

Engraftment

Engraftment kinetics of human CD34+ cells from cord blood and mobilized peripheral blood co-transplanted into NOD/SCID mice

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Summary:

We have reported short periods of post transplant neutropenia in human patients co-transplanted with cord blood (CB) and low numbers of haploidentical mobilized peripheral blood (MPB) CD34+ cells. To investigate the effect that the proportion of MPB to CB cells may have on engraftment kinetics, we have co-transplanted fixed numbers of human CB CD34+ cells mixed with different numbers of MPB CD34+ cells into NOD/SCID mice. We periodically quantified the proportion of human cells and the relative contribution of MPB and CB cells to the human engraftment on marrow aspirates. At the lowest MPB/CB ratios (5:1, 10:1), the contribution of CB cells predominated at all time points analyzed, and in three out of four experiments MPB cell contributions progressively decreased from day +15. At higher MPB/CB ratios, MPB cells had a more important contribution to both early and late engraftment, with the highest cell ratio resulting in only marginal CB cell engraftment. Therefore, our results showed greater potential, on a per cell basis, of human CB vs MPB cells for competitive sustained engraftment in the xenogeneic model used, which was only abrogated by the co-infusion of very high numbers of MPB cells.

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Cord blood (CB) transplantation has been shown to be an adequate source of hematopoietic stem cells for transplantation. Accumulated experience has shown that time to engraftment is longer than for transplants carried out with

bone marrow (BM) or mobilized peripheral blood (MPB) stem cells. This seems to be in part due to the relatively low number of stem cells infused in relation to the patient's body weight. The lower proportion of more mature precursors in the CB CD34+ cell population is also an important factor. This relatively long time to engraft may be an important contributing factor to the relatively high early transplant-related mortality of CB transplants.^{1–7}

We hypothesized that co-transplantation of a relatively low number of highly purified MPB stem cells could result in an early, although possibly transient, engraftment that could provide adequate hematological support to the patient, allowing time for the CB engraftment, a hypothesis that is supported by our clinical results. We have reported short post transplant neutropenia (median time to ANC > 0.5 × 10⁹/l 10 days; 100% in less than 17 days) in patients transplanted with a single CB unit and low numbers of MPB CD34+ cells from a third party donor carrying less than 10 000 CD3+ cells/kg of patient body weight.^{8,9} With this approach, the early rise of ANC after the transplant is mainly due to engraftment of the third party MPB CD34+ cells that neither interferes with the slower engraftment of CB cells, which are responsible for the sustained long-term engraftment (more than 90% full CB chimerism on day +100 with a median time of 58 days), nor produces any other unfavorable effects. In this strategy, the numbers of third party MPB CD34+ and CD3+ cells to be infused can be decided by the clinician before transplantation.

In order to obtain data that could help us improve our understanding on the engraftment kinetics and biology of dual transplants of CB and MPB stem cells, we have investigated the effect that the proportion of human MPB to CB cells may have on engraftment kinetics in the SCID/NOD xenogeneic transplant model, assaying the co-transplantation of different numbers of MPB cells and fixed numbers of CB CD34+ cells. As previously shown, this model allows us to functionally study the contribution of the different sources of human hematopoietic cells to the xenogeneic engraftment in the recipient mice, both in terms of timing and percentage.^{10–12} The results that we report here support the concept that MPB cells contribute to early engraftment, not interfering with the engraftment of the transplant of a fixed number of CB cells, except at high MPB/CB cell ratios.

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Material and methods

Human samples

CB units were collected from normal full-term deliveries after obtaining maternal informed consent and processed within 24 h as previously described.¹³ CB mononuclear cells were recovered by density gradient separation with Ficoll-Hypaque (1.077 g/ml, Pharmacia Biotech, Upsala, Sweden). Adult progenitors were collected after informed consent from selected voluntary donors by apheresis after mobilization with granulocyte colony-stimulating factor (G-CSF) (Amgen, Thousand Oaks, CA, USA).

Both CB and MPB CD34⁺ cells were selected using an indirect immunomagnetic separation method (MACS Miltenyi Biotech, Bergisch Gladbach, Germany, using the reagent kit #130-046-701, 2003-4 Catalogue). Median CD34⁺ cell content of the selected MPB and CB cells were respectively 98% (97.9–98) and 97.6% (93.2–99.4) and the respective median proportions of CD34⁺ and CD38⁻ cells were 15.9% (4.8–27.7) and 14.6% (12.9–21.77). CD3⁺ content of MPB cells ranged from 0.02 to 0.38% (median 0.09%, average 0.15%). These cell products were mixed with an equal volume of cryoprotectant solution containing 15% DMSO (Cryoserv, Edwards Lifesciences Research Medical Inc., Irvine, CA, USA), 45% phosphate-buffered saline (Gibco, Invitrogen SA, Paisley, Scotland) and 40% human albumin (Grifols, Barcelona, Spain) and cryopreserved using a programmable cell freezer (CM 25, Carubos Metálicos, Madrid, Spain). For transplantation into NOD/SCID mice, CD34⁺ cells were thawed in a 37°C water bath and immediately processed using the method described by the New York Blood Center.¹⁴ After cryoprotector elimination, CB and MPB CD34⁺ cells were diluted in TC199 medium (Gibco). Data of numbers of infused cells correspond to post thaw counts. Median cell viability at this stage was 99% (97–100) for CB and 98% (96–99) for MPB products. These were mixed at different ratios before infusion into the mice. Different pairs of MPB and CB donors were used for each experiment. In total, we used four pairs of MPB and CB donors: one pair was used for each of the three experiments represented in Figure 1a and b and the fourth for the experiment represented in Figure 2a and b.

Animals

We used 6–8 weeks old NOD.CB17-Prkdcscid/J (NOD/SCID) mice, bred at the CIEMAT Laboratory Animals Facility (Registration Number 28079-21 A) from breeding pairs originally obtained from Jackson Labs (Bar Harbor, Maine). The mice were routinely screened for pathogens, in accordance with FELASA (Federation of European Laboratory Animal Science Associations) procedures. They were housed in microisolators, individually ventilated cages, and allowed sterilized water and food *ad libitum*. All experimental procedures were carried out according to European and Spanish laws and regulations and internal biosafety and bioethics guidelines. Before transplantation,

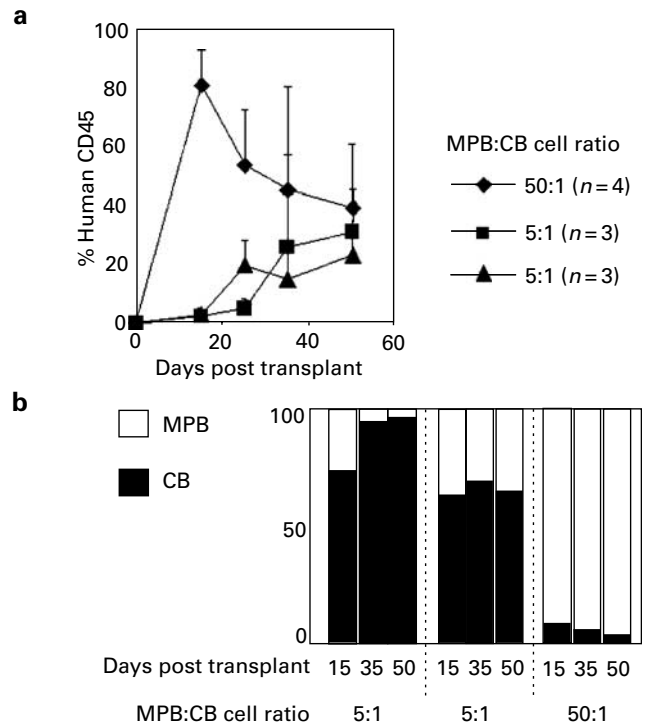


Figure 1 Engraftment kinetics of human CD34⁺ cells from cord blood and mobilized peripheral blood co-transplanted at low and high cell ratios into NOD/SCID mice. Groups of sublethally NOD/SCID received a fixed number of CB CD34⁺ cells plus MPB CD34⁺ cells at the indicated cell ratios. (a) The proportion (mean and s.d.) of CD45⁺ cells in the femoral marrow of NOD/SCID mice was determined by flow cytometric analysis. (b) The relative contribution of MPB and CB stem cells to the engraftment was assessed by PCR-STR.

the mice were total body irradiated with 2.5–3.0 Gy X-rays (300 kV, 10 mA; Philips MG-324, Hamburg, Germany).

Analysis of human cell engraftment

At intervals after transplantation, BM samples were aspirated from one femur by puncture through the knee joint, according to a previously described procedure.¹⁵ At the end of the experiments, mice were killed and BM cell suspensions were also obtained. All BM samples were analyzed by flow cytometry for the presence of human cells. Aliquots of $1-5 \times 10^5$ cells/tube were stained for 25 min at 4°C with anti-human-CD45-PECy5 (Clone J33, Immunotech, Marseille, France) in combination with different anti-human monoclonal antibodies. Thereafter, red blood cells were lysed by adding 2.5 ml of lysis solution (0.155 mol/l NH₄Cl + 0.01 mol/l KHCO₃ + 10⁻⁴ mol/l EDTA) and incubated at room temperature for 10 min. Cells were then washed in PBA (phosphate-buffered salt solution with 0.1% BSA and 0.01% sodium azide), resuspended in PBA + 2 µg/ml propidium iodide (PI), and analyzed by flow cytometry. For each analysis, a total of 10 000 viable (PI⁻) cells with appropriate forward scatter (FS)/side scatter (SSC) were collected. As controls of nonspecific staining, cells were labelled with conjugated nonspecific isotype antibodies.

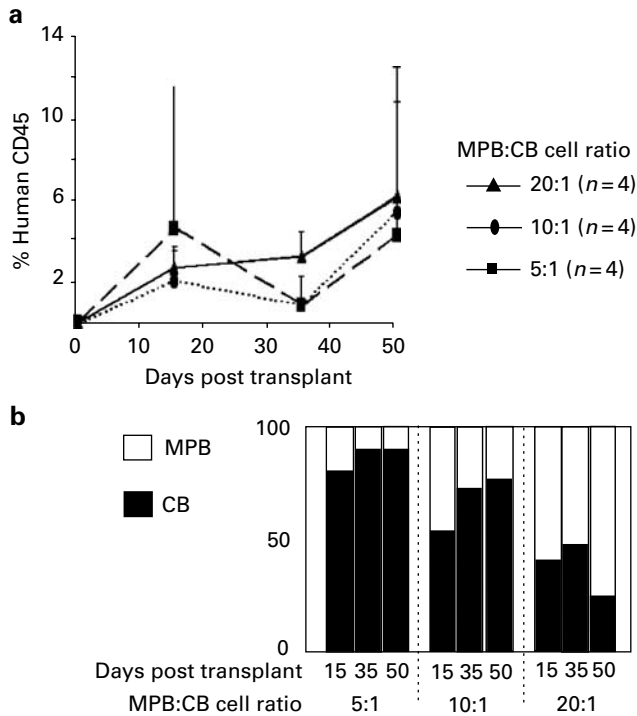


Figure 2 Contribution of MPB and CB stem cells to the hematopoietic engraftment is dose dependent. MPB stem cells from a single donor were mixed with a fixed number of CB stem cells from another single donor and transplanted into groups of four NOD/SCID mice. Samples from murine marrows were drawn periodically and the contribution of MPB and CB stem cells to the engraftment was assessed as explained. (a) The proportion (mean and standard deviation) of CD45⁺ cells in the femoral marrow of NOD/SCID mice was determined by flow cytometric analysis. (b) The relative contribution of MPB and CB stem cells to the engraftment was assessed by PCR-STR.

Analysis of the contribution of the two sources of human stem cells to the engraftment

Samples. Cells from CB and from apheresis products were collected before transplanting into NOD/SCID mice to identify polymerase chain reaction-short tandem repeats (PCR-STR) informative fragments. After transplanting, chimerism status was assessed on BM samples aspirated at days +15, +35 and +50.

DNA extraction. High-molecular-weight DNA was extracted from aliquots of $1-5 \times 10^6$ cells, using silica membranes (Qiamp Blood Kit, Qiagen), as recommended by the manufacturer. DNA was quantified using standard UV-absorption at 260 nm and all samples were diluted to a final concentration of $0.2 \mu\text{g}/\mu\text{l}$ in H₂O.

STR amplification. A quantitative multiplex PCR-based analysis of STRs was performed using the AmpFISTR Profiler Plus Kit (Applied Biosystems) as recommended by the manufacturer. The STR loci amplified were: D3S1358, Vw, FGA (labelled with 5-FAM); D8S1179, D21S11, D18S51, and amelogenin (labelled with JOE); D5S818, D13S317, and D7S829 (labelled with NED). Separation

and detection of amplified PCR products were performed on an ABI PRISM 377 automated DNA sequencer (Applied Biosystems). Fragment sizes were automatically determined using ABI PRISM GeneScan 2.0 (Applied Biosystems). Quantification was performed by determining the ratio areas from cord and apheresis informative alleles.¹⁶ Sensitivity of the method, determined on the basis of analysis of mixtures of cells of two sources, was 3%.

Results

Low MPB/CB cell ratios resulted in a low contribution of MPB cells to engraftment that tended to diminish along time

In two different experiments, mice ($n=6$) received a mixture of 1.5×10^6 MPB CD34⁺ cells and 3×10^5 CB CD34⁺ cells each (ratio 5:1). In each experiment, MPB and CB cells came from a single donor, with two pairs of MPB and CB donors being used. BM samples were obtained from the live animals on days +15 and +35 and on day +50 when the mice were killed. The proportion of human hematopoietic cells in the mouse marrows progressively increased during the post transplant period (Figure 1a). Human hematopoiesis was multilineage in every case (data not shown). CB stem cell contribution predominated at all time points assessed and MPB stem cell contribution did not increase or progressively decreased from day +15 to the end of the experiments (Figure 1b).

Increasing the MPB cell number and MPB/CB cell ratio may reduce the CB stem cells contribution to engraftment

In our experience with human patients, the MPB/CB CD34⁺ cell ratio varied from 6/1 to 69/1 (mean 22/1).⁹ In order to mimic in the *xenogeneic* model the highest MPB/CB cell ratios of our experience in humans, we transplanted a 10-fold higher number of MPB CD34⁺ cells (15×10^6) mixed with 3×10^5 CB CD34⁺ cells (ratio 50:1) to each of four mice. For all mice in this experiment MPB and CB cells came from single donors. BM samples were obtained at the same time intervals as in the previous experiment. The kinetics of human hematopoietic cell engraftment, shown in Figure 1a, indicated very high levels of human hematopoiesis at day +15 that progressively decreased at later time points. The initial high level of engraftment was probably related to the high number of cells transplanted.¹⁷ When we studied the contribution of each source of human stem cells, we found that CB stem cells only contributed marginally and tended to decrease over time. Therefore, at the high MPB/CB cell ratio of 50:1, MPB was the predominant source of human engraftment and progressively increased.

Contribution of MPB and CB stem cells to the hematopoietic engraftment is dependent on the number of MPB co-infused

The above results suggested that MPB and CB stem cells may compete for the stem cell niches made available

within the murine marrow by the sublethal conditioning irradiation. Therefore, tuning the number of co-infused MPB and MPB/CB cell ratios, both controllable variables in the clinical setting, could result in different contributions of each source of stem cells to the kinetics of human engraftment in the mouse model. To test this hypothesis we mixed a constant number of CB CD34+ cells with different numbers of MPB CD34+ cells and transplanted the resulting 5:1, 10:1, and 20:1 ratio mixtures into groups of four NOD/SCID mice. For this experiment we had to use 2×10^5 CB CD34+ cells per mouse (instead of the 3×10^5 used in the previous ones), in order to have enough CB cells from a single donor, so the total number of human cells received by the different groups of mice in this set of experiments were 1.2, 2.2, and 4.2×10^6 . The proportion of human cells in the mouse marrows showed a tendency to increase during the post transplant period (Figure 2a), although at lower levels compared to the above experiments. The relative contribution of the two sources of stem cells to the hematopoietic engraftment depended on the MPB cell dose and MPB/CB cell ratio (number of CB cells was constant) (Figure 2b). With lower MPB numbers and MPB/CB ratios (5:1 and 10:1), MPB cell contribution was less important than that of CB cells at the earliest time point (day +15) and tended to decrease thereafter. As said above, this was also observed in one of the two previous experiments also using the 5:1 ratio, but with higher numbers both of CB and MPB cells per animal. Only experiments with a higher number of MPB and MPB/CB cell ratio (20:1) resulted in early and late predominant contribution of MPB to the engraftment.

Discussion

Unrelated CB transplant (UCBT) has the disadvantage of relatively high early transplant-related mortality in part related to late engraftment due to the low number and primitive biological features of the transplanted hematopoietic stem cells.¹⁻⁷ Long periods of neutropenia imply a high risk of serious early infections that may result in death prior to engraftment. All of this represents a limitation to the use of UCBT for patients of high body weight. Several strategies are being investigated to reduce duration of post transplant neutropenia of UCBT. These include *in vitro* expansion of an aliquot of the transplanted CB unit, which so far has not resulted in significant clinical advantages, as well as transplantation of multiple CB units. This last approach does not appear to significantly reduce time to engraftment except if combined with submyeloablative conditioning, a strategy in which autologous recovery contributes to the initial rise of ANC, although complete CB chimerism may be finally achieved, very often exclusively or with much predominance by one of the transplanted units.¹⁸⁻²⁰ With our approach of co-transplantation of a single CB unit and a low number of MPB CD34+ cells from a third party donor (related or unrelated with different degrees of HLA mm), we have consistently obtained very short periods of post transplant neutropenia. This results from early predominant engraftment of the third party MPB cells, not interfering with CB

engraftment and final achievement of complete CB chimerism or causing other unfavorable effects.^{8,9} In this strategy, rejection by immunocompetent cells from the CB seems likely to be implicated in the effacement of the MPB graft.⁹

The study that we report here was designed to obtain information on the kinetics of human competitive engraftment of CB and MPB CD34+ highly selected cells in the NOD/SCID xenogeneic model. In this model there is actually competition between the autologous reconstitution and the two sources of the infused xenogeneic cells, supposedly without the intervention of T-cell dependent immune rejection effects.^{21,22} We have found that the relative contribution of human MPB and CB cells to the engraftment of NOD/SCID mice receiving a fixed dose of CB cells depended on the number of MPB CD34+ cells and MPB/CB cell ratio infused. At a low MPB cell number and MPB/CB stem cell ratios (5:1 and 10:1), CB-derived hematopoiesis predominated at any time point assessed. This reflects the higher engraftment potential that human CB stem cells have compared to that of MPB in this *in vivo* model.^{10,14} However, raising the cell ratio in favor of MPB by a factor of 10 (experiments using a 50:1 ratio of MPB/CB cells) resulted in almost complete abrogation of CB- engraftment (Figure 1b, cell ratio 50:1). This could be due to competition for a limited number of stem cell niches made available within the murine marrows by the conditioning irradiation, so we hypothesized that fine tuning of the MPB/CB stem cell ratio could result in the predominance of either source of stem cells. We confirmed this in experiments transplanting different numbers of MPB and a constant number of CB stem cells, lower than in the initial experiments because of the limited availability of these cells, from single donors into groups of NOD/SCID mice. In these experiments the rate of human engraftment was lower and had greater variability, as shown by the error bars, than in the previous ones. This could relate to several factors: (a) the lower total number of human MPB and CB cells infused to the mice; (b) the number of niches made available by the conditioning irradiation; (c) the intrinsic variability of different biological samples regarding proliferative/repopulating capacity; etc. Apart from this, we found that: (1) doubling the MPB/CB cell ratio from 5:1 to 10:1 resulted in a significant enhancement of the MPB contribution to the engraftment without displacing the CB predominance; and (2) by increasing the MPB cells four-fold (20:1 MPB/CB cell ratio), MPB-derived hematopoiesis predominated over that derived from CB, although not as extensively as seen in the 50:1 MPB/CB cell ratio experiment.

All together, the experiments with lower MPB/CB cell ratios (5:1 and 10:1), in which the relative contribution of CB cells was always higher at all time points assayed, the contribution of MPB cells was higher at the first point and progressively decreased at later stages in three out of four experiments, carried out with two levels of total human cells infused, and remained stable in the fourth. This is comparable to what we have observed in the clinical setting.^{8,9} In experiments with higher numbers of MPB stem cells and MPB/CB ratios, both the relative and absolute contributions of MPB to the early engraftment in

the mice increased. At a very high ratio (50:1), there was interference with the higher repopulating capacity of CB stem cells for short- and long-term engraftment shown by the experiments carried out with lower MPB/CB ratios. This interference is most likely a matter of competition for hematopoietic niches given the lack of T-cell engraftment that characterizes this *xenogeneic* model of hematopoietic stem cell transplantation^{20,21} and the very low total number of human T cells that were infused in our experiments. This was below 2500 for the mice that received the highest number of MPB cells, as the proportion of CD3+ in the infused MPB products was extremely low (median 0.09%, average 0.15%). The model is, thus, not fit to reproduce immunological mechanisms that could have important influence on the engraftment kinetics of joint transplants of hematopoietic stem cells from different donors in patients, in which there may be mutual rejection by grafts derived immune systems.^{8,17–19}

In summary, our data show that in the xenogeneic model of competitive engraftment that we have used, which can be reasonably regarded as free of T cell-dependent immune reactions, the number of third party MPB CD34+ necessary to interfere with the short- and long-term engraftment of CB CD34+ hematopoietic progenitors is, on a per cell basis, at least 10 times higher. We think that these data, together with reported results of engraftment in patients transplanted only with T-cell depleted CD34+ cells from haploidentical donors,²² help us to understand the biology of our clinical strategy of co-transplantation of CB and a relatively low number of MPB highly purified CD34+ cells from any HLA mm donor. The practical value of this strategy is being corroborated by our growing clinical experience (Takahashi *et al*⁷ and Fernández *et al*⁸ and unpublished results).

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