Cord Blood Units with Low CD34⁺ Cell Viability Have a Low Probability of Engraftment after Double Unit Transplantation

Andromachi Scaradavou,¹ Katherine M. Smith,² Rebecca Hawke,³ Allison Schaible,² Michelle Abboud,⁴ Nancy A. Kernan,¹ James W. Young,³ Juliet N. Barker³

Double unit cord blood (CB) transplantation (CBT) appears to augment engraftment despite only one unit engrafting in most patients. We hypothesized that superior unit quality, as measured by a higher percentage of viable cells postthaw, would determine the engrafting unit. Therefore, we prospectively analyzed 46 double-unit transplants postthaw using flow cytometry with modified gating that included all dead cells. Using a 75% threshold (mean viability minus 2 SD), 20% of units had low CD34⁺ cell viability, with viability varying according to the bank of origin. Further, in the 44 patients with single unit engraftment, CD34⁺ cell viability was higher in engrafting units (P = .0016). Although either unit engrafted if both had high CD34⁺ viability, units with <75% viability were very unlikely to engraft: in 16 patients who received one high and one low CD34⁺ viability unit, only 1 of 16 units with viability <75% engrafted (P = .0006). Further, in the single patient without engraftment of either unit, both had CD34⁺ viability <75%. Finally, poor CD34⁺ viability correlated with lower colony forming units (CFUs) (P = .02).

Our data suggests one mechanism by which double unit CBT can improve engraftment is by increasing the probability of transplanting at least one unit with adequate viability and the potential to engraft.


KEY WORDS: Allogeneic transplantation, Cord blood, Engraftment, Flow cytometry

INTRODUCTION

Although cord blood (CB) is increasingly used as an alternative hematopoietic stem cell (HSC) source that promises to extend transplant access to patients of racial and ethnic minorities, low total nucleated cell (TNC) dose is often limiting [1-4]. Double unit CB transplantation (CBT) can frequently overcome the cell dose limitations of single unit grafts in adults and large children [5-10]. This strategy improves engraftment compared with historic controls, despite only one unit giving rise to donor hematopoiesis in most patients [8]. Further, preliminary data suggest that the double unit approach may be associated with a reduced risk of relapse [11,12]. Therefore, the mechanisms that determine engraftment and unit predominance are of great interest, but have not been elucidated. Previous studies have compared the infused doses of TNC, CD34⁺ progenitors, or CD3⁺ T cells of the two units. Although one study found an association between higher CD3⁺ cell dose and the engrafting unit [8], this variable did not predict engraftment in individual patients. We hypothesized that superior unit quality, as measured by a higher percentage of viable cells postthaw, would determine the engrafting unit in double unit CBT. Processing, freezing, storage, transport, or thawing of a unit can damage CB cells at any point, and evaluation of cell numbers alone cannot accurately assess the degree of injury. We hypothesized that the proportion of dead cells in a unit reflects the degree of damage to the entire unit. Therefore, we evaluated the effect of viability (percent viable cells) of CB cell subpopulations (CD45⁺, CD34⁺, and CD3⁺ cells) postthaw on unit engraftment, in a prospective series of 46 consecutive double unit CBT recipients.

METHODS

Patients and Treatment Plan

All CBT recipients or their parents signed informed consent before transplantation. Patients had a median age of 37 years (range: 7-65 years) and
a median weight of 72 kg (range: 22-109). All had high-risk hematologic malignancies: acute myelogenous leukemia (AML; N = 11), acute lymphoblastic leukemia (ALL; N = 6), acute biphenotypic leukemia (N = 2), non-Hodgkin lymphoma (NHL; N = 14), Hodgkin lymphoma (HL; N = 9), chronic lymphocytic leukemia (CLL; N = 3), and prolymphocytic leukemia (N = 1). Conditioning was myeloablative (MA; N = 31) or nonmyeloablative (NMA; N = 15) according to age, diagnosis, extent of prior therapy, and comorbidities, with all regimens including fludarabine (Flu). All patients received immunosuppression with cyclosporine A (CsA) and mycophenolate mofetil (MMF), and engraftment support with posttransplant granulocyte-colony stimulating factor (G-CSF). Recipients of a prior allogeneic transplant were not included in this study.

CB Unit Selection

CB units were selected according to TNC dose and HLA match, and were obtained from domestic (N = 71) and international CB banks (N = 21 units). CB units were matched to the patient at 4-6/6 HLA-A,-B antigens, and -DRB1 alleles [6/6 (N = 5, 5%), 5/6 (N = 42, 46%), and 4/6 (N = 45, 49%)]. HLA-matching between the 2 units was 6/6 (N = 2), 5/6 (N = 11), 4/6 (N = 18), 3/6 (N = 12), and 2/6 (N = 3). High-resolution HLA typing at HLA-A, -B, -C, -DRB1, and -DQB1 alleles was performed on all patients and donor units, although the allele matching of loci other than HLA-DRB1 was usually not considered in unit selection. The donor-recipient HLA-match at high resolution ranged from 2-9/10. All patients received double unit grafts. The larger unit had a median infused TNC dose of 2.5 × 10^7/kg (range: 1.4-4.5), and the smaller had a median TNC of 1.9 × 10^7/kg (range: 0.9-3.7).

CB Unit Thaw and Infusion

Initially units were thawed with albumin-dextran dilution followed by centrifugation [13]. However, in an effort to reduce cell loss and unit manipulation, we subsequently changed our thaw strategy to a dilution without centrifugation for CBT recipients >20 kg [14]. Hence, the majority of CB units (N = 83, 90%) were thawed using the “no-wash” technique, whereas 9 CB units were thawed and washed with centrifugation. Units were infused within 2 hours of thawing with an interval of less than 45 minutes between the infusion of the first and second unit.

Postthaw Evaluations

Postthaw TNC

Postthaw TNC count was obtained using an automated hematology Coulter counter. Nucleated cell viability was assessed by trypan blue exclusion as per standard laboratory procedures.

Flow cytometric evaluation

Four-color flow cytometric evaluation of the CB units was performed using a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA) and CellQuest Pro software (BD Biosciences). Samples for flow cytometric analysis were taken from the final product prior to its release to the transplant floor and were stained within 1 hour of thaw. Duplicate aliquots containing 0.5 × 10^6 CB cells were incubated with anti-CD45 FITC (BD #340664), anti-CD34 PE (Beckman Coulter #IM1871), and anti-CD3 APC (BD #340661) at room temperature in the dark for 20 minutes. Red blood cells (RBCs) were lysed with fixative-free ammonium chloride (10 × NH4Cl Lysing Solution, Beckman Coulter #IM3514, diluted with reagent grade water to 1 x). 7-Amino-actinomycin D (7AAD) (Beckman Coulter #IM3422) was then added. Samples were vortexed, kept at room temperature in the dark for 15 minutes, and then stored for ≤1 hour on wet ice in the dark until acquisition. Because most units underwent albumin-dextran dilution, and cells were not washed during the staining, dead cells were not removed at any step during sample preparation.

Flow cytometric evaluation was performed using the International Society of Hematotherapy and Graft Engineering (ISHAGE) gating strategy [15] with several modifications to maximize the detection of live and dead cells. Compensations were set to avoid spectral overlap for CD34 PE and 7AAD. This was achieved by initially determining the appropriate photo multiplier tube (PMT) and compensation settings and then periodically checking the compensation settings with single stains. Heat killed or lysed cells were used to establish the 7AAD compensation, and compensation settings were confirmed with each sample by checking 2-color dot plots.

Although the essentials of the ISHAGE sequential gating strategy were retained, we lowered the forward scatter (FSC) threshold to include all 7AAD-positive dead cells and viewed the 7AAD versus FSC instead of the traditional 7AAD versus side scatter (SSC) dot plot. More debris was acquired with the lowered FSC threshold, but this was easily excluded with the modified 7AAD versus FSC dot plot because debris is 7AAD-negative with a lower FSC than cells. Therefore, a “Not Debris” region can be set so that debris is excluded from all subsequent dot plots. In figure 1A, plot 1 shows the FSC versus SSC dot plot in which the FSC primary threshold was adjusted to exclude most debris and RBC, but not the dead cells. Similarly, the FL1 secondary threshold was adjusted on the CD45 FITC versus SSC dot plot to exclude most red cells, platelets, and other debris but not any CD45^+ cells (not shown). The sequential Boolean
gating strategy as defined by ISHAGE was then applied to identify CD34<sup>+</sup> cells. Specifically, we gated on CD34<sup>+</sup> antigen expression, followed by a gate on CD45<sup>+</sup> low to intermediate antigen expression with fluorescence intensity characteristic of blast cells, and finally we set an FSC versus SSC gate with low SSC.

Figure 1. Flow cytometric evaluation of CB units postthaw. Live cells are shown in green, dead cells are shown in red, and debris is shown in black. (A) Modified gating strategy. Plot 1: the lower FSC primary threshold excludes most debris but no dead cells. Plot 2: viability is assessed in the FSC versus 7AAD dot plot. Debris is shown in R6 and is gated out in all subsequent plots. Dead cells (7AAD-positive) are in R7 whereas live cells (7AAD-negative) are in R5. Plot 3: for comparison traditional gating cannot adequately distinguish debris from viable cells in the 7AAD versus SSC dot plot. Live and dead cells are shown for CD45<sup>+</sup> (Plot 4), CD34<sup>+</sup> (Plot 5), and CD3<sup>+</sup> (Plot 6) cell populations. Viability was calculated from the total debris-free sample for CD45<sup>+</sup> cells (Plot 7), CD34<sup>+</sup> cells (Plot 8), and CD3<sup>+</sup> cells (Plot 9). An average of 300 events (range: 100-1000) were acquired per sample for the CD34<sup>+</sup> cell analysis. (B) Traditional gating strategy. The same plots as in A are shown with a high FSC threshold that excludes most dead cells, as frequently used in traditional gating. In CB units with a high percentage of dead cells the traditional methodology overestimates cell viability compared with our modified gating. (C) Comparison of CD34<sup>+</sup> cell viability using modified and traditional gating in CB units with a small percentage of dead cells. The difference between the 2 gating strategies is small.
and low to intermediate FSC characteristic of lymphocytes and blast cells. We assured that the lower boundary of the lymphoblast gate included the smallest lymphocytes. In this sequential gating strategy, monocytes are excluded because they express high levels of CD45 and increased SSC in comparison with true CD34+ cells, whereas granulocytes are excluded because they exhibit markedly higher SSC characteristics.

Viability was then evaluated on the FSC versus 7AAD dot plot (Figure 1A, Plot 2) instead of the traditional SSC versus 7AAD plot (shown in Figure 1A, Plot 3 for comparison). In our modified gating strategy shown in Figure 1A, Plot 2, to more clearly distinguish dead cells from debris, events were gated to encompass all 7AAD-negative (viable) cells in region 5 (R5), all 7AAD-positive (dead) cells in R7, and all low FSC 7AAD-negative debris in R6. Live and dead cells were evaluated as shown for CD45+ (Figure 1A, Plot 4), CD34+ (Figure 1A, Plot 5), and CD3+ (Figure 1A, Plot 6) cell populations. Notably, Figure 1A, Plot 5, clearly shows that the CD34+ cells are low SSC. Viability was calculated from the total debris-free sample for CD45+ (Figure 1A, Plot 7), CD34+ cells (Figure 1A, Plot 8), and CD3+ cells (Figure 1A, Plot 9). Initially, we performed isoclonic (CD34+) and isotype (CD3+) controls, but, because of the multiparameter gating using Boolean logic, they were found to be redundant, and were abandoned as recommended by Gratama et al. [16] and Keeney et al. [17]. Periodically, we stain controls to assure that nonspecific staining is minimal.

Our modified gating strategy allowed the acquisition of all dead (7AAD-positive) cells and viable (7AAD-negative) cells so that viability was determined from the total, debris-free populations. Figure 1B demonstrates the overestimation of the percent viability by traditional gating compared with the modified gating strategy in Figure 1A. This is especially evident in units with many dead cells as shown in this example. In contrast, both traditional and modified gating strategies resulted in similar CD34+ viabilities in CB units with few dead cells, as shown in Figure 1C. Absolute CD34+ and CD3+ cell numbers were determined using the two-platform method.

**Colony-forming unit (CFU) assays**

CFU assays were performed on all CB units using a total of $1 \times 10^5$ cells plated in duplicate. Colony growth was evaluated by light microscopy at 14 days.

**Engraftment, Donor Chimerism, and Unit Predominance**

Neutrophil engraftment was defined as the first of 3 consecutive days of an absolute neutrophil count (ANC) $\geq 0.5 \times 10^9$/L after the posttransplant nadir. Serial sampling of the bone marrow (BM) and/or peripheral blood (PB) at days 21, 28, 60, 100, 180, and 360 after transplantation determined donor chimerism using quantitative polymerase chain reaction of informative polymorphic DNA short tandem repeats (STR) in recipient and donor units (GenePrint Fluorescent STR Marker Kit, Promega, Madison, WI) [8]. The engrafting unit contributed $>50\%$ of the total donor chimerism in serial testing.

**Statistical Analysis**

Statistical analyses were performed using SPSS (v12.0, Chicago, IL) or Excel software. A value of
Non-engrafting units

Postthaw CD34+ (HHV)-6 infection. One patient, also a recipient of MA conditioning, had secondary graft failure in the setting of human herpes virus (CMV) disease at day 20. One additional septicemia on day 7, and the other had cytomegalovirus (CMV) disease early posttransplant. One developed Staphylococcus aureus septicemia on day 7, and the other had cytomegalovirus (CMV) disease at day 20. One additional patient also a recipient of MA conditioning, had secondary graft failure in the setting of human herpes virus (HHV)-6 infection.

Engraftment and Donor Chimerism

Forty-five of the 46 patients (98%) had donor engraftment in the BM by DNA analysis on day 21 posttransplant. One unit engrafted in 44 of these 45 patients, whereas a single patient had sustained engraftment of both units. The single patient without engraftment of either unit received NMA conditioning and had autologous recovery.

The median donor chimerism of the engrafting unit was 100% in recipients of MA conditioning at all time points tested. Recipients of NMA conditioning initially had mixed donor-host chimerism because of transient recovery of autologous hematopoiesis but attained 100% donor with the engrafting unit by day 60. Although a minority of patients had a small contribution from the nonengrafting unit early after transplant, the median percent chimerism of the nonengrafting unit was 0% at all time points with both types of conditioning.

Of the 45 patients with donor engraftment by DNA analysis, 43 had donor-derived neutrophil recovery with a median time to absolute neutrophil count (ANC) ≥0.5 x 10⁹/L of 25 days (range: 13-38) after MA and 11 days (range: 7-36) after NMA conditioning. The 2 patients who engrafted by DNA analysis but did not have sustained neutrophil recovery received MA conditioning and suffered lethal infections early posttransplant. One developed Staphylococcus aureus septicemia on day 7, and the other had cytomegalovirus (CMV) disease at day 20. One additional patient, also a recipient of MA conditioning, had secondary graft failure in the setting of human herpes virus (HHV)-6 infection.

Postthaw CD34+ Cell Viability and Unit Engraftment

Figure 2 depicts the flow cytometric cell viabilities of the engrafting and nonengrafting units in the 44 patients with single unit engraftment in the BM. The mean CD34+ cell viability of the 44 units that engrafted in these patients was 88% (SD = 6.5%). CD34+ cell viability was significantly higher in the engrafting units (P = .0016), and units with a high proportion of dead cells (ie, low viability) did not engraft. The numbers of engrafting and nonengrafting units were compared at various viability thresholds using a chi-squared analysis (Table 1). A clear distinction was seen at a CD34+ viability threshold of 75%, which represented 2 SDs below the mean of the overall distribution.

Among the 44 patients (88 units) with engraftment of a single unit, 16 received grafts consisting of one high and one low viability unit. In these 16 patients only 1 of 16 (6%) units with CD34+ cell viability <75% engrafted. In the other 28 patients, both units had viability over 75% and either engrafted. Thus, in these patients with engraftment of a single unit, 43 of 72 (60%) engrafted with a unit with high viability (Table 2). Notably, the single patient without evidence of any donor hematopoiesis posttransplant received two units, each of which had CD34+ viability <75% (74% and 36%, respectively). Thus, overall, only 1 of 18 units (6%) with CD34+ viability <75% engrafted. The patient with stable engraftment of both units received two units with CD34+ viability ≥75% (both were 86%).

CD3+ cell viability correlated with CD34+ cell viability (R² = 0.47, p < .01) and with unit engraftment (p = .0077). The mean CD3+ cell viability of the engrafting units was 84% (SD = 10.6%). An analysis was performed comparing the number of engrafting and nonengrafting CB units, using similar viability

<table>
<thead>
<tr>
<th>Engrafting units (CD34+ cells)</th>
<th>Non-engrafting units (CD34+ cells)</th>
<th>Mean viability</th>
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<tbody>
<tr>
<td>90%</td>
<td>70%</td>
<td>80%</td>
</tr>
</tbody>
</table>

Table 1. Analysis of CD34+ and CD3+ Cell Viability Thresholds for Unit Engraftment

<table>
<thead>
<tr>
<th>CD34+ Cell Viability of Engrafting CB Unit</th>
<th>Viability Threshold</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean viability</td>
<td>88.0%</td>
<td>.56</td>
</tr>
<tr>
<td>Mean—SD</td>
<td>81.5%</td>
<td>.016</td>
</tr>
<tr>
<td>Mean—2SD</td>
<td>75.0%</td>
<td>.0006</td>
</tr>
<tr>
<td>Mean—3SD</td>
<td>68.5%</td>
<td>.048</td>
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</tbody>
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<table>
<thead>
<tr>
<th>CD3+ Cell Viability of Engrafting CB Unit</th>
<th>Viability Threshold</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean viability</td>
<td>84.0%</td>
<td>.15</td>
</tr>
<tr>
<td>Mean—SD</td>
<td>73.4%</td>
<td>.039</td>
</tr>
<tr>
<td>Mean—2SD</td>
<td>62.8%</td>
<td>.11</td>
</tr>
<tr>
<td>Mean—3SD</td>
<td>52.2%</td>
<td>.048</td>
</tr>
</tbody>
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Table 2. Postthaw CD34+ Cell Viability and Unit Engraftment in 44 Recipients (88 Units) of Double Unit CB Grafts Who Engrafted with a Single Unit

<table>
<thead>
<tr>
<th>% CD34+ Cell Viability</th>
<th>Engrafting CB Unit (N, %)</th>
<th>Nonengrafting CB Unit (N, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;75%</td>
<td>1 (6%)</td>
<td>15 (94%)</td>
</tr>
<tr>
<td>≥75%</td>
<td>43 (61%)</td>
<td>29 (39%)</td>
</tr>
<tr>
<td>All units</td>
<td>44 (50%)</td>
<td>44 (50%)</td>
</tr>
</tbody>
</table>

The mean postthaw CD34+ cell viability for the engrafting units was 88%, SD was 6.5%. Using CD34+ viability of 75% (ie, mean ± 2SD), all but 1 of the engrafting units had CD34+ cell viability ≥75% (P = .0006). The single patient with engraftment of both units (both with high viability) is excluded.

thresholds of CD34+ cells as for CD34+ cells, but the results were not highly significant (Table 1). CD45+ viability correlated weakly with CD34+ cell viability ($R^2 = 0.3$, $P < .01$). There was no difference in CD45+ cell viability between the units that engrafted and those that did not ($P = .07$). Moreover, postthaw evaluation of nucleated cells by trypan blue exclusion had no correlation with CD34+ cell viability ($R^2 = .008$) or unit engraftment ($P = NS$).

Postthaw CD34+ cell viability correlated with postthaw total CFU output. CB units with CD34+ cell viability ≥75% had a significantly higher median total CFU of 224 (range: 18-722) compared to units with CD34+ cell viability <75% that had a lower median total CFU of 118 (range: 1-493) ($P = .02$).

Figure 3 shows the postthaw CD34+ cell viability of all CB units used in the study (N = 92) according to the CB bank of origin. Although units of high viability were obtained from both domestic and international banks, viability of units varied between banks with some banks (both domestic and international) providing a disproportionate number of low viability units. We did not detect a difference in CD34+ viability according to the method of thaw (data not shown).

Other Graft Characteristics and Unit Engraftment

Infused cell and CFU doses were examined for their relationship with engraftment in the 44 patients with engraftment of a single unit (Table 3). As in previous analyses of double unit engraftment [8], the absolute numbers of infused TNC/kg and infused viable CD34+ progenitor cells/kg were not associated with unit engraftment in univariate analyses. Engrafting units had significantly higher infused viable CD34+ cells/kg ($P = .03$), although the absolute CD34+ cells/kg dose did not always predict the engrafting unit in individual patients. CFU/kg and CFU-GM/kg were not associated with the engrafting unit except in 1 patient where 1 of the 2 units had no CFU growth and failed to engraft [18].

Also as previously reported [8], donor-recipient HLA-matching at HLA-A, -B antigens and -DRB1 alleles was not associated with unit engraftment in this study. In the 44 patients with single unit engraftment, 12 received units with different HLA match levels, and the better matched unit engrafted in only 3 cases. Each unit differed in the donor-recipient HLA-A, -B, -C, -DRB1, and -DQ allele match in 25 patients. The better HLA-matched unit engrafted in only 10 of them ($P = NS$). Notably, however, the patient with sustained engraftment of both donors received units that were each 9/10 HLA-matched to the recipient and to each other (each had a single mismatch at HLA-A). This was the only patient who received units so closely matched by high resolution HLA typing.

Finally, the order of unit infusion did not predict the engrafting unit. Of the 44 patients who had donor hematopoiesis from a single unit, the engrafting unit was infused first in 23 and second in 21 patients ($P = NS$).

**DISCUSSION**

We prospectively investigated the correlates of donor engraftment after double unit CBT in 46 consecutive patients. In contrast to the traditional gating where dead cells can be excluded [15], we used modified flow cytometric gating that excludes only...
We found the percentage of viable CD34+ cells as a surrogate of product quality is a novel finding. In fact, only 1 of the 18 units with CD34+ <75% had a low probability of engraftment (0.006). Further, the potential clinical importance of measuring the viable cell dose has been appreciated when administering hematopoietic grafts such as donor lymphocytes [21]. However, the significance of the percentage of viable cells in a cryopreserved product as a surrogate of product quality is a novel finding. We found the percentage of viable CD34+ cells as measured by the modified gating, but not the infused CD34+ dose/kg, proved to be the most critical determinant of the engraftment potential of a CB unit in double unit CBT (Figure 2, and Tables 1 and 2). This concept can be explained by the example in Figure 4 where two units with similar absolute CD34+ cell doses/kg differ widely in their percentages of viable CD34+ cells and thus their quality. Units with a low percentage of viable CD34+ cells have had a significant proportion of the CD34+ cells destroyed. The remaining cells, although viable as determined by 7AAD staining, are likely also damaged, thereby compromising the engraftment potential of the entire unit. The correlation of percent CD34+ cell viability with the respective values of CD45+ and CD3+ cell viability support this hypothesis, as does the association between lower CFU counts and units with poor CD34+ cell viability.

We, therefore, propose that double unit CBT increases the probability that the patient will receive at least one unit with adequate viability and thus with the potential to engraft. To facilitate clinical decisions, however, an appropriate viability “threshold” is needed. Using 75% viability, which represented the mean of the CD34+ distribution minus 2 SD, had clinical utility as units with CD34+ cell viability below 75% had a low probability of engraftment (0.006). In fact, only 1 of the 18 units with CD34+ cell viability <75% engrafted. Using this threshold a further major finding of our study was that low viability units were not uncommon: 18/92 (20%) had CD34+ cell viability <75%. This percentage is similar to the incidence of graft failure reported in many series of single unit CBT (reviewed in [21,22,23]). Therefore, although the significance of viability in single unit CBT cannot be determined from this study, the correlation of viability and engraftment in single unit CBT is of great interest.

We are not able to evaluate this as in an attempt to augment engraftment, and possibly protect against relapse [11,12], we have only performed CBT using double unit grafts to date.

It is intriguing to hypothesize that the only unit with CD34+ cell viability <75% that engrafted in our study may have sustained a different type of injury than the others. It is also likely that engraftment may be influenced by additional factors such as the infused TNC dose. Units with a very high TNC dose may engraft despite low CD34+ cell viability if they contain sufficient numbers of unaffected cells. Our patients received only a mean TNC dose of 2.2 × 10^7/kg (SD = 0.69). Thus, the effect of low CD34+ cell viability on the engraftment of high cell dose units could not be evaluated.

Our findings highlight the importance of CB unit quality and have significant implications for both CB banks and transplant centers. Notably, not only did we find variable viability from unit to unit, we also found marked differences in the CD34+ cell viability of units obtained from different individual CB banks (Figure 3), with some banks providing a disproportionate number of low viability units. This was despite the fact that units were stored in a uniform manner at our center and likely indicates the extent to which varying bank practices may alter product quality, and could not be attributed to shipping distance because many international units had high viability. What specific banking practices could lead to this finding could not be analyzed, as this information is not provided to the transplant centers. Future studies involving both banks...
and transplant centers need to investigate the events that could adversely affect viability during CB collection, processing, cryopreservation, long-term storage, and transport, as well as short-term storage at the transplant center. Criteria of product quality also merit standardization and implementation to optimize the global CB inventory.

These findings also have multiple ramifications for transplant centers. Centers must determine how they will assess unit quality and what threshold is acceptable. Based on the results of this study we now define an adequate graft as two units with at least one with CD34+ cell viability ≥75%. Our findings have prompted us to perform double unit transplants exclusively at MSKCC to date as the risk of having a single unit graft with poor viability on the day of transplant is not acceptable given the challenging logistics of urgently obtaining a second unit, which may not be readily available.

Although this analysis demonstrated that units with low CD34+ viability are very unlikely to engraft, it does not elucidate the mechanism of CB unit predominance when both units of a double unit graft have high viability. In this scenario, we postulate that either unit has the potential to engraft, and that immune mediated phenomena may dictate engraftment. This is supported by the engrafting unit having a higher CD3+ dose/kg in this analysis, as previously reported, as well as preliminary results of transplants in immunodeficient mice given aliquots from each unit of our patient's grafts as single unit grafts and in combination [24]. Further, it is interesting that the single patient with stable mixed donor engraftment was the only one who received two units with high viability that were both also unusually highly matched at 9/10 HLA-alleles to the patient and to each other. We propose that in this case each unit had engraftment potential, but each was tolerant of the other, permitting coengraftment.

In summary, units with low viability, and thus poor quality, are very unlikely to engraft in double unit CBT. Centers should ensure that at least one of the units of the graft has a significant proportion of viable CD34+ and CD3+ cells, without depending on trypan blue exclusion or traditional ISHAGE gating for evaluation of unit quality. Our viability assay is easily performed and available on transplant day, in contrast to CFU assay results that require 2 additional weeks. In the future, it would be preferable that a similar assay could be used to evaluate viability postcryopreservation, but prior to unit thaw, using the cells from an attached segment for example. Development of such an approach should be a major priority. Moreover, our results merit investigation in larger patient series of both single and double unit transplants in the multicenter setting. Important aspects of these studies will be the standardization of the CD34+ cell viability assay, the effect of the thaw method (wash versus no wash) on the accurate calculation of dead versus viable cells, and whether transplantation with high-quality units speeds neutrophil and platelet engraftment.

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Authorship Statement

Andromachi Scaradavou performed the research, analyzed and interpreted the data, and wrote the manuscript. Katherine M. Smith, Rebecca Hawke, Allison Schaible, Michelle Abboud, and Nancy A. Kernan performed the research, James W. Young wrote the manuscript, and Juliet N. Barker directed and supervised the research, analyzed and interpreted the data, and wrote the manuscript.

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