Chapter 3:

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

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Transfusion 2015, 55: 1772–1781

Abstract

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

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

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

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

Introduction

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

Materials and Methods

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

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

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

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

Clonogenic assays. To determine the clonogenic capacity of the cells, progenitor cell (CFU-GEMM) and megakaryocyte colony forming unit (CFU-Mk) assays were performed. The CFU-GEMM assay was performed as described by the manufacturer. In short, $2.7x10^4$ BM-WBC (bone marrow-white blood cells), $0.32x10^4$ $CD34^+$ or TPO expanded cells were added to Methocult™ (StemCell Technologies Inc.) in a final volume of 1.5 ml. 1.1 ml of the Methocult™ cell suspension was dispensed in 35 mm dishes (Greiner, Alphen a/d Rijn, The Netherlands) and cultured for 14 days at 37°C, 5% CO_2 in a humidified atmosphere (>95%). Each sample was tested in duplicate. After 14 days, the number of granulocyte, erythroid and monocyte colonies in the culture dishes was counted manually. The CFU-Mk assay was performed in MegaCult™-C medium with cytokines (StemCell Technologies Inc.). The assay was performed as described by the manufacturer. In short, $2x10^5$ BM-WBC cells or $0.25x10^4$ $CD34^+$ or TPO expanded cells per 3.3 ml were cultured in a chamber slide (StemCell Technologies Inc.) for 10 days at 37°C, 5% CO_2 in a humidified atmosphere (>95%). After 10 days, the number of megakaryocyte colonies in each well of the chamber slide was counted manually.

Expression of adhesion markers and migration of the cells cryopreserved before and after expansion. Expression of adhesion markers (CD11a-b, CD49d-e, CD62L) and the stem cell homing marker CD184 (CXCR4) on the three subpopulations was determined by flow cytometry in combination with CD61-PC7 and CD34-PC5 antibodies (all Beckman Coulter). Migration experiments were performed in transwell plates (Costar, Amsterdam, the Netherlands), with 5 μm pore filters. Before adding the cell suspensions, the lower compartment of the well and the bottom of the filter were coated for 15 minutes at 37°C with 2 ng/ml fibronectin (Sigma). $1x10^5$ TPO expanded cells were added to the upper compartment. The lower chamber contained media with or without human rCXCL12 (SDF-1α, 100 ng/ml; Sigma). Plates were incubated for 5 hours at 37ºC and 5% CO_2 in a humidified incubator, before harvesting the migrated cells from the lower chamber. Before and after incubation, all cells were analyzed by flow cytometry for the expression of CD45, CD34 and CD61 and cell number calculated in the presence of flow count beads (all from Beckman Coulter). The percentage of migrating cells was calculated by dividing the number of cells from the lower compartment by the number of cells that were added to the upper compartment.

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

Results

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

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

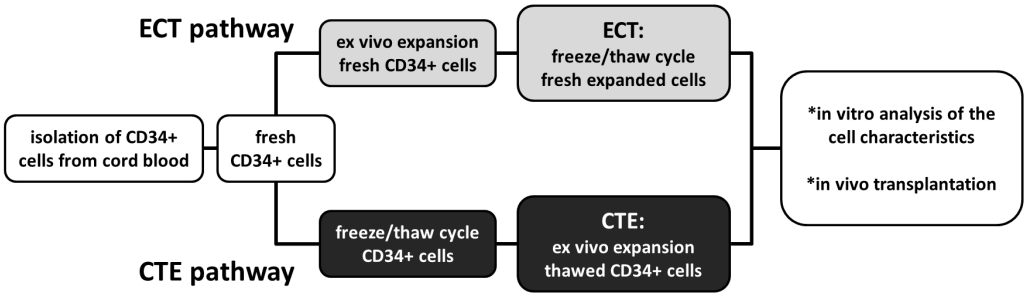


Figure 1: Flow chart of the experimental design. *Human $CD34^+$ cells were isolated from fresh CB and split in two fractions. The $CD34^+$ cells were either directly expanded with TPO for 10 days and cryopreserved (ECT pathway), or the $CD34^+$ cells were cryopreserved directly after isolation and TPO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

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

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

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

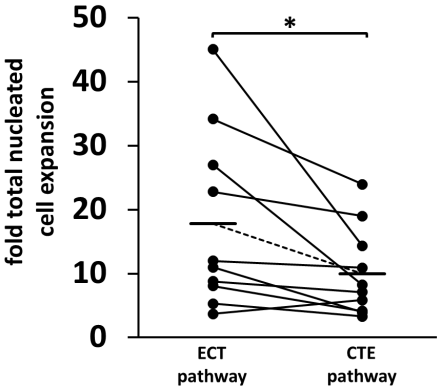


Figure 2: Fold total nucleated cell expansion comparing the ECT and CTE strategies. *Human CB CD34⁺ cells were isolated and ex vivo expanded for 10 days with TPO directly (ECT) or after cryopreservation (CTE) (n=10). Fold expansion after 10 days. Although variation in the fold expansion was observed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

Clonogenic progenitor content of TPO expanded cells

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

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

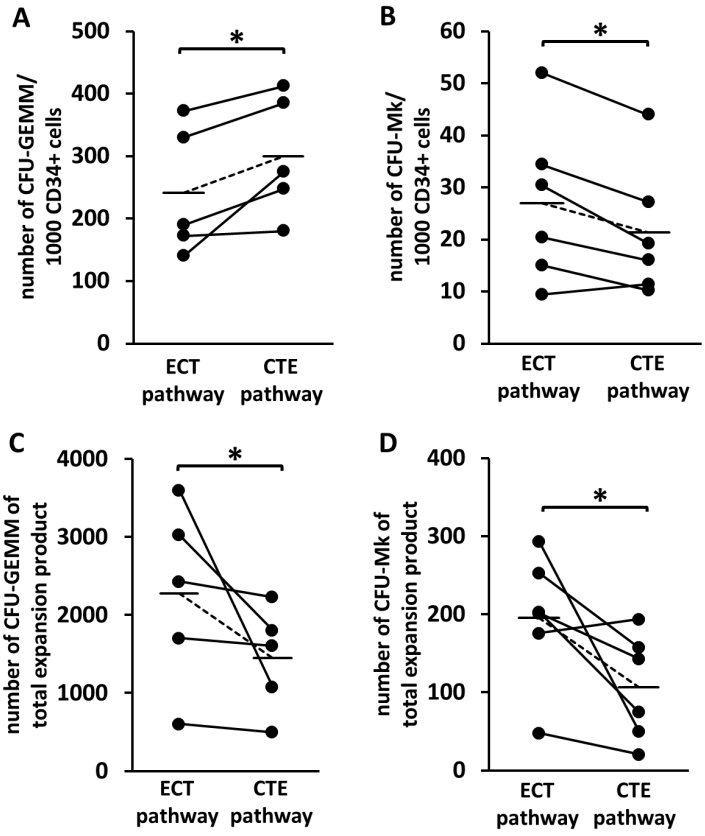


Figure 3: CFU-GEMM and CFU-Mk cultures from cells generated by the ECT vs. the CTE strategy. *Fresh human CB CD34$^+$ cells were TPO expanded and subsequently cryopreserved and then thawed (ECT), or were first cryopreserved and then expanded with TPO after thawing (CTE). Shown is the number of CFU-GEMM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

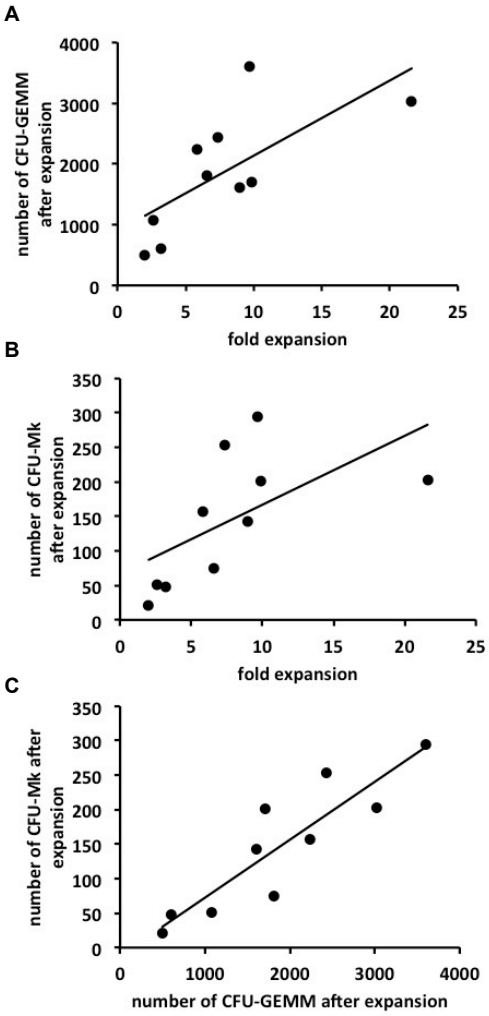


Figure 4. Correlation between fold expansion and formation of CFU-GEMM and CFU-Mk colonies from cells generated by the ECT vs. the CTE strategy. *The absolute number of CFU-GEMM* ***(A)*** *and CFU-Mk* ***(B)*** *in the total expansion product correlated significantly with the fold expansion in the presence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

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

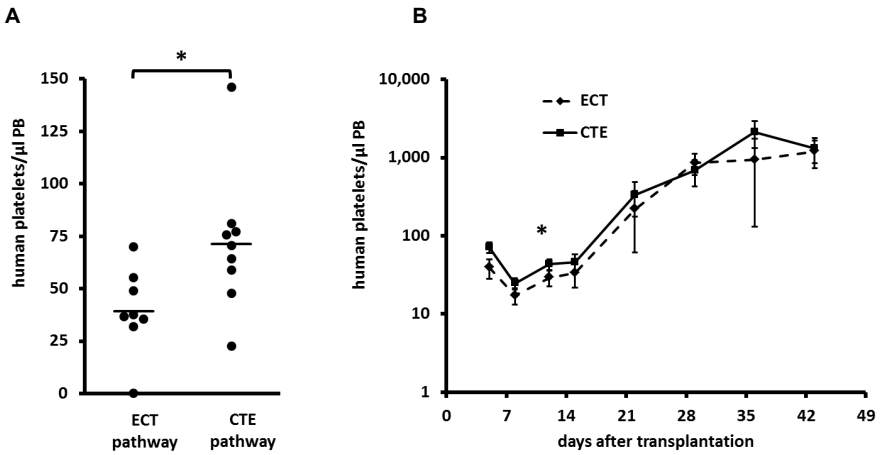
The $CD34^+$ cells of three different CB units were expanded via the ECT and CTE pathway and subsequently transplanted into NOD SCID mice (n=3 mice per group). Since the ECT pathway yields higher expansion numbers than the CTE pathway, the absolute cell numbers transplanted in NOD SCID mice were higher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

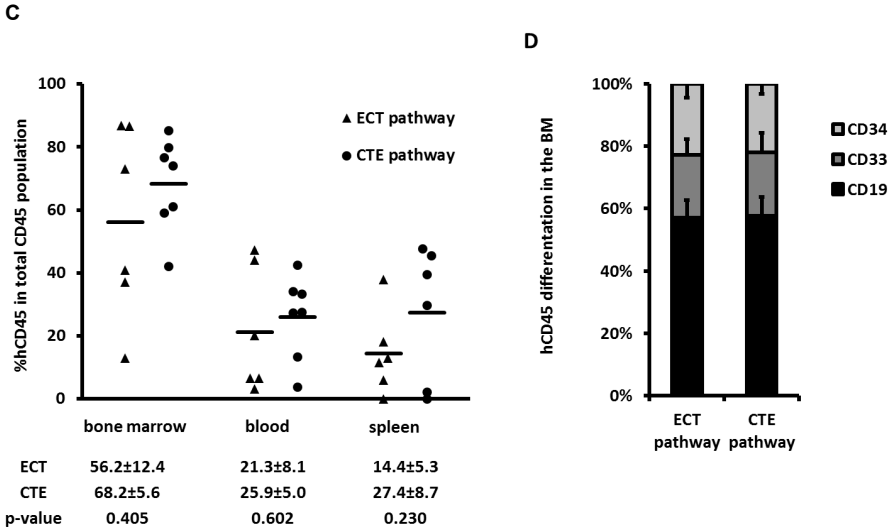
Adhesion marker expression and migratory capacity of cells expanded via the ECT or the CTE pathway

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

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

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





	bone marrow	blood	spleen
ECT	56.2±12.4	21.3±8.1	14.4±5.3
CTE	68.2±5.6	25.9±5.0	27.4±8.7
p-value	0.405	0.602	0.230

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

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

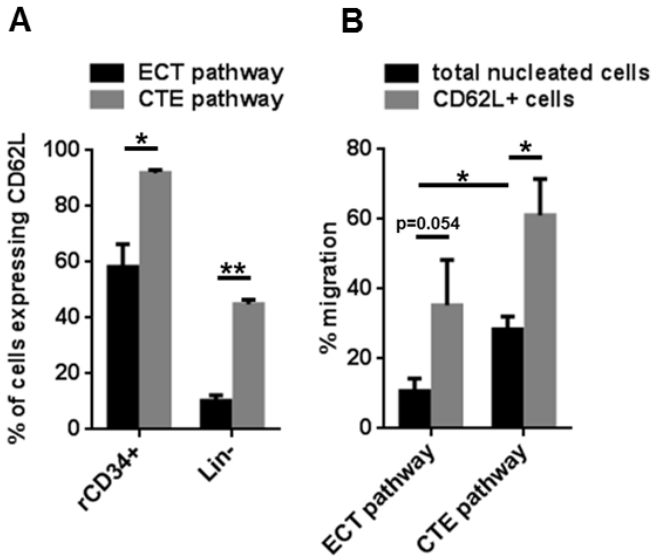


Figure 6: CD62L expression of the expanded cells (A) and percentage of cells that migrated towards an SDF-1α gradient in a trans-well plate (B). A: *Percentage of cells that express CD62L of the cell population responsible for bone marrow engraftment (rCD34+) and the population responsible for early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

Discussion

Expansion of CB CD34+ cells with TPO accelerates platelet recovery in vivo without hampering bone marrow engraftment at 6 weeks in immunodeficient mice.[8-11,23]. Anticipating clinical application, pre- or post-cryopreservation TPO expansion of hematopoietic stem/progenitor cells might influence subsequent in vivo platelet recovery and hematological reconstitution, an issue that has not been investigated so far. We compared the effect of TPO-expansion pre- versus post-cryopreservation of CB CD34+ cells in vitro and in the NOD SCID mice model.

The results show that TNC (total nucleated cell) expansion with TPO is higher when freshly isolated CB CD34+ cells are cultured for 10 days and subsequently cryopreserved (ECT), as compared to CD34+ cells from the same donor expanded after cryopreservation and thawing (CTE). Although the absolute number of rCD34+ cells on average was higher after the CTE protocol, the Lin- cell subset, which has been shown to correlate best with early platelet recovery in vivo in NOD SCID mice[8] was more abundant using the ECT protocol. Surprisingly, and in contrast to the higher expansion and the higher putative platelet precursor numbers observed after the ECT protocol, mice that received CTE cells had a significantly higher number of platelets early after transplantation. This was not at the expense of bone marrow engraftment, which tended also to be higher with the CTE; albeit engraftment as well as the lineage differentiation of the engrafted human cells in the mouse bone marrow at six weeks after transplantation in this respect was not significantly different between the two strategies. As mentioned, compromised viability after thawing of ECT cells is an unlikely explanation for the results as viability of all subsets, including the Lin- cell recovery, exceeded 75%.

However, CD34+ cells are heterogeneous[24] and more extensive studies are required to identify phenotypically the rCD34+ cells after TPO expansion from the non-TPO exposed CD34+ cells with respect to their sensitivity to cryopreservation.

An alternative explanation for the discrepancy between the quantitative in vitro and qualitative in vivo results between the two protocols might be found in the capacity of the cells to home to and engraft in specific bone marrow niches. Migration towards an SDF-1a gradient is a surrogate marker for this process. Additionally, recent studies by Niswander et al.[25,26] demonstrate an additive effect of SDF-1 after TPO treatment in vivo and implicate the SDF-1α in the movement of platelet progenitor cells to the bone marrow vasculature, where they can then promote a resulting increase in circulating platelet numbers. Indeed, we observed that a higher percentage of cells from the CTE pathway migrated towards SDF-1α. The higher CD62L expression, an important migration and homing receptor [27-29] on both rCD34+ and Lin- cells from the CTE pathway, may play a role in this. Indeed, CD62L expression has been correlated to speed of hematopoietic recovery,[30] and in particular with platelet recovery.[31] In this regard, the lower migration towards an SDF-1α gradient of the CD62L- population we observed seems compatible with previous reports. The lower number of CD62L expressing cells derived from the ECT pathway might in this regard explain why fewer cells from the ECT pathway are able to migrate towards or within the bone marrow. However, admittedly CD62L expression is unlikely to be the only explanation for the difference in the in vivo results between the ECT and the CTE pathway. Migration of CD62L+ cells derived from the CTE pathway, whether rCD34+ or Lin- was higher when compared to cells derived from the ECT pathway, indicating that other factors play a role in the migration as well. Important in this respect, no significant differences could be found in the expression of the SDF-1α receptor, CXCR4 (CD184), which was used as the chemo-attractant in these experiments,

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

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