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Chapter 3

Transplantation of human peripheral blood CD34-positive cells in combination with *ex vivo* generated megakaryocytes results in fast platelet formation

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Letter to the editor

Many types of cancers are currently treated with high-dose chemotherapy, which affects all (rapidly) dividing cells, including hematopoietic progenitor cells (HPCs). Therefore, even after stem cell transplantation, patients often suffer from a period of pancytopenia. Anemia and leukopenia, in this respect, can be successfully treated with Epo and G-CSF, respectively, by inducing a faster recovery of hemoglobin and neutrophil levels. Several attempts have been undertaken to shorten the period of thrombocytopenia by co-transplantation of autologous megakaryocytes (MKs) expanded from $CD34^+$ HPCs, but the effect of this treatment was variable and if present, minimal.[1,2,3,4] However, the MK expansion protocols used in these clinical trials vary considerably and optimization of the composition of the transplant may therefore increase the clinical benefit of co-transplantation of expanded MKs. We therefore investigated whether MKs generated from mobilized peripheral blood (MPB) $CD34^+$ could induce fast platelet production when combined with unmanipulated $CD34^+$ cells after transplantation in the NOD/SCID mouse. For this purpose, $CD34^+$ cells were cultured for 7 days with 100 ng/ml thrombopoietin (Tpo) and 10 ng/ml interleukin-1β (IL-1β), a cytokine cocktail, previously shown to efficiently generate MK progenitors and mature MKs (up to 32N) in our lab.[5]

The NOD/SCID mouse model has long been accepted as a model for human hematopoiesis.[6] Transplantation of human MPB $CD34^+$ cells can result in high levels of engraftment and differentiation along multiple lineages in the bone marrow (BM) of NOD/SCID mice.[7] In two previous studies in which human platelet formation after transplantation of mobilized MPB $CD34^+$ cells in NOD/SCID mice was investigated, the number of cells transplanted was 0.6-1 $x10^6$ per mouse.[8,9] Transplantation of such numbers of $CD34^+$ cells resulted in a variable and relatively low level (<10%) of human $CD45^+$ cells in the BM for most mice. Since the number of transplanted MPB $CD34^+$ cells has been shown to correlate with the observed chimerism in the mouse BM and blood,[7] we decided to transplant at least 2.25 $x10^6$ unmanipulated MPB $CD34^+$ cells in the groups that received combined grafts, to facilitate accurate measurement of human platelet formation.

Our 7-day culture protocol with Tpo and IL-1ß yielded an average fold increase in cell number of 6.6 (see supplementary information for full description of Materials and Methods). The average cell size was 12.5 µm. After culture 24% of the cells was $CD34^+CD41^-$, 22% was $CD34^+CD41^+$, and 28% was $CD34^-CD41^+$.

Sublethally irradiated (3.5 Gy) NOD/SCID mice were intravenously transplanted with grafts composed of different combinations of uncultured and/or cultured cells (Table 1). The kinetics of human platelet formation from day 3 till day 28 after transplantation is shown in Figure 1A. The absolute number of human platelets in mouse blood was determined with a flow cytometric assay. By titration of human platelets in mouse blood we established a detection threshold of 1 $x10^4$ human platelets/ml of blood (data not shown). Three mice that were irradiated but not transplanted served as internal controls.

Three days after transplantation, human platelets were detected in all groups that received grafts containing all the cells from the megakaryocyte cultures that were initiated with at least 2.25 $x10^6$ $CD34^+$ cells (= 50%). The blood of mice from group 3 (receiving 100% uncultured cells plus 100% expanded cells), group 4 (50% uncultured plus 50% expanded cells) and group 5 (100% expanded) contained a

Group	Cells	Cell numbers (NC + NC input culture/mouse)	Graft composition	Number of cells transplanted ($x10^6$/mouse)			Mice (n)
				$CD34^+CD41^-$	$CD34^+CD41^+$	$CD34^-CD41^+$	
0	Control	-	-	-	-	-	6
1	MPB $CD34^+$	**4.5 $x10^6$**	**100%** uncultured	4.3	0.16	<0.01	10
2	MPB $CD34^+$ + expanded $CD34^+$	**4.05 $x10^6$** + **0.45 $x10^6$**	**90%** uncultured + **10%** expanded	3.9 + 0.71	0.14 + 0.65	<0.01 + 0.83	10
3	MPB $CD34^+$ + expanded $CD34^+$	**4.5 $x10^6$** + **4.5 $x10^6$**	**100%** uncultured + **100%** expanded	4.3 + 7.1	0.16 + 6.5	<0.01 + 8.3	10
4	MPB $CD34^+$ + expanded $CD34^+$	**2.25 $x10^6$** + **2.25 $x10^6$**	**50%** uncultured + **50%** expanded	2.2 + 3.6	0.08 + 3.3	<0.01 + 4.2	10
5	expanded $CD34^+$	**4.5 $x10^6$**	**100%** expanded	7.1	6.5	8.3	10

MPB = Mobilized peripheral blood. NC = Nucleated cell.

Table 1: Grafts consisting of combinations of uncultured and/or 7-day expanded $CD34^+$ cells

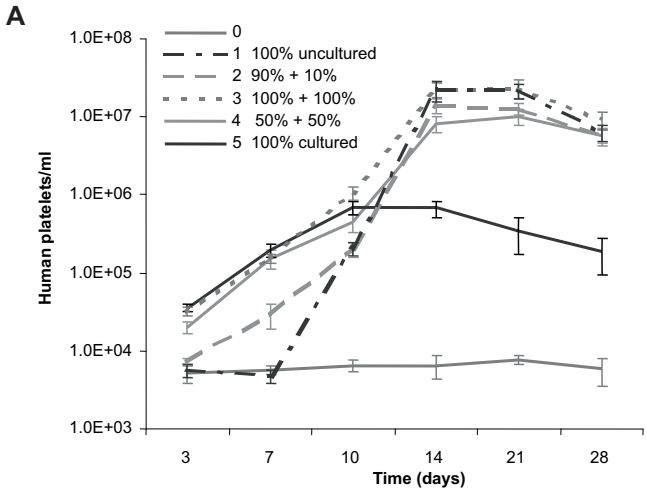
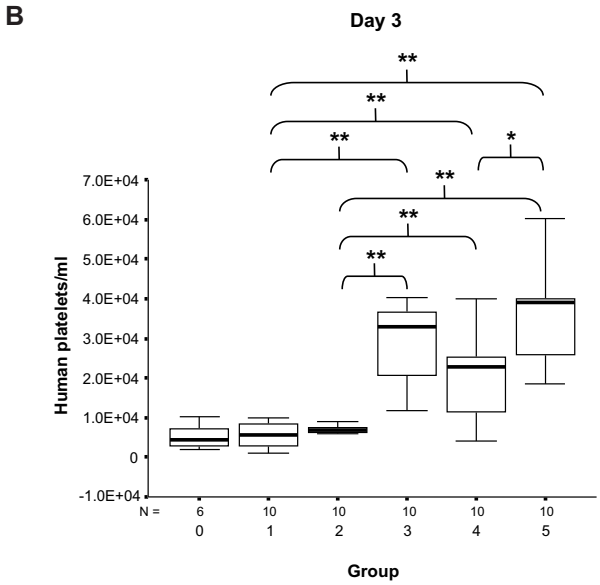


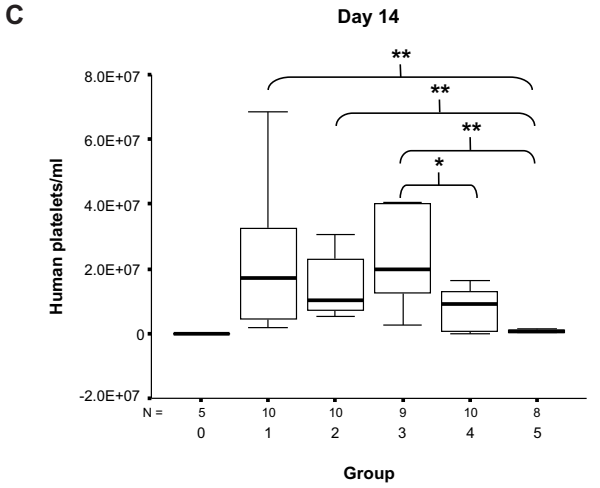
Figure 1: Human platelet formation in NOD/SCID mice after transplantation of grafts containing uncultured cells and/or cultured MKs

A. Graph showing the average number of human platelets detected per ml of mouse blood ± SEM. At day 3 and 7 after transplantation human platelets were only detected in the blood of mice receiving cultured cells. In the blood of mice receiving only uncultured cells (group 1) human platelets were detected from day 10 on. **B.** Box plot showing the number of human platelets per ml of mouse blood at day 3. The mice of group 1 and 2 did not show detectable numbers of human platelets. All mice receiving more than 10% expanded megakaryocytes (group 3, 4 and 5) showed significantly higher human platelet levels than did mice of group 1 and 2. **C.** Box plot depicting human platelet numbers at day 14. Mice that received only expanded MKs (group 5) showed significantly lower human platelet numbers per ml blood than mice from group 1, 2 and 3.

The statistical differences in platelet formation between groups were calculated with the non-parametric Wilcoxon-Mann-Whitney test. Differences were considered significant when $p<0.05$. *; $p<0.05$, **; $p<0.01$

median number of human platelets per ml of 3.3 x10^4, 2.3 x10^4, and 3.9 x10^4, respectively (Figure 1B). The difference between these groups and group 1 (100% uncultured) and group 2 (90% uncultured and 10% expanded), was significant. The median number of platelets between group 4 (50% uncultured plus 50% expanded cells) and group 5 (100% expanded cells) was significantly different at this time point as well (Figure 1B). At day 7 human platelets were also detected in the blood of mice from group 2 receiving MKs generated from 0.45 x10^6 $CD34^+$ cells (=10%; Figure 1A). Group 1 did not show human platelet formation at this point. At day 10 after transplantation and onwards, human platelets were also detected in the blood of the mice from this group. This indicates that between day 7 and 10 the transplanted HPCs had differentiated into MKs and started to produce platelets. After day 14, there was no difference in the number of human platelets detected between mice receiving at least 4.05 x10^6 uncultured $CD34^+$ cells, i.e. groups 1, 2, and 3 (median of 1.7 x10^7, 1.0 x10^7, and 2.0 x10^7/ml, respectively; Figure 1C). At this time, a small difference between the group receiving 4.5 x10^6 uncultured $CD34^+$ cells plus the MKs cultured





from 4.5 x10^6 $CD34^+$ cells (group 3) and the group receiving the graft containing 2.25 x10^6 uncultured $CD34^+$ cells plus MKs cultured from 2.25 x10^6 $CD34^+$ cells (group 4; median of 9.2 x10^6/ml) was still detected. However, at day 21 the levels of platelets detected in the mice of these groups were not statistically significant different (median of 2.0 x10^7/ml and 1.1 x10^7/ml, respectively). The mice in group 5, receiving grafts composed of only cultured cells, showed significantly lower levels of human platelets at day 14 (median of 6.0 x10^5/ml) than the mice from groups 1, 2, and 3 (Figure 1C), and this number declined thereafter. Group 4 did not show significant more human platelets at day 14 after transplantation, but from day 21 the difference between group 4 and 5 was significant.

Functionality of the human platelets produced in the NOD/SCID mice was tested by incubation with thrombin-receptor-activating peptide (TRAP-6). The resulting

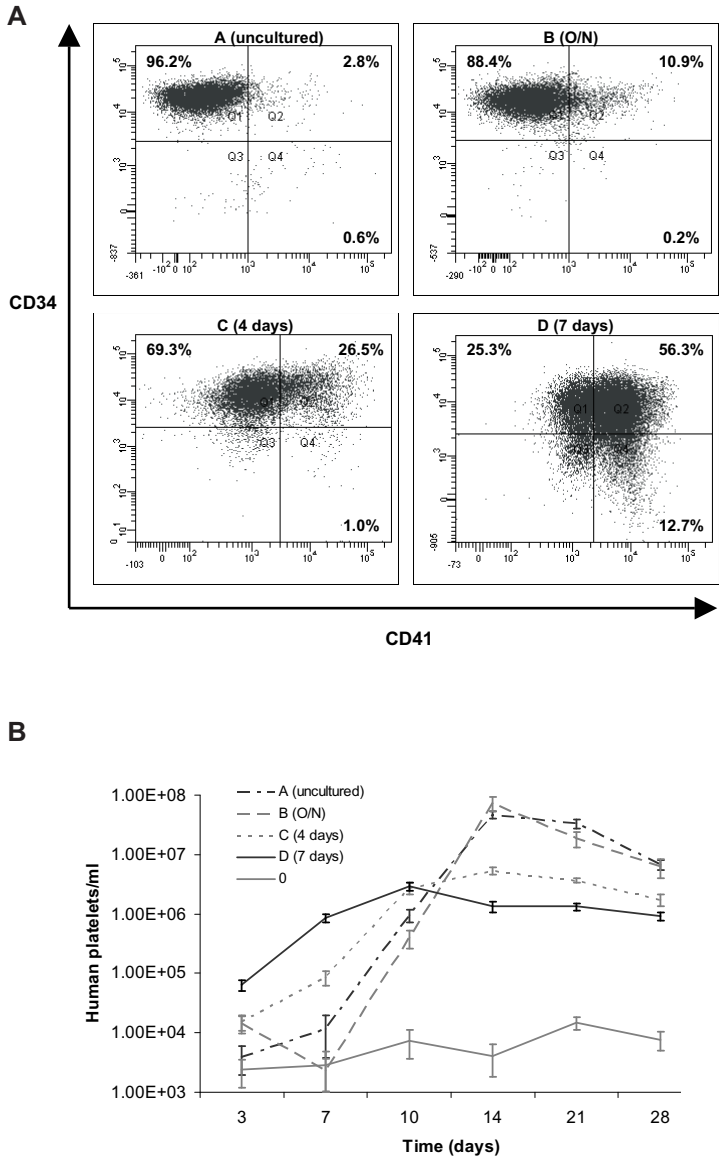


Figure 2: Human platelet formation in NOD/SCID mice after transplantation of uncultured cells or cells cultured for different periods

A. Flow cytometric analysis of CD34 and CD41 expression of the uncultured cells and cells cultured O/N, 4 days or 7 days. The expression of CD41 increased with culture time, and after 7 days a population of $CD34^{-}CD41^{+}$ cells could be observed.

B. Graph showing the mean ± SEM number of human platelets detected per ml of mouse blood. This graph demonstrates that *in vivo* human platelet formation was delayed when cells were cultured for a period shorter than 7 days.

clear increase in human CD62P surface expression indicated that the human platelets formed in NOD/SCID mice, transplanted with cultured or uncultured cells, were functional (supplementary information Figure 1).

Other groups have investigated the ability of MKs generated from cord blood (CB) $CD34^+$ cells to form platelets in NOD/SCID mice.[10,11,12,13] These four CB studies showed an advantage of transplantation of cultured CB $CD34^+$ cells compared to transplantation of an unmanipulated graft with respect to *in vivo* platelet formation. In a study in which MPB $CD34^+$ cell-derived MKs were transplanted, delayed and transient platelet formation was observed in NOD/SCID mice,[9] while in our study, human platelets were already detectable three days after transplantation of *ex vivo* generated MKs.

It might be argued that addition of cultured cells can have a negative effect on the formation of other blood cells than platelets. However, our results show that co-transplantation of expanded megakaryocytes did not significantly alter the extent of human engraftment or the lineage distribution at 5 weeks post-transplant in the mouse BM, spleen or blood (supplementary information Figure 2). Besides, in the clinical trials with *ex vivo* generated MKs published so far, only positive effects on neutrophil recovery and no negative effect on other blood cell lineages were observed.[1,2,3,4] Together, these data indicate that the addition of *ex vivo* generated MKs to the transplant does not influence the engraftment potential, engraftment pattern or the differentiation capacity of the unmanipulated $CD34^+$ cells in various mouse organs.

To investigate whether similar results of short-term platelet formation in NOD/SCID mice can be obtained with a shorter culture period, cells were cultured overnight, 4 days or 7 days in the presence of 100 ng/ml Tpo and 10 ng/ml IL-1ß, to obtain different MK (progenitor) populations (Figure 2A). In the O/N culture, cells were lost (proliferation factor of 0.77), but the percentage of $CD34^+CD41^+$ cells increased from 2.8% in the uncultured population to 10.9% after O/N culture. After 4 days, cell numbers had increased 1.9-fold, and 26.5% of the cell population consisted of $CD34^+CD41^+$ cells. After 7 days of culture, cells had expanded 7.9-fold. The percentage of $CD34^+CD41^+$ cells at that time was 56.3%, and in this culture a significant proportion of the cells was $CD34^-CD41^+$ (12.7%). The cells from these cultures were harvested and subsequently intravenously transplanted in sublethally irradiated NOD/SCID mice according to the dosing scheme shown in Table 2. Human platelet formation was again monitored for several weeks. Three days after transplantation, more human platelets were detected in the blood of mice of group D (7 day culture) as compared to the other groups (Figure 2B). The average platelet numbers in group A through C were 0.39 $x10^4$, 1.4 $x10^4$, and 1.5 $x10^4$ per ml, respectively, while it was 6.4 $x10^4$/ml in group D. This difference still existed at day 7 after transplantation. It should be noted that at this time group C (4 day culture) had a mean platelet number of 8.5 $x10^4$/ml, which is comparable to the number detected in group D after 3 days (6.4 $x10^4$/ml). Together, these results show that a shorter culture period results in delayed platelet formation. This implies that pre-differentiation of $CD34^+$ cells toward the megakaryocyte lineage for at least 7 days is necessary to achieve rapid *in vivo* platelet formation.

However, since the cell numbers in the graft were based on input numbers in the culture, the total number of cells transplanted varied for each group (Table 2), meaning that the total cell number transplanted was higher for groups receiving

Table 2: Grafts consisting of uncultured CD34$^+$ cells or CD34$^+$ cells cultured for different periods

			Number of cells transplanted ($x10^6$/mouse)			
Group	Cells	Cell numbers (NC or NC input culture/mouse)	CD34$^+$CD41$^-$	CD34$^+$CD41$^+$	CD34$^-$CD41$^+$	Mice (n)
0	Control	-	-	-	-	3
A	MPB CD34$^+$	**4.5 x10^6**	4.3	0.13	0.03	5
B	MPB CD34$^+$ cultured O/N	**4.5 x10^6**	3.1	0.38	<0.01	5
C	MPB CD34$^+$ cultured for 4 days	**4.5 x10^6**	5.9	2.3	0.09	4
D	MPB CD34$^+$ cultured for 7 days	**4.5 x10^6**	9.0	20.0	4.5	5

MPB = Mobilized peripheral blood. NC = Nucleated cell. O/N = overnight.

cells cultured for a longer period. However, the kinetics of platelet formation in the different groups of mice were comparable, implying that *in vivo* proliferation and differentiation compensates for the difference in culture time.

Five weeks after transplantation, the mice were sacrificed. The average percentage of human engraftment in the bone marrow was 56.4%, 33.5%, 15.3%, and 8.7% for group A through D, respectively (supplementary information Figure 3). Thus, the percentage of CD45$^+$ cells in the BM of the mice decreased with the culture time of the HPCs. Since equal numbers of input cells in the culture were transplanted, the mice in group C and D received more CD34$^+$ cells than the mice in group A and B (Table 2). The mice in group D received 6 times more CD34$^+$ cells than the mice in group A (29.9 x10^6 in group D versus 4.5 x10^6 in group A), but showed a more than six-fold decrease of human cells in the bone marrow 5 weeks after transplantation (8.7% in group D versus 56.4% in group A). This clearly demonstrates that the cultured cells are less effective in providing long-term engraftment. Similar to the above described transplantation experiments, the lineage distribution was not influenced by the difference in culture time (CD34$^+$ see supplementary information Figure 3; CD38$^+$, CD16$^+$, CD4$^+$CD8$^+$, CD19$^+$, and CD20$^+$; data not shown).

The observation that the engraftment potential decreased with the culture time, is in contrast with data obtained with CB CD34$^+$ cells expanded for 10 days in the presence of Tpo.[13] In that study, the total number of CD34$^+$ cells transplanted was comparable between the non-expanded and the expanded grafts and the percentage of CD45$^+$ cells in the BM after 6 weeks was slightly higher for the expanded graft compared to the non-expanded graft. The discrepancy with our results may be explained by differences in proliferation and maturation responses of CB and MPB HPCs.[14,15] Furthermore, in support of our data, it has been suggested that cell surface phenotype might not correlate with engraftment potential after culture.[16]

A decade ago, the first feasibility study with megakaryocytes generated *ex vivo* by a 7-day culture protocol with 20 ng/ml Macrophage Inflammatory Protein-1α (MIP-1α) and FLT-ligand, and 10 ng/ml Interleukin-3 (IL-3), IL-6, IL-11, Stem Cell Factor, and Megakaryocyte Growth and Development Factor (recombinant Tpo), demonstrated that *ex vivo* generated MKs can be administered safely and might

improve platelet recovery.[1] Less than 10% of the leukapheresis material was used for MK generation. Another clinical study with 10 lymphoma patients showed no positive effect of the addition of megakaryocytes, although higher $CD61^+$ cell numbers were infused.[2] In that study, however, cells were cultured for 10 days in the presence of MGDF and SCF. The longer culture period in this study might well have resulted in more mature, larger, and thus possibly more fragile MKs, which might therefore show less efficient homing and platelet production *in vivo*. In our study, more than 10% of the graft needed to be expanded and differentiated towards megakaryocytes to be able to detect platelets as early as three days after transplantation.

In terms of feasibility; in most autologous transplantation settings a graft of 4 $x10^6$ $CD34^+$ cells/kg is considered sufficient for full hematopoietic recovery and is normally easily harvested in one leukapheresis procedure. If another portion of 4 $x10^6$ $CD34^+$ cells/kg would be cultured in our 7-day expansion protocol, between 12 $x10^6$ and 18 $x10^6$ $CD41^+$ cells/kg would have to be co-transplanted, rendering our culture protocol clinically applicable.

To our knowledge, we are the first to demonstrate that MKs generated from MPB $CD34^+$ cells are able to significantly increase human platelet formation in NOD/SCID mice within the first 10 days following transplantation. The results of this study warrant new clinical trials on the use of *ex vivo* generated MKs to shorten the period of thrombocytopenia after stem cell transplantation.

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Reference list

1 Bertolini F, Battaglia M, Pedrazzoli P, Da Prada GA, Lanza A, Soligo D, Caneva L, Sarina B, Murphy S, Thomas T, della Cuna GR. Megakaryocytic progenitors can be generated ex vivo and safely administered to autologous peripheral blood progenitor cell transplant recipients. *Blood* 1997; **89**: 2679-2688.

2 Decaudin D, Vantelon JM, Bourhis JH, Farace F, Bonnet ML, Guillier M, Greissenger N, Marracho MC, Assari S, Bennaceur AL, Nemati F, Michon J, Turhan AG, Boccaccio C. Ex vivo expansion of megakaryocyte precursor cells in autologous stem cell transplantation for relapsed malignant lymphoma. *Bone Marrow Transplant* 2004; **34**: 1089-1093.

3 Prince HM, Simmons PJ, Whitty G, Wall DP, Barber L, Toner GC, Seymour JF, Richardson G, Mrongovius R, Haylock DN. Improved haematopoietic recovery following transplantation with ex vivo-expanded mobilized blood cells. *Br J Haematol* 2004; **126**: 536-545.

4 Scheding S, Bergmannn M, Rathke G, Vogel W, Brugger W, Kanz L. Additional transplantation of ex vivo generated megakaryocytic cells after high-dose chemotherapy. *Haematologica* 2004; **89**: 630-631.

5 van den Oudenrijn S, de Haas M, Calafat J, van der Schoot CE, von dem Borne AE. A combination of megakaryocyte growth and development factor and interleukin-1 is sufficient to culture large numbers of megakaryocytic progenitors and megakaryocytes for transfusion purposes. *Br J Haematol* 1999; **106**: 553-563.

6 Lapidot T, Fajerman Y, Kollet O. Immune-deficient SCID and NOD/SCID mice models as functional assays for studying normal and malignant human hematopoiesis. *J Mol Med* 1997; **75**: 664-673.

7 van der Loo JC, Hanenberg H, Cooper RJ, Luo FY, Lazaridis EN, Williams DA. Nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mouse as a model system to study the engraftment and mobilization of human peripheral blood stem cells. *Blood* 1998; **92**: 2556-2570.
8 Angelopoulou MK, Rinder H, Wang C, Burtness B, Cooper DL, Krause DS. A preclinical xenotransplantation animal model to assess human hematopoietic stem cell engraftment. *Transfusion* 2004; **44**: 555-566.
9 Perez LE, Rinder HM, Wang C, Tracey JB, Maun N, Krause DS. Xenotransplantation of immunodeficient mice with mobilized human blood CD34+ cells provides an in vivo model for human megakaryocytopoiesis and platelet production. *Blood* 2001; **97**: 1635-1643.
10 Bruno S, Gunetti M, Gammaitoni L, Dane A, Cavalloni G, Sanavio F, Fagioli F, Aglietta M, Piacibello W. In vitro and in vivo megakaryocyte differentiation of fresh and ex-vivo expanded cord blood cells: rapid and transient megakaryocyte reconstitution. *Haematologica* 2003; **88**: 379-387.
11 Bruno S, Gunetti M, Gammaitoni L, Perissinotto E, Caione L, Sanavio F, Fagioli F, Aglietta M, Piacibello W. Fast but durable megakaryocyte repopulation and platelet production in NOD/SCID mice transplanted with ex-vivo expanded human cord blood CD34+ cells. *Stem Cells* 2004; **22**: 135-143.
12 Feng Y, Zhang L, Xiao ZJ, Li B, Liu B, Fan CG, Yuan XF, Han ZC. An effective and simple expansion system for megakaryocyte progenitor cells using a combination of heparin with thrombopoietin and interleukin-11. *Exp Hematol* 2005; **33**: 1537-1543.
13 van Hensbergen Y, Schipper LF, Brand A, Slot MC, Welling M, Nauta AJ, Fibbe WE. Ex vivo culture of human CD34+ cord blood cells with thrombopoietin (TPO) accelerates platelet engraftment in a NOD/SCID mouse model. *Exp Hematol* 2006; **34**: 943-950.
14 Schipper LF, Brand A, Reniers N, Melief CJ, Willemze R, Fibbe WE. Differential maturation of megakaryocyte progenitor cells from cord blood and mobilized peripheral blood. *Exp Hematol* 2003; **31**: 324-330.
15 van den Oudenrijn S, von dem Borne AE, de Haas M. Differences in megakaryocyte expansion potential between CD34(+) stem cells derived from cord blood, peripheral blood, and bone marrow from adults and children. *Exp Hematol* 2000; **28**: 1054-1061.
16 Dorrell C, Gan OI, Pereira DS, Hawley RG, Dick JE. Expansion of human cord blood CD34(+)CD38(-) cells in ex vivo culture during retroviral transduction without a corresponding increase in SCID repopulating cell (SRC) frequency: dissociation of SRC phenotype and function. *Blood* 2000; **95**: 102-110.

Supplementary information

Materials and methods

Human cells

Peripheral blood stem cells were obtained (with approval of the Ethical committee of our hospital and after informed consent) from leukapheresis material of healthy donors treated with G-CSF (2 x 5 µg/kg/day subcutaneously; Filgastrim, Amgen, CA, USA) or from patients treated with chemotherapy and G-CSF (5-10 µg/kg/day). Material from patients diagnosed with Hodgkin's lymphoma, Non-Hodgkin's lymphoma, multiple myeloma, Ewing's sarcoma, breast cancer, Crohn's disease, mantle cell lymphoma or pineoblastoma was used.

$CD34^+$ cells were isolated from leukapheresis material provided by the stem cell laboratory of Sanquin Research (Dept of Experimental Immunohematology, Amsterdam, The Netherlands) by magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions within 48 hours

after leukapheresis. Purity of the samples was determined by flow cytometry with $CD34^{PE}$ (Immunotech, Marseille, France). The purity of all samples exceeded 90%. After isolation, cells were cryopreserved in IMDM (Cambrex Bio Science, Verviers, Belgium) supplemented with 20% FCS (Bodinco, Alkmaar, The Netherlands) and 5% DMSO.

Megakaryocyte expansion culture

Cells were thawed and subsequently seeded at 2.5 $x10^5$ cells/ml in CellGro (CellGenix, Freiburg, Germany) and cultured O/N, for 4 days, or 7 days with 100 ng/ml Tpo (Strathmann Biotec AG, Hamburg, Germany) and 10 ng/ml IL-1ß (Strathmann Biotec AG) in 6-well plates (Costar, Cambridge, MA, USA). Cultures were performed for each donor separately. Four batches of cells derived from the same donors were used for the experiment in which cells were cultured for different time periods. Cells were cultured in 2 ml of medium for the indicated periods at 37°C and 5% CO_2 in a humidified atmosphere. If the cell density of a culture reached an estimate of 2-2.5 $x10^6$ cells/ml, 0.5 ml of CellGro containing cytokines (day 3) or 0.5 ml of medium without cytokines (day 6) was added to the wells. On the day of transplantation, cells were harvested, counted and prepared for transplantation. Subsequently, a small sample was incubated with anti-human $CD41^{FITC}$ (Sanquin) and anti-$CD34^{PE}$ (Immunotech, Marseille, France) and phenotypically analyzed on a flow cytometer (LSRII or FACScanto; Becton Dickinson [BD], San Jose, CA, USA). The proliferation factor was defined as the fold-increase in absolute cell number.

Transplantation of human cells into NOD/SCID mice

Six-to nine-weeks-old NOD/LtSz-scid/scid mice were obtained from Charles River, Maastricht, The Netherlands (supplier for The Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed under specific pathogen-free conditions in a laminar air flow unit and supplied with sterile food and acidified drinking water containing 100 mg/l ciprofloxacine ad libitum. Housing, care and all animal experimentation were performed conform legal regulations in The Netherlands, which include approval by a local ethical committee. Before transplantation, 7- to 10-weeks-old NOD/SCID mice received a sublethal dose of 350 cGy total body irradiation. Grafts containing primary MPB $CD34^+$ cells and/or expanded cells were injected into the tail vein 2-6 hours after irradiation. In two separate experiments mice (n=4 and n=6, respectively) received a graft containing uncultured, cultured, or a combination of uncultured and cultured cells (Table 1 in the Letter), and in a third experiment, mice received a graft composed of uncultured cells or of cells cultured for different periods (Table 2 in the Letter). The number of cells from healthy donors and chemotherapy-mobilized patients was equally distributed among the experimental groups. On average 28.7% of the cells was obtained from healthy donors.

Analysis of human platelet formation in NOD/SCID mice

The presence of human platelets in mouse peripheral blood was determined twice a week during the first 2 weeks and weekly thereafter. Blood was collected in citrate. The total volume of collected blood was measured and platelet number per ml was corrected for the dilution by citrate. Subsequently anti-mouse $CD41^{FITC}$ (BD) and anti-human $CD41^{APC}$ (BD) were added. After incubation for 20 minutes at 4°C, 1 ml of lysisbuffer (0.155 M NH_4Cl, 0.01 M $KHCO_3$, 27 mM EDTA, 0.2% BSA; pH

7.2-7.6) was added. After 5 minutes on ice, 50 µl of Bright count microspheres (IQ Products, Groningen, The Netherlands) were added and samples were analyzed by flow cytometry (LSRII or FACScanto; BD).

Determination of platelet functionality

Mouse blood (collected as described above) or a human platelet concentrate in 30% human plasma (positive control) was diluted 1:10 in Hepes Tyrodes buffer (10 mM Hepes, 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.5 mM Na_2HPO_4; pH 7.25) plus 5 mM glucose. EDTA was added to a final concentration of 10 mM. Subsequently, 25 µM thrombin-receptor-activating peptide (TRAP-6; Bachem, Torrance, CA, USA) was added to 100 µl of diluted blood. After 10 minutes of activation at room temperature, anti-mouse $CD41^{FITC}$, anti-human $CD62P^{PE}$ (Sanquin), and anti-human $CD41^{APC}$ were added. After 15 minutes of incubation with antibodies, samples were analyzed by flow cytometry.

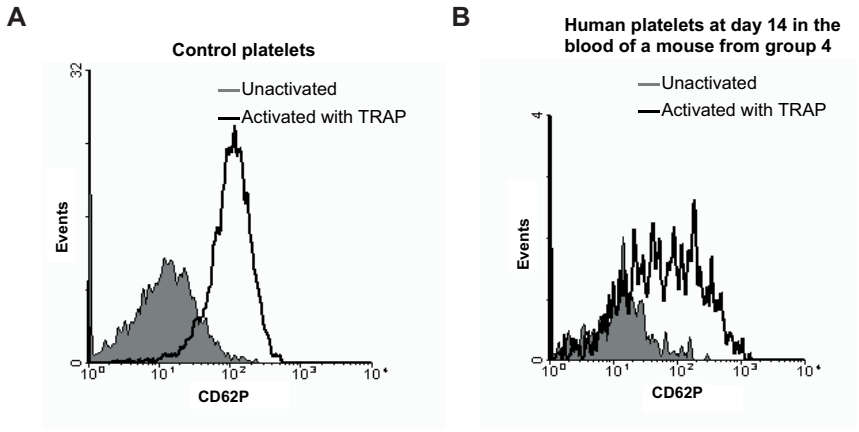
Analysis of engraftment

The mice were sacrificed 5 weeks after transplantation. Bone marrow, spleen, lung and peripheral blood were collected and cell suspensions were analyzed for human cell engraftment by flow cytometry. The cells were labeled with combinations of the following antibodies: $CD3^{FITC}$ (BD), $CD4^{PE}$ (BD), $CD8^{PE}$ (Sanquin), $CD13^{PE}$ (BD), $CD16^{FITC}$ (Sanquin), $CD19^{FITC}$ (Sanquin), $CD20^{PE}$ (BD), $CD33^{PE}$ (BD), $CD34^{PE}$, $CD38^{FITC}$ (Dako, Glostrup, Denmark), mouse-$CD41^{FITC}$ (BD), human-$CD41^{APC}$ (BD), $CD45^{FITC}$ (Sanquin), and $CD56^{PE}$ (Sanquin).

Statistical analysis

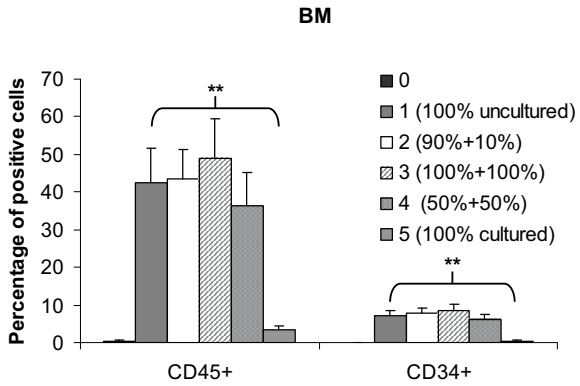
The human platelet numbers per ml of mouse blood are represented in a box plot showing the median and the upper and lower quartile of the data. In the third transplantation experiment one of the mice in group C had health problems and the platelet levels detected in the blood of this mouse deviated from those of the other mice in the group. This mouse was excluded from analysis. The statistical differences in platelet formation between groups were calculated with the non-parametric Wilcoxon-Mann-Whitney test using SPSS software (version 11.0 SPSS Inc., Chicago, IL). Differences were considered significant when $p<0.05$. In the figure showing the kinetics of human platelets in the mouse blood, data are plotted as average number of platelets per ml of blood ± SEM. The percentage of human cells in the BM is presented as mean ± SEM. Statistical differences in the presence of human cells in the BM were calculated with a non-paired two-sided *t*-test, assuming unequal variance.

Figures



Supplementary Figure 1: Activation of human platelets formed in NOD/SCID mice

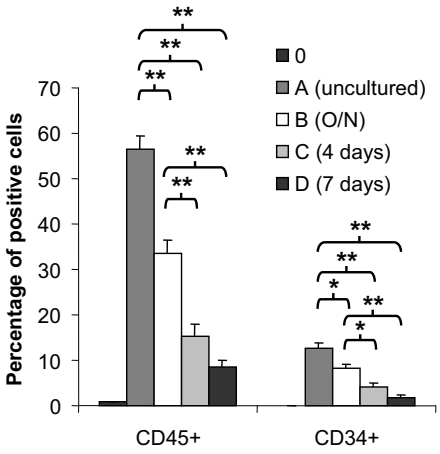
A. Control platelets in 30% human plasma were incubated with 25 µM TRAP-6 for 10 minutes. An increase in CD62P surface expression could be observed. **B.** When blood from NOD/SCID mice was treated with TRAP-6 at day 10 and 14 after transplantation an increase in CD62P expression on human CD41-expressing platelets was detectable. A representative example of 7 transplanted mice is shown.



Supplementary Figure 2: Presence of human cells in the bone marrow of NOD/SCID mice 5 weeks after transplantation of grafts containing uncultured cells combined with cultured MKs

Five weeks after transplantation mice were sacrificed and the presence of human cells in the BM was determined. The BM engraftment was not significantly different in group 1 through 4. The average level of chimerism reached within group 4 (36.3%) was only somewhat lower in comparison to the groups that received twice as many $CD34^+$ cells (group 1 and 3: 42.5% and 49.0%). When only cultured cells were transplanted (group 5) about 3.2% of the cells in BM expressed CD45, which is significantly less than the percentages in group 1 through 4. These data indicate that cultured MKs do not contribute significantly to long-term engraftment. This is in agreement with the observation that the mice in group 3, which received 100% cultured and 100% uncultured cells, only showed a small increase in chimerism compared to the mice that received only uncultured cells (group 1). A similar pattern in the presence of human cells was observed for the various cell lineages tested in the BM, i.e. $CD34^+$ (this figure), $CD38^+$, $CD16^+$ (NK), $CD4^+$ and $CD8^+$ (T-cells), and $CD19^+$ and $CD20^+$ (B-cells; data not shown). A *t*-test was used to determine statistical differences.**; $p<0.01$ as compared to group 1.

In the spleen and blood the percentage of $CD45^+$ cells was lower than in the BM (data not shown). In the spleen, the average percentage of CD45+ cells ranged from 0.4-7.1% and in the blood from 0.7-5.5%. No human cells were detected BM of mice in the control group (0) or in the lungs of the transplanted mice.



Supplementary Figure 3: Presence of human cells in the bone marrow of NOD/SCID mice 5 weeks after transplantation of uncultured cells or cells cultured for different periods

The mean percentage of $CD45^+$ cells in the mouse BM 5 weeks after transplantation of uncultured cells or cells cultured O/N, for 4 days or for 7 days was 56.4%, 33.5%, 15.3%, and 8.7% for group A through D, respectively. Thus, the percentage of $CD45^+$ cells in the BM of the mice decreased with the culture time of the HPCs. Since equal numbers of input cells in the culture were transplanted, the mice in group C and D received higher total cell numbers than the mice in group A and B. In Table 2 of the Letter the actual number of $CD34^+$ cells transplanted after culture is depicted. The mice in group D received 6 times more $CD34^+$ cells than the mice in group A (29.9 $x10^6$ in group D versus 4.5 $x10^6$ in group A), but showed a more than six-fold decrease of human cells in the bone marrow 5 weeks after transplantation (8.7% in group D versus 56.4% in group A). This clearly demonstrates that the cultured cells are less effective in providing long-term engraftment. The lineage distribution was not influenced by the difference in culture time ($CD34^+$ (this figure); $CD38^+$, $CD16^+$, $CD4^+CD8^+$, $CD19^+$, and $CD20^+$; data not shown).

A *t*-test was used to determine statistical differences. Human cells were not detected in the BM of mice in the control group (0).Comparison of the group A through D, showed significant differences between all groups, except for group D compared to group C. *; $p<0.05$ =, **; $p<0.01$data not shown). A *t*-test was used to determine statistical differences.**; $p<0.01$ as compared to group 1.

In the spleen and blood the percentage of $CD45^+$ cells was lower than in the BM (data not shown). In the spleen, the average percentage of CD45+ cells ranged from 0.4-7.1% and in the blood from 0.7-5.5%. No human cells were detected BM of mice in the control group (0) or in the lungs of the transplanted mice.