immune suppressive therapy applied post-transplantation[55]. Several therapeutic strategies have been proposed to enhance immune reconstitution following allo-SCT, however, most of these strategies have only been tested in preclinical settings [58, 59]. One of these approaches is the administration of the cytokine interleukine-7 (Il-7). In animal models and in humans, administration of Il-7 resulted in strong expansion of newly developed naïve T cells [60, 61]. In a phase I trial, administration of recombinant Il-7 enhanced T cell recovery, without increasing the incidence of GVHD [62]. Other approaches include adoptive T cell therapy by using either unselected donor lymphoid cells or donor T cells selected on the basis of antigen specificity, such as anti-cytomegalovirus (CMV) specificity [63, 64].

2.5 Donors and stem cell sources

Outcome after allo-SCT depends on many variables, including type and stage of underlying disease, patient co-morbidities, timing of the transplant and choice of donor. The potential benefit of allo-SCT and potential risks of the procedure are usually taken into account in the decision making whether or not allo-SCT is the preferred treatment in a particular patient or whether chemotherapy or autologous transplantation is to be preferred [65]. As mentioned earlier, matching of HLA-haplotypes between donor and patient is a key part of successful allo-SCT [66, 67]. An HLA-matched sibling of the patient is the preferred donor of choice, but 75% of the patients lack such a matched sibling donor. As a second option, HLA-matched unrelated donors can be identified using a worldwide donor database containing more than 20 million HLA-typed volunteers. For up to 50-60% of Caucasian patients a suitable matched unrelated donor may be identified, but for patients without such a donor, alternative donors can be considered, including haplo-identical donors and umbilical cord blood [68].

3. UMBILICAL CORD BLOOD STEM CELL TRANSPLANTATION

3.1 Umbilical cord blood as a source for hematopoietic stem cells

During fetal development, Hematopoiesis takes place in the yolk sac, the fetal liver and finally, in the bone marrow upon settling of HSCin their bone marrow niches. Hematopoietic stem and progenitor cells (HSPC) can be found in fetal blood [69] and especially in umbilical cord blood (UCB) around birth [2]. These UCB-derived HSPCappeared to be an alternative source of HSPC for allo-SCT [70], In 1988, the first transplantation with

HSPC from UCB was performed in a patient with Fanconi's anemia using the cord blood from his HLA-identical sibling [71]. The patient obtained full engraftment of the donor cells and has maintained hematological remission for more than 20 years. In this first patient, there were no signs of GVHD. Subsequent studies, using unrelated mismatched cord blood, showed encouraging results, with similar survival to HLA-identical sibling cord blood transplantation and less acute and chronic GVHD [72-74]. The Netcord group, an international cord blood bank network, was established in 1998 [75] and more than 700,000 cord blood units are currently stored in quality controlled public cord blood banks worldwide [76]. Currently, the number and broad availability of cord blood grafts as well as the organization of cord blood banks have resulted in a relative rapid and easy procedure of search and acquisition of cord blood grafts, which may even be faster than the search and acquisition for adult bone marrow and blood grafts [74]. In addition, less stringent HLA-matching was required compared to other donor sources [77], implying cord blood a suitable source of stem cells for many patients lacking a HLA-matched sibling or unrelated donor. By 2015, approximately 35,000 UCB transplantations (UCBT) have been performed [76].

Initial studies assessing cord blood transplantations in small groups of pediatric patients suffering from hematological malignancies, showed that the procedure was feasible and a potential therapeutic option [78]. These positive results were confirmed in several prospective multicenter studies, showing similar survival rates after cord blood transplantation compared to other donor sources used [79, 80]. In addition, GVHD rates were generally lower [79, 80]. These promising survival results, combined with a reduced risk of GVHD and the less stringent matching criteria made cord blood transplantation an attractive treatment modality for pediatric patients with hematological malignancies lacking a sibling or matched unrelated donor. These results in pediatric patients set the stage for a large study of cord blood transplantation in adults with hematologic malignancies receiving MA conditioning, showing cord blood transplantation is feasible and safe in adults as well [81]. When compared to matched unrelated stem cell transplantation after MA conditioning in patients with acute leukemia, cord blood transplantation displayed similar leukemia-free survival, chronic GVHD rates, transplant-related mortality and relapse rate[82]. Furthermore, acute GVHD rates were lower upon cord blood transplantation, but neutrophil recovery was delayed. Subsequently, several other studies showed similar rates of treatment-related mortality, treatment failure, overall mortality and leukemia-free survival for cord blood transplants (1 or 2 HLA-mismatches), mismatched related and unrelated donors [52, 83].

3.2 Umbilical cord blood stem cell transplantation: challenges and recommendations

Prolonged hematopoietic recovery and a higher incidence of graft failure were observed after cord blood transplantation, which appeared to be associated with the number of HSPC present in the graft and the degree of HLA mismatch] [54, 84, 85]. As a result, a higher risk for non-relapse mortality became apparent [79]. To avoid his, guidelines advocate the use of UCB grafts with at least 3.0 x 10^7/kg nucleated cells (NC) for single unit transplantations [79], and even higher cell numbers in the case of HLA-mismatches. However, these thresholds, combined with the criteria for HLA matching, severely limit the number of grafts suitable for transplantation. To improve the outcome of UCBT, the field has come up with various alternative strategies. We will discuss the most promising strategies, some of which are currently used in clinical practice.

4. DOUBLE UMBILICAL CORD BLOOD STEM CELL TRANSPLANTATIONS

4.1 Rationale and history of double umbilical cord blood stem cell transplantation

Co-infusing two cord blood units, the so-called double UCBT (dUCBT), was used as a strategy to increase the cell dose in order to improve engraftment and recovery, while using the same criteria for HLA histocompatibility. The first dUCBTs were performed in 1999 in two adult patients suffering from acute lymphoid and chronic myeloid leukemia, respectively. Donor engraftment was observed in both patients, who however both died three months post-transplant (due to relapse and hemorrhage, respectively) [86]. Barker *et al*. reported the first case of dual donor chimerism after dUCBT using two partially HLA-matched unrelated umbilical cord blood grafts [87]. In the years thereafter, it was shown that dUCBT was safe and feasible after both MA [88] and RIC regimens [89]. Following these studies, Eurocord advised to perform dUCBT if cell dose criteria could not be met using a single cord blood unit and since 2005, according to Eurocord reports, the number of dUCBT performed in adult patients has surpassed the number of single UCBT [86].

4.2 Graft predominance after double umbilical cord blood stem cell transplantation

In the majority of patients receiving a dUCBT, initial combined engraftment of both units is rapidly followed by single-unit-dominance in favor of one of the two grafts [88-91]. By day 21 post-transplantation, single-unit-dominance can be detected in more than 80% of the patients [92-94]. Although the exact mechanisms determining predominance are still largely unknown, an important hypothesis is that predominance is based on an immune interaction between the cord blood units [95, 96]. According to a study in

mice, mixed chimerism was established upon infusing of two $CD34^+$-selected units, whereas addition of the corresponding $CD34^-$ cells restored single-unit-dominance [96]. Furthermore, some studies showed that $CD3^+$ cell dose might be a predictor for cord blood predominance following both MA and RIC dUCBT [53, 95]. Early T cell chimerism of the predominating unit (day 7 and day 11 post-transplantation, respectively) predicts the long-term engrafting unit [93, 97] and in addition, administration of anti-thymocyte globulin shortly before infusion of the grafts, resulted in a higher incidence of mixed double chimerism [98], suggesting that an immunological mechanism involving T cells accounts for single-unit-dominance. Detailed engraftment kinetics studies showed that $CD4^+$ lymphocytes expand early after SCT, suggesting that $CD4^+$ T cells may play a pivotal role in the underlying graft-versus-graft response [93]. In addition, $CD4^+$ T cells of the predominating graft reactive against a non-matched HLA-class II antigen expressed by the disappearing unit were detectable in blood of most patients after dUCBT [99], suggesting a pivotal role for alloreactive $CD4^+$ T cells in immediate rejection. However, prediction of the winning unit prior to transplantation is still not feasible. Another factor may be the $CD34^+$ cell viability, which presumably reflects the quality of the overall graft, with grafts with a $CD34^+$ viability exceeding 75% showing a higher rate of engraftment compared to grafts with a lower $CD34^+$ viability [53, 100]. In addition, the number of ALDH-bright cells, as a reflection of viable progenitor cells, might influence donor chimerism, engraftment and recovery [101]. Order of UCB infusion or level of HLA matching with the patient did not appear to influence engraftment kinetics [95].

4.3 Clinical outcome following double umbilical cord blood stem cell transplantation

Sustained engraftment is usually observed in 85% to 100% of patients after dUCBT, both after MA and NMA conditioning [88, 91, 102, 103]. Median time to neutrophil recovery upon dUCBT varies from 12 days [91] to 36 days [94], depending on the conditioning regimen used and administration of granulocyte colony-stimulating factor (G-CSF) following transplantation. Conflicting results are reported on relapse and progression after dUCBT compared to other graft sources. Several studies report a lower incidence of relapse or progression upon dUCBT compared to single UCBT [104] and transplantation with grafts obtained from either matched-related or –unrelated donors [105]. Strikingly, there was no association with an increased risk of severe GVHD. However, others have reported no advantage in progression-free-survival following dUCBT [106-108]. It is important to emphasize that transplantation outcome is affected by many variables, including donor source, conditioning regimen used, characteristics of the underlying disease, post-transplant treatment and overall patient health. Therefore, conflicting results might be due to different treatment protocols and selected patient populations. In current clinical

practice, dUCBT is considered an appropriate alternative for patients lacking a single adequately dosed cord blood unit.

5. *IN VITRO* EXPANSION OF UCB-DERIVED HEMATOPOIETIC STEM CELLS

Because UCB grafts contain a relatively low number of HSPC and the dose of HSPC infused is related to patient survival and time required for engraftment [84, 105], it was hypothesized that expansion of HSPC *in vitro* prior to transplantation may result in improved engraftment and recovery compared to transplantation with non-expanded cord blood HSPC. Robust expansion of long-term repopulating HSC *ex vivo*, however, remains a challenge. Culturing HSPC with different combinations of hematopoietic cytokines such as stem cell factor (SCF), Fms-related tyrosine kinase 3 ligand (Flt3L), thrombopoietin (TPO) and granulocyte-macrophage colony-stimulating factor (GM –CSF) resulted in massive expansion of committed HPC which is accompanied by a loss or at best maintenance of primitive HSC with long-term repopulation ability [109-112]. In addition to these proliferation- and differentiation-inducing cytokines, *ex vivo* expansion of HSC may require additional signals that inhibit differentiation of HSC during culture. Expansion of HSC requires symmetric self-renewal cell divisions in which both daughter cells retain all HSC characteristics (**figure 2**). Asymmetric cell divisions in which one daughter cell is still a HSC and the other daughter cell is a more differentiated progenitor cell leads to maintenance of HSC number in culture, whereas symmetric differentiation cell divisions in which both daughter cells lost HSC characteristics results in a loss of HSC during culture. Decades of research has resulted in the identification of many developmental regulators and chemical modulators that have been implicated to be involved in the regulation of self-renewal, proliferation, differentiation and survival of HSC and that may increase the frequency of HSC symmetrical self-renewal cell divisions in culture [113]. Several of these factors have been evaluated for their potential to expand HSC in culture. *Ex vivo* HSC expansion protocols using some of these factors are currently evaluated in clinical trials. Several of these early phase clinical trials have reported promising results [7]. However, the search for the most optimal expansion protocol continues.

5.1 Nicotinamide

Nicotinamide (NAM) is a form of vitamin-B3, which inhibits several classes of ribosylase enzymes and sirtuin1 (SIRT1), a class III NAD+-dependent-histone-deacetylase, and is implicated to play an role in the regulation of cell adhesion, polarity, migration, proliferation and differentiation [114]. In addition, NAM modulates the fate of embryonic stem cells [115]. In primary growth factor-driven expansion cultures with UCB-derived $CD34^+$ cells, addition of NAM resulted in a higher number of immature $CD34^+CD38^-$ cells, a

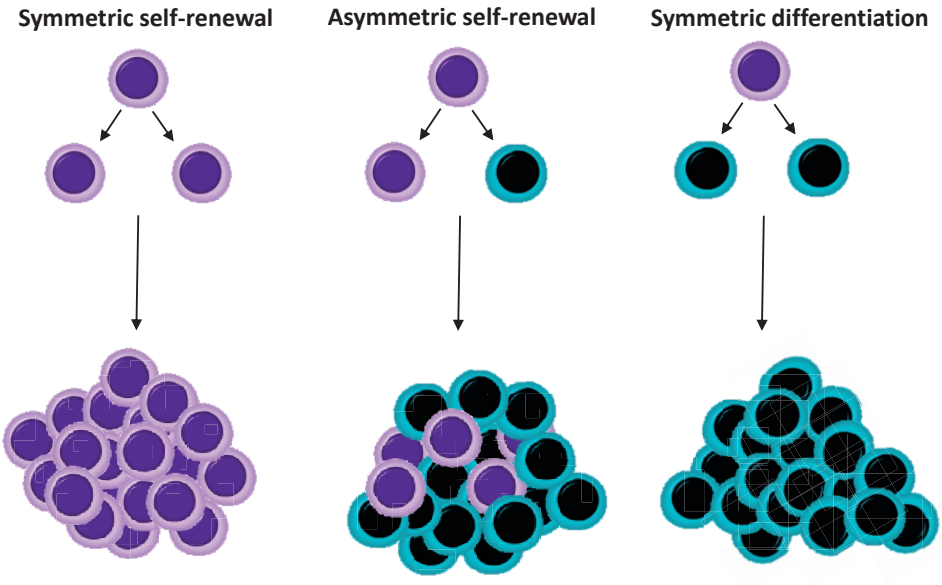


Figure 2. Symmetric and asymmetric cell division of a hematopoietic stem cells. Purple cells are HSC; green cells lost HSC characteristics.

lower number of differentiated cells and delayed cell cycling of $CD34^+$ cells. Furthermore, both *in vitro* and *in vivo* studies showed enhanced migration, homing and engraftment of the NAM-expanded $CD34^+$ cells compared to $CD34^+$ cells expanded in control condition (culture medium supplemented with fetal bovine serum (FBS), SCF, Flt3L, TPO and interleukin-6 (IL-6) [116]. In a phase I study, 11 patients received a NAM-expanded graft (consisting of both the expanded $CD133^+$ fraction and the non-expanded $CD133^-$ fraction) in a dUCBT setting, together with an unmanipulated graft. No adverse events were reported that were attributable to the infusion of the expanded product. In 8/11 patients, the expanded graft engrafted as the dominant graft, with stable engraftment upon a median follow-up of 21 months. In addition, patients engrafted with the expanded graft achieved a median neutrophil recovery of 13 days versus 25 days in the patients engrafted with the unmanipulated graft [117]. Currently, a phase I/II study investigating engraftment and recovery after transplantation with a single NAM-expanded graft is ongoing (ClinicalTrials.gov Identifier: NCT01816230).

5.2 Copper chelator tetraethylenepentamine

Copper has been implicated to be involved in the modulation of self-renewal and differentiation of HSPC. Deficiency of copper in patients mimics refractory anemia, which is manifested by an excess of immature cells and a reduction of differentiated cells in

the bone marrow [118]. *In vitro*, copper deficiency results in delayed differentiation and prolonged proliferation of HSPC, while increased cellular copper levels result in accelerated differentiation [119]. It appeared that reduction of the cellular chelatable copper content, rather than the overall copper content, resulted in the observed effects of copper deficiency [120]. Copper chelator tetraethylenepentamine (TEPA) can be used to modulate the level of cellular chelatable copper. Addition of TEPA to expansion cultures (containing FBS, SCF, Flt3L, TPO and IL-6) results in robust expansion of HSPC. These HSPC show both high levels of engraftment and multi-lineage differentiation potential upon transplantation in NOD/SCID mice [121]. In a phase I/II study, co-transplantation of TEPA-expanded $CD133^+$ CB-derived HSPC and unmanipulated HSPC from the same graft appeared to be safe and feasible [122]. Nine out of 10 patients engrafted with a median time to neutrophil and platelet recovery of 30 and 48 days respectively which is similar compared to recipients of unmanipulated single UCB grafts.

5.3 Notch ligands

Notch and its ligands (Jagged-1, Jagged-2, Delta-1) are expressed in the bone marrow micro-environment and by HSC, which may suggest that Notch is an important regulator in hematopoiesis [123]. The immobilized form of Delta-1 has been shown to promote expansion of murine and human HSPC in culture and to increase the number of SRC with secondary transplantation ability. The effects of Notch ligand appeared to be dose-dependent and HSPC expansion was only observed using lower Delta-1 concentrations [124, 125]. In a clinical phase I study, upon co-transplantation of a Notch ligand-expanded graft and an unmanipulated graft, patients engrafted with an accelerated time to neutrophil recovery of 16 days. In the first week after transplantation, hematopoietic recovery was mainly derived from the expanded graft, but upon day 80 all hematopoietic cells in blood and marrow were derived from the unmanipulated graft [126]. These data suggest that either long term HSC were lost during expansion or that the expanded graft, lacking T cells, was rejected by the unmanipulated graft.

5.4 Wingless-related integration site proteins

Wingless-related integration site (Wnt) signaling has been implicated to play a role in the regulation HSPC fate decisions *in vivo* and might promote HSPC self-renewal by inhibiting differentiation. However, the effects of Wnt on the *ex vivo* expansion of HSPC remain controversial. In primary cultures of Bcl2-mouse $Lin^-Sca^-c\text{-}Kit^+$ cells (LSK cells, enriched for HSPC in mice) supplemented with cytokines and serum, Willert *et al*. and Reya *et al*. showed induction of proliferation and inhibition of differentiation upon the addition of purified Wnt3a [127, 128]. However, other studies observed a decrease in mouse LSK cells upon addition of Wnt3a to the culture medium [129], which is also described in this thesis. In addition, we describe the effect of the addition of Wnt3a to expansion cultures

with human UCB-derived HSPC. To date, no clinical trials with Wnt protein expanded HSPC have been initiated.

5.5 Aryl hydrocarbon Receptor antagonist StemRegenin1

The Aryl hydrocarbon Receptor (AhR) pathway is involved in HSC self-renewal and inhibition of differentiation. AhR-KO mice have an increased number of hyperproliferative LSK cells [130], while treatment of donor mice with AhR agonist TCDD results in impaired competitive engraftment [131]. Using high throughput screening of a library of small molecules, purine derivative StemRegenin1 (SR1), an AhR antagonist, was identified promoting the *ex vivo* expansion of $CD34^+$ cells [132]. Addition of SR1 to expansion cultures supplemented with SCF, Flt3L, TPO and IL-6 resulted in a 50-fold increase in the number of $CD34^+$ cells and a 17-fold increase in SRC, the cells that are capable of hematopoietic reconstitution in sublethally irradiated mice [132]. Transplantation of SR1-expanded $CD34^+$ cells in a double cord blood setting appeared safe and feasible and resulted in engraftment in all 17 transplanted patients. The median time to neutrophil and platelet recovery was 15 and 49 days, respectively, which was faster compared to an historical control group receiving unmanipulated dUCBT. In 11/17 patients, hematopoiesis was primarily derived from the expanded unit and in those patients, neutrophil recovery was even more rapid with a median of 11 days versus 23 days in the 6/17 patients engrafting with the unmanipulated unit [133]. These results set the stage for clinical studies in a single UCBT setting (ClinicalTrials.gov Identifier: NCT01930162).

5.6 UM729 and UM171

In a recent study, a library-screen of more than 5000 low-molecular-weight compounds identified UM729 as an AhR-independent compound capable of expansion of human $CD34^+CD45RA^-$ cells [134]. Further characterization and optimization resulted in the synthesis of the analog UM171, which appeared to be 10 to 20 times more potent than UM729. When compared to SR1, UM171 resulted in similar $CD34^+$ and higher $CD34^+CD45RA^-$ cell frequencies and absolute numbers after 12 days of *in vitro* culture in the presence of SCF, Flt3L and TPO. Transplantation of UM171-expanded cells into NSG mice resulted in higher levels of human chimerism compared to transplantation of SR1-expanded cells. In addition, transplantation of the input equivalent of only 50-100 UM171-expanded cells resulted in long-term multilineage engraftment, which was not the case upon transplantation of the same amount of SR1-expanded cells. These data indicate that UM171 enables robust *ex vivo* expansion of human CB cells with long-term *in vivo* repopulating capacity. Currently, a phase I/II trial exploring the safety and feasibility of transplantation of UM171-expanded $CD34^+$ cells in a single graft setting is ongoing (ClinicalTrials.gov Identifier: NCT02668315).

5.7 Co-culture with mesenchymal stem cells

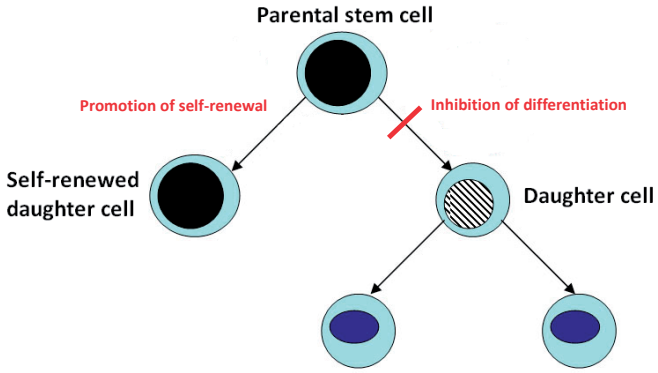
Most expansion protocols use selected $CD34^+$ or $CD133^+$ HSPC and subsequent culture with the addition of exogenous growth factors and differentiation-inhibiting factors. Another approach is to co-culture cord blood cells with cells that are present in the microenvironment of HSC in bone marrow, such as mesenchymal stem cells (MSC). Mimicking the *in vivo* 'niche' of the HSC is thought to provide the complex molecular signals that are important for HSC self-renewal, proliferation and differentiation. Co-culturing CB cells for 14 days on primary MSC cells in the presence of FBS, SCF, TPO and G-CSF resulted in a significant increase of $CD34^+$ cells and CFU [135]. Transplantation of MSC-expanded cells in a dUCBT setting together with a unmanipulated graft appeared to be safe and feasible [136]. Compared to historical controls that had received an unmanipulated dUCBT, the cumulative incidence of neutrophil engraftment at day 26 and platelet engraftment at day 60 was significantly increased. Although about half of the patients had hematopoiesis derived from both units in the first month after transplantation, long-term engraftment was produced primarily by the unit of the unmanipulated cord blood in all patients, suggesting either loss of self-renewing HSC in the expanded graft or rejection of the manipulated graft by T cells present in the unmanipulated graft.

The quest to the most optimal expansion protocol is still ongoing. An optimized protocol might involve a combination of several factors that act synergistically in order to induce cell proliferation while maintaining self-renewal capacities and inhibit differentiation [137].

6. AIMS AND OUTLINE OF THE THESIS

UCB has emerged as an important donor source in patients lacking a suitable sibling or matched unrelated donor. However, the lower number of HSPC in UCB and their relative primitivity combined with a higher degree of HLA-mismatches results in prolonged hematopoietic recovery and retracted immune reconstitution upon UCBT, which may predispose for infections and non-relapse mortality. To improve outcome following UCBT, various strategies have been developed including *ex vivo* expansion of UCB-derived HSPC. In the last decade, several factors have been reported to affect HSC fate decisions promoting self-renewal and inhibiting differentiation. The general aim of this thesis was to improve and optimize the ex vivo expansion and subsequent in vivo engraftment of HSPC. It was hypothesized that the combination of growth factors stimulating proliferation with promising factors that reportedly inhibit differentiation and enhance self-renewal of HSC (**figure 3**) would result in robust ex vivo HSC expansion.

The thesis starts with a study of recovery of thymopoiesis, immune reconstitution, the incidence of infections and clinical outcome in our own population of patients with



high-risk hematological malignancies who underwent dUCBT preceded by a RIC regimen without *in vivo* T cell depletion as described in **chapter 2.** Furthermore, as it is known that retracted thymopoiesis predicts for severe infections in patients receiving allo-SCT from a sibling or matched unrelated donor following MA condition, we evaluated the impact of recovery of thymopoiesis on clinical outcome in this dUCBT cohort.

In **chapter 3** we investigated the effect of Wnt3a on the proliferation and differentiation of mouse LSK cells in combination with early acting cytokines in stroma-free, serum-free cultures, as the Wnt signaling pathway has been implicated to play a role in the regulation of mouse HSC fate decisions between self-renewal and differentiation. Exogenous Wnt3a was found to decrease the number of LSK cells in expansion cultures. This was due to binding of Wnt3a to its receptor and subsequent activation of the canonical Wnt pathway and not due to an increase in apoptosis of LSK cells.

Next, we set out to evaluate the effects of exogenous Wnt3a in combination with hematopoietic growth factors on the *ex vivo* expansion of UCB-derived HSPC. The results of these studies are described in **chapter 4**. It was shown that exogenous Wnt3a protein suppresses rather than promotes the expansion of UCB-derived $CD34^+$ cells in serum free expansion cultures. In addition, we analyzed the effect of Wnt3a on $Lin^-CD34^+CD38^{low}CD45RA^-CD90^+$ cells which are highly enriched in HSC, and we observed the same effect. These data where further confirmed by *in vivo* transplantation assays in immunodeficient (NSG) mice.

In **chapter 5** we investigate the effects of the combination of hematopoietic growth factors and AhR antagonist SR1 on the *ex vivo* expansion of UCB-derived HSPC. We confirm that SR1 enables robust *ex vivo* expansion of UCB-derived HSPC retaining multilineage and long-term repopulation potential in NSG mice. Unexpectedly, we found human chimerism levels after transplantation of expanded cells to be lower than after transplantation of non-expanded cells which might be due to a combination of a

B cell-prone NSG mouse model and a reduced number of lymphoid progenitors in the expanded cell population.

To non-invasively monitor and quantify homing of transplanted HSPC in the bone marrow, we developed a ^{19}F-labeling protocol for HSPC as described in **chapter 6.** Our results show sufficient intracellular labeling of UCB-derived CD34^{+} cells using ^{19}F-containing Poly(Lactic-co-Glycolic Acid) (PLGA) nanoparticles. These labeled cells were detectable with both flow cytometry and magnetic resonance spectroscopy (MRS), while maintaining their ability to proliferate and differentiate.

In **chapter 7**, the development of a Good Manufacturing Practice (GMP)-compliant expansion protocol for clinical application, based on the results presented in **chapter 5**, is described. In addition, the protocol for a clinical study addressing the safety and feasibility of the transplantation of SR1-expanded HSPC in a single UCB unit setting is presented.

Finally, in **chapter 8**, the results, implications and future perspectives of the experimental chapters are discussed in a broader perspective.

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Chapter 2

Impaired thymopoiesis predicts for a high risk of severe infections after double umbilical cord blood transplantation

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Submitted

ABSTRACT

Background

Double umbilical cord blood transplantation is an important treatment option in patients in need of an allogeneic hematopoietic stem cell transplantation but lacking a matched related or unrelated adult donor. Hematopoietic recovery and immune reconstitution, in particular recovery of thymopoiesis, however, are retarded, which may predispose for infections and non-relapse mortality. Earlier, we reported that retarded thymic recovery strongly predicts for subsequent opportunistic infections in recipients of matched related and unrelated transplants. Here, we set out to assess whether impaired recovery of thymopoiesis also predicts for opportunistic infections in recipients of double umbilical cord blood transplantation and whether it is associated with infection-related mortality, non-relapse mortality, relapse-free survival and overall survival.

Patients and methods

Fifty-five patients with high-risk hematological malignancies receiving a double umbilical cord blood transplantation preceded by a reduced intensity conditioning regimen without *in vivo* T cell depletion were included. Recovery of B cells, NK cells, and T cells (including $CD4^+$ and $CD8^+$ subsets) was assessed at baseline and at 1, 2, 3, 6, 12 and 24 months post-transplantation by flow cytometry. In addition, recovery of thymopoiesis was assessed using signal joint T cell receptor rearrangement excision circle (sjTREC) analysis. The impact of recovery of lymphocyte subsets and thymopoiesis at 3 months post-transplantation on the incidence of subsequent infections, infection-related mortality, non-relapse mortality, relapse-free survival and overall survival was assessed by Cox regression analysis.

Results

T cell recovery was severely protracted in the majority of patients: the lower reference limit of absolute cell counts of $CD4^+$ and $CD8^+$ cells was not reached 24 months post-transplant in 64% and 73% of patients, respectively. $SjTREC^+$ T cells were undetectable in 38% and 27% of patients at 3 and 12 months post-transplantation, respectively. A total of 128 CTC grade 3-4 infections were observed in the first year post-transplantation, of which most infections occurred in the first 30 days post-transplant. The cumulative incidence of grade 2-4 and grade 3-4 infections was 89% and 83% at 6 months post-transplant and 95% and 87% at 12 months post-transplant, respectively. Non-relapse mortality at 12 months post-transplant was 16%, of which 78% infection-related mortality. One-year overall survival was 73%. Patients who failed to recover thymopoiesis at 3 months post-transplant were at a 3.3-fold higher risk of subsequent severe grade 3-4 infections. (HR 3.25, 95% CI 1.26-8.43, p=0.015).

Overall conclusion

Immune reconstitution, and especially T cell recovery and thymopoiesis, is impaired after double umbilical cord blood transplantation. Patients who fail to recover thymopoiesis at 3 months post-transplantation are at high risk for subsequent severe infections.

INTRODUCTION

Umbilical cord blood (UCB) is an important alternative hematopoietic stem cell (HSC) source for patients needing an allogeneic stem cell transplantation (allo-SCT) who lack a suitable matched related or unrelated donor (1). However, UCB transplantation (UCBT) is associated with retarded hematopoietic recovery and immune reconstitution, resulting in higher rates of infection compared to conventional HSC sources (2-4). Especially, the relatively low number of progenitor cells present in the graft is associated with a higher rate of graft failure and retarded recovery (5-7). Alternatively, two UCB grafts (double, dUCBT) instead of one were applied to increase the total nucleated cell (TNC) and progenitor cell dose (8-10). That approach appeared associated with less graft failure, however hematopoietic recovery is still slow and results most time from one predominating unit (8, 11).

Immune reconstitution upon allo-SCT is a complex process that consists of both the innate and the adaptive immune system. Recovery of innate immunity, characterized by restoration of mucosal barriers, neutrophil recovery and natural killer (NK) cell recovery occurs within the first weeks post- transplant (12). T cell reconstitution, which is part of the adaptive immune system, can take years to fully recover (13, 14). T cell recovery after allo-SCT occurs via two independent routes. Initially, lymphopenia-induced homeostatic proliferation of mature T cells (either present in the graft or residual host T cells) results in the expansion of a T cell population with a limited T cell receptor (TCR) repertoire (4). Long-term effective immune reconstitution with a broad T cell repertoire depends on thymic production of new naïve T cells (thymopoiesis) (15). The presence of these newly formed naïve T cells can be detected by the measurement of signal joint T-cell receptor rearrangement excision DNA circles (sjTREC), that are formed during TCR rearrangement. Previously, we showed that retarded thymopoiesis strongly predicts for opportunistic infections in recipients of matched related and unrelated transplants (16). Several studies showed higher rates of infectious morbidity and mortality upon single unit UCBT as compared to related or unrelated donor allo-SCT, which appeared to be due to delayed functional T cell recovery (17-19). Delayed immune recovery and a high incidence of infectious complications were observed in patients with high-risk haematological diseases after dUCBT preceded by either myeloablative (MA) or reduced intensity conditioning

(RIC) (20). However, it is still unclear to what extend impaired T cell recovery and thymopoiesis predicts for subsequent severe infections in dUCBT patients.

In the current study, hematopoietic recovery and immune recovery, including recovery of thymopoiesis, were evaluated in 55 poor-risk hematological patients receiving dUCBT upon conditioning using a RIC regimen, without T cell depletion. Additionally, it was explored whether impaired recovery of thymopoiesis predicted for subsequent infections or was associated with infection-related mortality (IRM), non-relapse mortality (NRM), relapse-free survival (RFS) and overall survival (OS).

METHODS

Patients and cord blood units

Patients receiving a dUCBT in the Erasmus MC Cancer Institute between September 2007 and December 2013 were included in this study. Patients were eligible for inclusion if they met the following criteria: (i) a diagnosis of a high-risk hematologic malignancy (as defined in (21)) in need for an allogeneic stem cell transplantation but lacking a matched related or unrelated donor and therefore eligible for dUCBT; (ii) receiving a RIC regimen without *in vivo* T cell depletion and (iii) presence of a peripheral blood sample to determine immune reconstitution and thymopoiesis available at 3 months post-transplantation. The institutional review board approved the protocols and all patients provided written informed consent. All patients were treated according to the HOVON 106 (11) and 115 study protocols (EudraCTnr.: 2008-000053-35 and 2012-001188-55 / Netherlands Trial Registry: NTR1573 and NTR3535).

Cord blood unit (CBU) selection was based on TNC dose and human leukocyte antigen (HLA) match. The minimum TNC dose required was set at 1.5 x 10^7/kg recipient body weight for each individual CBU and at 4.0 x 10^7/kg for both CBUs combined. HLA matching of CBUs and recipient was performed for HLA-A and -B antigens at the serological split resolution level and for HLA-DRB1 at the 4-digit resolution level. A minimal match grade of 4/6 between both individual CBUs and recipient and between CBUs was required.

Conditioning regimen, supportive care and transplantation procedures

Conditioning, supportive care and graft-versus-host disease (GVHD) prophylaxis were as previously described (11). In short, all patients received a RIC regimen, consisting of cyclophosphamide at day -7 (60 mg/kg), fludarabine at days -6, -5, -4 and -3 (total dose of 160 mg/m^2) and total-body irradiation (TBI) at days -2 and -1 (total dose 4 Gy). CBU were routinely infused on two consecutive days (day 0 and day +1). No hematopoietic growth factors were prescribed post-transplant per institution policy. GVHD prophylaxis consisted of cyclosporine A and mycophenolate mofetil. Patients received infection prophylaxis

by ciprofloxacin and fluconazole at least until neutrophil counts measured ≥ 0.2 x 10^9/L on two consecutive days. In addition, patients received valaciclovir for prevention of herpes infections starting at day 0. Cotrimoxazol for prevention of pneumocystis jirovecii was started after neutrophil recovery for at least one year following transplantation.

Infections

All infections diagnosed following transplantation between day +1 and day +365 post-transplant were evaluated and scored by grade, localization and causative micro-organism according to the NCI common toxicity criteria (CTC) version 3.0, as described before (16, 22, 23). CTC grade 3-4 infections were defined as severe (CTC grade 3) or life-threatening (CTC grade 4) with the need for admission and intravenous treatment. An infection was defined and scored as recurrent if an asymptomatic period of at least 4 weeks was documented between two infectious episodes. Patients were excluded from evaluation of infections when cytogenetic or morphological relapse was diagnosed, starting at the day the relapse was diagnosed. Death was considered associated with infection if findings consistent with an infection were present at the time of death or if the pathogen was detected in an autopsy specimen. In addition, death caused by an infection could be either direct (e.g. pneumonia) or indirect (e.g. sepsis leading to respiratory distress). Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) reactivations were diagnosed and treated as described previously (22). Both CMV and EBV reactivations were scored as infections.

Immune reconstitution analysis

Peripheral blood samples were acquired at baseline (after conditioning, but prior to dUCBT) and at 1, 2, 3, 6 and 12 months post-transplantation to determine post-transplant recovery of lymphocyte subsets i.e. $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$ and $CD3^-CD16/56^+$ cells, using four-color single platform flow cytometry as described before (16). In addition, the 3, 6 and 12 month samples were used to determine the frequency and absolute number of $sjTREC^+$ T cells.

Quantification of signal joint T cell receptor rearrangement excision circles

The frequency of $sjTREC^+$ cells per 10^5 $CD3^+$ T cell was determined at 3, 6 and 12 months post-transplant. $CD3^+$ cells were isolated from whole blood using immunomagnetic positive purification (RoboSep™, Stemcell Technologies, Grenoble, France) according to the instructions of the manufacturer. This resulted in a median purity of 99.0% (range 81.0% - 100%). DNA was purified from the $CD3^+$ T cells using the Illustra blood genomicPrep Mini Spin Kit (GE Healthcare Life Sciences, Eindhoven, the Netherlands) according to manufacturer's instructions. SjTRECs were quantified by a previously described 5'nuclease based real time quantitative polymerase chain reaction (RT-qPCR) assay using the

ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA, USA) (24). To compensate for variations in input DNA we used the constant gene segment of the TCRA gene (C-alpha) as an endogenous reference gene. The sequences for detection of both the C-alpha gene and of sjTREC$^+$ T cells are described earlier (16). The sjTREC frequency in CD3$^+$ lymphocytes was calculated by normalizing the sjTREC RT-qPCR data to the C-alpha RT-qPCR data. SjTREC frequency per 10^5 T cells was calculated by the following formula: [$(2x10^5/2^{\Delta CT})$/percentage of purity of T cells]. Since the frequency of sjTREC$^+$ T cells in peripheral blood is not only influenced by thymic output, but by peripheral expansion as well (25), we calculated the content of sjTREC$^+$ T cells per milliliter blood, which is not influenced by post-rearrangement expansion and may therefore be a better estimate of thymic output (26). SjTREC$^+$ T cell content (sjTREC$^+$ T cells/ml blood) was calculated by multiplying the absolute number of CD3$^+$ T cells (in 10^5/ml) by the sjTREC$^+$ frequency.

Statistical considerations

Endpoints of the study were neutrophil and platelet recovery, chimerism, acute GVHD (aGVHD), chronic GVHD (cGVHD), CTC grade 2-4 infections, IRM, NRM, relapse-related mortality (RM) and OS.

Time to neutrophil recovery was defined as the first two consecutive days on which neutrophils exceeded 0.5 x 10^9/L. Time to platelet recovery was defined as the first of three consecutive days on which platelets exceeded 50 x 10^9/L without platelet support for 7 days. Successful engraftment was defined as neutrophil recovery in association with donor hematopoiesis of > 10% in the bone marrow. Complete donor chimerism was defined as > 95% donor hematopoiesis (single or both donors) and < 5% recipient hematopoiesis in bone marrow; mixed chimerism was defined as 10% to 95% donor hematopoiesis and > 5% recipient hematopoiesis in the bone marrow. Chimerism analysis was performed as previously described (11). aGVHD was graded according to the Glucksberg criteria updated according to Przepiorka *et al*. (27, 28). cGVHD was scored according to the Seattle classification for limited and extensive chronic GVHD (29). Relapse before the occurrence of aGVHD or cGVHD was considered a competing risk at the day of one of these events for calculating the cumulative incidence of aGVHD and cGVHD. The time to occurrence of CTC grade 2-4, 3-4 and 4 infections was calculated from the day of transplantation (day 0) to the day of occurrence. Three time intervals were constructed, the first one starting at the day of transplantation and ending 30 days post-transplant, the second interval ranging from day +31 to +180 and the third interval from day +181 and day +365. Infections were expressed as 'infection rate' defined by the total number of infections per 100 patient days. OS was calculated from the date of transplantation until death. Patients still alive at the date of last contact were then censored. Causes of death were classified as RM or NRM and these were considered competing risks. IRM was defined as mortality in which any infection was involved, with relapse as a competing risk.

Risk scores used for the prediction of NRM included the European Group for Blood and Marrow Transplantation (EBMT) risk score (30) and the hematopoietic cell transplantation comorbidity index (HCT-CI), developed by the Seattle group (31). Univariate logistic regression was performed to evaluate factors associated with sjTREC$^+$ T cell presence (as a dichotomous outcome present versus absent). Parameters analyzed were age (continuous), gender, infused CD34$^+$ cells (continuous), infused CD3$^+$ cells (continuous), number of infused CD34$^+$ cells winning cord (continuous), number of infused CD3$^+$ cells winning cord (continuous), EBMT risk score (2-3 vs 4-5 vs 6-7), HCT-CI score (0 vs 1-2 vs >=3), aGVHD (grade 2-4) and HLA match of recipient versus winning cord (4/6 vs 5/6-6/6). Recovery of thymopoiesis was defined as the presence of detectable sjTREC$^+$ T cells. Univariate Cox regression analyses was performed to evaluate the impact of the recovery of thymopoiesis at 3 months (dichotomous; present versus absent), age (continuous), gender, infused CD34$^+$ cells (continuous), infused CD3$^+$ cells (continuous), infused CD34$^+$ cells winning cord (continuous), infused CD3$^+$ cells winning cord (continuous), total number of CD3$^+$ T cells, CD4$^+$ T cells, CD8$^+$ T cells, B cells and NK cells in PB at 3 months (all continuous), EBMT risk score (2-3 vs 4-5 vs 6-7), HCT-CI score(0 vs 1-2 vs >=3), CMV status of the patients (positive versus negative), HLA matching of the winning cord blood graft (4/6 versus 5/6-6/6) and preceding grade 2-4 aGVHD (present versus absent) on clinical outcome beyond that time point. Multivariate analysis of logistic regression and Cox regression was performed for parameters with a p-value <0.05 in univariate analysis. For parameters significant in Cox multivariate regression analysis hazard ratios (HR), corresponding 95% confidence intervals (95% CI), and p-values were calculated accordingly. Differences in Kaplan-Meier curves were calculated using the log-rank test, or the log-rank test for trend if appropriate. All reported p-values are two-sided, and a significance level $\alpha = 0.05$ was used. All data analyses were performed with Stata/SE version 12 (StataCorp LP, College Station, TX, USA).

RESULTS

Patient characteristics

Fifty-five patients were included. Patients' characteristics are shown in **Table 1**. The median age at inclusion was 51 years (range 23-68 years). Diagnoses included acute leukemia (53%), chronic leukemia (9%), lymphoma (11%), and myelodysplastic syndromes, myeloproliferative diseases or bone marrow failure (MDS/MPL/BMF; 27%). Seventeen percent of the patients had a high (6 or 7) EBMT risk score, while 34% of patients were at higher risk for NRM according to the HCT-CI. The majority of patients received either two 4/6 matched units (36%) or one 4/6 matched unit combined with a 5/6 matched unit (42%).

Table 1. Baseline characteristics (n=55)

Median age, years (range)	51 (23 – 68)
Sex, male/female (%)	31/24 (56/44)
Diagnosis, n. (%)	
Acute leukemia	29 (53)
Chronic leukemia	5 (9)
Lymphoma	6 (11)
MDS/MPL/BMF	15 (27)
HCT-CI score, n. (%)	
0	18 (33)
1 - 2	18 (33)
≥ 3	19 (34)
EBMT risk score, n. (%)	
2-3	15 (27)
4-5	31 (56)
6-7	9 (17)
Median number infused total nucleated cells x107/kg, (range)	
Both CBU together	4.98 (3.34 – 9.44)
Winning CBU	2.42 (1.38 – 5.72)
Median number infused CD34+ cells x105/kg, (range)	
Both CBU together	0.53 (0.08 – 2.00)
Winning CBU	0.24 (0.00 – 1.29)
Median number infused CD3+ cells x106/kg, (range)	
Both CBU together	0.88 (0.18 – 2.98)
Winning CBU	0.39 (0.05 – 2.10)
HLA match grade, n. (%)	
4/6 + 4/6	20 (36)
4/6 + 5/6	23 (42)
5/6 + 5/6	9 (17)
5/6 + 6/6	3 (5)
HLA matching recipient versus winning CBU, n. (%)	
4/6	27 (49)
5/6	26 (47)
6/6	2 (4)
CMV status of recipient, n. (%)	
Negative	23 (42)
Positive	32 (58)

Engraftment and chimerism

Fifty-two patients (95%) met the criteria for engraftment with a median time to neutrophil recovery of 34 days (**figure 1,** range 12-57 days). Of the patients that did not meet the criteria for engraftment, one suffered from primary graft failure and two from relapse. Platelet recovery (≥50 x 10^9/L) occurred at a median of 45 days (**figure 1,** range 23-139 days). Three patients did not achieve neutrophil recovery (5%), while four patients did not achieve platelet recovery (7%). At 3 months post-transplantation complete donor chimerism in the bone marrow, either single or double donor derived, was observed in 49/55 patients (**table 2**, 89%). Mixed chimerism was observed in 4 patients (7%), while 2 patients (4%) still showed full recipient chimerism. One of these patients suffered from relapse, the other from primary graft failure.

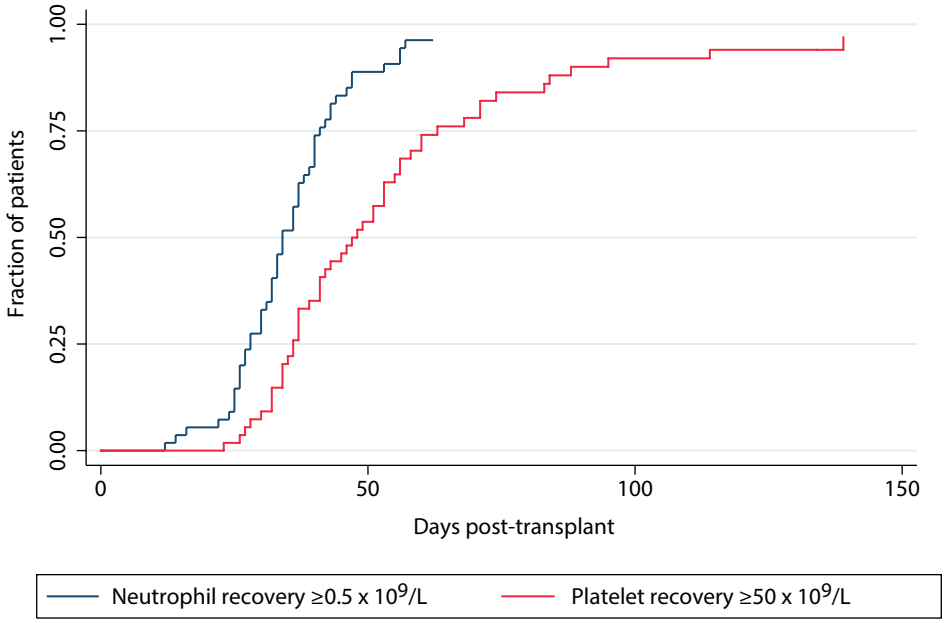


Figure 1. Recovery of neutrophils and platelets post-transplantation.

Immune reconstitution

Lymphocyte recovery was assessed at baseline and at 1, 2, 3, 6, 12 and 24 months post-transplantation.

Following conditioning, $CD3^+$ (both $CD4^+$ and $CD8^+$) T cells, $CD19^+$ B cells and $CD3^-CD16/56^+$ NK cells were virtually absent in all patients (**figures 2 & 3**). B cells showed an initial slow recovery during the first months post-transplantation (**figure 2A**), but reached normal levels in the majority of patients at 6 months post-transplantation. NK

Table 2. Levels of chimerism at 3 months post-transplantation

Bone marrow chimerism, n. (%)	
Single donor chimerism	46 (84)
Double donor chimerism	3 (5)
Mixed chimerism	4 (7)
Recipient chimerism	2 (4)
Peripheral blood chimerism, n. (%)	
Single donor chimerism	46 (84)
Double donor chimerism	3 (5)
Mixed chimerism	4 (7)
Recipient chimerism	2 (4)
Peripheral blood CD3+ fraction chimerism, n. (%)	
Single donor chimerism	46 (84)
Double donor chimerism	1 (2)
Mixed chimerism	5 (9)
Recipient chimerism	3 (5)

cells recovered rapidly and returned within the normal range between 1 and 2 months post-transplantation (**figure 2B**).

$CD3^+$ T cell reconstitution was severely retarded (**figure 3A**). At 24 months post-transplantation, the median level of $CD3^+$ T cells had not reached the lower normal range limit. Reconstitution of both $CD4^+$ helper T cells as $CD8^+$ cytotoxic T cells appeared to be very slow (**figure 3B and 3C**) and a significant number of patients did not reach the lower normal range limit at 24 months post-transplantation (64% and 73% respectively).

The presence, content and frequency of $sjTREC^+$ T cells were assessed at 3, 6 and 12 months post-transplantation. $SjTREC^+$ T cells were detectable in 62% of patients at 3 months, in 80% at 6 months and in 73% at 12 months post-transplantation. The median $sjTREC^+$ T cell content measured per milliliter blood was 150 (range 0-1070) at 3 months, 260 (range 0-24920) at 6 months and 410 (range 0-87000) at 12 months post-transplantation (**figure 3D**). The median frequency of $sjTREC^+$ T cells per 10^5 $CD3^+$ T cells was 63 (range 0-619) at 3 months, 62 (range 0 - 22402) at 6 months and 116 (range 0 - 5558) at 12 months (**figure 3E**). Despite this increase over time, at 12 months post-transplantation, $sjTREC^+$ cell frequency and content had not recovered to normal values as measured in healthy donors (median content 19044/ml, range 1873 – 77952; median frequency $1205/10^5$ $CD3^+$ T cells, range 147 - 3962(16)).

We evaluated whether pre-transplant variables, transplant characteristics and post-transplant variables would affect $sjTREC^+$ T cell recovery. In multivariate analysis the absence of $sjTREC^+$ T cells at 3 months was associated with higher recipient age (p=0.015). No differences were observed in $sjTREC^+$ T cell recovery at 3 months based on recipient

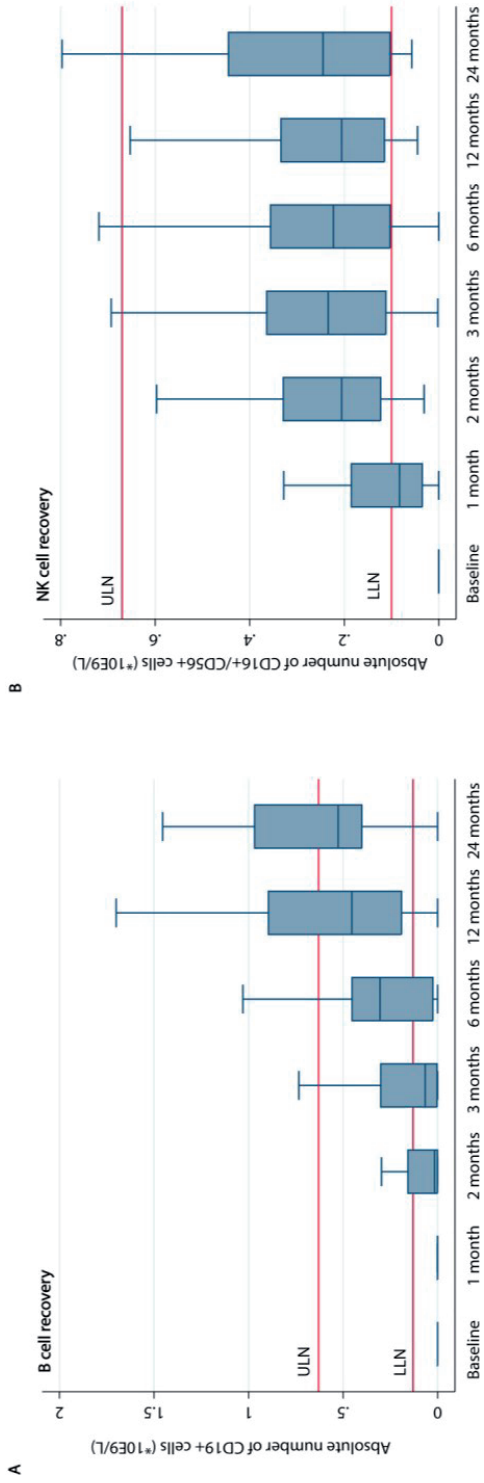


Figure 2. Recovery of B cells (panel A) and NK cells (panel B) following transplant. (Boxes show 25th percentile, median and 75th percentile, whiskers show the lower and upper adjacent values, according to Tukey). LLN = lower limit of normal; ULN = upper limit of normal.

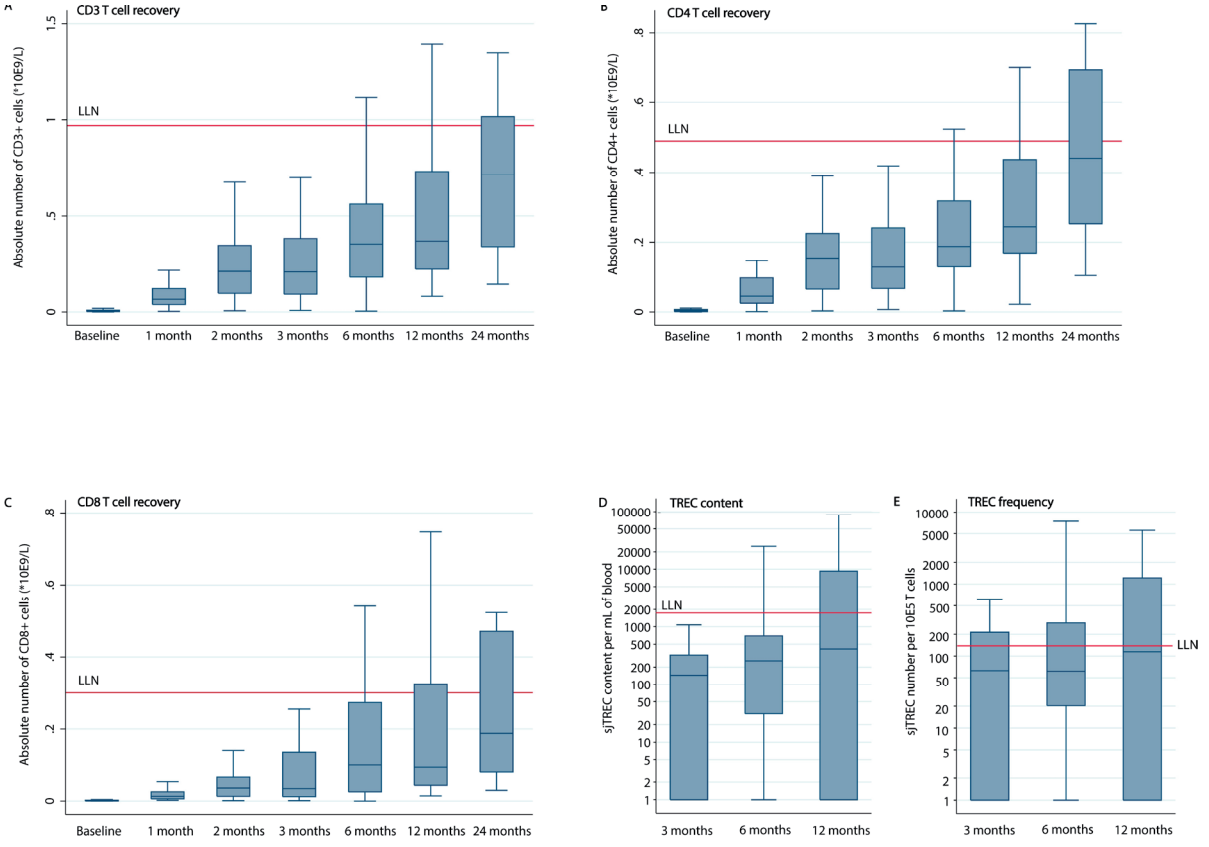


Figure 3. T cell reconstitution following transplantation. Panel A – C demonstrate respectively $CD3^+$, $CD4^+$ and $CD8^+$ T cell recovery. Panel D demonstrates recovery of TRECs expressed in TREC content, panel E demonstrates recovery of TRECs expressed in TREC frequency. (Boxes show 25th percentile, median and 75th percentile, whiskers show the lower and upper adjacent values, according to Tukey). LLN = lower limit of normal; ULN = upper limit of normal.

gender, number of infused $CD34^+$ cells (from either both grafts or predominant graft), number of infused $CD3^+$ cells (from either both grafts or predominant graft), EBMT risk score, HCT-CI score, preceding aGVHD or HLA-matching grade of the predominant graft. In addition, at 6 and 12 months post-transplantation, none of the tested variables were associated with the presence or absence of $sjTREC^+$ T cells.

Infections

The total number of infections was recorded in detail in surviving patients up to one year after dUCBT, resulting in a total of 197 CTC grade 2-4 and 128 CTC grade 3-4 infections. The cumulative incidence of CTC grade 2-4 and grade 3-4 infections, respectively, was 89% and 83% at 6 months and 95% and 87% at 12 months. In the first 30 days post-transplantation, the rate of infections was 3.16 CTC grade 3-4 infections per 100 patient-days (**table 3**), with 69% of patients experiencing at least one severe infection during that time period. Patients experienced a median of one CTC grade 3-4 infection in the first time period (range 0-3). 75% of the reported infections within the first month post-transplantation was either due to neutropenic fever, a central venous catheter infection or a positive blood culture without additional symptoms. The CTC grade 3-4 infection rate was much lower in the second (day 30-180) and third (day 180-365) time interval (0.74 and 0.29, respectively). In the second time interval, the majority of severe infections was caused by viral infections (38%), followed by bacterial (24%) and fungal (9%) infections. In 29% of the patients with severe infections in that time interval, no causative micro-organism could be identified. Out of the 32 CMV-seropositive patients (**table 1**), 14 developed at least one CMV reactivation (45%) at any time point in the first year post-transplantation. However, none of these patients developed clinical CMV disease. CTC grade 2 infections appeared to be scarce within the first and second time period compared to CTC grade 3-4 infections with a CTC grade 2 infection rate of 0.30 and 0.56 respectively (**table 3**). In the third time period, CTC grade 2 infections outnumbered the CTC grade 3-4 infections (CTC grade 2 infection rate 0.39 versus CTC grade 3-4 infection ate 0.29 respectively).

Table 3. Rate of infections per 100 patient days

Interval	CTC Grade 2	CTC Grade 3-4
0-30 days	0.30	3.16
31-180 days	0.56	0.74
181-365 days	0.39	0.29

Outcome

Median follow-up was 20 months. At one year post-transplantation, 40 of 55 patients (73%) were still alive (**figure 4A**). The EBMT risk score significantly impacted on OS (**fig-**

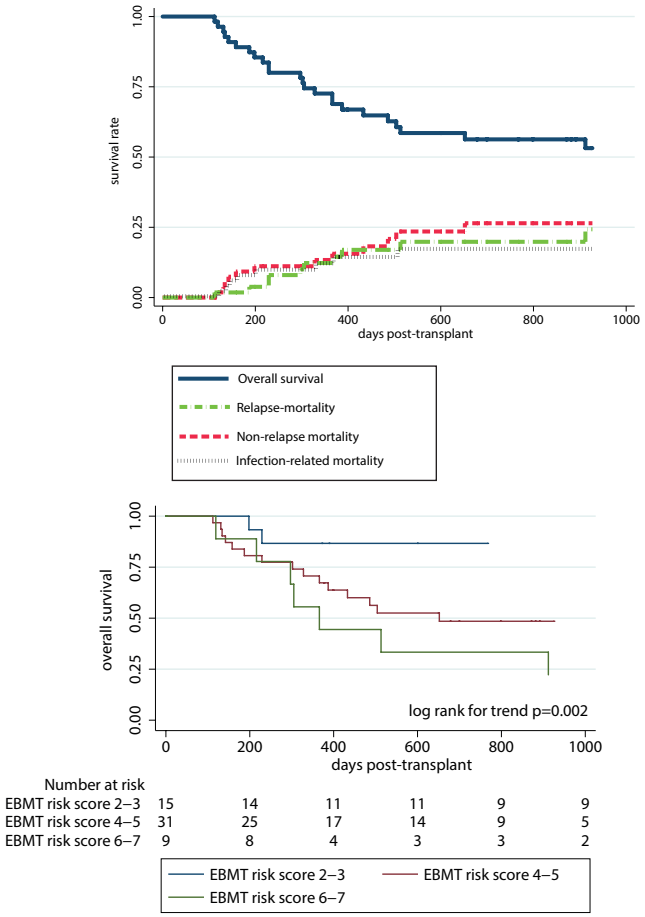


Figure 4. (A) Kaplan-Meier curves of OS, RM, NRM and IRM. (B) Overall survival according to EBMT risk score.

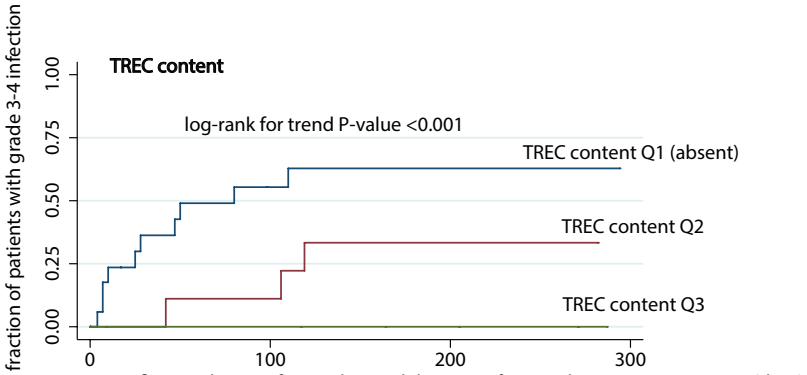
ure 4B, HR 2.36, 95% CI 1.35-4.12, p=0.003). The HCT-CI score was also associated with OS (HR 1.51, 95% CI 0.96-2.39), however this did not reach statistical significance (p=0.076). Fifteen patients had died within the first year after transplantation. Death was due to relapse in 6 patients (40%), allo-SCT related causes in 7 patients (47%) and other causes in 2 patients (13%; peritonitis of unknown cause and death of unknown cause, respectively). In the 7 patients who died from allo-SCT related causes, infectious complications contributed in all patients and were the primary cause of death in 6 out of 7 patients. In 4 patients the infection co-occurred with GVHD. By day 100 post-transplant, 28 patients (51%) had developed grade II-IV aGVHD, and 4 patients (7%) developed grade III-IV aGVHD. Limited or extensive cGVHD was observed in 9 and 15 out of 55 patients (16% and 27%, respectively) at 1 year post-transplant.

Table 4. Univariate analysis of the association of several factors with clinical outcome.

	CTC grade 3-4 infections		IRM		NRM		RFS		OS	
	HR(95% CI)	P=	HR(95% CI)	P=	HR(95% CI)	P=	HR(95% CI)	P=	HR(95% CI)	P=
Absent sjTREC$^+$ T cells at 3 months	3.25 (1.26-8.43)	0.015	1.77 (0.44– 7.07)	0.42	2.80 (0.91-8.56)	0.071	0.35 (0.08-1.60)	0.18	1.23 (0.57-2.65)	0.6
Age	1.01 (0.97-1.05)	0.6	1.00 (0.94- 1.05)	0.88	1.04 (0.99 - 1.09)	0.13	1.04 (0.99 - 1.09)	0.14	1.04 (1.01 - 1.08)	0.013
Sex	0.34 (0.11–1.06)	0.06	1.24 (0.31 - 4.96)	0.76	0.69 (0.23 - 2.15)	0.53	0.80 (0.25 - 2.56)	0.71	0.78 (0.36 - 1.68)	0.52
Tot. inf. CD34$^+$	1.40 (0.58–3.41)	0.46	1.27 (0.30 - 5.33)	0.74	1.66 (0.60 - 4.60)	0.32	0.72 (0.18 - 2.82)	0.65	1.20 (0.54 - 2.65)	0.16
Tot. inf. CD3$^+$	0.83 (0.40–1.71)	0.61	0.87 (0.30 - 2.52)	0.8	0.82 (0.36 - 1.88)	0.64	0.67 (0.27 - 1.68)	0.4	0.63 (0.33 - 1.19)	0.15
CD34$^+$ predom. graft	0.72 (0.15–3.32)	0.67	0.37 (0.02 - 5.89)	0.48	0.25 (0.02 - 2.65)	0.25	0.63 (0.09 - 4.29)	0.63	0.42 (0.10 - 1.77)	0.24
CD3$^+$ predom. graft	0.22 (0.05-1.09)	0.07	0.16 (0.01 - 2.49)	0.19	0.52 (0.11 - 2.45)	0.41	1.30 (0.39 - 4.40)	0.67	0.63 (0.23 - 1.73)	0.38
CD3$^+$ count at 3 months	0.78 (0.12 – 5.00)	0.79	0.02(0.00 – 4.20)	0.15	0.45 (0.04 – 6.06)	0.55	1.54 (0.24 – 9.96)	0.65	0.98 (0.22 – 4.41)	0.98
CD4$^+$ count at 3 months	0.02 (0.00 -2.83)	0.12	0.00(0.00 – 2.11)	0.07	0.23 (0.01 – 21.78)	0.53	2.12 (0.06 – 72.25)	0.68	0.58 (0.03 – 10.65)	0.72
CD8$^+$ count at 3 months	2.41 (0.19 – 30.4)	0.5	0.24(0.00 – 185.1)	0.41	0.47 (0.01 – 35.37)	0.73	2.03 (0.09 – 45.96)	0.66	1.56 (0.14 – 17.70)	0.72
B cell count at 3 months	0.72 (0.09 – 5.71)	0.76	0.29(0.00 – 16.81)	0.55	0.32 (0.01 – 7.32)	0.47	0.11 (0.00 – 5.91)	0.27	0.20 (0.02 – 2.35)	0.2
NK cell count at 3 months	0.09 (0.01 – 2.59)	0.16	0.41(0.01 – 14.81)	0.62	0.64 (0.05 – 8.60)	0.74	0.59 (0.04 – 8.12)	0.7	0.57 (0.08 – 4.06)	0.57
CMV status patient	1.04 (0.41-2.63)	0.94	2.52 (0.51-12.52)	0.26	2.95 (0.81-10.76)	0.1	2.01 (0.60 - 6.67)	0.26	2.17 (0.95 -4.96)	0.067
EBMT risk score	1.61 (0.83–3.10)	0.16	1.93 (0.68 - 5.50)	0.22	2.18 (0.97 - 4.90)	0.06	1.86 (0.84 - 4.13)	0.13	2.35 (1.35 - 4.09)	0.003
HCT-CI risk score	1.27 (0.72–2.25)	0.41	1.94 (0.78 - 4.83)	0.15	1.64 (0.84 - 3.19)	0.15	1.37 (0.70 - 2.68)	0.36	1.52 (0.96 - 2.40)	0.075
HLA-matching	2.13 (0.80–5.69)	0.92	0.28 (0.03 - 2.29)	0.23	0.38 (0.08 - 1.76)	0.22	1.19 (0.34 - 4.24)	0.79	0.77 (0.31 - 1.88)	0.57
Preceding aGVHD	1.51 (0.60–3.84)	0.39	7.5 (0.92 - 61.19)	0.06	1.94 (0.63 - 6.04)	0.25	2.63 (0.78 - 8.88)	0.12	2.18 (0.99 - 4.81)	0.055

Impact of the recovery of thymopoiesis on outcome

Next it was explored whether recovery of sjTREC$^+$ T cells at 3 months post-transplantation was associated with severe infections (CTC grade 3-4), IRM, NRM, RFS and OS determined from 3 months post-transplantation. In univariate analysis (**table 4**), the absence of sjTREC$^+$ T cells was the only factor associated with a higher risk of severe infection beyond 3 months post-transplantation (HR 3.25, 95% CI 1.26-8.43, p=0.015). Univariate analysis for NRM beyond 3 months post-transplantation also revealed the absence of sjTREC$^+$ T cells to be associated with NRM, however this did not reach statistical significance (HR 2.80, 95% CI 0.91-8.56, p=0.071). The absence of sjTREC$^+$ T cells was not associated with OS beyond 3 months post-transplant, but higher age (p=0.013) and the EBMT risk score (p=0.003) were. Multivariate analysis revealed that the EBMT risk score was more strongly associated with OS beyond 3 months post-transplant (HR 2.0, 95%CI 1.12-3.59, p=0.02) than the age at transplantation (HR 1.03, 95%CI 0.99-1.07), p=0.077). None of the tested parameters were associated with RFS or IRM beyond 3 months post-transplantation in univariate analysis. To further address the observed association between recovery of sjTREC$^+$ T cells and severe infections, patients were divided in three tertiles based on the sjTREC$^+$ T cell content per milliliter blood. A higher content of sjTREC$^+$ T cells was associated with a decreased risk for severe infection, with patients with the highest sjTREC$^+$ T cell content not developing any severe infections (**figure 5**).



Number at risk				
TREC content Q1 (absent)	17	6	5	4
TREC content Q2	11	8	6	5
TREC content Q3	11	11	8	5

Figure 5. Association between sjTREC$^+$ T cell content per milliliter blood (in tertiles) at 3 months post-transplantation and the occurrence of CTC grade 3-4 infection rate beyond 3 months post-transplantation.

DISCUSSION

Immune reconstitution upon UCBT is severely retarded and as a direct consequence, UCBT is associated with a significant risk of opportunistic infections (32). Kinetics of immune reconstitution upon UCBT have been widely studied, although most of these studies were performed in a single graft setting and are based on mixed populations of patients, receiving various conditioning and immunosuppressive regimens (15, 17-19, 33-35). Few studies reported on recovery of thymopoiesis upon dUCBT (20, 36, 37). This study, to our knowledge, is the first to describe immune reconstitution including thymopoiesis in a uniform population of 55 adult patients, receiving dUCBT upon a RIC regimen omitting *in vivo* T cell depletion. In addition, the present study is the first to correlate recovery of thymopoiesis with clinical outcome upon dUCBT and shows the predictive impact of impaired sjTREC$^+$ T cell recovery at 3 months on the risk of severe infections beyond that time point.

Although CD3$^+$ T cell recovery was severely protracted and the majority of patients had CD3$^+$ T cell levels below the normal range up to 24 months post-transplantation, sjTREC$^+$ T cells were detected at low levels in 62% of patients at 3 months post-transplantation. Our results are in contrast to several other studies, were sjTREC$^+$ T cells were detected in 20% (20), 11% (37) and 0% (38) of patients around the same time point upon dUCBT. However, in these studies, patients received either MA conditioning or a RIC regimen combined with antithymocyte globulin (ATG), a polyclonal antibody raised against thymocyte antigens resulting in profound *in vivo* T cell depletion (39). Cytotoxic chemotherapy and radiation negatively affect thymic reconstitution (40, 41). RIC regimens may therefore cause less damage to the thymus compared to MA conditioning, resulting in faster recovery of thymopoiesis. In addition, several studies showed an association between the use of a RIC regimen and enhanced immune reconstitution (42-44). The use of ATG results in a lower incidence of GVHD, but however, might result in delayed immune reconstitution as well. Enhanced B and T cell recovery is reported upon the use of ATG-free conditioning regimens in UCBT, both MA or RIC, in both pediatric and adult cohorts (45-47). Therefore, the high incidence of patients with detectable sjTREC$^+$ T cells at 3 months post-transplantation could be well explained by the relatively mild conditioning regimen used in our study. In a comparable cohort of 46 adult patients receiving a matched related (n=33) or unrelated (n=13) allo-SCT upon an ATG-free RIC regimen (Fludarabine 90 mg/m^2 and TBI 2 Gy) in our center, detectable sjTREC$^+$ T cells were observed in 67% of patients at 3 months and 86% at 12 months, which is similar to the above described proportions of sjTREC$^+$ T cell positivity in patients receiving dUCBT. These results indicate, that upon an ATG-free RIC regimen, dUCBT and adult donor allo-SCT show comparable reconstitution of thymopoiesis. Previously, both age and GVHD have been implicated to impact the reconstitution of thymopoiesis (44, 48-50). While an

association between higher age and undetectable sjTREC$^+$ T cells was observed in the current study, this association was not observed for the presence of GVHD.

In an earlier study, we showed that insufficient thymopoiesis predicted for severe infections and NRM in patients receiving a matched related or unrelated donor allo-SCT upon MA conditioning (16). In the current study, patients who failed to recover thymopoiesis at 3 months post-transplantation had a 3.3-fold higher risk for subsequent severe infections. This association was not observed between CD3$^+$, CD4$^+$ and CD8$^+$ T cell counts at 3 months post-transplantation and subsequent severe infections, indicating that the newly formed naïve T cells are pivotal for adequate infectious immunity. In addition, the content of sjTREC$^+$ T cells was associated with the risk for severe infection at that time point. These results compare well with Brown *et al*. (37) who demonstrated that clearance of CMV viremia after dUCBT depended on the recovery of thymopoiesis. Although several other studies assessed thymopoiesis upon dUCBT(20, 36-38) and infectious complications are a known problem upon UCBT(32, 46), severe infections after dUCBT were not yet linked to recovery of thymopoiesis.

In line with Sauter *et al*. (46) the highest infection rate was observed in the first 30 days post-transplantation. They described 61% of patients experiencing at least one infection in this time period, which compares well to the cumulative incidence of 69% of severe infections in our cohort. Of note is that most of the infections scored in the present study in the first month post-transplantation were neutropenic fevers, central venous catheter infections or positive blood cultures without an evident focus. These infections may have occurred and been reported more frequently because of longer hospitalization and therefore increased surveillance, leading to an overestimation of infections in the first 30 days post-transplantation. In a comparable adult donor recipient cohort, as mentioned earlier, the highest rate of CTC grade 2-4 infections was observed in the first 30 days post-transplantation as well, although this rate was markedly lower compared to the observed infection rate upon dUCBT (adult donor 1.09 CTC grade 2-4 infections per 100 patient-days versus 3.46 in the dUCBT group). In the second time interval of 30 days to 180 days post-transplantation, viral infections were the most common cause of infectious morbidity, which is in line with literature (4, 20, 32). In addition, 45% of the CMV-seropositive patients developed one or more CMV reactivation during the first year post-transplant. This is in line with the study performed by Sauter *et al*. (46), where a CMV reactivation rate of 51% was observed in 180 CMV-seropositive patients that received dUCBT upon conditioning omitting *in vivo* T cell depletion. However, clinical CMV disease was reported in 25 patients, whereas no cases of clinical CMV disease were observed in the current study. This difference might be due to the prophylactic use of valaciclovir for the prevention of herpes infections in our center. Although primarily used for the prevention of herpes infections, valaciclovir is a more potent CMV suppressor in patients receiving allo-SCT than acyclovir (51), which was used by Sauter and colleagues.

As mentioned earlier, this study is the first to assess immune reconstitution including thymopoiesis in a homogeneous cohort of 55 adult patients, receiving a RIC regimen omitting *in vivo* T cell depletion and to correlate recovery of thymopoiesis to clinical outcome. Although this population of 55 patients is larger than any other cohort described in this context, the limited cohort size was a limitation for the present study, especially for associations with clinical outcomes in which there were only a few events.

In conclusion, failure to recover thymopoiesis after RIC-dUCBT without *in vivo* T cell depletion resulted in a higher risk of developing subsequent severe infections. This may lead to increased NRM and adverse outcome. These results highlight the need to improve thymic function post-transplantation, for example by the administration of the cytokines interleukin-7 or keratinocyte growth factor (52-54). Furthermore, risk of infections might be reduced by adoptive transfer of virus or fungal specific T cells (55, 56). In addition, patients lacking thymic recovery at 3 months post-transplantation might benefit from intensified supportive measures in order to prevent infections and associated mortality. Therefore, we propose to consider monitoring sjTREC^{+} T cell recovery in dUCBT patients, to identify patients in need for intensified support.

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Chapter 3

Wnt3a protein reduces the number of mouse hematopoietic stem cells in serum-free cultures in an apoptosis-independent manner

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Submitted

ABSTRACT

The Wnt signaling pathway has been implicated to play a role in the regulation of mouse hematopoietic stem cell fate decisions between self-renewal and differentiation. However, the role of Wnt proteins in *ex vivo* cultures of hematopoietic stem cells remains controversial. Here, it is shown that exogenous Wnt3a combined with hematopoietic growth factors leads to a reduction in the number of Lin^-, $Sca1^-$, and c-Kit^+ (LSK) cells in a stroma-free, serum-free culture system. The same negative effect on the number of $CD48^-$, $CD150^+$, $CD34^{low}$ primitive LSK cells was observed when a more stable Wnt signal, provided in the form of a lipid-stabilized Wnt3a protein or the GSK3β inhibitor CHIR99021, was used. The decrease in mouse LSK number occurred at later stages of the culture. Inducible overexpression of the anti-apoptotic BCL2 protein did not prevent the Wnt-induced loss of primitive LSK cells, suggesting that the loss was not due to apoptosis. These data suggest that Wnt signals impede the maintenance of hematopoietic stem cells in serum-free cultures.

INTRODUCTION

Wingless-related integration site (Wnt) signals have been shown to act as self-renewal factors for both embryonic [1] and adult stem cells in a diversity of mammalian tissues, including intestines, skin and mammary gland [2]. They also provide critical cues for maintenance or expansion of stem cells in cultures of a variety of adult stem cells both from mouse and man [3–8]. Several signal transduction cascades may be induced upon binding of Wnt ligands to their receptors [9], the best-characterized one being the canonical or β-catenin pathway. In this pathway, β-catenin is phosphorylated by glycogen synthase kinase-3β (GSK-3β) to be targeted for proteasomal degradation. Upon binding of a Wnt protein to the Frizzled (Fz) family of receptors and to low-density lipoprotein receptor-related protein 5 (LRP5) or LRP6 co-receptors, the phosphorylation of β-catenin is inhibited. Stabilized β-catenin translocates into the nucleus where it interacts with TCF/LEF transcription factors to activate targeted gene expression [10].

Over the past decades, a variety of studies have implicated a possible role for canonical Wnt signaling in the control of mammalian hematopoietic stem cell (HSC) function. Mice carrying a null allele of Wnt3a [11] or overexpressing the Wnt inhibitor *Dickkopf1* in the bone marrow niche [12] display impaired HSC self-renewal. When stimulated by a constitutively active form of β-catenin or by purified Wnt3a protein, Wnt signaling increased self-renewal capacity of apoptosis-resistant, transgenic HSC *ex vivo* [13,14]. However, mouse models with constitutively activated Wnt signaling displayed a multilineage differentiation block and loss of HSCs [15,16], and later studies using purified Wnt proteins in *ex vivo* cultures of normal mouse HSC reported controversial outcomes. In one study, Wnt5a was suggested to improve repopulation capacity of mouse HSC by inhibiting canonical Wnt pathway induced by Wnt3a protein [17], while in another it was shown to impede maintenance of HSC [18]. Several other studies showed that active β-catenin induces apoptosis in HSPC [19,20]. Taken together, these studies underscore the controversies on the effect of Wnt activation on HSC proliferation, differentiation and apoptosis.

It has been known for some time that Wnt proteins have a very short half-life in serum-free cell culture media, losing its activity within several hours [21]. This complicates the interpretation of the results obtained for the role of exogenous Wnt proteins on *ex vivo* HSC proliferation. Thus, addition of Wnt3a protein to cell cultures would result in intermittent rather than continuous activation of the pathway, which may be insufficient to inhibit HSC differentiation. In this study, the effect of Wnt signals in combination with hematopoietic growth factors on *ex vivo* cultures of mouse HSC is studied and the role of apoptosis and of Wnt3a protein stability on the response of HSC to Wnt signals is investigated.

METHODS AND MATERIALS

Mice

All animal procedures were performed in accordance with institutional and national guidelines and regulations, and approved by the Erasmus MC Animal Experiment Committee (DEC). Animals were euthanized using carbon dioxide. C57BL/6 and TRE-BCL2 mice used in this study were bred and maintained in the animal facility at Erasmus Medical Center (Erasmus MC), and handled according to institutional guidelines. All procedures were carried out in compliance with the Standards for Care and Use of Laboratory Animals. All mice were used at 12-24 weeks of age.

For generation of TRE-BCL2 mice, a human BCL2 cDNA (accession number BC027258) was amplified, and a Kozak sequence and C-terminal FLAG tag added. The vector p2Lox.GFP [22] was digested with XhoI and NotI to remove the GFP sequence, and the XhoI/NotI-digested BCL2-FLAG sequence ligated into its place to create p2Lox.BCL2. 25 µg of p2Lox.BCL2 was electroporated into A2Lox.Cre ES cells, followed by induction of the cells with 1 µg/ml doxycycline overnight to induce recombination, and the cells selected on 250 µg/mL of G418, as previously described [23]. The cells were then used for blastocyst injections into C57Bl/6 blastocysts. Chimeras were mated against C57Bl/6 mice and readily gave germline transmission. Mice containing the BCL2 transgene and the reverse tetracycline transactivator (rtTA, expressed from the Rosa26 locus in A2Lox.Cre ES cells) were identified by PCR using the following primers:

BCL2: forward GGATGCCTTTGTGGAACTGT, reverse GCTCACTTGTCGTCATCGTC; rtTA: forward GGACGAGCTCCACTTAGACG, reverse GGCATCGGTAAACATCTGCT.

Isolation of LSK and LSK CD48$^-$ cells

For LSK or LSK CD48$^-$ cell isolation, femurs and tibias from mice were removed, BM flushed, and mononuclear cells were isolated by Lymphoprep™ according to manufacturer's instructions (Stem Cell Technologies, Grenoble, France). Lineage-positive (Lin$^+$) cells were depleted by magnetic cell sorting (MACS) with the use of a lineage cell depletion kit according to manufacturer's instructions (Miltenyi Biotech, GmBH, Bergisch Gladbach, Germany). Lin depleted cells were further stained with a Lin-PE cocktail (anti-CD3e-PE, anti-CD45R-PE, anti-CD11b-PE, anti-Gr1-PE, and anti-Ter119-PE) as well as with anti-c-Kit-APC and anti-Sca-1-PE-Cy7 (all from BD Pharmingen, Heidelberg, Germany) where indicated. Hoechst (BD Biosciences, San Jose, CA, USA) was added to allow live/dead cell discrimination. Cell sorting for Lin$^-$, c-Kit$^+$, Sca-1$^+$ (LSK) cells was performed using BD FACSAriaIII™ (BD Biosciences, San Jose, CA, USA).

For sorting LSK CD48$^-$ population, cells were stained with Lin-PE cocktail, anti-CD48-PE (BD Pharmingen, Heidelberg, Germany) anti-c-Kit-APC-Cy7 (eBioscience, Vienna, Austria) and anti-Sca-1-PE-Cy7. For further analysis of the starting population, these cells were

also stained with CD34-FITC (BD Pharmingen, Heidelberg, Germany), and/or CD150-APC (Biolegend, London, UK) where indicated.

Expansion cultures

Sorted LSK or LSK CD48$^-$ cells were cultured in StemSpan™ Serum-Free Expansion Medium (SFEM, Stem Cell Technologies, Grenoble, France) supplemented with 1% penicillin/streptomycin (Sigma, St Louis, MO, USA), 10 µg/ml low molecular weight heparin (Abbott, Wiesbaden, Germany), SCF (10 ng/ml, Biovision, Milpitas, CA, USA) and TPO (20 ng/ml, Cell Sciences, Canton, MA, USA). Cells were cultured in a volume of 200 µl medium in a U-bottom 96-well plate at a range of 3000-30.000 cells/ml at 37 °C in 5% CO2. Where indicated purified Wnt3a (250 ng/ml, if not indicated otherwise), liposomal Wnt3a (250 ng/ml), Wnt antagonist Fz8CRD (5 µg/ml) and CHIR99021 (1µM, Stemgent Cambridge, MA, USA) were added to the cultures daily by half medium refreshment or splitting.

To induce BCL2 expression in sorted cells derived from TRE-BCL2 mice, doxycycline (1µg/ml, Sigma, St Louis, MO, USA) was added to cultures at the start of the experiment as well as during the refreshments or splitting.

Flow cytometry

For analysis of cultures after the indicated culture period, cells were harvested, washed and resuspended in PBS containing 10% FCS. Absolute numbers of viable subpopulations (LSK, LSK CD48$^-$, and LSK, CD48$^-$, CD150$^+$, CD34$^{low)}$ cells were determined by a single platform flow cytometric analysis, using the same panel of monoclonal antibodies that was used for sorting and a calibrated number of Cyto-Cal™ Control Count counting microspheres (Thermo Scientific, Waltham, MA, USA). For apoptosis analysis, cells were simultaneously stained with anti-Annexin V-APC in binding buffer (BD Biosciences, San Jose, CA, USA) in PBS containing 10% FCS. Flow cytometric analysis was performed using a BD FACSFortessa™ (BD Biosciences, San Jose, CA, USA) and data was analyzed using FlowJo software (Tree Star Inc, Ashland, OR, USA).

Purification of Wnt3a protein and preparation of liposomal Wnt3a

Mouse Wnt3a was purified from Wnt3a-conditioned medium, collected from *Drosophila* S2 cells, using Blue Sepharose affinity and gel filtration chromatography as described previously [14]. Liposomes containing DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine), DMPG (1,2-dimyristoyl-sn-glycero-3-phospho-rac-glycerol) (both Lipoid AG, Ludwigshafen, Germany) and Cholesterol (Sigma, St Louis, MO, USA) at a 10:1:10 molar ratio were prepared as described in chapter 4 of this thesis. Briefly, purified Wnt3a was mixed with liposomes and incubated for one hour by rotating at 4°C. CHAPS was removed by dialysis (10 kDa molecular weight cut-off) in Hepes-buffered saline at 4°C. The Wnt liposomes were stored at 4°C until use.

Luciferase reporter assay

Mouse LSL cells, which express luciferase in response to TCF promoter binding [24], were routinely cultured at 37°C and 5% CO_2 in culture medium containing DMEM (Invitrogen, Life Technologies, Bleiswijk, The Netherlands), 10% FCS (HyClone, Thermo Scientific, Waltham, MA, USA), 100 U/ml Penicillin and 100 µg/ml Streptomycin (both from Invitrogen, Life Technologies, Bleiswijk, The Netherlands). Prior to the activity assays, 25,000 LSL cells were plated in each well of a 96-well plate and grown for 24 hours. Wnt3a reagents were separately incubated in culture medium with or without serum for various periods of time at 37°C in 96-well plates. After the incubation intervals, media with Wnt3a reagents were added to LSL cells. Upon an additional overnight incubation with the indicated reagents, relative luciferase units were measured with a Glomax multiplate reader.

Western blotting

Lineage-negative (Lin^-) cells from TRE-BCL2 mice were isolated by FACS sorting as described above. 100,000 cells were seeded for each condition and were cultured for 24h with and without doxycycline (1µg/ml, Sigma, St Louis, MO, USA) treatment. Whole-cell extracts were then isolated from total cells. Cells were washed twice in PBS and lysed in RIPA buffer (Thermo Scientific, Waltham, MA, USA) including protease and phosphatase inhibitors. Protein concentrations were measured by BCA protein assay kit (Thermo Scientific, Waltham, MA, USA) and equal amounts of protein were subjected to SDS-PAGE, followed by western blot analysis using anti-Flag antibody (1:1500, Sigma, St Louis, MO, USA).

Statistical analysis

Data are presented as mean ± SD. Statistical significance was determined by a Student's two-tail t-test (unpaired and ratio-paired), performed using GraphPad Prism software (version 6.0; GraphPad), and $p<0.05$ was defined as statistically significant.

RESULTS

Wnt/beta-catenin signals reduce the number of mouse LSK cells in stroma- and serum-free cultures

The effect of the combination of hematopoietic growth factors and exogenous Wnt3a on HSPC in stroma- and serum-free cultures was assessed. For this, FACS-sorted LSK cells were cultured for 10 days in serum-free medium containing stem cell factor (SCF) and Thrombopoietin (TPO). Addition of Wnt3a protein did not significantly affect the expansion of total nucleated cells (**figure 1A**). However, treatment with purified Wnt3a protein resulted in a 2-fold decrease in the number of Lin^- cells relative to control condition

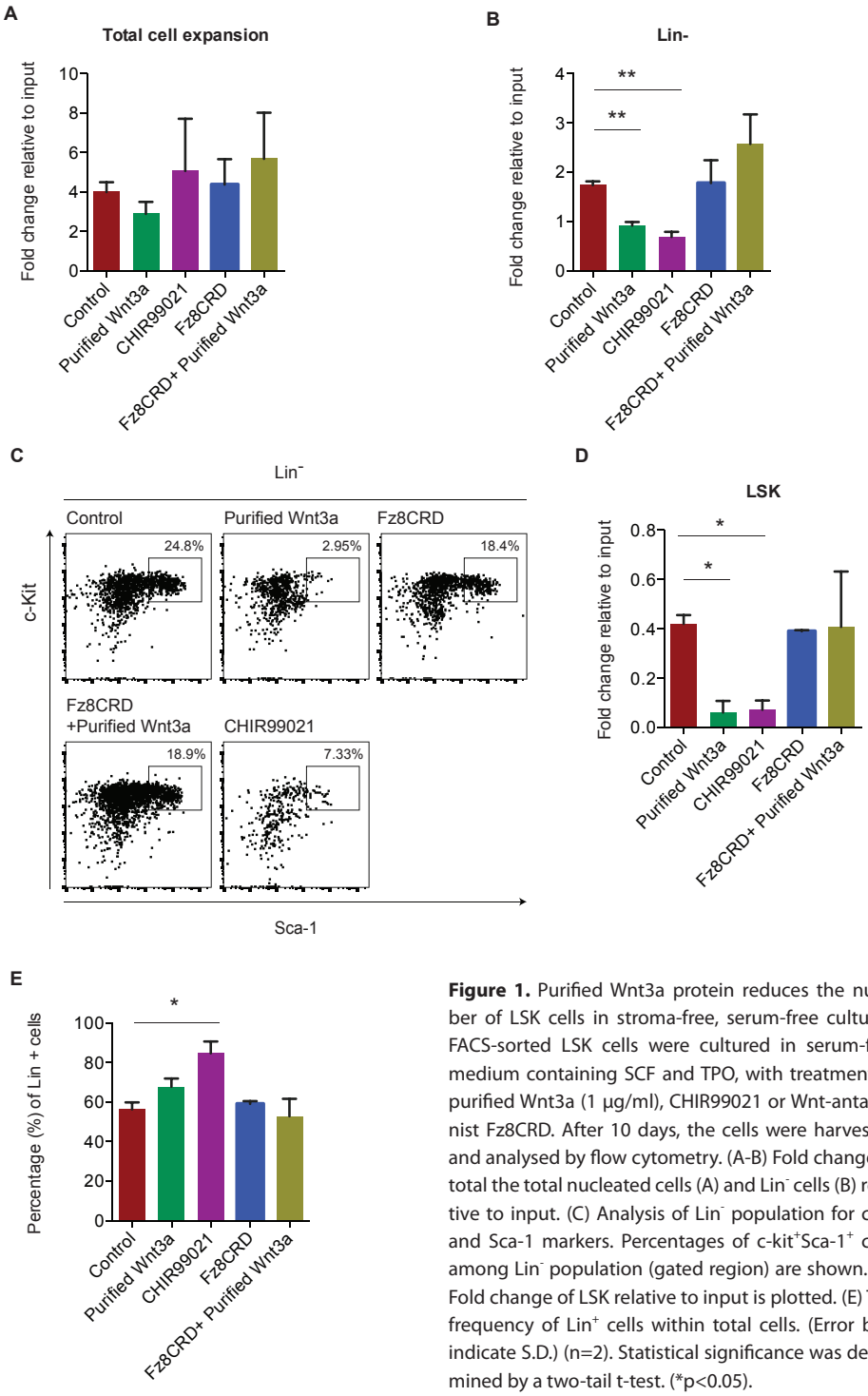

Figure 1. Purified Wnt3a protein reduces the number of LSK cells in stroma-free, serum-free cultures. FACS-sorted LSK cells were cultured in serum-free medium containing SCF and TPO, with treatment of purified Wnt3a (1 µg/ml), CHIR99021 or Wnt-antagonist Fz8CRD. After 10 days, the cells were harvested and analysed by flow cytometry. (A-B) Fold change of total the total nucleated cells (A) and Lin^- cells (B) relative to input. (C) Analysis of Lin^- population for c-kit and Sca-1 markers. Percentages of $c\text{-}kit^+Sca\text{-}1^+$ cells among Lin^- population (gated region) are shown. (D) Fold change of LSK relative to input is plotted. (E) The frequency of Lin^+ cells within total cells. (Error bars indicate S.D.) (n=2). Statistical significance was determined by a two-tail t-test. (*$p<0.05$).

(**figure 1B**, p<0.01). Moreover, while approximately half of the LSK cells were maintained in control conditions, treatment with Wnt3a led to a 6-fold decrease in the number of LSK cells (**figure 1C+D**, p=0.01). These effects were reversed upon simultaneous addition of Fz8CRD, a soluble domain of the Wnt receptor that sequesters Wnt proteins [25], indicating they were specific for the Wnt3a protein (**figure 1A-D**). A similar effect on the number of total nucleated cells, Lin^- cells and LSK cells was observed when the canonical Wnt pathway was activated using the GSK3 inhibitor CHIR99021 (**figure 1A-D**), indicating that the effect was specific for the beta-catenin pathway. The frequency of Lin^+ cells was not significantly affected by Wnt3a protein (**figure 1E**, p=0.09). However, a higher frequency of Lin^+ cells when cells were induced with CHIR99021 was observed, which might be due to a stronger or more prolonged activation of the Wnt pathway, or by effects other than Wnt/β-catenin induction (**figure 1E**, p<0.05). These data collectively show that the addition of Wnt3a protein decreased the number of LSK cells in serum-free, stroma-free cultures and that this is a consequence of the binding of Wnt3a to its receptor and subsequent activation of the canonical Wnt pathway.

Recent data indicates that stability of purified Wnt proteins depends on the presence of detergent or serum [21], implying that its activity may be rapidly lost in serum-free culture systems. This may explain its inability to support LSK cells in serum-free culture. Luciferase reporter assays demonstrated that purified Wnt3a protein, while retaining its activity for several days in medium containing serum, loses its activity within a few hours in serum-free medium (**figure 2A**). Even daily addition of purified Wnt3a to serum-free cultures would therefore result in brief pulses of Wnt3a activity instead of a sustained Wnt signal, which may be insufficient to support LSK cells. It was recently shown that association with lipid vesicles prolongs the stability of Wnt ligands [26,27], allowing us to achieve a more sustained Wnt signal in serum-free culture (**figure 2B**). It was therefore investigated whether lipid-stabilized Wnt3a protein would be able to support LSK, $CD48^-$ cells in stroma-free, serum-free conditions. After 7 days of culture, no significant differences between regular and liposomal Wnt3a for total nucleated cell expansion was observed (**figure 2C**), and the frequency of Lin^+ $CD48^+$ cells remained similar in all conditions (**figure 2D**). Moreover, like purified Wnt3a, liposomal Wnt3a induced a decrease in the more immature LSK, $CD48^-$, $CD150^+$, $CD34^{low}$ population, which is highly enriched for HSCs (**figure 2E**, p<0.05 and p<0.01 for purified and liposomal Wnt3a, respectively). Thus, the failure of purified Wnt3a to support mouse LSK cells in our serum-free culture system was not due to insufficient stability of the Wnt ligand since it also occurred in the presence of lipid-stabilized Wnt3a protein.

Wnt3a signaling does not induce apoptosis in short-term cultures

Previous studies suggest that Wnt signals may induce apoptosis in HSC, and that combined activation of Wnt signaling and inhibition of apoptosis may be required to sup-

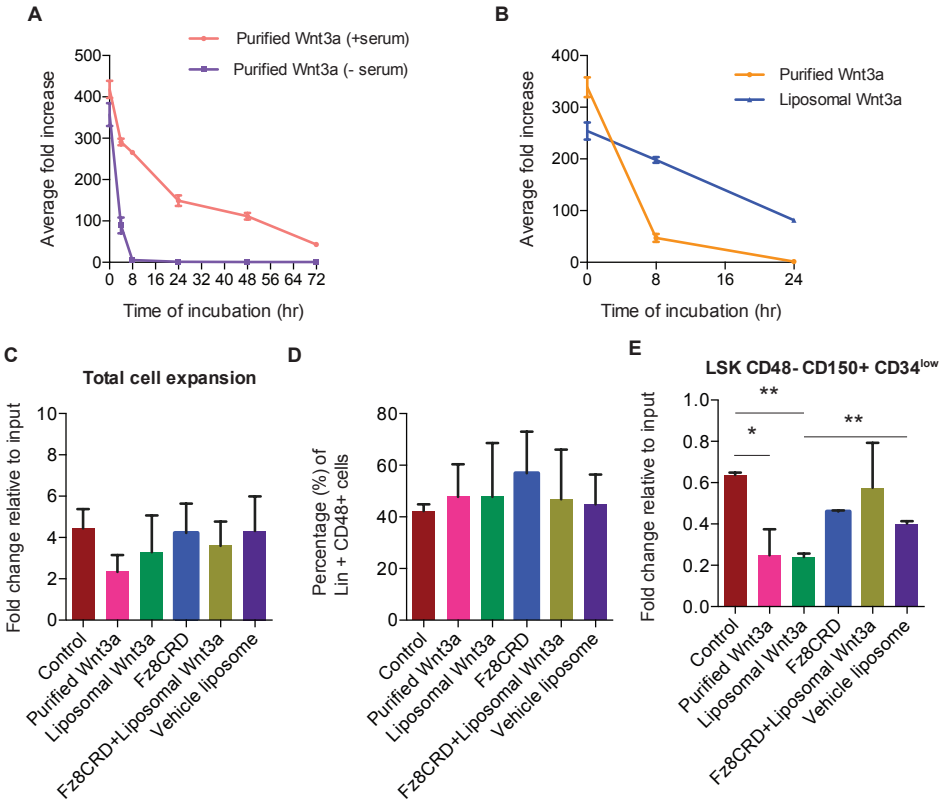

Figure 2. Stable Wnt activity by liposomal Wnt3a reduces the number of primitive LSK cells *in vitro*. (A) Quantification of Wnt activity retained after incubation for 0, 8, 24, 48 and 72 hours at 37°C in cell culture media with or without serum. Activity plot displays average increase of luminescence relative to background. Purified Wnt3a was added at 250 ng/ml (Error bars indicate S.E.M., n=6). (B) Quantification of Wnt3a activity retained after incubation of the indicated reagents in serum-free cell culture medium at 37°C for 0,8 and 24 hours. Purified and liposomal Wnt3a were added at 250 ng/ml. (Error bars indicate S.E.M., n=10). (C-E) FACS sorted LSK CD48$^-$ cells, were cultured with purified Wnt3a, liposomal Wnt3a, and Fz8CRD. Total cells were analysed by flow cytometry after 7 days of culture. The graphs display the total nucleated cell expansion relative to input (C), the frequency of Lin$^+$ cells within total cells (D), and the fold change of LSK CD48$^-$CD150$^+$CD34^{low} cells compared to input (E). (Error bars indicate S.D.) (n=2). Statistical significance was determined by a two-tail t-test (*: p<0.05; **: p<0.01).

port HSC expansion [17,19]. To assess whether Wnt activation leads to early apoptosis, FACS-sorted LSK CD48$^-$ cells were cultured in our stroma-free, serum-free system in the presence or absence of exogenous Wnt3a protein. After 4 days of culture, no difference in total cell numbers or in the frequency of Hoechst$^-$, Annexin V$^+$ early apoptotic cells was observed (**figure 3A-C**). In contrast to longer-term cultures, as described above, addition of Wnt3a protein did not significantly affect the number of LSK CD48$^-$ or LSK CD48$^-$ CD34^{low} cells (**figure 3D-E**). Moreover, no increase in levels of early apoptotic LSK cells in

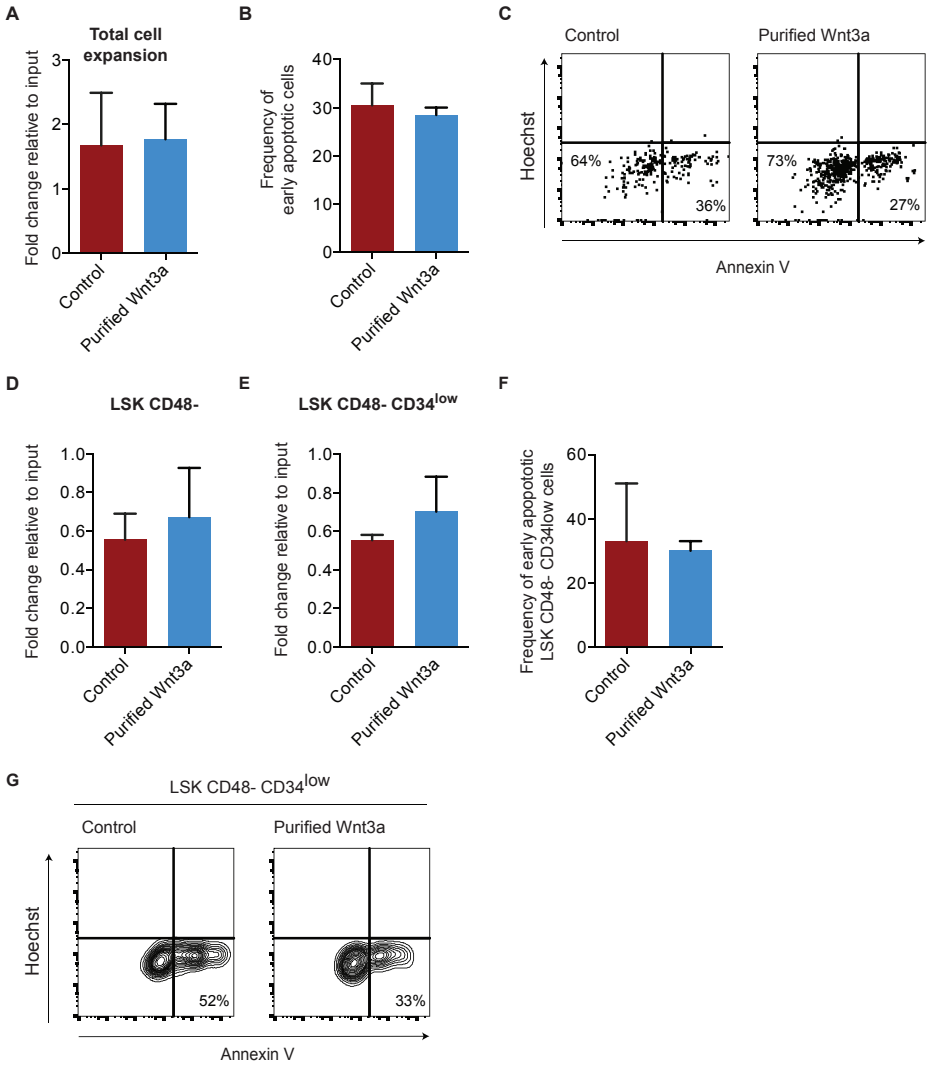

Figure 3. Wnt3a protein does not induce apoptosis of LSK cells in short-term stroma-free, serum-free cultures. FACS-sorted LSK cells were cultured in serum-free conditions with the addition of purified Wnt3a. Cells were analysed by flow cytometry after 4 days of culture. (A-B) The graphs display fold change of total nucleated cells (A) and the frequency of Annexin-V^{+}Hoechst^{-} early apoptotic cells among the same population (B). (C) Representative flow cytometry plots for Annexin-V analysis within Hoechst^{-} viable total cells. Percentages of Annexin-V^{-}Hoechst^{-} and Annexin-V^{+}Hoechst^{-} cells are shown. Shown are graphs for fold change of LSK CD48^{-} (D), and LSK CD48^{-}CD34^{low} cells (E). (F) The graph displays the frequency of Annexin-V^{+}Hoechst^{-} early apoptotic LSK CD48^{-}CD34^{low} cells among the same population. (G) Representative flow cytometry plots for Annexin-V analysis within LSK CD48^{-}CD34^{low} cells. Percentages of Annexin-V^{+}Hoechst^{-} cells are shown. (Error bars indicate S.D.) (n=2).

the presence of Wnt3a was observed (**figure 3F-G**). This suggests that Wnt-mediated reduction in the number of LSK occurs at later stages of culture and cannot be explained by an increase in early apoptotic events.

Inhibition of apoptosis does not rescue Wnt3a-induced loss of LSK cells

To further investigate a possible role for apoptosis in Wnt-induced loss of LSK cells in our cultures, a novel transgenic anti-apoptotic mouse model was developed. Using Cre-mediated transgenesis in embryonic stem cells, an anti-apoptotic FLAG-tagged human BCL2 transgene was placed under control of a doxycycline-responsive promoter targeted in the HPRT locus. The reverse tetracycline transactivator was constitutively expressed from the Rosa locus. Thus, the cells could be induced to express BCL2 upon treatment with doxycycline, inhibiting the induction of apoptosis. Transgenic mice were generated using blastocyst injection of the cells, which allowed us to obtain LSK cells carrying the transgenes. It was verified that doxycycline indeed induced BCL2 in primitive hematopoietic Lin^- progenitors by Western blot analysis (**figure 4A**). This system enabled us to investigate the effect of BCL2 overexpression in otherwise fully comparable cell populations. Doxycycline treatment substantially enhanced total nucleated cell expansion upon culture of LSK $CD48^-$ cells for 10 days (**figure 4B**, $p<0.05$), confirming the effectivity of BCL2 induction. Moreover, a strong increase in LSK $CD48^-$ cells and of the more immature LSK $CD48^-$ $CD150^+$ $CD34^{low}$ population was observed upon doxycycline induction (**figure 4C+D**, $p<0.05$ and $p<0.01$, respectively), indicating that apoptosis was indeed an important factor limiting the expansion of these cells in culture. However, with combined doxycycline and liposomal Wnt3a treatment, the total nucleated cell expansion was reduced compared to doxycycline only condition (**figure 4B**, $p<0.05$). Likewise, the expansion of LSK $CD48^-$ cells and of LSK $CD48^-$ $CD150^+$ $CD34^{low}$ cells in response to BCL2 induction was repressed by liposomal Wnt3a treatment (**figure 4C**, $p<0.05$, and **figure 4D**, $p<0.01$ and $p<0.001$). Combined, our data showed no evidence that Wnt3a induced early apoptosis in LSK cells, or that inhibition of apoptosis by BCL2 supported LSK cell expansion in response to Wnt3a protein.

DISCUSSION

This study investigated the role of Wnt3a-induced signaling in regulation of mouse HSC *ex vivo*. Purified Wnt3a protein combined with hematopoietic growth factors induced a decrease in the number of LSK cells in stroma- and serum-free culture systems. This effect was reversed by a competitive Wnt antagonist, indicating that it was due to binding of Wnt3a to its receptor. The hypothesis that the failure of Wnt3a protein to support LSK cells in a serum-free context was due to its very short half-life in these conditions was

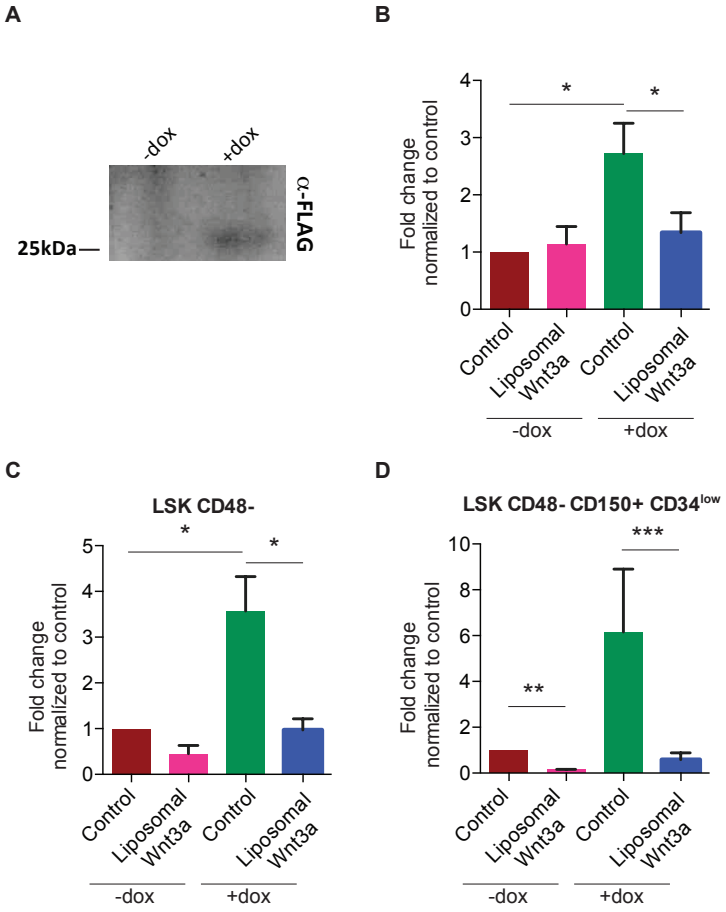

Figure 4. The negative effect of Wnt signals on LSK cell number is not reversed by BCL2 overexpression. (A) Western blotting for FLAG in Lin$^-$ cells from TRE-BCL2 mice cultured for one day with doxycycline. (B-D) LSK CD48$^-$ cells from TRE-BCL2 mice were cultured with and without liposomal Wnt3a and doxycycline treatment for 10 days. The graphs display fold change of the total nucleated cells (B), LSK CD48$^-$ (C) and LSK CD48$^-$CD150$^+$CD34^{low} cells (D) normalized to control. (Error bars indicate S.D.) (n=3). Statistical significance was determined by a two-tail t-test. (*p<0.05, ** p<0.01 and 0.001).

explored, by developing a lipid-stabilized Wnt3a formulation able to provide a sustained Wnt signal. However, lipid-stabilized Wnt3a protein induced a similar reduction in the number of LSK cells, as did GSK3β inhibition, strongly suggesting that the effect was due to activation of the canonical Wnt pathway. Finally, it was investigated whether the induction of apoptosis by Wnt signals caused the loss of LSK cells. However, no evidence that Wnt signals induced apoptosis was found. Moreover, a transgenic mouse model in which apoptosis can be inhibited by the inducible expression of BCL2 was developed. While on its own this greatly supported the expansion of LSK cells, it did not prevent their

loss in response to Wnt signals. Since the loss of LSK cells in response to Wnt signals was not mediated by apoptosis, the most likely explanation would be that Wnt signals induce differentiation of the cells.

A number of studies indicate an important role for Wnt ligands in the maintenance/expansion of HSPC *in vitro* [13,14]. Willert *et al*. and Reya *et al*. report that Wnt signaling activation by either purified Wnt3a protein or a constitutively active form of β-catenin *in vitro* induced expansion of mouse BCL2-overexpressing HSCs [13,14]. In contrast, Nemeth *et al*. observed that activation of Wnt signaling by purified Wnt3a proteins induced a decrease in the number of wild-type mouse HSCs during culture in serum-free medium [17]. These studies did not account for the rapid loss of Wnt protein activity that occurs in the absence of serum [21]. In the present study, it is shown that lipid-stabilized Wnt3a protein, which retains activity for more than 24 hours in the absence of serum, also induced loss of mouse LSK cells. Several *in vivo* studies indicated increased apoptosis of HSC by activated Wnt signaling, suggesting the need for additional survival signals [19,20]. This could explain the observations of Reya *et al*. and Willert *et al*. as they used HSC carrying a constitutively expressed BCL2 transgene [13,14]. In our stroma- and serum-free culture system however, the level of apoptotic cells did not respond to Wnt agonists. Moreover, induction of the BCL2 anti-apoptotic protein could not rescue the negative impact of Wnt signals on LSK cells. Although the different outcomes of our studies and the ones indicating increased numbers of HSC upon Wnt3a treatment [13,14] might still be attributed to the differences in the culture systems, the latter studies lack direct comparisons to control conditions: while HSCs cultured with Wnt3a protein were shown to repopulate recipient mice, the repopulation efficiency in the absence of Wnt3a protein was not reported.

A tripartite balance of proliferation, apoptosis and differentiation is of vital importance to maintain functional HSC *in vivo*. Skewing the balance towards proliferation to achieve *ex vivo* expansion of functional HSC, therefore, requires inhibition of both apoptosis and differentiation. Since our data indicates that Wnt3a has no apparent effect on the total cell proliferation and that the Wnt-mediated loss of LSK cells is not mediated by apoptosis, the most likely explanation would be that Wnt signals induce differentiation of the LSK cells. Indeed, a small increase in the number of differentiated cells in the presence of Wnt signaling agonists was observed (**figure 1E**).

Luis and colleagues considered the dosage of canonical Wnt signaling on HSCs *in vivo*, using a series of mutations in APC to constitutively activate the Wnt pathway at a range of intensities [28]. This showed that low level Wnt activation resulted in enhanced HSC function, while high level constitutive activation impaired repopulation capacity. Moreover, two other studies report that *in vivo* constitutive activation of Wnt signaling blocks differentiation of HSC and causes loss of repopulation capacity [15,16]. In the current setting however, using transient Wnt3a activation, a block in differentiation of LSK cells

which would have caused their numbers to increase was not observed. Therefore, it is unlikely that the negative effect on the number of LSK cells was due to too high levels of Wnt activation in our *in vitro* culture system.

Collectively, this study shows that Wnt signaling induced by Wnt3a protein reduces the expansion of mouse LSK cells in serum-free expansion cultures by apparently promoting their differentiation without affecting their survival. Future studies may address whether additional differentiation-blocking signals may promote HSC expansion.

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Chapter 4

Wnt3a protein reduces growth factor-driven expansion of human hematopoietic stem and progenitor cells in serum-free cultures

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ABSTRACT

Ex vivo expansion of hematopoietic stem and progenitor cells (HSPC) is a promising approach to improve insufficient engraftment after umbilical cord blood stem cell transplantation. Although culturing HSPC with hematopoietic cytokines results in robust proliferation, it is accompanied with extensive differentiation and loss of self-renewal capacity. Wingless-related integration site (Wnt) signaling has been implicated in regulating HSPC fate decisions *in vivo* and in promoting HSPC self-renewal by inhibition of differentiation, but the effects of Wnt on the *ex vivo* expansion of HSPC are controversial. Here, we demonstrate that exogenous Wnt3a protein suppresses rather than promotes the expansion of UCB-derived $CD34^+$ cells in serum free expansion cultures. The reduced expansion was also observed in cultures initiated with $Lin^-CD34^+CD38^{low}CD45RA^-CD90^+$ cells which are highly enriched in HSC and was also observed in response to activation of beta-catenin signaling by GSK3beta inhibition. The presence of Wnt3a protein during the culture reduced the frequency of multilineage CFU-GEMM and the long-term repopulation ability of the expanded HSPC. These data suggest that Wnt signaling reduces expansion of human HSPC in growth factor-driven expansion cultures by promoting differentiation of HSPC.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-SCT) is an important part of treatment for patients suffering from hematological disorders, including leukemia, myelodysplastic syndromes, and aplastic anemia. However, many patients lack a suitable sibling or human leucocyte antigen (HLA) matched unrelated donor (MUD). Because of its rapid availability and less stringent matching criteria[1], umbilical cord blood (UCB) is an important alternative source for hematopoietic stem and progenitor cells (HSPC). However, UCB-derived HSPC significantly differ from bone marrow- and peripheral blood-derived HSPC quantitatively and qualitatively. UCB grafts contain a relatively low number of HSPC which are relatively more primitive, resulting in impaired engraftment and a delayed hematopoietic recovery [1-5], during which patients are at increased risk for severe complications, including infections and bleeding. Several approaches have been pursued to improve engraftment after UCB transplantation, including the *ex vivo* expansion of HSPC.

HSC are defined by their self-renewal capacity and the ability to generate all different hematopoietic lineages. Although *in vivo* studies demonstrated that HSPC expand after transplantation [6], robust *ex vivo* expansion of long-term repopulating HSC remains a challenge. Culturing HSPC with different combinations of hematopoietic cytokines such as stem cell factor (SCF), Fms-related tyrosine kinase 3 ligand (Flt3L), Thrombopoietin (TPO) and granulocyte-macrophage colony-stimulating factor (GM –CSF) resulted in massive expansion of committed HPC which is accompanied by a loss or at best maintenance of primitive HSC with long-term repopulation ability [7-11]. Additional signals are needed to support the expansion of primitive HSC in *ex vivo* culture systems. Several novel factors, such as the immobilized Notch-ligand Delta1, copper chelator tetra-ethylenepentamine (TEPA) and signals derived from mesenchymal stromal cells, were identified that may affect self-renewal of HSC and inhibit differentiation, thereby having the potential to improve *ex vivo* expansion protocols [12-14]. In addition, numerous promising factors have been tested in a pre-clinical setting, including developmental regulators such as fibroblast growth factor signaling, insulin-like growth factor, Angiopoietin-like proteins and Pleiotrophin and chemical modulators like all-trans retinoic acid, stemregenin1 and prostaglandin E2 (reviewed by Walasek *et al*. [15]).

The Wingless-related integration site (Wnt)/beta-catenin signaling pathway regulates cell fate decisions in many developmental processes in embryo and adult. Stimulation of cells with Wnt signaling proteins induces the stabilization and accumulation of the signal transducer protein beta-catenin, which then localizes into the nucleus where it regulates target gene expression (reviewed by Clevers *et al*. [16]). When combined with other growth factors, Wnt proteins can promote self-renewal in several types of stem cells, such as mammary, intestinal and embryonic stem cells [17-20]. Several studies, using different

approaches to inhibit the Wnt signaling pathway, showed that Wnt signaling is pivotal for normal HSC function in mouse [21-23]. In addition, some reports show that treatment with recombinant Wnt3a protein or overexpression of activated beta-catenin enhances the self-renewal capacity of mouse HSC *ex vivo* [24-26]. These studies offer hope that Wnt signals may be of use in the expansion of human UCB-derived HSPC. However, other studies show that constitutive activation of beta-catenin blocks multilineage differentiation [27] and that active beta-catenin induces apoptosis in HSPC [28, 29].

In this study we investigate the effect of Wnt signals on growth factor-driven *ex vivo* expansion of human HSPC. We show that Wnt3a signaling reduces growth factor driven expansion of human HSPC by promoting differentiation.

MATERIAL AND METHODS

Cord blood processing, $CD34^+$ cell selection and HSC sorting

Umbilical cord blood was collected in several hospitals using Stemcare/CB collect blood bag system (Fresenius Kabi Norge AS) containing citrate phosphate dextrose (CPD) as an anticoagulant. Approval for collection was obtained from the Medical Ethical Committee of the Erasmus University Medical Centre (MEC-2009-410) and written informed consent from the mother was obtained prior to donation of the cord blood. Within 48 hours after collection, mononuclear cells were isolated using ficoll (Lymphoprep, Fresenius Kabi Norge AS). $CD34^+$ cells were isolated with double positive immunomagnetic selection using Magnetic Activated Cell Sorting (MACS) technology according instructions of the manufacturer (Miltenyi Biotech GmBH, Bergisch Gladbach, Germany). MACS-selected $CD34^+$ cells were either used directly in experiments or stained with anti-Lin-FITC, anti-CD38-PerCP-Cy5.5, anti-CD90-PE (all from eBioscience, Vienna, Austria), anti-CD34-PE-Cy7, anti-CD45RA-APC-H7 (both from BD Biosciences, San Jose, CA, USA) and DAPI (Sigma-Aldrich, St Louis, MO, USA) after which viable $DAPI^-Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$ cells, highly enriched for hematopoietic stem cells (HSC)[30], were sorted using BD FACSAria Cell Sorting System (BD Biosciences, San Jose, CA, USA).

Expansion cultures

Selected $CD34^+$ cells and sorted $DAPI^-Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$ cells were cultured in serum free Glycostem Basic Growth Medium (GBGM, Glycostem, Oss, The Netherlands) or StemSpan Serum-Free Expansion Medium (SFEM, Stemcell Technologies, Grenoble, France) supplemented with 20 µg/ml low molecular weight heparin (Abbott, Wiesbaden, Germany) and the early acting growth factors SCF (50 ng/ml, Cellgenix, Freiburg, Germany), Flt3L (50 ng/ml, Cellgenix, Freiburg, Germany) and TPO (50 ng/ml, Cellgenix, Freiburg, Germany) (from now on referred to as 'SFT medium') with or without

the addition of 250 ng/ml purified Wnt3a unless indicated otherwise. Cells were cultured in a volume of 1 ml in 24-well plate at a concentration of 10^5/ml at 37 °C in 5% CO_2. Every 2 to 3 days, wells were split or half of the medium was refreshed. In some experiments, we used GSK3β inhibitor CH99021 (1 μM, Stemgent, Cambridge, MA, USA) as an alternative activator of the canonical Wnt pathway. Frizzled8CRD (Fr8CRD, which blocks the binding of Wnt3a to its receptor) was produced as described[31] and used at a concentration of 15 μg/ml. Wnt3a was combined in some experiments with the Aryl hydrocarbon Receptor (AhR) antagonist StemRegenin1 (SR1, 1 μM, Cellagen Technology, San Diego, CA, USA).

Purification of Wnt3a and preparation of liposomal Wnt3a

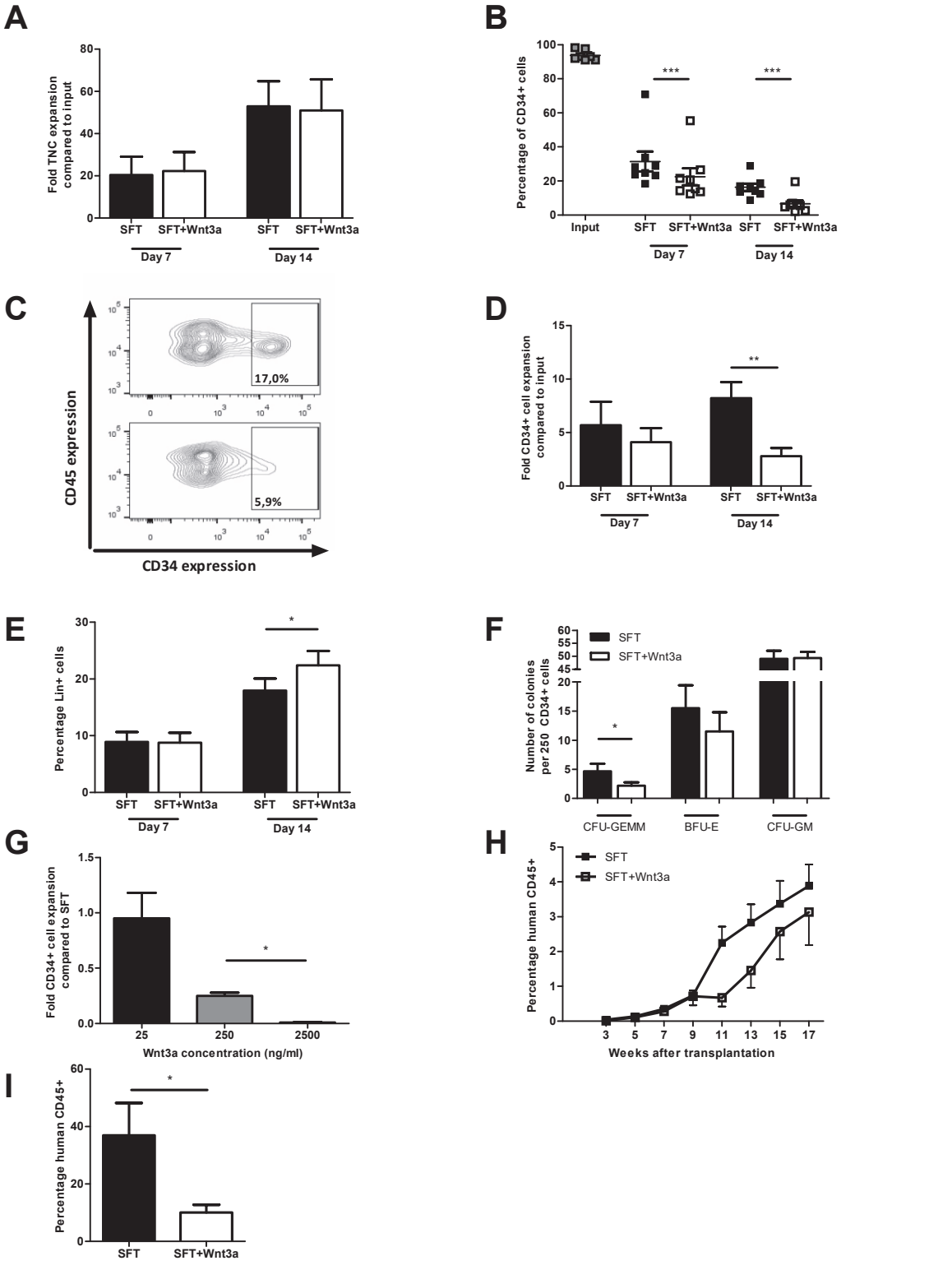
Wnt3a-conditioned medium was collected from Drosophila S2 cells grown in suspension culture. Wnt3a was further purified using Blue Sepharose affinity and gel filtration chromatography as described [24]. Liposomes containing DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine), DMPG (1,2-dimyristoyl-sn-glycero-3-phospho-rac-glycerol) (both Lipoid AG) and Cholesterol (Sigma-Aldrich, St Louis, MO, USA) at a 10:1:10 molar ratio were prepared by extrusion method. Purified Wnt3a was mixed with liposomes at a 1:7.5 ratio to achieve a total concentration of 7-10 μg/ml of Wnt3a. After mixing, the Wnt liposomes were incubated for at least one hour on the roller coaster at 4 °C. Next, CHAPS was removed from the Wnt liposomes by dialysis at least three times in PBS 1 hour each, using dialysis membrane with molecular weight cut-off of 10 kDa at 4 °C. The Wnt liposomes were stored at 4 °C. Activity of purified Wnt3a protein and liposomal Wnt3a was determined in a luciferase reporter assay (see below).

Luciferase reporter assay

Mouse LSL cells, which express luciferase in response to TCF promoter binding, were routinely cultured at 37 °C and 5% CO2 in culture medium composed of DMEM (Invitrogen, Life Technologies, Bleiswijk, The Netherlands), 10% FCS, and 1% Penicillin/Streptomycin. For the activity assays, Wnt3a reagents at a concentration of 250 ng/ml were incubated in culture medium without FCS for various periods of time at 37 °C in U-bottom 96-well plates. These media were then transferred to F-bottom 96-well plates containing LSL cells, which were plated the day before at a density of 25,000 cells/well. After overnight incubation with the indicated Wnt reagents, relative luciferase units were measured with Glomax multiplate reader.

Flowcytometry

At serial time points in culture, absolute numbers of viable $CD34^+$ cells were determined by a single platform flowcytometric assay, using anti-CD45-FITC, anti-CD34-PE, DAPI and a calibrated number of Stem-Count Fluorospheres (all from Beckman Coulter, Fullerton, CA, USA). Within the $CD34^+$ population, the frequency of


A
Fold TNC expansion compared to input
SFT
SFT+Wnt3a
Day 7
Day 14
B
Percentage of CD34+ cells
Input
***
C
CD45 expression
CD34 expression
17,0%
5,9%
D
Fold CD34+ cell expansion compared to input
**
E
Percentage Lin+ cells
*
F
Number of colonies per 250 CD34+ cells
CFU-GEMM
BFU-E
CFU-GM
G
Fold CD34+ cell expansion compared to SFT
Wnt3a concentration (ng/ml)
H
Percentage human CD45+
Weeks after transplantation
I
Percentage human CD45+

← **Figure** 1. Exogenous Wnt3a reduces growth factor-driven expansion of $CD34^+$ cells. UCB-derived $CD34^+$ cells were cultured in serum free SFT medium with or without the addition of Wnt3a. Cells were analyzed using flow cytometry at 7 and 14 days of culture. Shown are (A) the total nucleated cell expansion compared to input (n=8), (B) the frequency of $CD34^+$ cells within the TNC population during culture (n=8), (C) expression of CD45 and CD34 after 14 days of culture in SFT with (lower panel) or without (upper panel) Wnt3a (representative experiment out of 8), (D) the expansion of $CD34^+$ cells compared to input (n=8) and (E) the frequency of cells expressing lineage markers after 7 and 14 days of culture (n=6). (F) Frequency of CFU-GEMM, BFU-E and CFU-GM in 250 $CD34^+$ cells cultured for 2 weeks in SFT or SFT+Wnt3a (n=2, 3 dishes per experiment). (G) $CD34^+$ cell expansion compared to SFT medium after 14 days of culture with different dosages of Wnt3a (n=2). (H) Levels of human chimerism at several time points after transplantation with the progeny of 10^5 $CD34^+$ cells cultured for 7 days in SFT or SFT+Wnt3a medium (n=5 mice per group). (I) Levels of human chimerism in bone marrow 17 weeks after transplantation with the progeny of 10^5 $CD34^+$ cells cultured for 7 days in SFT or SFT+Wnt3a medium (n=5 mice per group). ** $p<0.05$, ** $p<0.01$, *** $p<0.001$*

$DAPI^-Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$ cells was determined using the antibody panel as described above for the sorting of these cells. Absolute numbers of $DAPI^-Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$ cells were determined by multiplying the absolute number of $CD34^+$ cells obtained in the single platform analysis by the percentage of $DAPI^-Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$ cells within the $CD34^+$ cell population. Flowcytometric analysis was performed using a BD FACSCanto (BD Biosciences, San Jose, CA, USA) and data was analyzed using FlowJo software (Tree Star Inc, Ashland, OR, USA).

Transplantation of human hematopoietic cells into NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice

This study was carried out in accordance to the Dutch law on Animal Welfare and Experiments. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Erasmus University Medical Centre Rotterdam, The Netherlands. Intrabone transplantations were performed under isoflurane anesthesia. All animals were housed in groups in individually ventilated cages. Food and water were available ad libitum. NSG mice were sublethally irradiated (3 Gy) and subsequently transplanted with the progeny generated from 1,00E+05 UCB-derived $CD34^+$ cells cultured in our SFT medium, with or without the addition of 250 ng/ml Wnt3a for 7 days. Each group contained 5 mice. Engraftment was assessed every 2 weeks starting at 3 weeks after transplantation by flowcytometric analysis of the peripheral blood, using a flowcytometric panel including anti-mouseCD45-eFluor450, (eBioscience, Vienna, Austria) and anti-humanCD45-APC-Cy7 (BioLegend, London, UK). Mice were considered engrafted when human CD45 levels were higher than 0.1%. At 17 weeks after transplantation, the mice were sacrificed by cervical dislocation and cells from femurs were analysed.

RESULTS

Wnt3a reduces growth factor-driven expansion of UCB derived CD34$^+$ cells

To assess whether Wnt signals affect expansion of human HSPC in culture, UCB-derived CD34$^+$ cells were cultured in serum-free medium supplemented with SCF, Flt3L and TPO (SFT medium) with or without purified Wnt3a protein. After 14 days, no significant change in total nucleated cell expansion was observed in response to Wnt3a protein (**figure 1A**, p=0.74). However, Wnt3a accelerated the decline in the frequency of CD34$^+$ cells that was observed during culture (**figure 1B**, p<0.001 for both 7 and 14 days of culture and **figure 1C**), resulting in a significantly reduced expansion of CD34$^+$ cells after 2 weeks of culture (**figure 1D**). In addition, we observed a higher frequency of cells expressing lineage markers after 14 days of culture in the presence of Wnt3a protein compared with SFT medium only (**figure 1E**, p<0.05). Next, we assessed the functionality of the cultured CD34$^+$ cells by performing colony forming unit (CFU) assays. The presence of Wnt3a during culture reduced the frequency of multi-lineage CFU-GEMM (granulocyte, erythrocyte, monocyte, megakaryocyte), while no effect was seen in the frequency of lineage committed BFU-E (Burst Forming Unit-Erythrocyte) and CFU-GM (granulocyte, macrophage) (**figure 1F**). The reduction in frequency of most immature CFU and the higher frequency of lineage positive cells suggest that exogenous Wnt3a protein promotes rather than inhibits growth factor-driven differentiation of CD34$^+$ cells in expansion cultures. Next, we evaluated the dose-response relationship of Wnt signaling on the *in vitro* expansion of CD34$^+$ cells. At the lowest concentration of 25 ng/ml, Wnt3a had no effect on the expansion of CD34$^+$ cells relative to control SFT cultures (**figure 1G**). On the other hand, the highest concentration of 2500 ng/ml Wnt3a protein resulted in a decline of total cell number (not shown) and a complete loss of CD34$^+$ cells (**figure 1G**). The effect of Wnt3a on the repopulating ability of the expanded CD34$^+$ cells was assessed by transplantation of the expanded population of cells into sublethally irradiated NSG mice after 7 days of culture. All transplanted mice showed engraftment (defined as >0.1% human CD45$^+$ cells in the peripheral blood at 7 weeks after transplantation). However, the kinetics of human chimerism development in peripheral blood appeared delayed when cells were cultured in the presence of Wnt3a (**figure 1H**). Lower levels of human chimerism were also observed in the bone marrow of mice 17 weeks after transplantation of cells cultured in the presence of Wnt3a (**figure 1I**, 36.9% versus 10.1% respectively, p<0.05). This indicates that Wnt3a protein reduces the long-term repopulation ability of cultured CD34$^+$ cells.

Wnt3a reduces growth factor-driven expansion of HSC

CD34$^+$ cells constitute a heterogeneous population, including only a minor fraction of the most immature HSC subset. Wnt3a may act differentially on primitive HSC and CD34$^+$ cells with committed progenitor properties A putative differentiation-inhibiting effect of

Wnt3a on HSC may be obscured by a differentiation-inducing effect on the large population of committed progenitor cells in culture. To study the effects of Wnt3a on the most immature HSC subset, we expanded Lin$^-$CD34$^+$CD38^{low} CD45RA^{low}CD90$^+$ cells, highly enriched for HSC [30] in the presence or absence of Wnt3a. A similar effect of Wnt3a was observed on the expansion of the sorted Lin$^-$CD34$^+$CD38^{low}CD45RA^{low}CD90$^+$ cells. A robust total nucleated cell expansion, approximately 100-fold, was observed regardless of the presence of Wnt3a (**figure 2A**). However, Wnt3a again accelerated the decline in the frequency of CD34$^+$ cells (**figure 2B**, $p<0.05$ and $p<0.01$ for 7 and 14 days of culture, respectively) and led to a significantly reduced expansion of CD34$^+$ cells (**figure 2C**, $p<0.01$). Moreover, Wnt3a strongly reduced the number of Lin$^-$D34$^+$CD38^{low}CD45RA^{low}CD90$^+$ cells obtained after culture, while these cells were maintained in the absence of Wnt3a (**figure 2D**). These data suggest that Wnt3a inhibits the expansion of both multilineage committed progenitors and HSC.

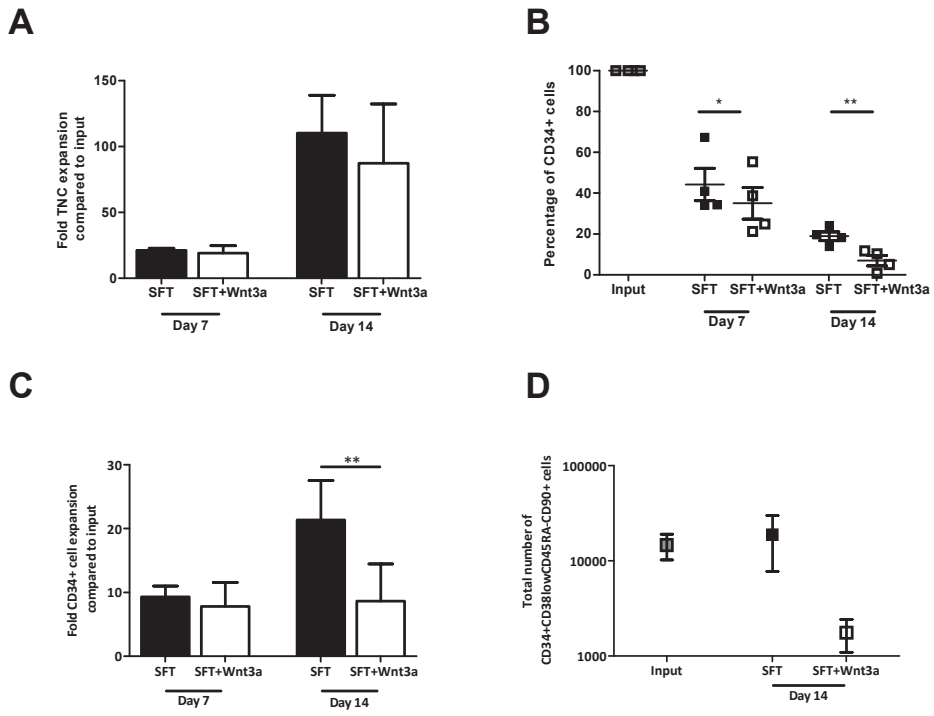


Figure 2. Wnt3a reduces growth factor-driven expansion of HSC. UCB-derived DAPI$^-$Lin$^-$CD34$^+$CD38^{low}CD45RA^{low}CD90$^+$ cells were sorted out of CD34-selected cells and were cultured in SFT medium with or without Wnt3a. Flowcytometric analysis was performed at day 7 and 14. Depicted are (A) the total nucleated cell expansion in SFT and SFT+Wnt3a medium at 7 and 14 days of culture (n=4), (B) the CD34$^+$ cell frequency during culture (n=4), (C) the fold expansion of CD34$^+$ cells at day 7 and 14 of culture (n=4) and (D) the total number of DAPI$^-$Lin$^-$CD34$^+$CD38^{low}CD45RA^{low}CD90$^+$ cells at input and after 14 days of culture in SFT or SFT+Wnt3a medium (n=4). ** $p<0.05$, ** $p<0.01$*

Prolongation of Wnt3a activity does not result in increased expansion of CD34$^+$ cells.

Purified Wnt3a has been shown to have a half-life that is considerably shorter than 24 hours upon dilution in serum free media [32]. Thus, daily addition of Wnt3a protein to cell cultures would result in intermittent rather than continuous activation of the pathway. A possible explanation for our observations of reduced HSPC expansion in response to daily Wnt3a addition is that these intermittent pulses are unable to inhibit HSC differentiation. At the same time, Wnt signals may promote the differentiation of more mature CD34$^+$ cells, leading to an overall reduction of HSPC. The stability of Wnt3a protein can be increased by association with liposomes [33, 34], and we therefore tested whether such stabilized Wnt ligands were able to prevent the decline in HSPC that we observed in response to regular Wnt3a protein. We compared the stability of purified Wnt3a protein and of Wnt3a protein associated with liposomes by incubating the proteins in serum free medium at 37°C and assessing the remaining Wnt-activity at several time points by a luciferase reporter assay. Whereas purified Wnt3a lost its activity within 8 hours, liposomal Wnt3a retained significant activity after 24 hours (**figure 3A**). Despite this increased stability however, liposomal Wnt3a induced a 3.3-fold decline of CD34$^+$ cell expansion (**figure 3B**), similar to purified Wnt3a. In conclusion, prolongation of Wnt3a activity does not result in increased expansion of CD34$^+$ cells.

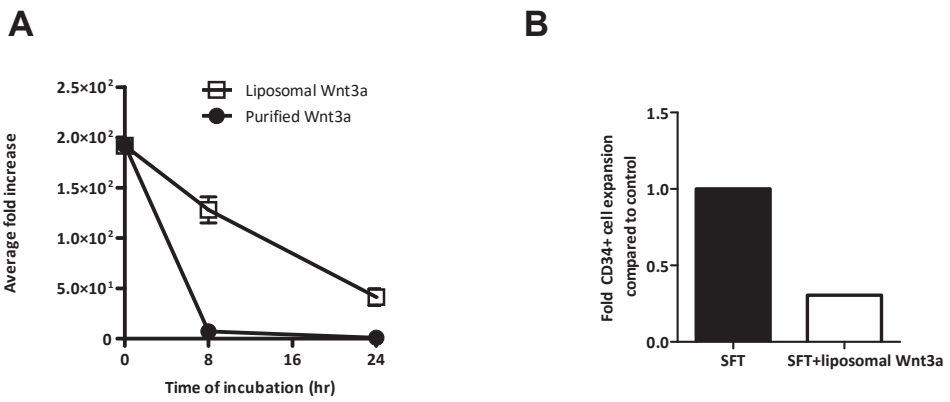


Figure 3. Liposomal Wnt3a reduces expansion of CD34$^+$ cells. (A) Purified Wnt3a and liposomal Wnt3a were incubated for 0, 8, and 24 hours at 37°C in cell culture media, and transferred to LSL cells. Remaining Wnt activity was assayed by luminescence measurements. Activity plot displays average increase of luminescence over incubation time relative to background (n=10). (B) CD34$^+$ cell expansion in SFT medium with or without liposomal Wnt3a after 7 days of culture (n=1).

Enhanced differentiation of CD34$^+$ cells is dependent on activation of the canonical Wnt pathway

Binding of Wnt3a to its receptor can be blocked with the Wnt antagonist Fz8CRD, a soluble domain of the Wnt receptor that sequesters Wnt proteins [31]. To demonstrate that the observed effects of Wnt3a were indeed dependent on binding to its receptor on HSPC, CD34$^+$ cells were cultured with or without Wnt3a and/or Fz8CRD. The negative effect of exogenous Wnt3a on the expansion of CD34$^+$ cells and the decline in frequency of CD34$^+$ cells during culture was reversed by the addition of Fz8CRD (**figure 4A+B**). An alternative way to activate the canonical Wnt pathway is by inhibiting glycogen synthase kinase 3β (GSK3β) which results in stabilization of the cytoplasmic β-catenin pool and subsequent transfer to the nucleus. To confirm that activation of the canonical Wnt pathway by exogenous Wnt3a underlies the inhibitory effects of Wnt3a on the expansion of CD34$^+$ cells we used a synthetic GSK3β-inhibitor (CHIR99021). Addition of CHIR99021 supressed the expansion of CD34$^+$ cells (**figure 4C**) to a similar extend as purified Wnt3a protein (**figure 1D**). Collectively these data show that the reduced expansion of CD34$^+$ cells is due to binding of Wnt3a protein to its receptor on HSPC and subsequent activation of canonical Wnt pathway.

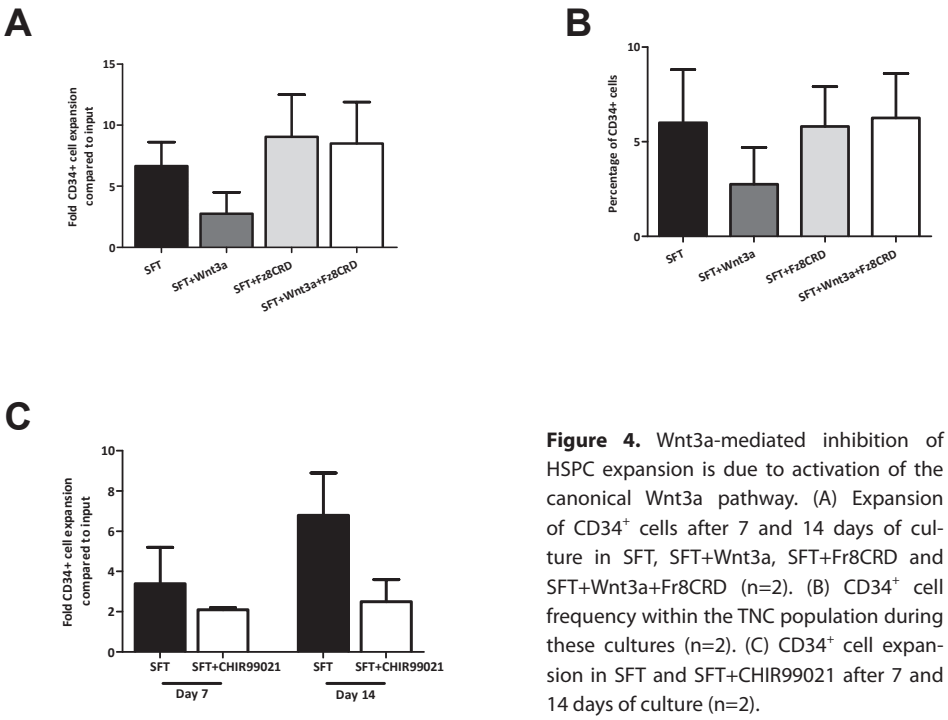


Figure 4. Wnt3a-mediated inhibition of HSPC expansion is due to activation of the canonical Wnt3a pathway. (A) Expansion of CD34$^+$ cells after 7 and 14 days of culture in SFT, SFT+Wnt3a, SFT+Fr8CRD and SFT+Wnt3a+Fr8CRD (n=2). (B) CD34$^+$ cell frequency within the TNC population during these cultures (n=2). (C) CD34$^+$ cell expansion in SFT and SFT+CHIR99021 after 7 and 14 days of culture (n=2).

Wnt3a inhibits expansion of CD34$^+$ cells driven by the Aryl hydrocarbon receptor antagonist Stemregenin1

The Aryl hydrocarbon Receptor (AhR) is implicated to play a role in the proliferation and differentiation of HSPC. AhR-KO mice have increased number of LSK cells in the bone marrow and these LSK are hyperproliferative [35] and hematopoietic progenitors of donor mice treated with the AhR agonist TCDD show impaired competitive engraftment [36]. In addition, the AhR antagonist StemRegenin1 (SR1) has been shown to effectively enhance the expansion of human HSPC [37, 38]. SR1 was included in our cultures as a positive control for enhancing growth factor-driven CD34$^+$ cell expansion and in addition, to evaluate putative cooperative effects of the Wnt and AhR signalling pathways. Expectedly, SR1 indeed promoted CD34$^+$ cell expansion to a similar extent as reported before [37], indicating the suitability of our SFT medium, while Wnt3a reduced CD34$^+$ cell expansion (**figure 5**). Addition of both Wnt3a and SR1 resulted in an increased expansion compared to Wnt alone, but a reduced expansion compared to SR1 alone (**figure 5**, $p<0.01$ and $p<0.05$ respectively), showing that even in the presence of AhR pathway inhibition, Wnt3a suppresses the expansion of CD34$^+$ cells.

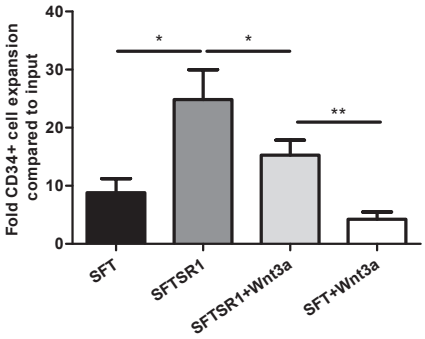


Figure 5. Wnt3a inhibits SR1-enhanced CD34$^+$ cell expansion. UCB-derived CD34$^+$ HSPC were cultured for 14 days in SFT or SFTSR1 medium with or without the addition of Wnt3a. Flowcytometric analysis was performed at day 14. Depicted is the CD34$^+$ cell expansion after 14 days of culture (n=5). ** $p<0.05$, ** $p<0.01$*

DISCUSSION

The effects of Wnt signaling on human HSPC proliferation, differentiation and survival are still poorly understood. In this study, we show that Wnt3a protein suppresses rather than promotes growth factor-driven expansion of UCB-derived CD34$^+$ cells in serum-free expansion cultures. Wnt3a induced accelerated differentiation of both HSC and multipotent progenitors, resulting in the production of more differentiated cells. The reduced expansion of human HSPC, appeared to be dependent on activation of the canonical Wnt signaling pathway upon binding of Wnt3a to its receptor at the cell surface.

Our findings compare well to those by Nemeth *et al*., who observed a decrease in expansion of mouse LSKI (Lineage negative, Sca-1$^+$, c-kit$^+$, IL-7Rα$^-$) cells after culture in

serum-free medium containing SCF, Flt3L and Wnt3a compared to culture conditions with growth factors only. In addition, they showed no enhanced repopulation capacity of Wnt3a-cultured LSKI cells compared to control-cultured cells[25]. Earlier, several studies showed that overexpression of beta-catenin in HSC resulted in a functional defect of hematopoiesis and loss of repopulating activity [27, 39]. These results are in line with our observations of enhanced differentiation of HSPC upon stimulation of the canonical Wnt pathway. However, Malhotra *et al*. reported that overexpression of activated beta-catenin may expand the pool of HSC, both phenotypically and functionally in long term cultures [40] . In addition, overexpression of the Wnt inhibitor Dickkopf-related protein 1 (Dkk1) was shown to significantly impair the self-renewal capacity of adult HSC[21, 22]. How to reconcile these contradictory results? Although at first sight contradictory, the many differences in types of cultures, cells growth factors added may explain some of the observed differences.

Willert *et al*. and Reya *et al*. earlier showed that purified Wnt3a induced proliferation, while inhibiting differentiation of HSC in growth-factor based cultures of mouse LSKT (Lineage negative, Sca-1^{+}, c-kit^{+}, Thy-1.1^{lo}) [24, 26]. However, they used cells derived from BCL2 transgenic mice. The BCL2 anti-apoptotic signal in HSPC may counteract possible apoptotic signals induced by Wnt3a signaling. Moreover, they used serum, which may supply additional signals that allow canonical Wnt signaling to exert a differentiation inhibitory effect or a more pronounced effect on self-renewal. It would compare well to several other studies [28, 41], which show that the presence of additional factors is required to balance the activated Wnt pathway. Trowbridge *et al*. showed enhanced long-term repopulation after treating mice with a GSK3beta inhibitor, which regulates the canonical Wnt pathway and several other pathways as well[41]. Perry *et al*. showed that the combination of a PTEN deletion and activation of beta-catenin results in enhanced self-renewal and expansion of HSC [28]. Another important pathway involved in HSC self-renewal and inhibition of differentiation is the pathway initiated by the aryl hydrocarbon Receptor (AhR). It is now well established that AhR-KO mice have increased number of LSK cells in the bone marrow and these LSK are hyperproliferative [35], while hematopoietic progenitors of donor mice treated with AhR agonist TCDD show impaired competitive engraftment[36]. In addition, the AhR antagonist StemRegenin1 (SR1) promotes expansion of human hematopoietic stem cells [37, 38]. We evaluated whether combined inhibition of the AhR pathway and activation of the Wnt pathway would result in enhanced expansion of HSPC. However, Wnt signaling also reduced the expansion of CD34^{+} HSPC in the presence of the aryl hydrocarbon receptor antagonist SR1. Another important factor that may affect the response of HSPC to Wnt signaling is the level of oxygenation. Expansion cultures are usually performed at normoxic levels. However, it is well established that the response of HSPC to hematopoietic cytokines is different under normoxic and hypoxic conditions, which mimics the in situ bone marrow environment

[42]. The effect of stimulation of the canonical Wnt pathway on HSC expansion may be modulated by the level of oxygenation, which was already shown for the effect of the Wnt4-dependent pathway on the functional capacities of mesenchymal stem cells [43].

Apart from other cytokines and pathways involved, the dose and timing of Wnt activation might play an important role. Our data show that both intermittent pulses of Wnt3a and more continuous exposure to Wnt3a have a similar negative effect on the expansion of $CD34^+$ cells in serum-free expansion cultures. Luis *et al* reported that Wnts are tightly regulated in a dose-dependent fashion [44], which may affect their biological activity. Thereby, varying results (exerted via the canonical Wnt pathway) may result from different levels of Wnt signaling, such as can be achieved in different experimental conditions. The optimal level of Wnt signaling for HSC was suggested to be only slightly increased over normal physiological values, while higher levels resulted in impaired engraftment potential of HSC fashion [44].

Collectively, we show that exogenous Wnt3a proteins reduces the expansion of human HSPC in serum-free growth factor-driven HSPC expansion cultures by promoting their differentiation without apparently affecting their proliferation or survival. It cannot be excluded that additional signals, such as the induction of a hypoxic cellular response, are needed for a possible positive effect of canonical Wnt signaling on HSPC expansion. Future studies may address the fragile balance between canonical Wnt signaling and other pathways to determine whether combined activation of Wnt signaling and other signaling pathways may promote human HSPC expansion.

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Chapter 5

In vitro and *in vivo* evaluation of StemRegenin1-expanded umbilical cord blood-derived hematopoietic stem and progenitor cells

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Submitted

ABSTRACT

The slow hematopoietic recovery following umbilical cord blood stem cell transplantation (UCB-SCT) is considered to be primarily due to the relatively low number of hematopoietic stem and progenitor cells (HSPC) in the graft. Aryl hydrocarbon receptor (AhR) antagonists, such as StemRegenin1 (SR1), have been shown to promote *ex vivo* expansion of UCB-derived $CD34^+$ cells. Here, the effects of SR1 on the expansion of the most primitive subset of UCB-derived $CD34^+$ cells were evaluated. It is shown that the combination of SR1 and the hematopoietic growth factors Stem Cell Factor (SCF), FMS-like tyrosine kinase 3 ligand (Flt3L) and Thrombopoietin (TPO) resulted in a 27-fold expansion of the phenotypically most primitive $CD34^+$ cell subset (i.e. $Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$ cells) following serum-free culture of 2 weeks. That combination also resulted in a 105-fold expansion of the functionally most primitive subset as assessed by the long-term culture initiating cell (LTC-IC) assay. Transplantation of SR1-expanded cells into sublethally irradiated NSG mice resulted in robust long-term multilineage human engraftment in blood and bone marrow, although mice transplanted with expanded cells, with or without SR1, developed lower levels of human chimerism as compared to mice transplanted with non-expanded cells. The number of $CD34^+C10^+$ lymphoid progenitors was strongly reduced in the expanded population of cells, cultured with or without SR1, which might account for the lower level of human B cell chimerism, being the primary endpoint in the B cell-prone NSG mouse model. In conclusion, the combination of proliferation inducing cytokines combined with the differentiation inhibiting factor SR1 resulted in strong augmentation of phenotypic HSC with long-term repopulating capacity *in vivo*.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-SCT) is an important treatment modality in many patients suffering from severe hematological disease. Umbilical cord blood (UCB) has become an attractive alternative source of hematopoietic stem and progenitor cells (HSPC) for patients lacking a suitable HLA-matched related or unrelated donor. Recently, interest in haplo-identical allo-SCT as an alternative source of stem cells has met increasing attention (1, 2). As compared to haplo-identical allo-SCT, UCB transplantation (UCBT) is associated with prolonged hematopoietic recovery due to the relatively low number of hematopoietic stem and progenitor cells (HSPC) present in the graft (3-5). The retarded recovery may explain a somewhat higher non relapse mortality (NRM) rate (6) upon UCBT as compared to matched unrelated donor (MUD) transplantation. Given the relatively low number of HSPC in UCB, several strategies were studied to expand the progenitor cells *ex vivo* prior to transplantation (7-16). *Ex vivo* expansion of HSPC was initially explored using hematopoietic cytokines, including stem cell factor (SCF), Fms-related tyrosine kinase 3 ligand (Flt3L), thrombopoietin (TPO) and granulocyte-macrophage colony-stimulating factor (GM –CSF) (7-10). While strong expansion of total cell numbers were observed, expansion appeared accompanied by rapid differentiation, and the most primitive subsets of HSC were hardly expanded or even exhausted (7-10). Therefore, in order to expand the true multipotent self-renewing HSC, expansion protocols were developed in which hematopoietic growth factors were combined other factors that might inhibit HSC differentiation. We earlier explored the inhibitory effect of Wingless-related integration site protein 3a (Wnt3a) in combination with hematopoietic cytokines, but were unable to demonstrate inhibition of differentiation (17). Alternative factors explored included agents that may influence HSC fate decisions, such as the Notch ligand Delta1, nicotinamide and the copper chelator tetraethylenepentamine (TEPA) (18-20). The Aryl hydrocarbon Receptor (AhR) antagonist StemRegenin1 (SR1) combined with early acting hematopoietic cytokines was also explored based on the hypothesis that induction of proliferation accompanied by inhibition of differentiation might result in expansion of HSC (15). The AhR pathway is critically involved in in HSC self-renewal and inhibition of differentiation: AhR-KO mice have an increased number of hyperproliferative $Lin^{-}Sca^{+}c\text{-}Kit^{+}$ cells (enriched for HSC) (21), while treatment of donor mice with AhR agonist TCDD resulted in impaired competitive engraftment (22). *In vitro* studies suggested that SR1 combined with SCF, Flt3L, TPO and IL-6 resulted in a 17,100-fold and 47-fold increase of $CD34^{+}$ cells compared to input and control cultures supplemented with the cytokine cocktail but lacking SR1, respectively, after 5 weeks of culture (15). Subsequently *in vivo* studies showed that SR1-expanded HSPC were capable of hematopoietic reconstitution in both mice (15) and men (23) and neutrophil and platelet recovery were significantly shortened upon transplantation of

an SR1-expanded graft in a double UCBT (dUCBT) setting compared to unmanipulated dUCBT (23). In addition, UCB-HSPC cultured in the presence of an AhR antagonist retained the potential to differentiate towards the T cell lineage(24), which is pivotal for full immune reconstitution post-transplantation (25). Recently, Gu *et al*. showed that *in vitro* culture of adult HSPC in the presence of an AhR antagonist maintained but failed to increase the number of *in vivo* engrafting cells (26). As a result, it was suggested that SR1 does not affect the true long-term HSC, responsible for long-term multilineage engraftment, but rather a population of cells with short-term repopulation potential (27, 28). However, all studies were performed with $CD34^+$ HSPC, which comprise a heterogeneous population, including only a minor subset with the most immature HSC. Given these conflicting findings, we set out to study the effect of SR1 on the expansion of the most primitive $Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$ cells, which are highly enriched for HSC (29).

MATERIAL AND METHODS

Cord blood processing, $CD34^+$ cell selection and cell sorting

Umbilical cord blood was collected in several hospitals using Stemcare/CB collect blood bag system (Fresenius Kabi Norge AS) containing citrate phosphate dextrose (CPD) as an anticoagulant. Approval for collection was obtained from the Medical Ethical Committee of the Erasmus University Medical Centre (MEC-2009-410) and written informed consent from the mother was obtained prior to donation of the cord blood. Within 48 hours after collection, mononuclear cells were isolated using ficoll (Lymphoprep, Fresenius Kabi Norge AS). $CD34^+$ cells were isolated with double positive immunomagnetic selection using Magnetic Activated Cell Sorting (MACS) technology according to instructions of the manufacturer (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). MACS-selected $CD34^+$ cells were either used directly in experiments or stained with anti-Lin-FITC, anti-CD38-PerCP-Cy5.5, anti-CD90-PE (all from eBioscience, Vienna, Austria), anti-CD34-PE-Cy7, anti-CD45RA-APC-H7 (both from BD Biosciences, San Jose, CA, USA) and DAPI (Sigma-Aldrich, St Louis, MO, USA) after which viable $DAPI^-Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$ cells, highly enriched for hematopoietic stem cells (HSC)(29), were sorted using BD FACSAria Cell Sorting System (BD Biosciences, San Jose, CA, USA).

Expansion cultures

Selected $CD34^+$ cells and sorted $DAPI^-Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$-cells were cultured in serum free Glycostem Basic Growth Medium (GBGM, Glycostem, Oss, The Netherlands), StemSpan Serum-Free Expansion Medium (SFEM, Stemcell Technologies, Grenoble, France) or Serum-free Stem Cell Growth Medium (SCGM, CellGro, CellGenix, Freiburg, Germany). Culture media were supplemented with 20 µg/ml low molecular

weight heparin (Abbott, Wiesbaden, Germany) and the early acting growth factors SCF, Flt3L and TPO (50 ng/ml each, all from Cellgenix, Freiburg, Germany) (from now on referred to as 'SFT medium') with or without the addition of 1 μM StemRegenin1 (SR1, Cellagen Technology, San Diego, CA, USA or Axon Medchem, Groningen, The Netherlands) unless indicated otherwise. Cells were cultured in a volume of 1 ml in a 24 well plate at a concentration of 10^5/ml at 37 °C in 5% CO2. Every 2 to 3 days, wells were split with addition of an equal volume of culture medium.

Flow cytometry

At serial time points in culture, absolute numbers of viable $CD34^+$ cells were determined by a single platform flow cytometric assay, using anti-CD45-FITC, anti-CD34-PE, DAPI and a calibrated number of Stem-Count Fluorospheres (all from Beckman Coulter, Fullerton, CA, USA). Within the $CD34^+$ population, the frequency of $DAPI^-Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$-cells was determined using the antibody panel as described above for the sorting of these cells. Absolute numbers of $DAPI^-Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$-cells were determined by multiplying the absolute number of $CD34^+$ cells obtained in the single platform analysis by the percentage of $DAPI^-Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$-cells within the $CD34^+$ cell population.

For cell division tracking during culture, the CellTrace™ Violet Cell Proliferation Kit (Life Technologies, Eugene, OR, USA) was used. Prior to culture, selected and sorted cells were labeled using CellTrace™ Violet, according to instructions of the manufacturer. Flow cytometry was performed at day 5 of culture. Flow cytometric analysis was performed using a BD FACSCanto (BD Biosciences, San Jose, CA, USA) and data was analyzed using FlowJo software (Tree Star Inc, Ashland, OR, USA).

Long term culture-initiating cell (LTC-IC) assays

The LTC-IC assay was performed as described(30). In short, confluent MS-5 stromal layers in 96-well plates were overlaid with sorted subsets of (expanded) $CD34^+$ cells in a limiting dilution set-up, with 3000 cells per well as the highest concentration and titrating down by 1/3, using 20 wells per condition. Cells were cultured for 5 weeks at 37 °C in 5% CO2 in IMDM medium, containing 12% horse serum, 12% fetal calf serum, 2mM L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin, with medium change every week. After 5 weeks, the medium was removed and methylcellulose containing medium (Methocult GF H84434, Stemcell Technologies, Vancouver, BC, Canada) was added to each well. Upon another 14 days of culture, the percentage of wells containing a colony (=positive wells) was determined. The frequency of LTC-IC within each cell population was calculated using the number of negative wells per dilution and Poisson statistics.

Transplantation of human hematopoietic cells into NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice

NSG mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and housed under specific pathogen-free conditions in the Erasmus MC animal facility. The mice experiments were carried out in accordance to the Dutch law on Animal Welfare and Experiments. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Erasmus University Medical Centre Rotterdam, The Netherlands. All animals were housed in groups in individually ventilated cages. Food and water were available ad libitum. All NSG mice were sublethally irradiated (2.5 – 3.5 Gy) and transplanted intravenously with the indicated number of $CD34^+$ cells, the progeny thereof or sorted subsets of $CD34^+$ cells. Engraftment was assessed by flow cytometric analysis of the peripheral blood, using a panel including anti-mouseCD45-eFluor450, (eBioscience, Vienna, Austria) and anti-humanCD45-APC-Cy7 (BioLegend, London, UK). For the assessment of multilineage engraftment, peripheral blood and bone marrow cells were stained with anti-humanCD56-PE-Cy7, anti-humanCD33-PerCP-eFluor710, anti-humanCD3-APC and anti-humanCD19-PE (all from eBioscience, Vienna, Austria). Mice were considered engrafted when human $CD45^+$ cell levels were higher than 0.1% in the bone marrow. The mice were sacrificed by cervical dislocation and cells from femurs were analyzed at the time points described in the results section.

Statistics

Paired or unpaired two-tailed t-tests were performed to test the differences between the different culture conditions, as appropriate. Differences were considered to be statistically significant is $p < 0.05$. Statistical tests were performed in GraphPad Prism 5.0 (GraphdPad software, San Diego, CA, USA).

RESULTS

Serum-free cultures combining hematopoietic growth factors and StemRegenin1 result in expansion of the phenotypically most immature $CD34^+$ cells that are enriched for LTC-IC

To confirm the effect of Stemregenin1 (SR1) on the expansion of HSPC in culture, purified UCB-derived $CD34^+$ cells were cultured in serum-free medium supplemented with SCF, Flt3L and TPO (SFT medium), with or without the addition of SR1. No significant difference was observed in expansion of $CD45^+$ cells after 14 days of culture in either SFT or SFT+SR1 medium (**Table 1, figure 1A**, p=0.23). During expansion, the frequency of $CD34^+$ cells gradually declined due to concomitant differentiation of the expanding cells. Addition of SR1 delayed the decline in frequency of $CD34^+$ cells, resulting in a higher frequency of $CD34^+$ cells at both 7 and 14 days compared to SFT medium alone (**Table**

Table 1. Fold expansion and frequency of $CD45^+$, $CD34^+$ and $CD34^+CD90^+$ cells upon culture in SFT or SFT+SR1 medium

	Day 7			Day 14		
	SFT	**SFT+SR1**	**p-value**	**SFT**	**SFT+SR1**	**p-value**
Fold expansion compared to input						
$CD45^+$ cells	47.3	47.6	0.88	100.3	113.3	0.23
$CD34^+$ cells	19.9	31.2	<0.01	23.3	55.2	<0.01
$CD34^+CD90^+$ cells	10.8	21.6	0.09	8.9	26.9	<0.001
Frequency						
$CD34^+$ cells (within $CD45^+$ cells)	36.9%	63.0%	<0.0001	19.2%	42.5%	<0.0001
$CD90^+$ cells (within $CD34^+$ cells)	5.8%	7.1%	<0.05	5.5%	4.5%	0.18

1, figure 1B, p<0.001 for both time points), suggesting inhibition of differentiation by SR1. The increased frequency of $CD34^+$ cells led to an increased expansion of $CD34^+$ cells after 1 and 2 weeks of culture (**Table 1, figure 1C**, p=0.0083 and p=0.006 respectively). A 55-fold (SD ±44.46) expansion of $CD34^+$ cells upon addition of SR1 after 2 weeks of culture was observed, compared to a 23-fold (SD ±20.78) expansion upon culture in SFT medium alone. These results are in line with the results reported by Boitano *et al*. (15).

$CD34^+$ cells are a heterogeneous population, of which the most immature HSC are only a minor subset. Next, it was evaluated whether the expanded $CD34^+$ cells still contained cells with the most immature phenotype, i.e. $Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$ cells (referred to as "$CD34^+CD90^+$ cells" hereafter), which are highly enriched for HSC (29). Before culture, $CD34^+CD90^+$ comprised 4.0% (SD ±2.1) of the $CD34^+$ cell population (**figure 1D**). After 14 days of culture, the frequency of $CD34^+CD90^+$ cells within the $CD34^+$ cell population remained stable in both culture conditions (**Table 1, figure 1D**, p=0.38 for SFT; p=0.18 for SFT+SR1). The number of cells with the most immature phenotype expanded 26.9-fold (SD ±9.6) in the presence of SR1 and 8.9-fold (SD ±9.5) in the absence of SR1 (**Table 1, figure 1E**, p=0.0003).

During expansion, a population of $CD45RA^+CD90^+$ cells appeared within the $CD34^+$ cell population that was not present in unexpanded $CD34^+$ cells (**figure 1F**). To assess whether after *ex vivo* expansion in the presence of SR1, the functionally most immature cells were primarily present within the phenotypic most immature $CD45RA^{low}CD90^+$ subset or also present in the new $CD45RA^+CD90^+$ subset, 3 populations of cells were sorted, namely $Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$ cells, $Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^{low}$ cells and $Lin^-CD34^+CD38^{low}CD45RA^+CD90^+$ and long term culture-initiating (LTC-IC) assays were performed. At present, the LTC-IC assay is the best *in vitro* surrogate assay for primitive HSPC. The highest frequency of LTC-IC was found in the $CD45RA^{low}CD90^+$ population (**figure 1G**, 1 in 23.3 cells), indicating that after culture, the phenotypically most immature cells are still enriched for LTC-IC. The new $CD45RA^+CD90^+$ subset contained a

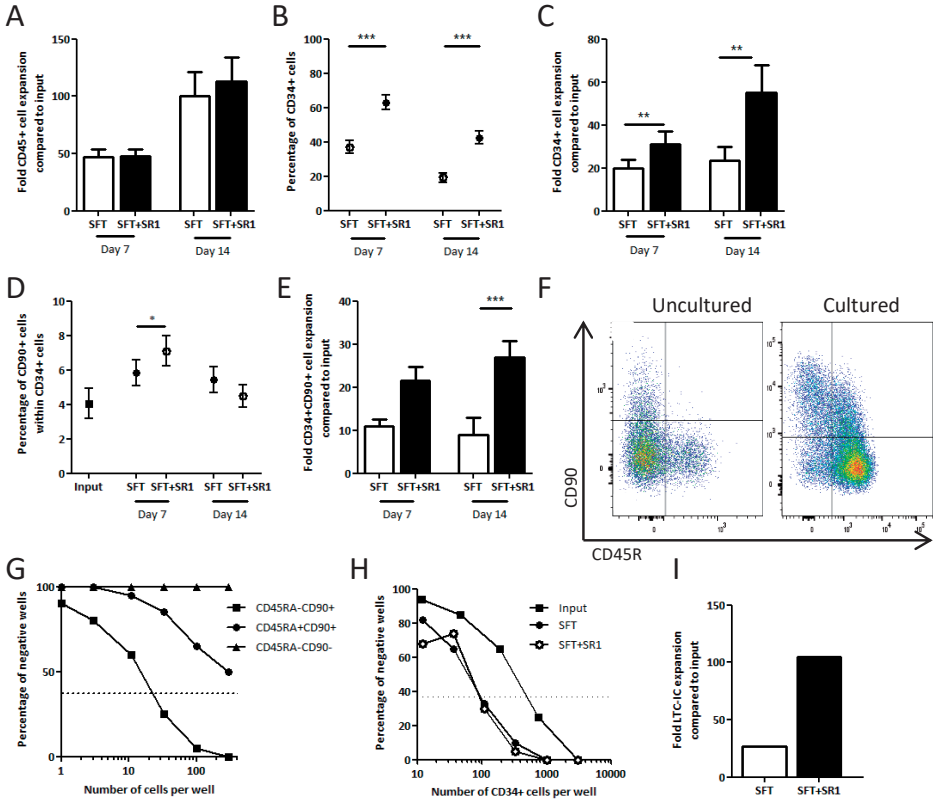

Figure 1. Serum-free cultures combining hematopoietic growth factors and StemRegenin1 result in expansion of the phenotypically most immature CD34$^+$ cells that are enriched for LTC-IC. UCB-derived CD34$^+$ cells were cultured in serum free SFT medium with or without the addition of SR1. Cells were analyzed using flow cytometry at 7 and 14 days of culture. Shown are (A) the CD45$^+$ cell expansion compared to input (n=10), (B) the frequency of CD34$^+$ cells within the CD45$^+$ cell population (n=10), (C) the fold expansion of CD34$^+$ cells compared to input (n=10), (D) the frequency of CD90$^+$ cells within the CD34$^+$ cell population (n=6) and (E) the expansion of CD34$^+$CD90$^+$ cells compared to input (n=6).(F) shows the expression of CD45RA and CD90 in either uncultured (left panel) or cultured (right panel) Lin$^-$CD34$^+$CD38^{low} cells (representative out of 6). (G) and (H) show the frequency of negative wells versus the number of cells plated in a LTC-IC assay. In (G), LTC-IC frequency for several sorted populations upon SFT+SR1 culture are shown. (H) shows the LTC-IC frequency in uncultured and cultured CD34$^+$ cells. (I) Fold expansion of LTC-IC after 14 days of culture compared to input. * p<0.05, ** p<0.01, *** p<0.001

much lower frequency of LTC-IC (~1/400), whereas the CD90^{low} population contained no detectable LTC-IC. To quantify the increase in LTC-IC in expansion cultures in the presence and absence of SR1, LTC-IC frequencies upon 14 days of culture in either SFT or SFT+SR1 medium, without prior cell sorting, were determined. Upon 14-day culture in either the absence or presence of SR1, an increase in the frequency of LTC-IC in CD34$^+$ cells was observed (**figure 1H,** input: 1 in 466 cells; SFT: 1 in 109 cells; SFT+SR1: 1 in 90 cells), resulting in a 105-fold expansion of LTC-IC upon culture in SFT+SR1 medium (**figure 1I**).

To specifically assess the effect of the combination of SR1 and hematopoietic growth factors on the phenotypically most immature cells in the absence of more mature $CD34^+$ cells, sorted $CD34^+$ $CD90^+$ cells were expanded in SFT medium in the presence and absence of SR1. Similar to our results in cultures with $CD34^+$ cells, robust expansion of total nucleated cells, with no difference between both conditions after 14 days of culture, was observed (**figure 2A**, 100-fold versus 128-fold for SFT and SFT+SR1 respectively, p=0.36). The gradual decline in the frequency of both $CD34^+$ cells and $CD34^+$ $CD90^+$ cells during culture was retarded in the presence of SR1, again indicating inhibition of differentiation by SR1 (**figure 2B+C**). The expansion of $CD34^+$ cells (**figure 2D**, p<0.05) and $CD34^+CD90^+$ cells (**figure 2E**, p<0.05) after 14 days of culture in SFT+SR1 was increased compared to SFT alone. Taken together, our data show that the combination of hematopoietic growth factors and SR1 not only results in robust expansion of $CD34^+$ cells, but also of the phenotypically most immature $Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$ cells and the functionally most immature cells as assessed by the *in vitro* LTC-IC assay.

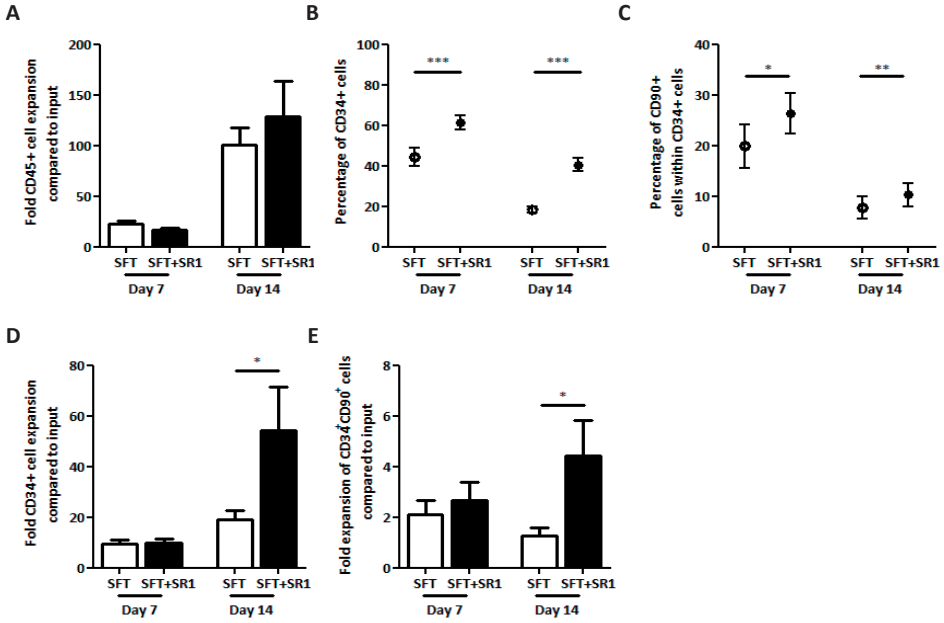

Figure 2. SR1 combined with hematopoietic growth factors promotes expansion of $CD34^+CD90^+$ cells. UCB-derived $Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$ cells were sorted out of CD34-selected cells and were cultured in SFT medium with or without SR1. Flow cytometric analysis was performed at day 7 and day 14. Depicted are (A) the expansion of $CD45^+$ cells (n=8), the frequency of (B) $CD34^+$ cells (n=8) and (C) $Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$ cells (n=7) within the $CD34^+$ cells population and the expansion of (D) $CD34^+$ cells (n=8) and (E) $Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$ cells (n=7). * p<0.05, ** p<0.01, *** p<0.001

SR1 induces retention of CD34- and CD90-expression following cell division

The phenotypically most immature $CD34^+CD90^+$ subset expanded in the presence of SR1, but the frequency of those immature cells within $CD34^+$ cells remained similar during culture. To address the question how many cell divisions the immature phenotype is retained in expansion cultures, sorted $CD34^+CD90^+$ cells were cultured for 5 days in cultures supplemented with hematopoietic growth factors in the absence or presence of SR1. Prior to culture, cells were labeled with a CellTrace™ Violet labeling. Using flow cytometry, populations of cells could be distinguished based on the number of cell division. Most cells had undergone 3 or 4 cell divisions in both culture conditions (SFT: 26,5% and 41%; SFT+SR1: 35% and 38% respectively). Addition of SR1 resulted in a lower frequency of cells that had undergone 5 or 6 cell divisions, suggesting that SR1 might slightly delay cell division in the total cell population. While CD34 expression was retained in the majority of cells up to the 5^{th} cell division in SFT+SR1 cultures, the frequency of $CD34^+$ cells rapidly declined after the 4^{th} cell division in SFT cultures (**figure 3A**). CD90 expression was already lost in the majority of the cells after 4 cell divisions both in the presence or absence of SR1 (**figure 3B**). The loss of CD90 expression was somewhat delayed in cells cultured with SR1 (**figure 3A-C**). The percentage of cells retaining their $CD90^+$ phenotype within the $CD34^+$ population after 3 and 4 cell divisions was 40.1% and 16.8% in SFT-cultured cells versus 49.5% and 24.2% in SFT+SR1 cultured cells, respectively (**figure 3C**). Next, it was evaluated whether cells that retained their immature phenotype after 3 and 4 cell divisions also retained their LTC-IC frequencies. $CD34^+CD90^+$ cells that had undergone 3 or 4 cell divisions in SFT+SR1 culture were sorted and evaluated in a LTC-IC assay. The frequency of LTC-IC appeared to be higher in the cell population that had undergone 3 cell divisions compared to the population that had undergone 4 cell division (**figure 3D**, 1 in 1,8 cells versus 1 in 9,9 cells respectively). Thus, part of the cells that retained their immature phenotype after 4 cell divisions had lost their immature functional characteristics (LTC-IC activity).

Engraftment potential and hematopoietic repopulation ability of $CD34^+$ cells expanded by the combination of hematopoietic growth factors and SR1

To evaluate the engraftment potential and repopulation ability of UCB-derived $CD34^+$ cells expanded in the presence of hematopoietic growth factors and SR1, sublethally irradiated NSG mice were transplanted with either 10^5 purified unmanipulated $CD34^+$ cells or the input-equivalent (i.e. the number of expanded cells that is derived from 10^5 $CD34^+$ cells) after expansion in SFT or SFT+SR1 medium. In time, the level of human chimerism in blood gradually increased (**figure 4A**). All mice engrafted, with human chimerism levels 72.9% for non-expanded cells and 33.3% and 28.2% for SFT- and SFT+SR1 expanded cells, respectively (**figure 4B**). Multilineage engraftment was established both in the peripheral blood and bone marrow of all transplanted mice (**figure 4C-E**). More

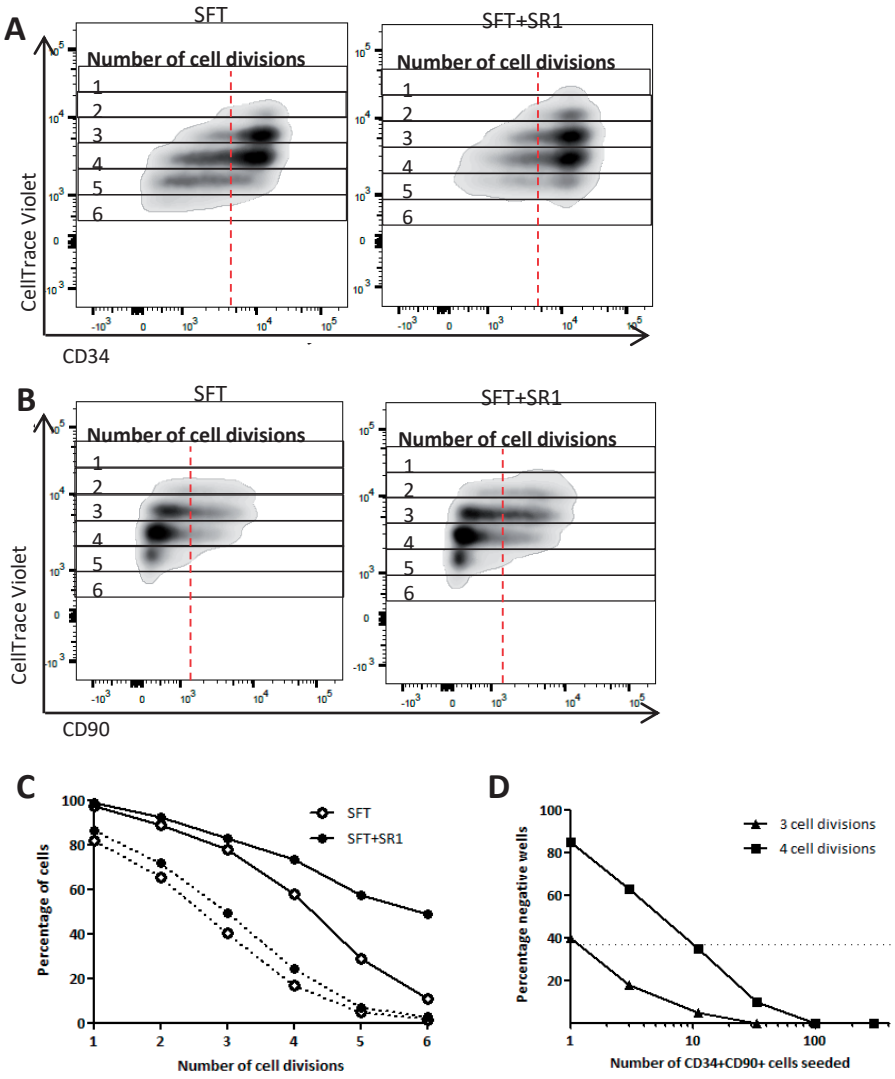

Figure 3. SR1 induces the retention of CD34- and CD90-expression following cell division. Sorted $CD34^+CD90^+$ cells were labeled with CellTrace™ Violet and subsequently cultured for 5 days in SFT or SFT+SR1 medium. On day 5, flow cytometric analysis and a LTC-IC assay were performed. Shown are expression of CellTrace™ Violet, indicating the number of cell divisions undergone versus (A) CD34 expression and (B) CD90 expression for cells cultured in SFT (left panels) or SFT+SR1 (right panels) medium. The red dotted lines represent the cutoff point of either $CD34^+$ or $CD90^+$ cells (representative experiment out of 2). (C) shows the percentage of cells expressing CD34 (solid lines) within total life cells and CD90 (dotted lines) within $CD34^+$ cells. (D) depicts the percentage of negative wells in a LTC-IC assay versus the number of sorted $CD34^+CD90^+$ cells seeded that had undergone 3 (triangle) or 4 (square) cell divisions.

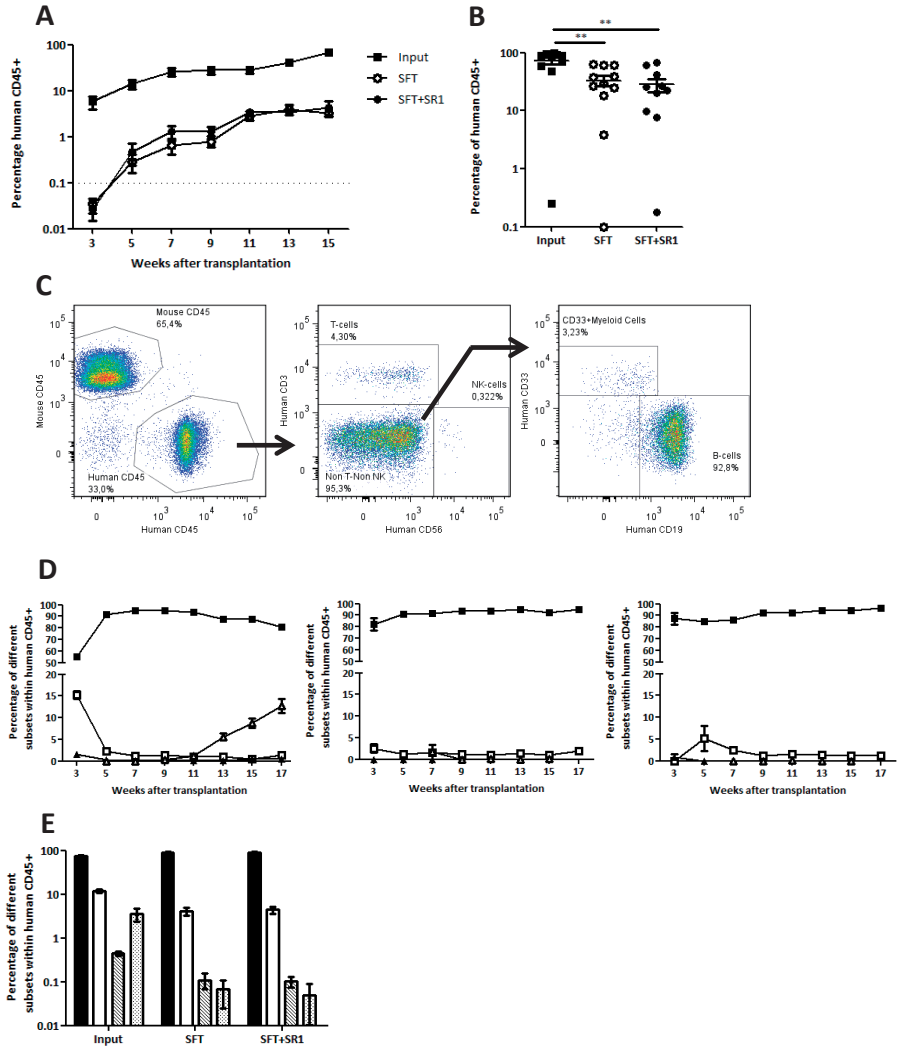

Figure 4. Engraftment potential and hematopoietic repopulation ability of CD34$^+$ cells expanded by the combination of hematopoietic growth factors and SR1 10^5 Selected, non-expanded CD34$^+$ cells and the input-equivalent of those cells upon 8 days of culture in SFT of SFT+SR1 medium were intravenously transplanted into NSG mice (input: n=25; SFT: n=18; SFT+SR1; n=15). Short-term repopulation was measured in the peripheral blood every 2 weeks, starting 3 weeks after transplantation. At week 15 or 17, mice were sacrificed and long-term human chimerism was measured in the bone marrow. Shown are (A) levels of human chimerism in the peripheral blood (Y-axis) over time (X-axis), (B) levels of human chimerism at 15 or 17 weeks post-transplantation in the bone marrow , (C) representative FACS blots showing the gating strategy determining the different subsets within the human CD45$^+$ population, (D) the representation of CD19$^+$ B cells (black squares), CD33$^+$ myeloid cells (white squares), CD56$^+$ NK cells (black triangles) and CD3$^+$ T cells (white triangles) within the human CD45$^+$ population in the peripheral blood over time upon transplantation of non-expanded (left panel), SFT-expanded (middle panel) or SFT+SR1-expanded cells (right panel) and (E) the distribution of CD19$^+$ B cells (black bar), CD33$^+$ myeloid cells (white bar), CD56$^+$ NK cells (striped bar) and CD3$^+$ T cells (dotted bar) in the bone marrow 15 or 17 weeks after transplantation . ** p<0.01, *** p<0.001

than 80% of human cells in blood were CD19$^+$ B-cells, but CD33$^+$ myeloid cells, CD3$^+$ T cells and CD56$^+$ NK-cells were also detected, albeit at much lower levels (**figure 4D**). Multilineage engraftment was also established in bone marrow of mice transplanted with either input or expanded CD34$^+$ cells (**figure 4E**). Thus, expanded CD34$^+$ cells retain their multilineage hematopoietic repopulation capacity in NSG mice. However, the levels of human chimerism observed upon transplantation of expanded CD34$^+$ cells (either in SFT or SFT+SR1 medium) were lower compared to the levels observed after transplantation of non-expanded cells.

The SR1-enhanced expansion of the phenotypically most immature subset, as assessed by flowcytometry, and of the functionally most immature subset, as assessed by the LTC-IC assay, in expansion cultures supplemented with hematopoietic growth factors, did not result in a corresponding increase in repopulation capacity in NSG mice. The repopulation ability of a stem cell graft depends on its subset composition. Human cell development in NSG mice is skewed towards the B-lymphoid lineage. Therefore, reduced numbers of B cell progenitors in the expanded graft may cause lower levels of human chimerism in NSG mice. Non-expanded CD34$^+$ HSPC contain a subpopulation (5 - 10%) of committed CD10$^+$ lymphoid precursors (**figure 5A,** left panel). Our expansion protocol however induced a shift in the subset composition of CD34$^+$ HSPC. After expansion, either in the presence or absence of SR1, the CD34$^+$CD10$^+$ committed lymphoid precursor subset was markedly reduced (**figure 5A,** right panel). To demonstrate that the high levels of human chimerism levels in blood after transplantation of unexpanded CD34$^+$ cells were indeed derived from lymphoid progenitors in the graft, CD34$^+$CD10$^-$CD90$^+$

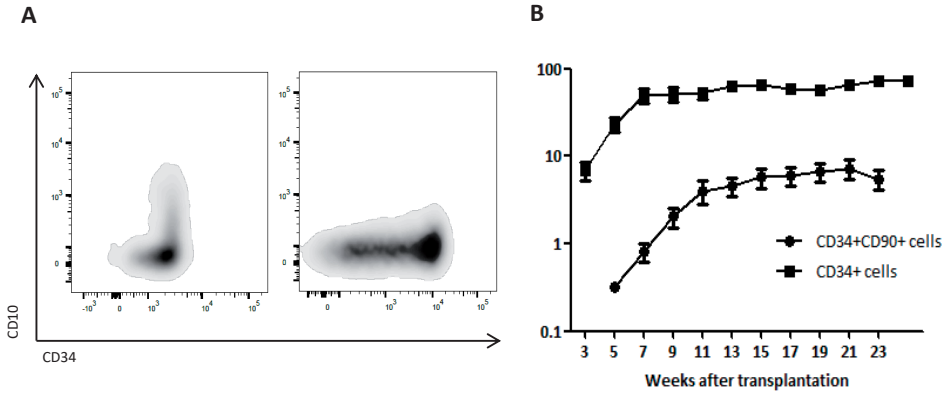

Figure 5. Lymphoid progenitors contribute to early hematopoietic recovery and are markedly reduced upon expansion cultures (A) shows the expression of CD34 (X-axis) and lymphoid progenitor marker CD10 (Y-axis) in uncultured cells (left panel) and in cells cultured for 1 week in SFT+SR1 medium (right panel) and (B) shows levels of human chimerism in the peripheral blood of NSG mice upon transplantation with10^5 non-expanded CD34$^+$ cells or 5000 non-expanded sorted CD34$^+$CD10$^-$CD90$^+$ cells.

cells were sorted and transplanted into NSG mice (5000 cells per mice, total of 15 mice). As expected, the removal of committed progenitors from the fresh $CD34^+$ HSPC resulted in a delayed recovery of human cells and lower levels of human chimerism in blood of transplanted mice (**figure 5B**). These results demonstrate that the observed high levels of chimerism after transplantation of non-expanded $CD34^+$ cells are mainly derived from lymphoid progenitors, which are markedly reduced after culture in SFT+SR1 medium.

DISCUSSION

SR1 has been shown to promote the expansion of human HSPC capable of hematopoietic reconstitution in mice (15) and men (23). However, some studies suggested that SR1 predominantly affects committed progenitor cells with short-term repopulation potential, rather than the true long-term repopulating HSC (27, 28). Therefore, this study evaluated whether the combination of SCF, Flt3L and TPO (SFT) with SR1 would be able to expand a very immature subset of human UCB-derived phenotypic HSPC, characterized as $Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$ cells, which are highly enriched for HSC (29). In addition, functional evaluation included LTC-IC assays as well as an *in vivo* mice model. It is shown that the combination of SFT and SR1 expands purified immature $Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$ cells in serum-free expansion cultures. Most studies claiming expansion used either the number of $CD34^+$ cells or the more immature population of $CD34^+CD38^{low}$ cells as an endpoint (11-13), but few studies addressed the effect on the phenotypically most immature subset ($Lin^-CD34^+CD38^{low}$ $CD45RA^{low}CD90^+$ cells). Boitano *et al*. observed a ten-fold expansion of human $CD34^+CD90^+$ cells in cultures supplemented with SCF,Flt3L, TPO, IL-6 and SR1 compared to cultures supplemented with SFT6 only. Fold expansion compared to input was not shown. These cultures were initiated with mobilized peripheral blood-derived $CD34^+$ cells from adult patients (15). However, Gu *et al*. (26) reported a reduced frequency of $CD34^+CD90^+$ cells upon 1 week culture of mobilized adult peripheral blood $CD34^+$ cells in a serum-free culture system with a combination of SR1 and the growth factors SCF, Flt3L, TPO and IL-6. Despite that reduced frequency, absolute number of $CD34^+CD90^+$ cells were increased compared to input cell numbers due to strong expansion of the total $CD34^+$ population. Fares *et al*. observed only a two-fold increase in CD34+CD38lowCD45RAlowCD90+ cells in cultures using a combination of SFT and SR1 compared to cultures initiated with growth factors only (27). In contrast to those studies, cell sorting of a more primitive subset ($Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$ cells) was performed prior to initiate cultures in the present study enabling the evaluation of proliferation and inhibition of differentiation of HSPC more closely. It is shown that a combination of SFT and SR1 enhanced both the frequency and absolute number of $Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$ cells in cultures initi-

ated with purified immature cells. Cell division analysis showed a retention of both CD34 and CD90 expression following cell division, suggesting a delay of the differentiation of actively dividing cells by SR1. Moreover, a robust expansion of LTC-IC was observed following evaluation of expanded cells in a 6-week LTC-IC system. Virtually all LTC-IC activity resided in the phenotypically most immature $Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$ cells. The subset of $Lin^-CD34^+CD38^{low}CD45RA^+CD90^+$ cells, which was generated during culture as a more differentiated subset, hardly contained any LTC-IC activity. These results suggest that expansion of phenotypically immature $CD34^+CD90^+$ cells cannot be taken as a surrogate marker for expansion of functional HSC with LTC-IC capacity. Apart from expanding the subset of $Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$ cells our data indicate that SR1 is able to inhibit the differentiation of actively dividing $Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$ cells with retention of most LTC-IC activity.

Although SR1-expanded cells retained long-term multilineage repopulation ability in NSG mice, lower levels of human chimerism were observed upon intravenous transplantation of SR1-expanded cells compared to transplantation of unexpanded cells. These results may seem in contrast to those by Boitano *et al*., who reported a 17-fold increase in SCID-repopulating cells (SRC) (15). In addition, several other groups have reported that SR1 induces at best maintenance of SRC rather than an increase (26, 27) and Fares *et al*. proposed that SR1 does not affect the true long-term HSC, but rather a less primitive subtype, the short-term HSC (28). How to explain these contradictory results? A possible clue may come from the differences of cytokines and time periods used in expansion cultures. The growth factors used in the present study included SCF, FLT3L and TPO, while IL-6 was left out considering its predominant effect on committed lymphoid progenitors and the absence of the IL-6 receptor on human HSC. Fares *et al*. evaluated the combination of SCF, Flt3L, TPO and SR1, which compares more closely to the present study (27). Their results indicated a two-fold expansion of $CD34^+CD38^{low}CD45RA^{low}CD90^+$ cells compared to the growth factor only condition. Gu *et al*. (26), however, also included IL-6 in their expansion cultures and reported a 10-fold decrease in SRC upon 7 day expansion cultures using SFT6+SR1 compared to overnight SFT6+SR1-culture. This clearly differs from the 17-fold increase in SRC that was described by Boitano *et al*. Of note, transplantation of non-expanded cells was not included in the latter study, excluding the comparison of human chimerism levels to unmanipulated cell transplantation, as presented here. Several publications have suggested that IL-6 has a positive effect on the maintenance of long-term HSC in *in vitro* expansion cultures (31, 32). However, these results may need to be interpreted cautiously, because the endpoints are related to either committed progenitor cells (CFC activity directly upon culture (32)) or related indirectly to HSC by evaluating secondary transplantations (31). SRC numbers assessed in primary transplantations did not differ between the conditions with and without IL-6 in the study by Duchez *et al* (31),

while the effect on the expansion of $Lin^{-}CD34^{+}CD38^{low}CD45RA^{low}CD90^{+}$ cells and LTC-IC was not addressed. Collectively, these results suggest that the contributory effect of IL-6 in expansion cultures is questionable. In addition, the use of IL-6 might explain the different *in vivo* results in the present study as compared to the study by Boitano. The effect of IL-6 on the *in vivo* repopulating capacity of expanded UCB-derived progenitor cells needs to be interpreted cautiously, because of severe limitations of the model to assess SRC numbers. Increased SRC numbers are generally considered the 'golden standard' to address whether the true long-term HSC has actually been expanded. However, early hematopoietic recovery after transplantation in NSG mice originates from committed progenitor cells. In the NSG mouse model human repopulation rapidly starts after transplantation and 80-90% of human cells are $CD19^{+}$ B cells, indicating that committed lymphoid progenitors predominantly account for human engraftment in NSG mice. The present study showed that *ex vivo* $CD34^{+}$ cell expansion without IL-6 resulted in an almost complete loss of lymphoid progenitors, which may explain the lower levels of human chimerism in NSG mice as compared to transplantation of uncultured cells and as compared to earlier studies that included IL-6 in their expansion protocol (15). In order to assess the contribution of lymphoid progenitors to early recovery, $CD34^{+}CD90^{+}CD10^{-}$ cells were sorted and thereby the $CD10^{+}$ lymphoid progenitor subset was removed to specifically study repopulation capacity of immature HSPC. A delayed recovery and lower levels of human chimerism were observed demonstrating early hematopoietic recovery and chimerism is predominantly accounted for by lymphoid progenitors in the graft. Collectively, these results question whether numbers of SRC as assessed in NSG mice upon transplantation reflect the number of HSC in the graft. As a result, increasing SRC number cannot be interpreted as evidence of expansion of HSC and the LTC-IC analysis may be preferred to functionally evaluate expanded cell fractions. However, secondary transplantation and long-term T cell reconstitution might alternatively be considered as primary endpoint in NSG mice and might reflect more closely the number and functional capacity of expanded human HSC. In addition, the use of a more myeloid skewed mouse model, such as the MISTRG mice (33) may be preferred over the current NSG mouse model, allowing to evaluate both myeloid and lymphoid recovery in addition to long-term recovery, which might yield a more complete picture of HSC content of the transplanted and expanded graft. Despite the limitations of the *in vivo* model, both the expansion of the immature subset of Lin-CD34+CD38lowCD45RAlowCD90+ cells and the strong increase of LTC-IC in the present study suggest effective expansion of HSC.

Recently, the safety and feasibility of SR1-expanded HSPC was evaluated clinically in a dUCBT setting. Wagner and colleagues observed rapid neutrophil and platelet recovery upon transplantation of SR1-expanded cells (15 days and 49 days, respectively) in 17 patients. Recovery originated from the expanded graft in 11 out of 17 patients. Of note,

no graft failures were observed, indicating long-term engraftment capacity of the SR1-expanded cells. These favorable results have set the stage to study SR1-expanded UCB in a single UCBT setting, which is currently underway by us and others. Ultimately, prospective randomized trials comparing transplantation of (SR1-)expanded and non-expanded cells are needed to assess whether transplantation with expanded UCB may result in more rapid and qualitative better recovery that may result in a reduction of non-relapse mortality. The encouraging results of the present study, showing robust expansion of the phenotypic most immature cells and of LTC-IC cells, combined with the work by Wagner *et al*. in a dUCBT setting, may lead the way to improved hematopoietic recovery following UCBT, that may match the recovery currently observed with haplo-identical allo-SCT.

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Chapter 6

Magnetic resonance detection of $CD34^+$ cells from umbilical cord blood using a ^{19}F label

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ABSTRACT

Impaired homing and delayed recovery upon hematopoietic stem cell transplantation (HSCT) with hematopoietic stem cells (HSC) derived from umbilical cord blood (UCB) is a major problem. Tracking transplanted cells *in vivo* will be helpful to detect impaired homing at an early stage and allows early interventions to improve engraftment and outcome after transplantation. In this study, we show sufficient intracellular labeling of UCB-derived $CD34^+$ cells, with ^{19}F-containing Poly(Lactic-co-Glycolic Acid) (PLGA) nanoparticles which were detectable with both flow cytometry and magnetic resonance spectroscopy (MRS). In addition, labeled $CD34^+$ cells maintain their capacity to proliferate and differentiate, which is pivotal for successful engraftment after transplantation *in vivo*. These results set the stage for *in vivo* tracking experiments, through which the homing efficiency of transplanted cells can be studied.

INTRODUCTION

Cell transplantation is an important therapeutic strategy for various malignant and non-malignant diseases. Migration of transplanted cells to their designated organs ('homing') is pivotal for treatment success. Information about transplanted cell localization can be of great value in the evaluation and development of stem cell-based therapies [1]. This information follows from magnetic resonance imaging (MRI) data, when cells of interest are labeled so that they can be discriminated from surrounding tissue. The stable nature of MRI cell labels facilitates longitudinal measurements, respecting the dynamic process of stem cell homing. Multiple studies have shown effective magnetic labeling and subsequent *in vivo* imaging in a variety of medical fields, including cardiovascular disease [2], neurodegenerative disease [3], neurological trauma [4], diabetes [5] and others. Using fluorine (^{19}F) as a label has the advantage that ^{19}F [6] has no detectable background *in vivo* [7]. Therefore, detection of ^{19}F in cell labels is highly specific.

Labeling cells with ^{19}F is mostly done using perfluorocarbons (PFCs), because PFCs are high in fluorine content [8]. Because of their insolubility in both lipophilic and hydrophilic solvents, PFCs need to be incorporated in emulsion droplets, nanoparticles or micelles before they can be used for cell labeling. Another reported ^{19}F labeling strategy is to fluorinate sugars or peptides [9-11] but then the ^{19}F content of the label is low compared to the ^{19}F content of PFCs.

Transplantation of hematopoietic stem and progenitor cells from umbilical cord blood (UCB) is an example of the need for information about homing. UCB is an important alternative stem cell source for patients lacking a sibling or matched unrelated stem cell donor, because of its rapid availability and less stringent matching criteria [12]. However, adult patients who receive a UCB transplantation have a delayed neutrophil and platelet recovery time and a higher incidence of graft failure as compared to patients who receive CD34$^+$ cells from adult donors [13, 14]. During the delayed recovery period, patients are at high risk for severe complications such as infections and bleeding, resulting in a high mortality rate. Several factors may contribute to the delayed hematopoietic recovery following UCB transplantation. Besides the relative higher immaturity of UCB-derived CD34$^+$ cells as compared to adult bone marrow-derived CD34$^+$ cells [15] , delayed recovery may also be due to the relatively low number of CD34$^+$ cells in UCB grafts [12, 16]. In addition, it is known that CD34$^+$ cells derived from UCB do not home as efficiently to the bone marrow as their adult-donor-derived counterparts, due to a lack of binding of UCB-HSC to the P- and E-selectin adhesion molecules expressed by the recipients bone marrow endothelial cells [17]. Tracking stem cell homing after transplantation, as a means to study engraftment kinetics, will be helpful to detect impaired homing at an early stage after transplantation, allowing interventions to improve engraftment and outcome after transplantation.

In this preclinical study, the aim is to label umbilical cord blood (UCB)-derived $CD34^+$ cells with fluorine (^{19}F)-containing nanoparticles while maintaining cell viability and functionality. This will set the stage for further *in vivo* studies in order to track the homing of $CD34^+$ cells upon hematopoietic stem cell transplantation.

MATERIAL AND METHODS

Synthesis and characterization of ^{19}F- Poly(Lactic-co-Glycolic Acid) (PLGA) nanoparticles

Nanoparticles were produced as described [18]. Subsequently, the nanoparticles were resuspended in PBS (Invitrogen, the Netherlands) and stored at 4°C until use. The final concentration of ^{19}F-PLGA nanoparticles was 76 mg/ml, as measured from a lyophilized sample.

Analysis of the ^{19}F -PLGA nanoparticles using dynamic light scattering (Zetasizer Nano Series, Malvern Instruments, Worcestershire, UK) showed a mean particle diameter ± SD (n = 4) of 290 nm ± 56 nm. The mean polydispersity index ± SD was 0.17 ± 0.04, indicating particles were fairly homogenous in diameter.

Umbilical cord blood processing and cell selection

Umbilical cord blood was collected in several hospitals using Stemcare®/CB collect blood bag system (Fresenius Kabi Norge AS) containing citrate phosphate dextrose (CPD) as an anticoagulant. The Medical Ethical Committee of the Erasmus University Medical Centre approved collection of the cord blood (MEC-2009-410) and written informed consent from the mother was obtained prior to donation. Within 48 hours after collection, mononuclear cells were isolated using ficoll (Lymphoprep™, Fresenius Kabi Norge AS). $CD34^+$ cells were isolated with positive immunomagnetic selection using Magnetic Activated Cell Sorting (MACS) technology according instructions of the manufacturer (Miltenyi Biotech GmBH, Bergisch Gladbach, Germany).

Labeling $CD34^+$ cells with ^{19}F -PLGA nanoparticles

Cells were resuspended at 200,000 cells/ml in serum-free Glycostem Basal Growth Medium (GBGM®, Glycostem Therapeutics, 's Hertogenbosch, The Netherlands) supplemented with thrombopoietin (TPO), stem cell factor (SCF) and Flt3 ligand (Flt3L) (Cellgenix, Freiburg, Germany) at 50 ng/ml each. Labeling of cells was performed at concentrations ranging from 5 to 40 µl/ml of ^{19}F -PLGA nanoparticles. Standard concentration was 20 µl/ml. After addition of the label, cells were incubated in the dark at 37°C and 5.0% CO_2 for 4 or 20 hours. Control cells were mock-labeled, i.e. treated identically until the end of the incubation time, but without the addition of nanoparticles. After incubation, cells were processed as required for further analysis.

Flow cytometry

Labeling efficiency and median labeling intensity were determined using flow cytometry. Cells were stained with anti-CD45-APC-H7, anti-CD34-PE-Cy7 (both from BD Biosciences, San Jose, CA, USA) and diamidinophenylindole (DAPI) (Sigma-Aldrich, St Louis, MO, USA). Only viable ($DAPI^-$) $CD45^+CD34^+$ cells were included in the analysis. The maximal fluorescence intensity of mock-labeled control cells in the FITC-channel was set as threshold for considering a cell labeled. Flow cytometric analysis was performed using a BD FACSCanto™ (BD Biosciences, San Jose, CA, USA) and data was analyzed using FlowJo software (Tree Star Inc, Ashland, OR, USA).

To assess ^{19}F-PLGA labeling intensity in cells that divided 0-3 times, cells were labeled with 5μM CellTrace Violet (Molecular Probes, Eugene, Oregon, USA) upon ^{19}F-PLGA labeling and subsequently cultured for 4 days in our culture medium as described above.

Confocal microscopy

Cells were labeled at a ^{19}F -PLGA nanoparticle concentration of 20 μl/ml with an incubation time of 4 hours. Labeled-cells were separated from free ^{19}F -PLGA particles using ficoll separation. Washed labeled or mock-labeled control cells were transferred to microscopy slides by centrifugation. Subsequently, slides were air dried and mounted in Prolong® Gold Antifade Reagent with DAPI (Molecular Probes, Eugene, Oregon, USA). Cells were imaged using a Leica SP5 CLSM equipped with Ar-He/Ne lasers (Leica Microsystems, Wetzlar, Germany) and a Zeiss 63x Plan-Apochromat oil immersion objective (Carl Zeiss, Oberkochen, Germany). A 405 nm laser was used for DAPI excitation (with a 413-476 nm acousto-optical beam splitter (AOBS)), and a 488 nm laser was used for FITC excitation (with a 503-596 nm AOBS).

Colony forming unit (CFU) assays

Washed labeled or mock-labeled cells were resuspended in methylcellulose containing medium (Methocult GF H84434, Stemcell Techonologies, Vancouver, BC, Canada) and seeded in triplicate at 500 cells per 35 mm dish. Dishes were incubated for 14 days in a humidified tray at 37°C and 5.0% CO2, after which two trained non-blinded observers enumerated the colonies. We accepted an interobserver variation of 10%. Three types of colonies were distinguished: burst forming unit-erythroid (BFU-E), colony forming unit-granulocyte/monocyte (CFU-GM) and colony forming unit-granulocyte/erythrocyte/monocyte/megakaryocyte (CFU-GEMM).

Magnetic resonance spectroscopy

Cells were labeled at a concentration of 20 μl/ml for 20 hours and subsequently fixed in 4% formaldehyde solution for at least 15 minutes at room temperature, washed in PBS, and resuspended in agar solution (0.3%). An MR 901 Discovery 7T magnet (Agilent

Technologies, Santa Clara, CA, USA) with a preclinical front-end (GE Healthcare, Little Chalfont, UK) was used for MRS acquisition. The system is equipped with a gradient set with a maximum gradient strength of 300 mT m-1, a rise-time of 600 T m-1 s-1 and an inner diameter of 310 mm. For transmission and reception, an in-house-built dual tuned $^{1}H/^{19}F$ single channel surface coil with a diameter of 2 cm was used. The ^{19}F MRS spectrum was recorded using a EchoSCI sequence (TR/TE = 1250/15 ms, NEX = 128, FOV = 6 cm, slice thickness = 2,5 cm).

MRS processing was performed in SAGE 7.6.2 (GE Healthcare, Little Chalfont, UK) on the MR 901 Discovery system. For processing of the data, time domain signals were apodized with a 17.6 Hz line broadening function, after which the signal was zerofilled to 4096 points. Subsequently the time domain signal was Fourier transformed and the resulting spectrum was properly phased to show an absorption mode resonance line. For signal intensity the maximum intensity of the resonance line was determined, and noise was estimated from the standard deviation of the signal intensity of the baseline. ^{19}F in the sample was quantified by reference to a standard curve, which was obtained by measuring a dilution series of PFCE with known ^{19}F content.

Statistics

Unpaired two-tailed t-tests were performed to test the difference in median labeling intensity between different incubation times and to test the difference in number of colonies between labeled and mock-labeled $CD34^+$ cells for both incubation times in CFU experiments. Differences were considered to be statistically significant if $p < 0.05$. Statistical tests were performed in Excel (Microsoft Corporation, Redmond, WA, USA).

RESULTS

$CD34^+$ cells are efficiently labeled with ^{19}F -PLGA nanoparticles

Cells were labeled with 5, 10, 20 and 40 µl/ml ^{19}F -PLGA nanoparticles for 4 or 20 hours respectively, in order to address whether $CD34^+$ cells could be labeled using ^{19}F -PLGA nanoparticles and which labeling time and concentration would be most optimal. In all conditions tested, incubation of $CD34^+$ cells with ^{19}F -PLGA nanoparticles resulted in labeling of nearly all cells. The lowest percentage of labeled cells (94.5%) was observed following incubation with a ^{19}F -PLGA nanoparticle concentration of 5 µl/ml for 4 hours; all other conditions resulted in > 99% labeled cells (**Table 1**). However, the median fluorescence intensity of labeled cells varied across labeling conditions (**Table 1, figure 1A**). Higher ^{19}F -PLGA nanoparticle concentrations were associated with higher median fluorescence intensity of viable $CD45^+CD34^+$ cells. At each labeling concentration, a longer incubation time was associated with higher median fluorescence intensity of

Table 1. Labeling efficiency of CD34+ cells in relation to 19F -PLGA NP concentration and incubation time

Labeling concentration (µl/ml)	Percentage of cells labeled (%)		Median fluorescence intensity	
	4 hours incubation	*20 hours incubation*	*4 hours incubation*	*20 hours incubation*
5	94.5	99.2	461	733
10	99.1	99.9	781	1169
20	99.7	99.9	1618	1879
40	99.7	99.9	2628	2709

PLGA: poly(lactic-co-glycolic acid); NP: nanoparticle

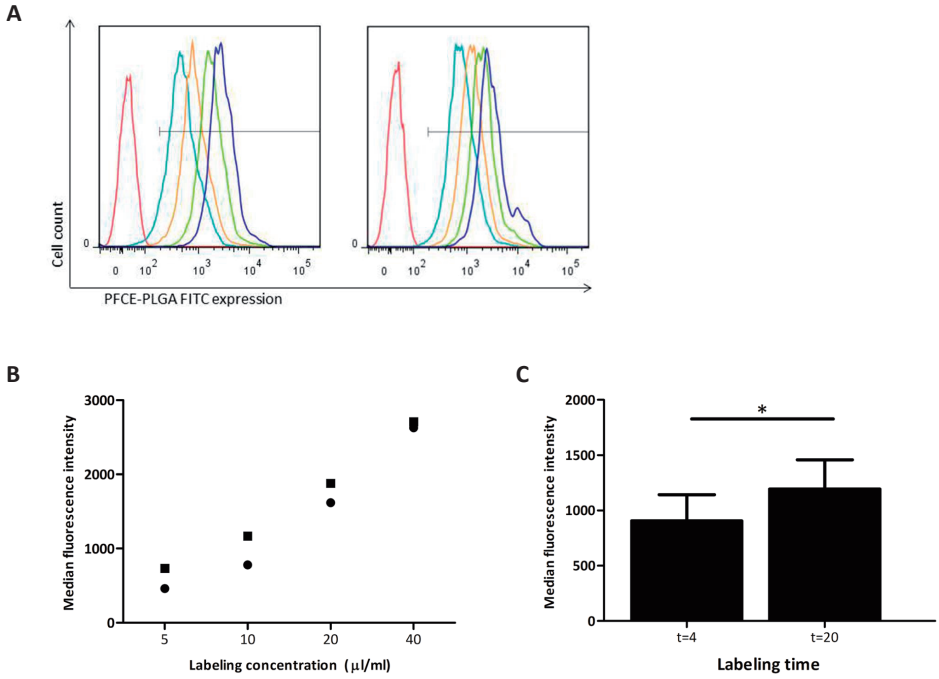

Figure 1. CD34+ cells can be labeled efficiently with 19F -PLGA nanoparticles with the intensity increasing with longer incubation time and higher labeling concentration. (A) Fluorescence histograms of cells labeled with 0 (red), 5 (turquoise), 10 (orange), 20 (green) and 40 (blue) µl/ml nanoparticles at incubation times of 4 (left panel) or 20 (right panel) hours. Horizontal axes show the intensity of the FITC signal, representing the 19F -PLGA nanoparticles. (B) Median fluorescence intensity per labeling concentration after 4 (circle) and 20 (square) hours of labeling. Figures A and B show a representative experiment out of 2 experiments. (C) Median fluorescence intensity of cells labeled with 20 µl/ml 19F -PLGA nanoparticles for 4 and 20 hours (n=5). * = $p<0.05$.

CD34+ cells (**figure 1B**). However, the increase in median fluorescence caused by longer incubation time consistently decreased with increasing labeling concentration. An incubation time of 20 hours led to a statistically significantly higher median labeling intensity than 4 hours of labeling at 20 µl/ml (**figure 1C**, $p<0.05$). In summary, we can discriminate

labeled CD34$^+$ cells from mock-labeled control cells using FACS in each of the conditions tested, with the labeling intensity increasing with longer incubation time and higher labeling concentration. We chose a labeling time of 20 hours with a concentration of 20 µl/ml for further experiments.

Detection of ^{19}F-PLGA labeled CD34$^+$ cells by magnetic resonance spectroscopy

We recorded a ^{19}F MRS spectrum of 2 agar gel phantoms containing 10^5 and 10^4 labeled CD34$^+$ cells in 150 µl respectively. The respective signal to noise ratio (SNR) was 58,6 for the sample containing 10^5 labeled CD34$^+$ cells and 5,3 for the sample containing 10^4 labeled CD34$^+$ cells (**figure 2**). Data acquisition took 65 minutes to recover sufficient SNR in the sample containing 10^4 cells. 5,06x10^{19} ^{19}F spins were measured in the sample with 10^5 labeled CD34$^+$ cells and 6,65x10^{18} ^{19}F spins in the sample with 10^4 labeled CD34$^+$ cells.

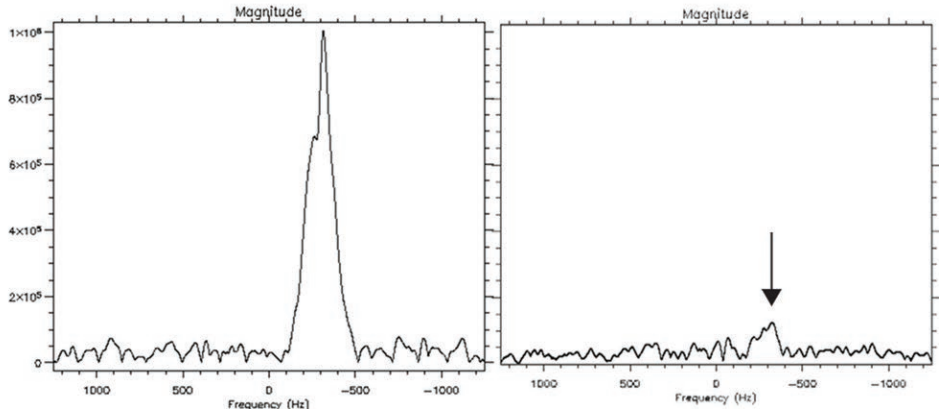

Figure 2. Detection of labeled CD34$^+$ cells by magnetic resonance spectroscopy and imaging. Left and right panel show the ^{19}F MRS spectrum of 2 agar gel phantoms containing 10^5 and 10^4 labeled CD34$^+$ cells in 150 µl respectively. Shown is the ^{19}F resonance line, the horizontal axes shows the frequency offset from the transmitter. Here the transmitter frequency has been set to the resonance frequency of the ^{19}F in PFCE. Labeled cells were labeled with 20 µl/ml ^{19}F -PLGA nanoparticles with an incubation time of 20 hours.

Uptake of the label is an active process and results in intracellular accumulation of the label

To discriminate between active uptake of the label and binding of the label to the membrane, we performed labeling with 20 µl/ml ^{19}F -PLGA nanoparticles at both 4°C and 37 °C. We observed a decrease in frequency of labeled cells from 99.9% at (37°C) to 28.2% (at 4°C). In addition, the median fluorescence intensity decreased from 2061 after labeling at 37°C to 82.2 after labeling at 4°C (**Table 2** and **figure 3A**). This indicates that CD34$^+$ cells actively incorporate the ^{19}F -PLGA nanoparticles. To further investigate the intracellular accumulation of the label, we performed confocal microscopy of ^{19}F -PLGA-labeled CD34$^+$ cells. **Figure 3B** clearly shows FITC-positive ^{19}F -PLGA nanoparticles within the

Figure 3. Uptake of the label is an active process and results in intracellular accumulation of the label. (A) Fluorescence histogram for mock-labeled control cells (red) and cells labeled 20 hours at 37 °C (turquoise) or 4 °C (orange). Horizontal axes show the intensity of the FITC signal, representing the ^{19}F -PLGA nanoparticles. (B) Differential Interference Contrast image (left) and fluorescent image (right) of CD34$^+$ cells labeled with ^{19}F -PLGA nanoparticles, showing the blue DAPI-signal (nucleus of the cell) and the green FITC-signal (^{19}F -PLGA nanoparticles).

Table 2. Effect of incubation temperature on uptake of ^{19}F -PLGA NPs by CD34$^+$ cells.

Incubation temperature	Percentage of cells labeled (%)	Median fluorescence intensity
37 °C	99.9%	2061
4 °C	28.2%	82.2

PLGA: poly(lactic-co-glycolic acid); NPs: nanoparticles

cytoplasm of the labeled CD34$^+$ cells. Mock-labeled control cells showed no FITC-signal (image not shown). Combined, these data show active uptake of the label by the CD34$^+$ cells, leading to intracellular accumulation of the label.

^{19}F-PLGA labeling does not affect the relative proportion of committed hematopoietic progenitors and cell viability

To test the ability of the labeled cells to proliferate and differentiate, colony forming unit (CFU) assays were performed. ^{19}F -PLGA labeled and mock-labeled CD34$^+$ cells were plated on the basis of baseline cell counts (counted prior to labeling) and after 14 days of culture, three different colony types were scored by 2 trained observers. Relevant to the interpretation of CFU results are the total number of colonies and the relative distribution of colony types, indicating the relative proportion of distinct committed hematopoietic progenitors, compared between labeled and mock-labeled conditions. With an incubation time of 4 hours, the average total number of colonies and the relative distribution of the colony subtypes are similar among the labeled and control conditions (**figure 4A**, p=0.96 for the total number of colonies and p=0.31, p=0.53 and p=0.83 for the frequency of BFU-E, CFU-GM and CFU-GEMM respectively). With an incubation time of 20 hours, the total number of colonies in the labeled condition is lower than in the mock-labeled control (90.6 versus 79.7 colonies per 500 CD34$^+$ cells in control and

labeled cells respectively, p=0.04), although no significant decrease is observed in any of the colony types. Irrespective of this small decrease in total colony number after 20 hours of labeling, these results show that both after 4 and 20 hours of labeling, cells are still capable of proliferation and differentiation and the relative proportions of committed hematopoietic progenitors are similar.

To study whether ^{19}F-PLGA labeling affects cell viability, flow cytometric analysis was performed using diamidinophenylindole (DAPI). The percentage life CD34^{+} cells prior to labeling and after labeling with ^{19}F -PLGA for 4 and 20 hours were similar (**figure 4B**), indicating no toxic effect of the internalization of the ^{19}F -PLGA nanoparticles.

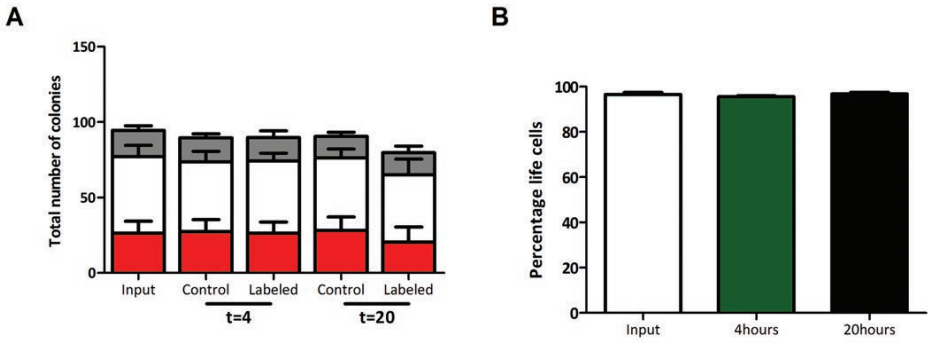

Figure 4. Labeling with ^{19}F-PLGA does not affect the relative proportion of committed hematopoietic progenitors and cell viability. (A) Total number of colonies per 500 CD34^{+} cells for BFU-E (red), CFU-GM (white) and CFU-GEMM (gray). (B) Percentage of life cells at input (white), and after 4 (green) and 20 (black) hours of labeling with 20 µl/ml ^{19}F-PLGA (n=5).

^{19}F-PLGA labeled cells remain detectable over time and upon cell division, although label intensity decreases

To assess the stability of the label in non-dividing cells and loss of label intensity upon cell division, we labeled ^{19}F -PLGA-labeled CD34^{+} cells with the cell division tracker CellTrace and evaluated the fluorescence intensity upon 4 days of culture. At day 0, all cells showed a high intensity of both the CellTrace label and the ^{19}F -PLGA label (**figure 5A**). After 4 days of culture, cells had divided 0, 1, 2 or 3 times respectively (**figure 5B**). We observed a decrease in fluorescence intensity in cells that had not divided, compared to the day 0 population (**figure 5C,** left panel), indicating some leakage of the label in time. However, labeled cells were still easily detectable using flow cytometry. In addition, upon every cell division we observed a halving of the fluorescence intensity, indicating an equal distribution of the ^{19}F -PLGA nanoparticles over both daughter cells upon cell division. Despite the decrease in label intensity, labeled cells were still detectable by flow cytometry upon 3 cell divisions (**Figure 5C,** right panel).

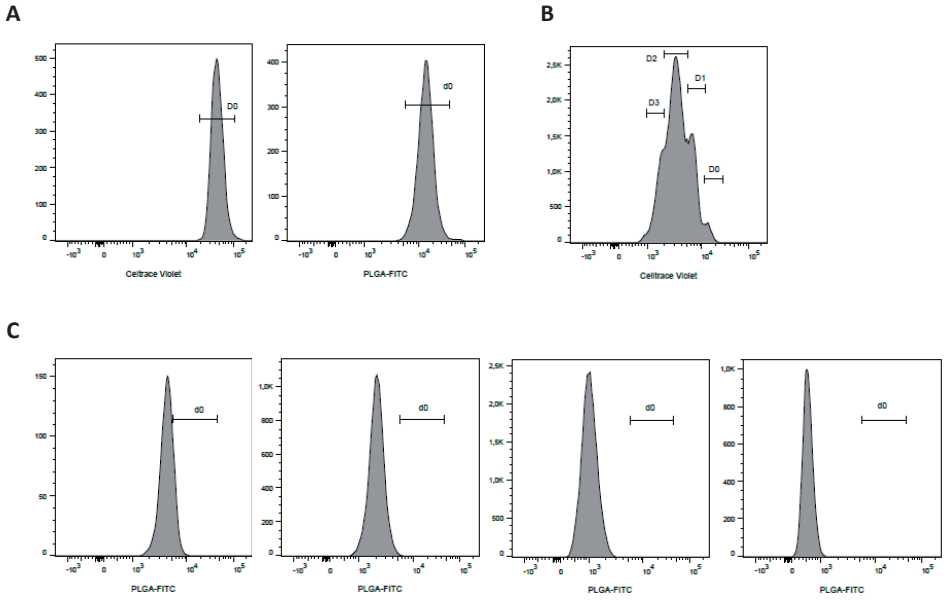

Figure 5. ^{19}F-PLGA labeled cells remain detectable over time and upon cell division, although label intensity decreases. Fluorescence histograms for CellTrace and ^{19}F -PLGA-labeled CD34$^+$ cells (A) Population shown is labeled cells at day 0. Horizontal axes show the intensity of the CellTrace Violet signal (left panel) and of the FITC signal, representing the ^{19}F -PLGA nanoparticles (right panel). (B) Population shown is labeled cells after 4 days of culture. The horizontal axes show the intensity of the CellTrace Violet signal, indicating the number of cell divisions. (C) Populations shown are labeled cells after 4 days of culture who had undergone 0, 1, 2 or 3 cell division respectively (depicted in the panels from left to right). Horizontal axes show the intensity of the FITC signal, representing the ^{19}F -PLGA nanoparticles.

DISCUSSION

This study shows the feasibility of labeling CD34$^+$ cells with ^{19}F-containing PLGA nanoparticles. In addition, labeled CD34$^+$ cells maintain viable and retain their capacity to proliferate and differentiate, which is pivotal for successful engraftment after transplantation *in vivo*. In the future, this technique can be used to monitor homing evaluating efficacy of hematopoietic stem cells transplantation and the information obtained may have implications to further improve treatment outcome.

Several conditions must be met in order to consider cell labeling and subsequent MR detection as feasible. Firstly, MR detection of the labeled cells should be feasible *in vivo*. MR-based imaging has advantages regarding its high spatial resolution, the absence of ionizing radiation and the ability to provide anatomical information [1]. However, as compared to nuclear imaging of isotopes, MRI may be a less sensitive technique, with a higher detection limit in a reasonable measurement time. Higher MR detection sensitivity may provide more detailed information about the distribution of the labeled cells

after transplantation. Since MRS has a higher sensitivity than MRI, we decided to first perform MRS experiments in order to create a starting point from which our MRS results can serve for increasing signal and cell detection sensitivity. This may be achieved by optimizing the ^{19}F content of the nanoparticles, increasing the cellular uptake of the label and refining MR hardware and acquisition [19]. A second prerequisite involves preservation of viability and functionality of the cells during the labeling process. We observed comparable percentage of life cells and total colony numbers after an incubation period of 4 and 20 hours compared to control samples. In addition, all different types of colonies were formed by the labeled cells, in similar frequencies as by non-labeled cells. Thirdly, stable cell-label association is pivotal for successful MR detection of labeled cells. The stability of the cell-label association is partly determined by the localization of the label. Both surface labels and intracellular labels may be applied, but intracellular labels may be preferred due to a lower risk of detachment. Our data indicates that cellular label uptake is an active, energy-dependent process, resulting in a stable intracellular localization of the label. This finding is consistent with the results of previous studies, which identified endocytosis as the cellular mechanism responsible for uptake of PLGA NPs in other cell types [20-22]. Future research should address the question whether such intracellular labels are useful to investigate homing of transplanted $CD34^+$ cells in the first 24 hours after transplantation. We observed decrease in label intensity over time and dilution of the signal upon cell division. This dilution of the signal may have consequences in imaging sensitivity, provided the ^{19}F concentration per imaging voxel is reduced by migration of cells out of the area of interest. However, when cells do not migrate upon division dilution through cell division would not affect detection sensitivity since for ^{19}F MRI/MRS the total amount of ^{19}F in a voxel determines detection sensitivity and not how it is spread within a voxel. This was already shown in previous studies using gadolinium-DTPA containing liposomes to label mesenchymal stem cells. Guenoun *et al*. showed a stable amount of label in all cells over time, even though the amount per cell decreased as a result of mitosis [23]. In our studies, the voxel dimensions were dictated by the area of interest and a similar approach could be followed for clinical applications. Lastly, in order to develop this technique further also for clinical applications, it is crucial that the label is biocompatible. All components of our nanoparticles are biocompatible and are already used in other applications in humans. PLGA polymer is approved by the FDA and European Medicine Agency for use in humans as a drug delivery system [24, 25]. Because of their susceptibility to hydrolysis and subsequent clearance by the Krebs cycle [26], PLGA polymers have very minimal systemic toxicity[27]. PFC emulsion droplets are cleared by macrophages of the reticuloendothelial system and eliminated from the body by exhalation [28]. Possible adverse effects are caused by stimulation of the macrophages and are dose-dependent. Therefore, they may not apply to cell tracking studies using low doses of PFC[8]. Finally, PVA is used as an emulsifier in the production of PFCE-PLGA

nanoparticles. Some PVA remains despite extensive washing of the nanoparticles [29]. PVA is biocompatible and applied in humans through oral administration [30] or implantation [31].

Helfer *et al*. [32]labeled CD34$^+$ cells from adult bone marrow using a ^{19}F label in emulsion droplets. Similar to our results, they found an increase in labeling intensity and frequency of labeled cells with increasing labeling concentration. However, we found no evidence of a detrimental effect of labeling on viability or functionality after 4 hours of labeling, whereas Helfer *et al*. found a slight decrease in viability. Both studies measured comparable ^{19}F payloads. In addition, Partlow *et al*. showed internalization of ^{19}F label in emulsion droplets in UCB mononuclear cells that were grown towards endothelial cells[6], which are different from the CD34$^+$ cells we used in our study. The cells remained functional *in vivo* as well. We preferred to incorporate ^{19}F in PLGA nanoparticles, because these are more stable than emulsion droplets, easier to store and the association with fluorescent dyes is more stable.

In a recent study, Ahrens *et al*. efficiently labeled human dendritic cells with a clinical grade PFC agent without changes in viability or phenotype. In this phase 1 study, patients suffering from stage IV colorectal cancer subsequently received intradermal administration of 1x10^6 or 1x10^7 labeled dendritic cells and underwent a MRI scan at 2 and 24 hours after administration. In the patients that received 1x10^6 dendritic cells, no ^{19}F signal was observed. However, 1x10^7 administered dendritic cells could be detected by MRI at both the 2 and 24 hour time point, although the number of dendritic cells decreased to approximately half of the original values at 24 hours, due to either cell efflux, cell migration or cell death [33]. Although dendritic cells are different from CD34$^+$ cells as used in our experiments and the required number of injected cells is high, these results are very promising first steps in *in vivo* tracking of labeled human cells.

In conclusion, CD34$^+$ cells can be labeled efficiently with PFCE-PLGA NPs without affecting cell functionality or viability. Labeled cells can be detected using MRS on a 7T MRI scanner. These results set the stage for *in vivo* tracking experiments, through which homing efficiency of transplanted cells can be studied.

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Chapter 7

Towards clinical application of StemRegenin1-expanded umbilical cord blood-derived hematopoietic stem and progenitor cells: manufacture and clinical study design

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The preclinical development studies described in chapter 5 of this thesis served as the basis for the development of an expansion protocol for clinical application. In this chapter, the translation of our experimental expansion protocol into a 'Good Manufacturing Practice' (GMP)-compliant expansion protocol for StemRegenin1 (SR1)-expanded umbilical cord blood (UCB)-derived hematopoietic stem and progenitor cells (HSPC) products is described. In addition, the design and objectives of the clinical feasibility study to evaluate umbilical cord blood transplantation (UCBT) using a single SR1-expanded UCB unit is described.

MANUFACTURE OF GMP-GRADE SR1-EXPANDED UCB-DERIVED HSPC PRODUCTS

Development of manufacturing process

In the experimental expansion protocol, research-grade SFEM medium was used in combination with research-grade cytokines and SR1. To adapt the experimental protocol to GMP requirements, two commercially available media were compared with the research-grade SFEM medium: i.e. StemSpan ACF medium, which is used in FDA-approved cell therapy trials in the USA, and CellGro SCGM medium, which is manufactured in compliance with GMP guidelines. The three media were found to be more or less equally potent in total nucleated cell expansion and $CD34^+$ cell expansion (**figure 1**). Also the yields of $Lin^-CD34^+CD38^{low}CD45RA^-CD90^+$ cells, which are highly enriched for primitive HSC, were similar. Based on these results and the fact that the CellGro SCGM medium is manufactured according to GMP guidelines, it was decided to use the Cellgro SCGM medium in the GMP-compliant expansion protocol. Overall a stable and reproducible expansion of $CD34^+$ cells of at least 30-fold in Cellgro SCGM medium within 12 days was observed.

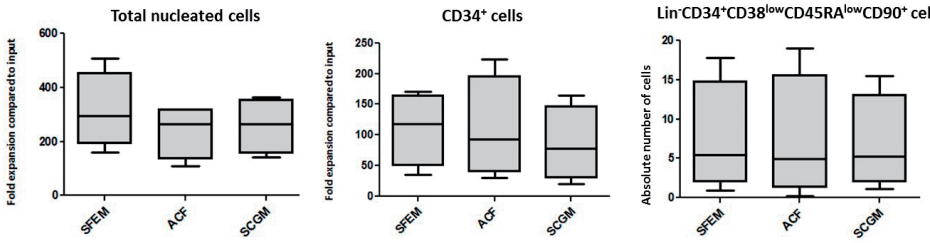

Figure 1. Expansion of total nucleated cells (left panel), $CD34^+$ cells (middle panel) and $Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$ cells (right panel) after 12 days using StemSpan SFEM medium, StemSpan ACF medium and CellGro SCGM medium supplemented with SCF, Flt3L, TPO and 1μM of SR1 (n=3).

Next, the research-grade cytokines Stem Cell Factor (SCF), FMS-like tyrosine kinase 3 ligand (Flt3L) and Thrombopoietin (TPO) were replaced by GMP-grade cytokines and a

similar fold expansion of total cells and CD34$^+$ cells was found (data not shown). Finally, the research-grade SR1 which was used in the experimental protocol was changed into a custom-made GMP-grade SR1, produced by ChemConnection BV which holds a GMP license. The fold expansion of total nucleated cells and CD34$^+$ cells and the yield of Lin$^-$CD34$^+$CD38^{low}CD45RA$^-$CD90$^+$ cells after 12 days of culture with research-grade and GMP-grade SR1 were found to be similar (**figure 2**).

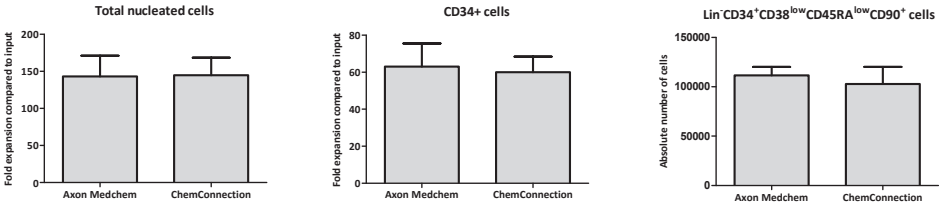

Figure 2. Expansion of total nucleated cells (left panel), CD34$^+$ cells (middle panel) and Lin$^-$CD34$^+$CD38^{low}CD45RA^{low}CD90$^+$ cells (right panel) after 11 or 12 days in expansion cultures supplemented with SCF, Flt3L, TPO and 1µM of SR1 from Axon Medchem or ChemConnection (n=3)

After showing that replacement of research-grade materials by GMP-grade materials resulted in similar folds expansion of UCB-derived CD34$^+$ cells, the production process was up-scaled and the open culture system in wells and flasks was transferred into a closed culture system in Vuelife® 290AC gas-permeable culture bags. The median CD34$^+$ cell expansion after 12 days of culture in bags was 85.6 ± 28.8 fold. The fold expansion in bags was similar to the fold expansion in wells/flasks (**figure 3**). These results demonstrate that UCB-derived CD34$^+$ cells can be efficiently expanded during 2 weeks of culture in Vuelife® culture bags.

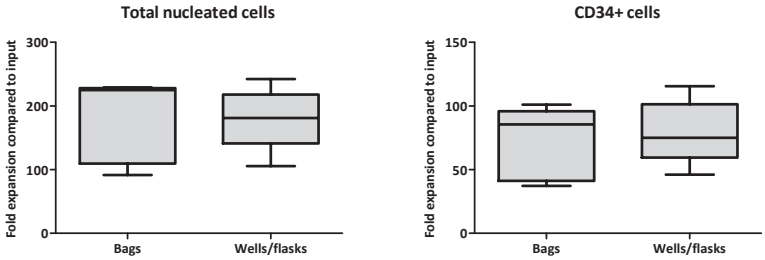

Figure 3. Expansion of total nucleated cells (left panel) and CD34$^+$ cells (right panel) in 12 days using Vuelife® 290AC bags and wells and flasks (n=5). Cultures were supplemented with SCF, Flt3L, TPO and 1µM of SR1.

Description of the manufacturing process

A flowchart of all successive steps in the manufacturing process is shown in **figure 4**. The manufacturing process of SR1-expanded UCB-derived HSPC starts with a minimal manipulation of the selected cryopreserved UCB product. The acceptance criteria of

UCB units for *ex vivo* expansion and UCB units for direct infusion into patients are identical. The cryopreserved UCB product is thawed and washed, after which the $CD34^+$ cell population is selected using CE-marked CliniMACS technology. The $CD34^+$ cell fraction is released by the Responsible Person of the Tissue Establishment for further process-

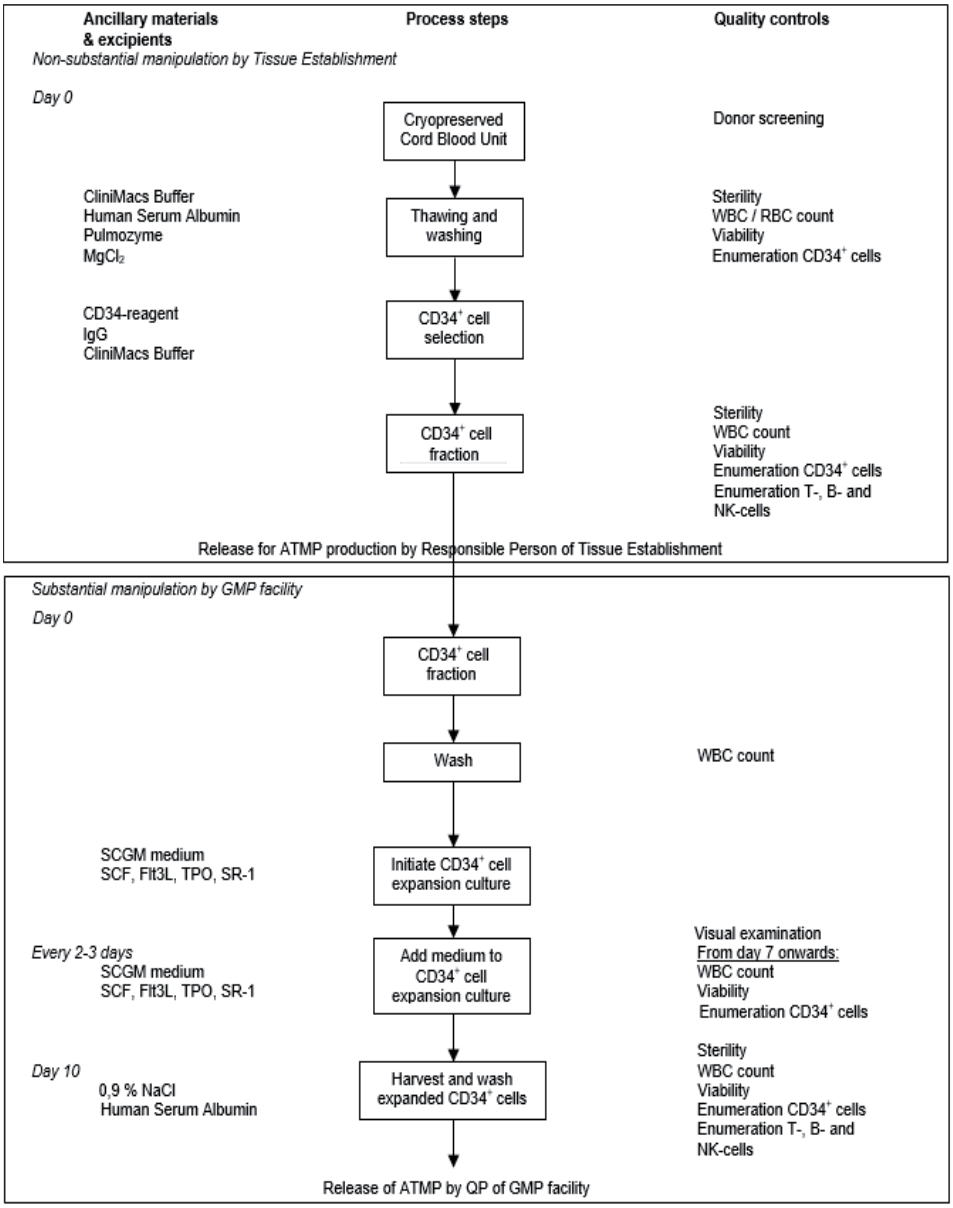

Figure 4. Flowchart of the production process of the SR1-expanded UCB-derived HSPC product including ancillary materials and excipients and in process controls.

ing into an Advanced Therapy Medicinal Product (ATMP) on basis of predefined criteria, including viability, purity and number of $CD34^+$ cells.

The manufacturing process continues with a substantial manipulation (*ex vivo* expansion) which falls under the GMP license for the production of an ATMP. All ancillary materials, e.g. media and cytokines, used in the production process are either manufactured in compliance with GMP guidelines or registered medicinal products. The selected $CD34^+$ cells are cultured in serum-free CellGro SCGM expansion medium with hematopoietic cytokines (SCF, FLT3-L and TPO) and the aryl hydrocarbon receptor (AhR) antagonist SR1 in a closed system using gas-permeable Vuelife™ culture bags for 10 days. Cultures are refreshed with expansion medium every 2-3 days to achieve the desired HSPC expansion. At several critical points during the production process, in process quality controls are performed (see **figure 4**). Quality controls include: bacterial and fungal contamination tests, White Blood Cell (WBC) count, overall viability and enumeration of viable $CD34^+$ cells. The analytical procedures for the quality controls have been validated and are performed in accordance with the European Pharmacopeia. The criteria that have to be met are defined for each quality control at each time point.

At the end of the culture at day 10, cells are harvested, extensively washed to decrease ancillary material contamination in the final cell product below threshold levels. The final cell product is resuspended in 0,9% NaCl with 5% Human Serum Albumin. In addition to the standard in process quality controls, the final cell product is also analyzed for frequencies of phenotypic HSC, and mature T-, B- and NK-cells. The final UCB-derived *ex vivo* expanded HSPC product, when meeting the release criteria (see **Table 1**), is released by the Qualified Person (QP) of the GMP facility.

Table 1. Validation of expansion protocol

Exp. nr.	Cell number at start of culture (x 10^6)		Cell number at end of culture (x 10^6)		Fold expansion	
	WBC	$CD34^+$ cells	WBC	$CD34^+$ cells	WBC	$CD34^+$ cells
1	1,84	1,70	421	172	229	101
2	2,14	1,93	488	175	227	91
3	1,77	1,74	399	149	225	86
4	0,83	0,82	105	37	127	45
5	2,01	2,00	184	74	92	37
6	2,09	2,01	375	183	180	91
7	2,27	2,20	157	86	69	39
8	2,67	2,60	679	261	254	100

Exp. 1, 2 and 6 were started with selected $CD34^+$ cells from a fresh UCB product and exp. 3, 4, 5, 7 and 8 were started with selected $CD34^+$ cells from a cryopreserved UCB product.

Validation of manufacturing process

To demonstrate that the GMP-compliant manufacturing process is robust and reproducibly yield SR1-expanded UCB-derived HSPC products that meet the predefined release criteria, 8 validation runs were performed in which clinically relevant numbers of UCB-derived $CD34^+$ cells (i.e. > 0,5 x 10^6 $CD34^+$ cells) were expanded in Vuelife® 290AC culture bags in SCGM medium with GMP-grade cytokines (SCF, Flt3L and TPO) and SR1. **Table 1** summarizes the yields of WBC and $CD34^+$ cells and the fold expansion of WBC and $CD34^+$ cells in these validation procedures. The results of these 8 validation runs demonstrate that the end-product of the GMP-compliant manufacturing process always met the predetermined release criteria (see **Table 2**).

An investigational medicinal product dossier (IMPD) including summaries of information on the development, quality, manufacturing process, controls and risk assessment of the SR1-expanded UCB-derived HSPC product has been developed.

Table 2. Release criteria for UCB-derived *ex vivo* expanded HSPC product

In process test	Analysis method	Criteria
Release of UCB-derived $CD34^+$ cell fraction for *ex vivo* expansion by the Responsible Person of Tissue Establishment	-	Present.
Sterility (intermediate and end-products)	Visual examination	No signs of bacterial or fungal contamination.
Sterility (end-product)	Microbial culture: BacTec™ PEDS-culture bottle	No bacterial or fungal contamination.
Viability (end-product)	Trypan Blue exclusion	≥ 70 % trypan blue negative.
WBC count (end-product)	Bürker counting chamber	≥ 50 x 10^6 nucleated cells.
$CD34^+$ cell enumeration (end-product)	Flow cytometry	≥ 20 x 10^6 viable $CD34^+$ cells.

CLINICAL STUDY DESIGN

Currently, double UCBT is generally applied in adult patients qualifying for cord blood transplantation in the Netherlands. Double UCBT was originally developed to improve engraftment by administering higher numbers of progenitor cells[1]. It has since been shown that sustained engraftment is observed in 85-100% of patients, both after myeloablative (MA) and after nonmyeloablative (NMA) conditioning regimens [1-5]. However, hematopoietic recovery generally originates from only a single unit and receovery times for neutrophils, platelets, and lymphocytes are still prolonged as compared to other alternative donor types.

Preceding studies: HOVON studies 106 and 115

Recently, results from the HOVON 106 study were published, showing the feasibility of double (dUCBT) preceded by a 4 Gy TBI containing reduced intensity conditioning (RIC) regimen in patients with poor-risk underlying hematological malignancies [6,7]. Data indicated that dUCBT following the above mentioned RIC regimen is feasible and may provide an important alternative source of hematopoietic stem cells in recipients lacking a sibling or well matched unrelated donor (MUD). With a median follow-up of 35 months, 2-year OS was 57%, 2 year TRM was 19%, and 2 year PFS was 42%. Thereafter, the HOVON-106 protocol has become the standard protocol for application of UCBT in adult patients in The

Netherlands. Sustained hematopoiesis in dUCBT recipients was derived from a single donor in 90% of patients and early predominance was already discernible at days 7-14 [6,7]. The mechanism of predominance was unclear, but subject of investigation in the succeeding HOVON 115 study. It was hypothesized that HLA class II specific $CD4^+$ T cells of the ultimate engrafting UCB unit elicit an immediate and targeted alloreactive immune response towards mismatched HLA class II molecules of the non-engrafting UCB cells, resulting in rapid rejection of the non-engrafting unit. The HOVON-115 trial was designed to test that hypothesis. It was shown that the majority of patients indeed developed an early expansion of $CD4^+$ T-cells, including effector T-cells with specificity for mismatched class II alleles of the rejected cord. The majority of mismatched class II alleles between the 2 cords in each individual patient evoked an alloreactive response, which appeared most prominent early after transplantation [8]. These findings suggested that matching of the 2 cords at the allele level for class II is no longer obligatory and can be omitted, which expands the choices for dUCBT. Despite the low incidence of graft failure following dUCBT, hematopoietic recovery is usually slow with neutrophil recovery (>0.5 x 10-9/L) around day 26 after transplantation and platelet recovery (>50) around day 50. Retarded hematopoietic recovery is mainly associated with the low number of HSPC in the graft. In order to increase these numbers, several investigators have initiated studies to expand HSPC *ex vivo* and improve hematopoietic recovery [9-21].

Expansion of hematopoietic stem cells

In this thesis, the history and current practice on the expansion of HSC are comprehensively described. Currently, the hypothesis that *ex vivo* expansion of HSC requires a combination of proliferative signals provided by hematopoietic growth factors with a factor that inhibits differentiation during culture has been studied extensively. Compounds including StemRegenin1 [20-21], copper chelation [14-15], mesenchymal stem cells [18-19], Nicotinamide [12-13], and Notch ligand Delta-1 [16-17], were studied in order to test that hypothesis. Of these, especially SR1 was shown to be associated with inhibition of

differentiation and when used with early acting cytokines to result in strong expansion of HSPC (this thesis (Chapter 5); [20-21]).

Rationale of the study

Transplantation of SR1-expanded $CD34^+$ cells in a clinical double cord blood setting appeared safe and feasible and resulted in engraftment in all 17 transplanted patients [21]. The median time to neutrophil and platelet recovery was 15 and 49 days, respectively, which is faster compared to an historical control group receiving unmanipulated dUCBT. In 11/17 patients, hematopoiesis was primarily derived from the expanded unit and in those patients, neutrophil recovery was even more rapid with a median of 11 days versus 23 days in the 6/17 patients engrafting with the unmanipulated unit [21]. These results have set the stage for clinical studies in a single UCBT setting, which is proposed here. Collectively, in view of 1. the rejection of one UCB graft in the majority of recipients of double UCBT, 2. the absent contribution of the rejected unit to hematopoietic recovery, and 3. the potency of SR1 to expand hematopoietic progenitor cells and thereby to improve engraftment and recovery, it is proposed to perform a feasibility study in 10-15 patients evaluating single UCBT using one SR1 expanded unit.

Study objectives

- To study the feasibility of single UCBT using one SR1-expanded unit
- To assess engraftment and engraftment kinetics; to evaluate immune reconstitution, acute and chronic Graft-Versus-Host Disease (GVHD), chimerism, toxicity, progression-free survival and overall survival after single unit UCBT, using with one *ex vivo* expanded unit.

Study design

This is a prospective phase I-II feasibility study. Patients lacking a matched unrelated donor and patients for whom a matched unrelated donor cannot be identified within 2 months and for whom an allogeneic transplant is urgently needed, are eligible for UCBT if a suitable UCB unit is available. Transplantation will be preceded by a reduced-intensity conditioning regimen, irrespective of patient age. Post grafting immunosuppression is performed by mycophenolate mofetil (30 days) and cyclosporine A (90 days, taper thereafter).

Study population

Eligibility for registration

All patients must be registered before start of treatment and must meet all of the following eligibility criteria.

Inclusion criteria

- Age 18-70 years inclusive
- Diagnosis of poor-risk hematological malignancy or (Very) Severe Anaplastic Anemia relapsing after or failing immunosuppressive therapy and meeting the criteria for a MUD allo-SCT
- Lacking a sufficiently matched volunteer unrelated donor or lacking such a donor within the required time period of ≤ 2 months in case of urgently needed allo-SCT
- Availability of 1 (≥5/6) matched UCB graft with a nuclear cell count > 2,7 x 10^7/kg (see paragraph 8.2).
- Availability of a back-up autograft, harvested and frozen earlier in the course of treatment, (harvest according to local apheresis policies)
- WHO performance status 0-2
- Written informed consent

Exclusion criteria

- Bilirubin and/or transaminases > 2.5 x normal value
- Creatinine clearance < 40 ml/min
- Cardiac dysfunction as defined by:
- Reduced left ventricular function with an ejection fraction < 45% as measured by MUGA scan or echocardiogram (another method for measuring cardiac function is acceptable)
- Unstable angina
- Unstable cardiac arrhythmias
- Pulmonary function test with VC, FEV1 and/ or DCO < 50%
- Active, uncontrolled infection
- History of high dose (≥ 8 Gy) total body irradiation
- Pregnant or lactating females
- HIV positivity

UCB unit selection

UCB units will be selected according to the following criteria:

1. The total amount of total nucleated cells present in the UCB unit must be > 2.7 x 10^7/kg recipient body weight.
2. HLA-matching is performed for HLA-A and HLA-B at the serological split resolution level and for HLA-DRB1 at the 4-digit resolution level. The minimal match grade required is a 5/6 match both between recipient and the UCB unit.
3. Absence of HLA-antibodies in the recipient directed against HLA class I and class II mismatches on the cord blood cells is required.
4. Preferably, RBC- and plasma reduced UCB units are selected.

5. Preferably, ABO-compatible or minor ABO-mismatched UCB units are selected.
6. Preferably, UCB units are selected from NETCORD/FACT accredited UCB banks

Note: If recipient anti-A/anti-B titer >512, (NaCl gel card (Diamed, Switzerland)); incubation either at room temperature (IgM) or after treatment with 0.01M DTT at 37° C (IgG), major incompatible non-RBC reduced / non-plasma reduced CBUs are not administered.

Treatment

Conditioning regimen

A reduced-intensity conditioning regimen is used (**table 3**).

Table 3. Reduced-intensity conditioning regimen used

Agent	Dose/day	Route of administration	Days
Cyclophosphamide	60 mg/kg	i.v.	-7
Fludarabine	40 mg/m^2	i.v.	-6, -5, -4, -3
TBI	2 Gy		-2, -1

Conditioning regimen and immunosuppression schedule

See **table 4**.

Table 4. Conditioning regimen and immunosuppression schedule

Day	-7	-6	-5	-4	-3	-2	-1		0	+1
Cyclophosphamide	X									
Fludarabine		X	X	X	X					
TBI						X	X			
Infusion of *ex vivo* expanded HSPC product									X	
Infusion of UCB-CD34- fraction										X
Cyclosporine A			X	X	X	X	X		X	X→
MMF									X	X→
(Val)acyclovir									X	X→
Benzylpenicilline									X	X→

Cord blood processing, expansion and infusion

As outlined in the IMPD, the selected cord blood will be thawed, washed and CD34$^+$ cells will be purified using immunomagnetic positive selection with a CliniMACS device according to Standard Operating Procedures (SOP's) of the Transplantation Laboratory. The CD34 negative fraction that contains donor T-cells will be frozen again, according to SOP's of the Transplantation Laboratory. The CD34 positive fraction will be released on basis of specific criteria, as described in the IMPD for further processing into an ATMP by the Responsible Person of the Tissue Establishment Transplantation Laboratory.

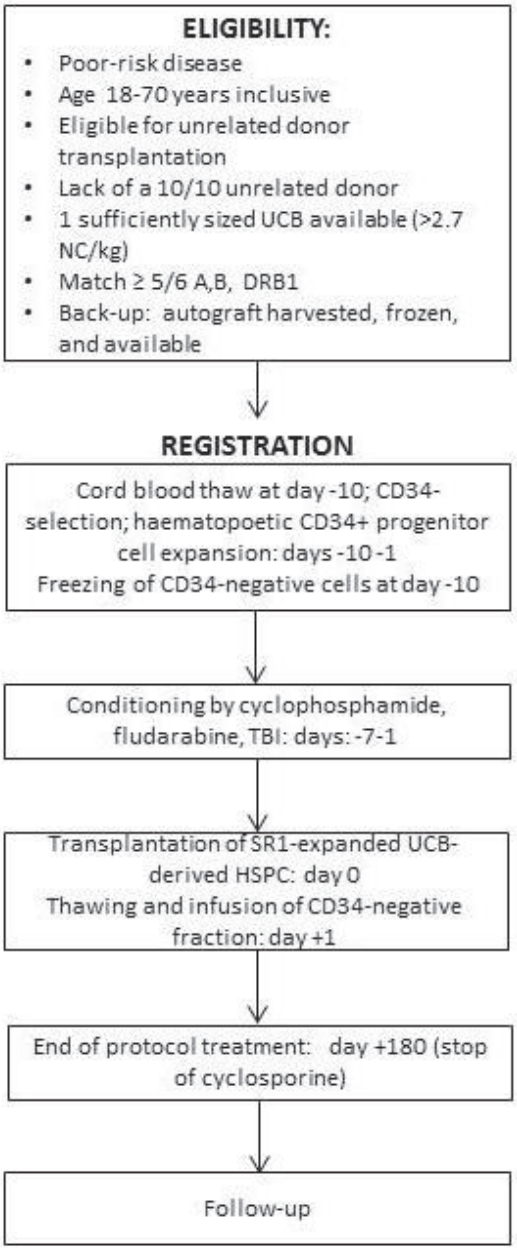

Figure 5. Scheme of study

Subsequently, $CD34^+$ cells will be expanded in *ex vivo* cultures using a combination of cytokines and SR1, as detailed in the IMPD. The UCB-derived *ex vivo* expanded HSPC end product will be released by the QP of the GMP facility on basis of prespecified criteria (as outlined in detail in the IMPD). The manipulated UCB unit will contain after culture no red blood cells and will also be devoid of donor plasma, which obviates the need for additional precautions in case of major and minor ABO-incompatibility. The expanded graft will be infused on day 0, followed by thawing and infusion of the cryopreserved CD34 negative fraction on day +1.

The CD34 negative fraction contains RBC and residual donor plasma, which necessitates the need for monitoring of hemolysis (haptoglobin, bilirubin, LDH, creatinine, CBC, DAT and plasma-Hb) in case of major- or minor AB0 blood group incompatibility.

Approval

As outlined above, a clinical study protocol and the IMPD have been submitted for approval to the competent authorities in the Netherlands, the Centrale Commissie Mensgebonden Onderzoek (CCMO).

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Chapter 8

General discussion

INTRODUCTION

Umbilical cord blood (UCB) has emerged as an important alternative stem cell source for patients in need of an allogeneic hematopoietic stem cell transplantation (allo-SCT) but lacking a HLA-matched related or unrelated donor. Compared to other donor sources, UCB is rapidly available because of the well-organized storage by public cord blood banks that are affiliated with international donor registries (1, 2) and require less stringent HLA-matching (3). However, the relatively low number of hematopoietic stem cells (HSC) in UCB units results in retarded hematopoietic recovery and a higher incidence of graft failure (4-6). Co-infusion of two UCB units, the so-called double UCB transplantation (dUCBT) to increase the number of HSC infused, results in sustained engraftment and less graft failure compared to single UCBT. However, hematopoietic recovery appeared still retarded and derived from a single unit with the other unit being rejected (7-10). *Ex vivo* expansion of HSC, as a means to increase the number of cells in the graft, has been studied extensively from bench to bedside (11-23). Other types of stem cells, such as embryonic stem cells and epithelial stem cells, can be expanded in cultures supplemented with a combination of growth factors to stimulate proliferation and a factor to inhibit differentiation. Based on this hypothesis, the combined effects of proliferation-inducing cytokines and a differentiation-inhibiting factor were studied with the aim to expand HSC. The studies described in this thesis were based on that hypothesis: two potential inhibitors of HSC differentiation, Wingless-related integration site protein 3a (Wnt3a) and the Aryl hydrocarbon Receptor (AhR) StemRegenin1 (SR1), were studied with the aim to develop a clinically applicable expansion protocol. While Wnt3a failed to inhibit hematopoietic differentiation in our *in vitro* culture system, SR1 combined with early acting hematopoietic cytokines showed effective proliferation of hematopoietic progenitor cells accompanied with limited differentiation, resulting in the expansion of hematopoietic stem and progenitor cells (HSPC). Based on these studies, a clinical phase I study was developed. In the current chapter, we discuss the main findings of this thesis, the potential challenges and the future prospects of UCB transplantation using expanded HSPC.

IMPROVING *EX VIVO* HSPC EXPANSION STRATEGIES

The *ex vivo* expansion of HSPC has progressed remarkably in the last decade but robust *ex vivo* expansion of the long term repopulating HSC remains a formidable challenge. The optimal HSPC expansion protocol should (1) generate a sufficient number of HSPC to perform a single unit UCBT, (2) shorten the time to hematopoietic and immune recovery, (3) achieve durable long-term engraftment and (4) be cost-effective. The currently used

expansion protocols for HSPC are based on extensive studies that have resulted in the identification of developmental regulators and chemical modulators that play a role in the regulation of self-renewal, differentiation and survival of HSC. Although fate decisions of HSC, balancing self-renewal, proliferation and differentiation, are controlled *in vivo* by a variety of factors (i.e. signaling molecules, direct cell-cell contact, circadian rhythm and oxygen level), most of the reported expansion protocols use, in addition to hematopoietic cytokines, only a single differentiation-inhibiting or self-renewal promoting factor, including the protocols tested in chapters 4 and 5 of this thesis. Few studies investigated simultaneous activation or inhibition of multiple pathways known to be important in HSC fate decisions (21, 24, 25). In 2011, Perry *et al*.(25), studied the simultaneous activation of the PI3K pathway and the canonical Wnt pathway combined with hematopoietic growth factors in mouse HSPC expansion cultures. While neither pathway alone was sufficient to promote self-renewal, combined stimulation of both pathways resulted in robust expansion of immature HSPC which were capable of long-term engraftment in serial transplantations in immunodeficient mice. More recently, AhR antagonist SR1 was used in two studies, combining SR1 with Notch ligand Delta1 (24) and pyrimidoindole derivative UM171 (21). Combination of SR1 and Delta1 resulted in a threefold increased expansion of severe combined immune deficient mice (SCID) repopulating cells (SRC) compared to either agent alone. Delta1 increases the expression of the Notch target gene HES1, which is a transcriptional repressor that influences the differentiation of HSC. The authors hypothesized that the increase of HES1, which was not observed in cultures with SR1 alone, may be responsible, at least in part, for the delay in differentiation observed in their culture system. Fares *et al*. discovered UM171, a pyrimidoindole derivative, working independently of AhR suppression. Their studies suggested that UM171 and SR1 both affect very immature, but yet different, cell populations. Both compounds appeared to cooperate to enhance *ex vivo* expansion of progenitor cells and suppressed differentiation. In this setting, interaction between the pathways is key, with each component making cooperative as well as unique contributions to HSC expansion.

Expansion protocols using mesenchymal stem cells (MSC) as stromal layer are based on the same principle, since mesenchymal stem cell provide multiple signals for *ex vivo* expansion of HSPC (23). However, it is still unknown which MSC-derived signals are pivotal for *ex vivo* HSPC expansion. Moreover, co-culture of HSPC with MSC appears to preferentially expand committed progenitors at the expense of long-term repopulating HSC with self-renewal capacity.

Extracellular vesicles (EVs) are a newly uncovered means of intercellular communication that mediate cell-to-cell communication and regulate cell fate via the horizontal transfer of bioactive lipids, proteins and RNA. Recently, it has been described that MSC-derived EVs contain microRNAs (miRNAs) and Piwi-interacting RNAs (piRNAs) which were able to modify the gene expression profile of UCB-derived $CD34^+$ cells, resulting in more vi-

able, less differentiated cells (26). Furthermore, human osteoblast-derived EVs promoted proliferation and cell cycle progression of human UCB-derived HSPC and enhanced the *ex vivo* expansion of human UCB-derived HSPC, including the most immature subset in growth factor supported expansion cultures. EV-expanded HSPC retained their full differentiation capacity *in vitro* and successfully engrafted in immune-deficient NSG mice (27). Interestingly, the expansion promoting effects of osteoblast-derived EVs on UCB-derived HSPC remained evident even in the presence of the differentiation inhibiting factor SR1. However, the molecular mechanisms underlying the EVs mediated expansion of HSPC still remain to be explored.

Another strategy to improve engraftment and time to hematopoietic and immune recovery is to expose HSPC to factors that improve homing of HSPC towards the bone marrow niche. It is known that UCB-derived HSPC do not home as efficiently to the bone marrow as HSPC derived from adult bone marrow (28) and in addition, cytokine exposure of HSPC leads to a rapid loss of bone marrow homing capacity (29). Several strategies have been reported to enhance the homing capacity of HSPC and thereby enhance hematopoietic recovery post-transplantation, such as fucosylation of the HSPC ligands binding to P- and E-selectin adhesion molecules expressed on bone marrow endothelial cells (30, 31) or activation of 'homing receptor' CXCR4 on HSPC by treatment with 16,16-dimethyl prostaglandin E2 (dmPGE2) (32, 33). Practical advantage of these techniques is that they require minimal processing as the maximum incubation time is 120 minutes. Strategies aiming to combine an optimized expansion protocol with an approach to improve the homing capacity of the expanded cells may result in a graft with optimal engraftment capacity and hematopoietic recovery potential.

IMMUNE RECONSTITUTION AFTER EXPANDED (D)UCBT

Of all the different expansion protocols, only few were tested clinically (14, 16, 18, 20, 23). The studies by Horwitz *et al*. (14), Wagner *et al*. (20) and de Lima *et al*. (23), with limited patient numbers, showed significant enhanced hematopoietic recovery in terms of neutrophil and platelet recovery. Neutrophil recovery was accelerated by approximately 10 days, while the time to platelet recovery (> 50 x 10^9/L) was reduced with 4 to 5 days. However, apart from protracted neutrophil and platelet recovery, especially retarded immune recovery is of concern, as non-relapse mortality (NRM) is especially associated with insufficient T cell recovery rather than protracted neutrophil recovery(34). T cell recovery after dUCBT was studied and described in chapter 2. A higher risk of severe infections in patients failing to recover thymopoiesis at 3 months post-transplantation was described, which compares well to our earlier observations in unrelated donor allo-SCT recipients (34). Sofar, only one clinical study addressed the recovery of B and T

cells after expanded UCB transplantation (20), which failed to demonstrate improved T cell recovery upon engraftment of the expanded UCB unit compared to T cell recovery originating from the unmanipulated unit in a dUCBT setting. Of note, T cell recovery was even suggested to occur at a slower rate after transplantation using an expanded UCB unit (20). How to explain the latter findings? Different factors may be involved. First, it is still possible that expansion of true HSC was limited, while more committed progenitors were expanded at a greater degree, especially myeloid committed progenitors (35). The rapid neutrophil recovery rather than lymphoid recovery might support that explanation. Secondly, T cell recovery not only depends on hematopoietic HSC engraftment but also depends on generation of lymphoid precursors, their entry into the thymus, and effective thymopoiesis. It is well known that thymopoiesis in adult patients can be severely compromised (36-38) despite efficient engraftment and early hematopoietic recovery. However, Wils *et al*. (36) showed that T cell recovery may be improved upon expansion of HSC and lymphoid progenitors *in vivo* by treatment with Flt3ligand of immunodeficient mice having received a bone marrow transplantation. Lastly, many transplant centers use G-SCF post-transplant to accelerate neutrophil recovery. That myeloid growth factor may skew hematopoietic recovery towards the myeloid lineage. Therefore, a slow rate of T cell recovery after UCBT with expanded HSC/HPC may be due to a combination of insufficient thymic function and insufficient generation and supply of lymphoid progenitors. Since T cell recovery is pivotal to reduce the toxic and potential lethal complications of allo-SCT, it is of the utmost importance to develop expansion strategies that also expand T cell precursors. Apart from the cytokines we applied in our expansion protocol (SCF, Flt3L and TPO), lymphopoietic cytokines including IL-7 and IL-15 might be considered. Both cytokines are acting as survival factors for (developing) T cells by the prevention of apoptosis through up- and downregulation of anti- and pro-apoptotic factors, respectively (39, 40). An alternative approach might the use of stromal cells, such as DL-1 expressing cells, in the expansion protocol. Interaction of HPC with DL-1 results in the generation of T cell progenitors, that may directly enter the thymus (41). Given the pivotal role of the thymic epithelium in T-cell recovery, another strategy to improve T cell recovery might be the application of strategies that favorably affect the thymic epithelium. Possible strategies may include protection of the nursing stromal compartment by keratinocyte growth factor or by regeneration of the thymic epithelium (34). Altogether, it is clear that expansion of true hematopoietic stem cells is an important first step, but it might be necessary to add strategies specially aiming to improve T cell recovery and thymic function. Therefore, in future clinical trials with *ex vivo* expanded stem cell grafts, endpoints should include improvement of T cell reconstitution and thymopoiesis, apart from neutrophil and platelet recovery. In addition, immune reconstitution data should be correlated to HSC and HPC numbers infused as well as to subsets of lymphoid and myeloid progenitors obtained after expansion.

ALTERNATIVE DONORS

HLA-matched related and HLA 10/10 matched unrelated donors are currently considered as preferred donors for patients eligible for allo-SCT. However, many patients lack such a preferred donor and depend on alternative stem cell sources. Currently, alternative donors or stem cell sources include mismatched unrelated donors, haplo-identical family donors, and UCB. While the large retrospective registry study by Brunstein *et al*. suggested that disease free survival (DFS) in recipients of UCBT may come close to DFS following matched unrelated allo-SCT (51% versus 48%, respectively), NRM remains of concern (34% versus 14%, respectively)(42). The use of UCB as an alternative stem cell source for adult patients is hampered by the limited number of HSPC in most of the UCB grafts available, leading to susceptibility for infections and also NRM. In order to increase the number of HSPC, double UCBT was developed , which however insufficiently accelerated recovery and also appeared to be associated with high costs. Other alternative donor sources for patients lacking a matched related or unrelated donor include HLA-mismatched unrelated donors (MMUD, defined as 1 or 2 mismatches at class I and/or class II) or haplo-identical family donors (haplo-SCT). Currently, no consensus has been reached as regards the hierarchy of alternative donors. Recently, several studies have been published discussing and comparing alternative transplant possibilities (43-47). An important concern for the use of MMUD grafts is the higher risk of GVHD due to HLA-incompatibility. However, HLA-incompatibility may also be associated with a somewhat higher graft versus leukemia (GVL) effect, preventing disease relapse. A recent meta-analysis comparing overall survival after MUD and MMUD showed an increased mortality risk of 27% for patients receiving a MMUD (43). It indicates that the excess rate of NRM was insufficiently compensated by a lower risk of relapse, leading to significantly reduced OS. In the past, results obtained with haplo-SCT suggested a similar pattern of increased NRM associated with increased degree of HLA-incompatibility, which is insufficiently counteracted by a reduced relapse rate (48, 49). As a result of observed high NRM, also autologous transplantation has been brought forward as an alternative stem cell source (50, 51). While the latter stem cell source lacks allogenic T cells and thereby is devoid of the allogeneic GVL-effect, the very low rate of NRM may favor that mode of transplantation. Especially in leukemia patients, autografting may provide for an alternative stem cells source if no HLA-identical sibling or MUD is available (52). However, the last decade has witnessed an enormous surge in haplo-SCT, because of significant progress in the prevention of GVHD and thereby reduction of NRM. Especially the use of post-transplant cyclophosphamide, as explored by the Baltimore group (53, 54) appeared associated with effective prevention of GVHD and limited NRM. Of note, an accompanying increase of relapse, as may be expected by inhibition and elimination of allogeneic haplo-identical T-cells, has sofar not been demonstrated. These results suggest that the

net result of low NRM and an acceptable risk of relapse may favor haplo-SCT. In addition, benefits of haplo-SCT include the rapid availability of the donor, the option for subsequent donor lymphocyte infusions and the relatively low costs. When compared to UCBT, clinical outcome after haplo-SCT was reported to be largely similar in terms of relapse incidence, NRM and relapse-free survival (RFS) in a retrospective study including patients with acute leukemia (46). However, higher rates of relapse and lower rates of NRM, resulting in similar OS, have been reported as well (47). Preliminary findings in a large group of high-risk AML patients showed a lower relapse rate and similar NRM upon haplo-SCT (using post-transplant cyclophosphamide) compared to UCBT, resulting in better OS and RFS at 2 years post-transplantation (55). Collectively, recent studies have shown a clear improvement in haplo-SCT, while NRM following UCB remains of concern. Future studies using expanded UCB will answer the question whether manipulation of UCB grafts may be associated with less NRM and to what extent that will compare to haplo-SCT. Until results of those studies become available, allo-SCT using sibling and MUD donors are still to be preferred, while haploidentical donors and UCB can be considered as preferred alternative donors and mismatched unrelated donors as third alternative, which then, however, should also be weighed versus autologous transplantation.

CONCLUSION

The field of alternative donor transplantation is currently rapidly evolving. Although hampered by the small size of the grafts, UCB is an important stem cell source for patients in high need of an allogenic HSC donor. *Ex vivo* expansion protocols using a combination of signals to inhibit differentiation in combination with proliferation inducing growth factors are promising. Preclinical studies as described in this thesis and clinical studies in dUCBT settings have set the stage for a clinical feasibility study of expanded HSPC in a single unit setting. Results of that study as well as those addressing other approaches to expand HSC are eagerly awaited.

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Appendices

SUMMARY

The first allogeneic hematopoietic stem cell transplantation (allo-SCT) using umbilical cord blood (UCB) was performed in 1988 in a patient with Fanconi's anemia. Over the past decades, UCB has emerged as an important alternative stem cell source in patients in need of an allo-SCT but lacking a matched related or unrelated donor. To date (2016), more than 35,000 UCB transplantations (UCBT) have been performed. Advantages of UCB as a stem cell source are its rapid availability and less stringent HLA-matching criteria. However, adult patients receiving UCBT show retracted hematopoietic recovery and a higher incidence of graft failure compared to recipients of other stem cell sources. An association between time to hematopoietic recovery and numbers of nucleated cells (NC) in the graft resulted in guidelines discouraging the use of UCB grafts with low numbers of NC. The total number of NC is generally used as a reflection of the number of hematopoietic stem cells (HSC) in the UCB graft. However, the NC threshold adhered to severely limits the number of grafts suitable for transplantation, especially for adult patients. Double UCBT (dUCBT) was developed in order to increase the number of HSC and thereby to improve engraftment and recovery. Although less graft failure was observed, hematopoietic recovery appeared still retarded and is generally accounted for by a single unit with the other unit being rejected. In order to increase the number of HSC per UCB unit, the expansion of HSC and hematopoietic progenitor cells (HPC) *in vitro* has been studied extensively (**Chapter 1**). Studies in the eighties and nineties of the last century focused on the application of early acting hematopoietic growth factors, which exert proliferative, anti-apoptotic, and differentiation inducing effects in HSC. The latter phenomenon proved to be an overriding effect resulting in the generation of large numbers of differentiated cells accompanied by loss of HSC. Thereafter, the combined effects of proliferation inducing cytokines and a differentiation inhibiting factor was studied with the aim to expand HSC and prevent their differentiation and exhaustion. The underlying hypothesis was based on observations in embryonic and epithelial stem cells, which could effectively be expanded by a combination of early acting cytokines and a differentiation inhibiting factor (see **Figure 2, Chapter 1**) The studies described in this thesis were based on exactly that hypothesis. Two potential inhibitors of HSC differentiation were studied, namely Wnt3a and the Aryl hydrocarbon Receptor antagonist StemRegenin1 (SR1). Only studies with SR1 ultimately supported our hypothesis and, therefore, provided a basis for the development of a clinical study.

In **Chapter 2** a retrospective study on hematopoietic and immune reconstitution in recipients of dUCBT is described. Clinical outcome, including infection rate and non-relapse mortality (NRM), were correlated to immune reconstitution in a group of 55 high-risk patients receiving dUCBT. While B and NK cell recovery was relatively rapid (values returned within the normal range at 6 and 2 months respectively), T cell recovery was

severely retarded. At 24 months post-transplantation, the median level of $CD3^+$ T cells had not reached the lower normal range limit. Although T cell numbers were very low, newly formed $sjTREC^+$ T cells were detectable in the majority of patients at 3, 6 and 12 months post-transplantation. Median frequency of $sjTREC^+$ T cells per 10^5 $CD3^+$ T cells and median $sjTREC^+$ T cell content per milliliter blood increased over time, but at 12 months post-transplantation, both frequency and content had not recovered to normal values. The absence of $sjTREC^+$ T cells at 3 months post-transplantation appeared associated with a higher risk for severe infections after that time point. These results show that a retarded T-cell recovery and subsequent susceptibility for infections are a pronounced feature of dUCBT.

In **Chapter 3** the protein Wingless-int3a (Wnt3a) in combination with the cytokines Stem Cell Factor (SCF) and Thrombopoietin (TPO) is studied using mouse HSPC. Wnt proteins have been implicated to play an important role in the regulation of stem cells, including embryonal stem cells and HSC. Based on the potential differentiating inhibitory effects of Wnt3a, we investigated whether the combination of the tyrosine kinase receptor activating growth factors SCF, Flt3L, and TPO and Wnt3a would result in expansion of HSC. Addition of Wnt3a to our culture system resulted in a reduced number of HSPC, as measured by flow cytometry. To increase the stability of Wnt3a and prolong its half-life, Wnt3a was tethered onto liposomes resulting in the formation of 'liposomal Wnt3a'. Similar to purified Wnt3a, liposomal Wnt3a resulted in a reduced number of HSPC. Overexpression of the anti-apoptotic BCL2 protein failed to rescue the negative impact of Wnt signals on HSPC numbers, suggesting that the loss of HSPC was not due to apoptosis. An alternative explanation for the reduced number of HSPC is increased differentiation by the combination of the growth factors used and Wnt3a.

Chapter 4 describes the evaluation of the combined effects of Wnt3a and early acting cytokines, SCF, TPO and FMS-like tyrosine kinase 3 ligand (Flt3L) with respect to the expansion of human UCB-derived HSPC. Similar to observations with murine HSPC, combining Wnt3a with the hematopoietic growth factors SCF, Flt3L and TPO resulted in less expansion of $CD34^+$ cells compared to these growth factors alone. The reduced expansion was also observed in cultures initiated with $Lin^-CD34^+CD38^{low}CD45RA^{low}CD90^+$ cells, which are highly enriched for HSC. The effect was due to the activation of the canonical Wnt pathway by binding of Wnt3a to its receptor, as was shown by blockade of Wnt3a binding to its receptor using a Wnt antagonist. Stabilization of Wnt3a in liposomes effectively increased its half-life, but failed to increase expansion of HSPC. In addition, Wnt3a reduced the frequency of multilineage CFU-GEMM and the long-term repopulation ability of the expanded cells in immuno-deficient mice. In conclusion, the results described in **Chapters 3 and 4** show that, in contrast to our hypothesis, Wnt3a failed to inhibit the enormous differentiation induced by the growth factors. Rather, the combination of Wnt3a and the growth factors SCF, TPO and Flt3L resulted in increased differentiation.

Additional signals might be needed to inhibit growth factor induced proliferation and differentiation.

In **Chapter 5** the Aryl hydrocarbon Receptor (AhR) antagonist StemRegenin1 (SR1), a strong inhibitor of hematopoietic cell differentiation, is described. An earlier study by Boitano and coworkers suggested that SR1 combined with hematopoietic growth factors could result in expansion of HSPC. Here, we show that SR1 in combination with the early acting hematopoietic growth factors SCF, Flt3L and TPO induces a more than 25-fold expansion of the most primitive subset, as measured by flow cytometry. It is important to emphasize that flow cytometric techniques are still not able to identify true HSC, capable of self-renewal and multilineage differentiation. The expanded population of cells retained their functional properties as assessed in long-term culture systems (LTC-IC assays), which currently is the best *in vitro* surrogate assay for primitive HSC. Transplantation of SR1-expanded UCB cells into sublethally irradiated NSG mice resulted in long-term multilineage engraftment. Strikingly, human chimerism levels in blood were reduced as compared to levels obtained after the transplantation of non-expanded cells. Of note, the mouse model used, the NSG mouse, results in a preferable outgrowth of B cells. Expansion using a combination of cytokines and SR1 results in expansion of HPC, but with relative low numbers of B cell progenitors. A preferential increase of myeloid precursors at the expense of lymphoid precursors upon expansion cultures might explain reduced human chimerism levels as was observed *in vivo*. In addition, impaired homing of the expanded cells could play a part as well. Expansion may result in decreased homing capacity by the loss of essential homing (surface) proteins.

To visualize the homing of transplanted HSPC to the bone marrow, a ^{19}F-labeling protocol was designed. **Chapter 6** describes the successful labeling of UCB-derived CD34^{+} cells using poly(lactic-co-glycolic acid) (PLGA) nanoparticles that contain ^{19}F and can be detected by both flow cytometry and magnetic resonance spectroscopy. These intracellularly labeled CD34^{+} cells maintained their capacity to proliferate and differentiate, characteristics pivotal for successful engraftment upon transplantation. These results set the stage for *in vivo* tracking experiments, studying the homing capacity of expanded and non-expanded cells.

The research expansion protocol for HSPC described in **Chapter 5** was translated into a Good Manufacturing Practice (GMP) expansion protocol, that can be used for the expansion of UCB-derived HSPC intended for transplantation in patients suffering from hematological malignancies. This process is described in **Chapter 7**. Using the GMP expansion protocol, similar levels of expansion of HSPC were obtained as compared to the expansion levels using the research grade protocol. The setup of a clinical study is described, aiming to assess the safety and feasibility of transplantation of SR1-expanded UCB-derived HSPC in a single graft setting in a total of 10 patients.

Finally, the implications of the findings described in this thesis for the future are discussed in **Chapter 8**. The quest for the optimal protocol for *ex vivo* expansion and the study of its effects on subsequent *in vivo* engraftment will continue. In addition, early clinical studies are pivotal to address whether the current practice using double transplants can be abandoned and if transplantations using expanded single units result in similar, or better, clinical outcome. Finally, UCB should be compared with other alternative donor sources, such as adult mismatched unrelated donors and non-matched (haplo-identical) family donors.

SAMENVATTING

De eerste allogene hematopoëtische stamceltransplantatie (allo-SCT) waarbij gebruik gemaakt werd van navelstrengbloed werd in 1988 uitgevoerd bij een patiënt met Fanconi Anemie. De afgelopen decennia heeft navelstrengbloed zich ontwikkeld tot een belangrijke alternatieve stamcelbron voor patiënten die een allo-SCT nodig hebben, maar waarbij geen passende verwante of niet-verwante donor beschikbaar is. Momenteel (2016) zijn er wereldwijd al meer dan 35.000 navelstrengbloedtransplantaties uitgevoerd. Bij volwassen patiënten, die een navelstrengbloedtransplantatie krijgen, duurt het herstel van witte bloedcellen en bloedplaatjes vaak lang en komt uitblijvend herstel ("graft failure") vaker voor in vergelijking tot andere stamcelbronnen voor allo-SCT. Dit bleek terug te voeren op het geringe aantal bloedstamcellen (verder 'stamcellen') in navelstrengbloed en heeft geleid tot richtlijnen, waarin het gebruik van navelstrengbloedeenheden met een klein aantal stamcellen afgeraden wordt. Hierdoor vermindert het aantal eenheden dat geschikt is voor transplantatie, met name in volwassen patiënten, echter aanzienlijk. Teneinde het aantal stamcellen te verhogen werd de dubbele navelstrengbloedtransplantatie ontwikkeld. "Graft failure" werd minder vaak waargenomen, maar het hematopoëtisch herstel bleek niet verbeterd, mogelijk als gevolg van afstoting van een van de twee gegeven eenheden. Teneinde het aantal stamcellen per navelstrengbloed eenheid te doen toenemen, is internationaal veel onderzoek gedaan naar expansie van navelstrengbloedstamcellen in het laboratorium (**hoofdstuk 1**). In de jaren '80 en '90 werden expansieprotocollen ontwikkeld met daarin hematopoëtische groeifactoren, welke zorgden voor proliferatie en overleving, maar ook uitrijping van de stamcellen. Dit resulteerde in de productie van grote aantallen uitgerijpte voorlopercellen, terwijl de stamcellen niet meer aanwezig waren. Op basis hiervan, werd de combinatie proliferatie stimulerende groeifactoren met een factor die de uitrijping remt bestudeerd met als doel stamcellen te expanderen zonder dat zij uitrijpen of uitputten. De onderliggende hypothese was gebaseerd op observaties gedaan in studies met embryonale en epitheliale stamcellen, welke geëxpandeerd konden worden door de combinatie van specifieke groeifactoren en een factor die de uitrijping remt (zie figuur 2, **hoofdstuk 1**) De in dit proefschrift beschreven studies zijn gebaseerd op exact die hypothese. Twee potentiele remmers van stamcel uitrijping zijn onderzocht, namelijk Wnt3a en Aryl hydrocarbon Receptor antagonist StemRegenin1 (SR1). Alleen het onderzoek naar SR1 ondersteunde onze hypothese en heeft de basis gelegd voor de ontwikkeling van een protocol voor een klinische studie.

In **hoofdstuk 2** wordt een retrospectief onderzoek naar het hematopoëtisch en immunologisch herstel na transplantatie met twee navelstrengbloedeenheden beschreven. Klinische uitkomstmaten, inclusief het aantal infecties, werden gerelateerd aan het immunologisch herstel in 55 ontvangers van een dubbele navelstrengbloedtransplantatie.

Terwijl herstel van B- en NK-cellen relatief vlot plaatsvond na transplantatie (respectievelijk na 6 en na 2 maanden), bleek het T-cel herstel erg traag. Na 24 maanden lag het mediane aantal T-cellen nog steeds onder de normaalwaarde. Hoewel de T-cel aantallen erg laag waren, werden bij de meeste patiënten op 3, 6 en 12 maanden na transplantatie nieuwe, in de thymus gevormde sjTREC$^+$ T-cellen aangetroffen. De mediane sjTREC frequentie per 10^5 CD3$^+$ T-cellen en het mediane aantal sjTREC$^+$ T-cellen per millimeter bloed nam in de loop van de tijd toe, maar 12 maanden na transplantatie waren zowel de frequentie als het aantal nog niet terug op de normale waardes. Onderzocht werd of het herstel van de thymusfunctie, gemeten aan de aanwezigheid van sjTREC$^+$ T-cellen, verband hield met ernstige infecties en sterfte als gevolg van complicaties. Met name uitblijven van herstel van nieuwgevormde T-cellen in de thymus op 3 maanden na transplantatie bleek voorspellend voor ernstige opportunistische infecties daarna. Hoe hoger het aantal sjTREC$^+$ T-cellen in het bloed, hoe lager de kans op infectie. Deze resultaten tonen aan dat het trage hematopoëtische herstel na dubbele navelstrengbloedtransplantatie het meest uitgesproken is wat betreft het T-cel herstel. Naast het geven van meer stamcellen zouden ook interventies gericht op verbetering van thymusfunctie verder onderzocht moeten worden.

In **hoofdstuk 3** wordt effect van het eiwit Wingless-int3a (Wnt3a) op de proliferatie en differentiatie van muizen stam- en voorlopercellen onderzocht. Wnt eiwitten spelen een belangrijke rol bij de deling en rijping van stamcellen, waaronder embryonale stamcellen en bloedstamcellen. De hypothese was dat een combinatie van tyrosine kinase receptor activerende groeifactoren in combinatie met Wnt3a expansie van stamcellen zou kunnen geven, zoals eerder gezien met embryonale stamcellen, waarbij het Wnt-effect berustte op remming van de uitrijping. Toevoeging van Wnt3a aan ons kweeksysteem, (met daarin ook hematopoëtische groeifactoren die aangrijpen op tyrosine kinase receptoren) leidde echter tot een vermindering van het aantal stam- en voorlopercellen, zoals gemeten door middel van flowcytometrie. Teneinde de stabiliteit te verhogen en de halfwaardetijd te verlengen werd Wnt3a geladen op liposomen (liposomaal-Wnt3a), hetgeen echter opnieuw leidde tot een vermindering van het aantal stam- en voorlopercellen. Overexpressie van het anti-apoptotische BCL2 proteïne in stam- en voorlopercellen had geen effect op de vermindering van het aantal stam- en voorlopercellen door Wnt3a. Dit suggereert dat de Wnt3a-geïnduceerde vermindering van het aantal stam- en voorlopercellen niet het gevolg is van apoptose. Een mogelijke verklaring voor het lagere aantal stam- en voorlopercellen is onvoldoende remming en zelfs toename van uitrijping onder invloed van de combinatie van groeifactoren en Wnt3a.

In **hoofdstuk 4** wordt het effect van Wnt3a beschreven op expansie van humane CD34$^+$ cellen, een populatie van cellen bestaande uit stam- en voorlopercellen. Gelijk aan de observatie bij muizen stam-en voorlopercellen, bleek te combinatie van groeifactoren en Wnt3a te leiden tot minder expansie van het aantal CD34-positieve cellen in vergelijking

met stimulatie d.m.v. groeifactoren alleen. Dit effect werd ook waargenomen in kweken met $Lin^-CD34^+CD38^{low}CD45RA^-CD90^+$ cellen, die in hoge mate verrijkt zijn voor stamcellen. Het waargenomen effect was te wijten aan de activering van de Wnt signaalroute, waarbij Wnt3a aan diens receptor bond en stabilisering van Wnt3a tot liposomaal Wnt3a leidde andermaal niet tot een verhoogde expansie van stam- en voorlopercellen. Wnt3a verminderde ook het aantal multilineage kolonies (CFU-GEMM) en het vermogen tot succesvolle transplantatie in immuun gecompromitteerde NSG muizen. Concluderend wordt gesteld dat de in de **hoofdstukken 3 en 4** beschreven data, in tegenstelling tot onze hypothese, aantonen dat Wnt3a de uitrijping geïnduceerd door de groeifactoren niet kan remmen. Daarentegen zagen we juist iets meer uitrijping door de combinatie van groeifactoren en Wnt3a. De door hematopoëtische groeifactoren geïnduceerde celdeling en celrijping benodigen derhalve aanvullende signalen om de rijping te blokkeren.

In **hoofdstuk 5** wordt een studie beschreven, waarin gebruik gemaakt werd van een sterke remmer van de celrijping, namelijk de Aryl hydrocarbon Receptor (AhR) antagonist StemRegenin1 (SR1). Een eerdere studie in een ander centrum suggereerde dat SR1 in combinatie met hematopoëtische groeifactoren wel zou kunnen leiden tot stamcelexpansie. Wij laten zien dat SR1 de expansie van humane stam- en voorlopercellen inderdaad bevordert. De celpopulatie die het meest verrijkt is voor echte stamcellen ($Lin^-CD34^+CD38^{low}CD45RA^-CD90^+$ cellen) expandeerde meer dan 25 maal in 2 weken. Overigens dient benadrukt te worden, dat de werkelijke bloedstamcel, die zichzelf kan vermeerderen en ook alle cellijnen als nakomelingen kan genereren, nog steeds niet met flowcytometrische technieken precies aangetoond kan worden. De geëxpandeerde stam- en voorlopercellen behielden hun functionele eigenschappen, zoals o.a. gemeten in lange termijn kweeksystemen (de zogenaamde LTC-IC testen), welke momenteel de beste *in vitro* surrogaattest voor primitieve stamcellen vertegenwoordigt. Transplantatie van SR1-geëxpandeerde cellen in bestraalde NSG-muizen resulteerde in herstel van rijpe humane cellen in het perifere bloed. Opvallend genoeg bleek het percentage humaan chimerisme na transplantatie van SR1-geëxpandeerde humane stam- en voorlopercellen lager dan na transplantatie van niet-geëxpandeerde humane stam- en voorlopercellen. De meest waarschijnlijke verklaring hiervoor is het gekozen muizenmodel, waarin met name rijpe humane B-cellen ontstaan. Expansie met groeifactoren en SR1 leidt weliswaar tot stam- en voorlopercel expansie, maar met een relatief laag aantal voorloper B-cellen en juist een hoog aantal myeloïde voorlopercellen. Deze verdeling van voorlopercellen na expansie is een mogelijke verklaring van het lagere percentage humaan chimerisme. Daarnaast zou ook de nesteling ("homing") van de stam- en voorlopercellen een rol kunnen spelen. Expansie zou kunnen leiden tot een verminderde homing-capaciteit, als gevolg van verlies van daartoe benodigde (oppervlakte) eiwitten op de geëxpandeerde stam- en voorlopercellen.

Om homing van getransplanteerde stam- en voorlopercellen naar het beenmerg zichtbaar te kunnen maken, werd een Fluor-19-labeling protocol voor stam- en voorlopercellen ontworpen. In **hoofdstuk 6** wordt beschreven hoe stamcellen uit navelstrengbloed met succes gelabeld kunnen worden met poly(lactic-co-glycolic acid) (PLGA) nanodeeltjes die Fluor-19 bevatten. Fluor-19-gelabelde cellen kunnen worden opgespoord met behulp van zowel flowcytometrie als magnetische resonantie spectroscopie (MRS). Deze intracellulair gelabelde stamcellen behielden hun capaciteit om *in vitro* te prolifereren en differentiëren, eigenschappen die essentieel zijn voor een geslaagde uitkomst na transplantatie. Deze studie heeft de weg vrijgemaakt voor toekomstige *in vivo* experimenten, waarbij de homing-capaciteit van geëxpandeerde en niet-geëxpandeerde cellen naar het beenmerg wordt onderzocht.

In **hoofdstuk 7** wordt beschreven hoe het in **hoofdstuk 5** ontwikkelde laboratorium expansieprotocol voor het expanderen van stam- en voorlopercellen werd vertaald in een Good Manufacturing Practice (GMP)-expansieprotocol dat gebruikt kan worden voor transplantatie in patiënten met bloedkanker. Met het GMP-expansieprotocol werd een vergelijkbare expansie van stam- en voorlopercellen verkregen als het met laboratorium expansieprotocol als beschreven in **hoofdstuk 5**. Daarnaast beschrijven we de opzet van een klinische studie waarvan het doel is om de veiligheid van transplantatie van SR1-geëxpandeerde van stam- en voorlopercellen afkomstig van één navelstrengbloedeenheid te evalueren. In deze studie zullen 10 patiënten geïncludeerd worden.

Ten slotte worden in **hoofdstuk 8** van dit proefschrift de implicaties van onze bevindingen voor de toekomst beschreven. De zoektocht naar het optimale *ex vivo* expansie protocol gaat verder. Daarnaast zijn vroeg-klinische studies nodig om na te gaan of de huidige praktijk met dubbele navelstrengbloedtransplantaten verlaten kan worden in volwassen patiënten en transplantaties met enkele geëxpandeerde navelstrengbloed eenheden dezelfde, zo niet betere, klinische resultaten zullen geven. Tenslotte zal navelstrengbloed afgewogen dienen te worden tegen andere alternatieve stamcelbronnen zoals die van volwassen onverwante donoren of niet-passende (haplo-identieke) familiedonoren.

PUBLICATIONS IN THIS THESIS

1. **Duinhouwer LE**, ter Borg MND, Rombouts WJC, Cornelissen JJ, Braakman E. In vitro and in vivo evaluation of StemRegenin1-expanded UCB-derived hematopoietic stem and progenitor cells. *Submitted*

2. **Duinhouwer LE***, Beije N*, van der Holt B, Rijken-Schelen A, Lamers CH, Somers J, Braakman E, Cornelissen JJ. Impaired thymopoiesis predicts for a high risk of severe infections after double umbilical cord blood transplantation **.** *Submitted*. *Shared first authorship

3. Tüysüz N, **Duinhouwer LE**, Rombouts WJC, Kurek D, van Bloois L, van der Linden R, Mastrobattista E, Cornelissen JJ, Braakman E, ten Berge D. Wnt3a protein reduces the number of mouse phenotypic hematopoietic stem and progenitor cells in serum-free cultures. *Submitted*

4. **Duinhouwer LE**, van Rossum BJM, van Tiel ST, van der Werf RM, Kotek G, ter Borg M, Rombouts WJC, Braakman E, Cornelissen JJ, Bernsen MR. Magnetic Resonance Detection of CD34+ Cells from Umbilical Cord Clood Using a 19F Label. *PLoS ONE. 2015 Sep 22;10(9)*

5. **Duinhouwer LE**, Tüysüz N, Rombouts WJC, ter Borg MND, Spanholtz J, Mastrobattista E, Cornelissen JJ, ten Berge D, Braakman E. Wnt3a protein reduces growth factor-driven expansion of human hematopoietic stem and progenitor cells in serum-free cultures. *PLoS ONE. 2015 Mar 25;10(3)*

OTHER PUBLICATIONS

1. **Duinhouwer LE***, van Mil SR*, Mannaerts GH, Dunkelgrun M, Biter LU, Apers JA. The standardized postoperative checklist for bariatric surgery: what are the predictors of complications? *Submitted.* *Shared first authorship

2. **Duinhouwer LE**, Deerenberg E, Rociu E, Kortekaas RTJ. Herniation of the colon through the foramen of Winslow: a case report. *Int J Surg Case Rep. 2016 May 4;24:14-17*

3. **Duinhouwer LE**, Biter LU, Wijnhoven BP, Mannaerts GH. Treatment of giant hiatal hernia by laparoscopic Roux-en-Y gastric bypass. *Int J Surg Case Rep. 2015 Feb 19;9C:44-46*

4. Lodewyck T, Oudshoorn M, van der Holt B, Petersen E, Spierings E, von dem Borne PA, Schattenberg A, Allebes W, Groenendijk-Sijnke M, **Duinhouwer L**, Willemze R, Lowenberg B, Verdonck LF, Meijer E, Cornelissen JJ. Predictive impact of allele-matching and EBMT risk score for outcome after T-cell depleted unrelated donor transplantation in poor-risk acute leukemia and myelodysplasia. Leukemia. 2011 Oct;25(10):1548-54

PHD PORTFOLIO

Summary of PhD training and teaching activities

Name PhD Candidate:	Lucia E. Duinhouwer
PhD period:	September 2010 – November 2016
Erasmus MC department:	Hematology
Promotor:	prof. dr. J.J. Cornelissen
Co-promotor:	dr. E. Braakman
Research School:	Molecular Medicine

Activities	Year	ECTS
1. PHD TRAINING		
General academic/research skills		
Biomedical Research Techniques (MolMed)	2010	1.6
Basic and Translation Oncology (MolMed)	2010	1.6
Course on Molecular Medicine (MolMed)	2011	0.7
Laboratory Animal Science (Article 9 course; Erasmus MC)	2011	3
Workshop Presenting Skills for PhD students (MolMed)	2011	1
Research Management for PhD Students (MolMed)	2011	1
Workshop on Photoshop and Illustrator CS (MolMed)	2013	0.3
Research Integrity (Erasmus MC)	2013	2
In-depth courses		
Workshop Optical and Molecular Imaging (MolMed)	2013	0.6
2nd Scientific Workshop on Leukemic and Cancer Stem Cells, Mandelieu, France (EHA)	2013	1
National and international conferences		
Annual Dutch Hematology Congress (4x; Arnhem, the Netherlands)	2010-2013	4
Biannual World Cord Blood Congress (2x; Rome, Italy; Monaco, France)	2011, 2015	2
17th Annual European Hematology Association Congress (Amsterdam, the Netherlands)	2012	1
54th American Society of Hematology Meeting and Exposition (Atlanta, USA)	2012	1
Molecular Medicine Day (3x; Rotterdam, the Netherlands)	2011-2013	1

Scientific presentations		
Dutch Hematology Congress (2x; oral)	2012-2013	2
Top Institute Pharma Spring Meeting (poster)	2012	1
54th American Society of Hematology Meeting and Exposition (Atlanta, USA; poster)	2012	1
Scientific meetings		
Work discussions at the Department of Hematology, Erasmus MC	2010-2013	3
AIO/Post doc meetings at the Department of Hematology, Erasmus MC	2010-2013	2
Erasmus Hematology Lectures	2010-2013	2
2. TEACHING ACTIVITIES		
Co-supervising Master of Science student 'Clinical Research' (52 weeks)	2012-2013	7.5
Medical student VO Bone marrow and Leukemia (2x)	2012-2013	0.5
Total	**40.8**	

CURRICULUM VITAE

Lucia Duinhouwer werd op 18 april 1988 geboren in Rotterdam en bracht haar gelukkige jeugd door onder de rook van deze prachtige stad. In 2006 behaalde zij cum laude haar gymnasiumdiploma aan het Einstein Lyceum te Hoogvliet Rotterdam. In datzelfde jaar begon zij aan de studie Geneeskunde aan de Erasmus Universiteit Rotterdam. Zij behaalde haar doctoraal diploma in augustus 2010 en startte aansluitend met een promotietraject op de afdeling Hematologie onder leiding van dr. E. Braakman en prof. dr. J.J. Cornelissen, waarvan de resultaten staan beschreven in dit proefschrift. In oktober 2013 pakte zij haar studie Geneeskunde weer op en startte met haar coschappen. In november 2015 behaalde zij cum laude haar artsexamen, waarop zij als ANIOS Chirurgie aan de slag ging in het IJsselland Ziekenhuis te Capelle aan den IJssel (dr. I. Dawson). Op 1 januari 2017 start zij met haar opleiding tot chirurg in de regio Rotterdam (dr. B. Wijnhoven).

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