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 Translational Research

The impact of transportation time on apoptosis in allogeneic stem cell grafts and the clinical outcome in malignant patients with unrelated donors



Tengyu Wang^{1,2,*}, Mats Remberger³, Andreas Björklund⁴, Emma Watz^{1,2,*}

¹ Department of Clinical Science, Intervention and Technology (CLINTEC), Karolinska Institute, Stockholm, Sweden

² Department of Clinical Immunology and Transfusion Medicine, Karolinska University Hospital, Stockholm, Sweden

³ Department of Medical Sciences, Uppsala University and KFUE, Uppsala University Hospital, Uppsala, Sweden

⁴ Unit for Cell Therapy and Allogeneic Stem Cell Transplantation (CAST), Karolinska University Hospital Huddinge, Sweden

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ABSTRACT

Background: The quality of cells in peripheral blood stem cell (PBSC) grafts is important for allogeneic stem cell transplantation outcome. The viability of PBSC grafts may decrease during transportation time between donor and transplant center. We hypothesize that the graft viability based on apoptosis and necrosis in the graft may better reflect graft quality and clinical outcome.

Methods: PBSC graft viability from unrelated donors was analyzed in 91 patients. Viable cells were defined as 7-aminoactinomycin D- and Annexin V-negative. The clinical outcome, including survival, transplant-related mortality and graft-versus-host disease (GvHD), was correlated to graft viability.

Results: Grafts transported for 1 day had a median viability of 86.4% (range 63.8 to 98.9%), and grafts transported for 2 days had median viability of 83.2% (range 52.8% to 96.2%) ($P = .003$). Grafts were divided into two groups based on the median graft viability of 85.1%. Patients who received low viability grafts had lower 1-year survival of 63.7% compared with 88.9% for those who received high viability grafts ($P = .007$). In the multivariate analysis, transplant-related mortality (TRM) was higher in the low viability group ($P = .03$), whereas overall survival was not significantly associated with graft viability. The incidence of acute GvHD grade II to IV, chronic GvHD and relapse risk remained comparable between the groups.

Conclusion: Low graft viability was an independent predictor of 1-year survival and TRM after adjusting for multiple confounders. Better graft quality markers are important for the detection of clinically important variations in the stem cell graft.

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Introduction

Peripheral blood stem cell (PBSC) grafts are the most common source for allogeneic hematopoietic stem cell transplantation (HSCT) [1]. Stem cell graft quality has been correlated to transplantation outcomes such as overall survival (OS), transplantation-related mortality (TRM), relapse risk, and acute and chronic graft-versus-host disease (aGvHD and cGvHD) [2–4]. The quality parameters assessed are most often the quantity and ratio of CD34⁺ stem cells and other lymphocyte populations [5]. The viability of PBSC grafts in relation to clinical outcome is less well characterized. The most common viability markers measured in stem cell laboratories are nonviable, late apoptotic, or

necrotic cells. Limited data are available on whether early apoptotic cells in the PBSC grafts influence allogeneic HSCT outcomes.

Apoptotic cells in the PBSC graft exist because of physiological and nonphysiological cell stress [6–8]. The handling and transportation of PBSC grafts expose cells to a metabolically, physically and chemically stressful environment [7,9]. It is well known that viability, as measured with colony-forming units (CFU) and 7-aminoactinomycin D (7AAD), decreases throughout transportation, especially >48 h [10,11]. However, graft transportation studies have not measured the early apoptotic content of PBSC grafts.

Different markers exist to measure various apoptotic stages. Early apoptotic cells express cellular markers such as phosphatidylserine (PS), a negatively charged phospholipid [8,12]. These cellular changes can be detected through Annexin V, which binds to PS on the external plasma membrane. If early apoptotic cells are not cleared, the cells

* Corresponding authors: Tengyu Wang, Emma Watz, Karolinska University Hospital, Huddinge C2:66, SE-141 86 Stockholm, Sweden. Phone: +46 8-585 800 00.
 E-mail address: tengyu.wang@ki.se (T. Wang).

start to lose membrane integrity and become late apoptotic cells [13]. This stage can be detected with the intracellular stain 7AAD. If late apoptotic cells are still not cleared by phagocytes, they can further transform into secondary necrotic cells expressing DAMPS, inducing an inflammatory response [14,15]. Apoptotic cells have a clear immunological function and can modify the downstream immune response.

Apoptotic donor cells have been used in allogeneic stem cell transplantation to modify immunologic responses. A clinical trial has shown promising results in reducing aGvHD risk with apoptotic donor cells [16]. In animal models of solid organ transplants, infusion of apoptotic donor cells can prolong graft survival [17,18]. Phagocytosis of apoptotic cells by antigen-presenting cells (APCs) promotes more tolerogenic cytokine release, with more transforming growth factor- β (TGF- β) [19–21]. At the same time, costimulatory receptors are downregulated, and the presentation of apoptotic cell antigens induces a more anergic or tolerogenic T cell response.

We hypothesized that a higher frequency of apoptotic cells in the PBSC graft would lead to lower GvHD risk while lowering OS owing to increased relapse risk. A lower-quality graft may influence time to engraftment. The primary outcome measurements were time to engraftment, infectious complications, GvHD risk and survival.

Table 1
Pretransplant data

	Low viability ($<85.1\%$); n = 46	High viability ($>85.1\%$); n = 45	P Value
Patient characteristics			
Age (y)	59 (14 to 75)	54 (1 to 73)	NS
Sex (M/F)	24/22	30/15	
Diagnosis			
AML	22 (48)	13 (29)	.09
ALL	6 (13)	7 (16)	NS
CML	2 (4)	2 (4)	NS
Lymphoma + CLL	4 (9)	6 (13)	NS
Myeloma/ Waldenstrom	2 (4)	7 (16)	.09
MDS/MF	10 (22)	10 (22)	NS
Follow-up time (d)	313 (22 to 1322)	478 (136 to 1336)	.09
Donor characteristics			
Age (y)	28 (19 to 53)	26 (19 to 54)	NS
Sex (M/F)	28/18	33/12	
F:M ratio	5	5	NS
Fully HLA- matched (6/6)	43	45	NS
HLA-DR1 mismatch	3	0	NS
Conditioning (MAC/ RIC)	9/37	7/38	NS
GvHD prophylaxis			
ATG	44	44	NS
CsA + MTX	45	42	NS
Tacrolimus + MTX	0	1	NS
Other	1	2	NS
Graft characteristics			
CD34 dose ($\times 10^6$ / kg)	7.2 (3.6 to 10)	7.5 (3.2 to 23)	NS
TNC ($\times 10^8$ /kg)	9.0 (4.6 to 19.6)	10.0 (4.6 to 36.3)	NS
Early apoptotic cells (%)	17.3 (12.5 to 36.1)	10.3 (1.1 to 14.2)	$<.001$
Necrotic cells (%)	1.4 (0.3 to 13.8)	0.7 (0.1 to 3.0)	$<.001$
Temperature at arrival ($^{\circ}\text{C}$)	6.1 (3.6 to 15)	5.6 (2.5 to 19.5)	NS

Data are median (range) or n (%) unless noted otherwise. ALL, acute lymphatic leukemia; CML, chronic myeloid leukemia; CLL, chronic lymphatic leukemia; CsA, Cyclosporine A; MDS, myelodysplastic syndrome; MF, myelofibrosis; MAC, myeloablative conditioning; MTX, methotrexate; NS, not significant; RIC, reduced-intensity conditioning; TNC, total nucleated cells in graft.

Materials and Methods

In this retrospective, single-center study, patient data were collected from the Unit for Allogeneic Stem Cell Transplantation (CAST) at Karolinska University Hospital for patients receiving transplants from 2014 to 2017. Clinical patient data were collected from a quality registry regarding outcomes and complications. No major changes in standard treatment and supportive care were conducted during this period. A total of 91 patients transplanted with PBSC grafts from unrelated donors were included. Data regarding Annexin V and 7-aminoactinomycin D (7AAD), to identify early apoptotic cells and other cell markers in the graft, were retrieved from the Department of Clinical Immunology and Transfusion Medicine at Karolinska University Hospital. The local ethics committee approved the study (2017/2421).

Donor typing and apheresis

All donors and recipients underwent routine HLA class I and II typing using sequence-specific oligonucleotides and confirmatory 4-digit typing using sequence-specific primers [22]. G-CSF was used for 4 to 6 days to mobilize stem cells before apheresis collection. The standard target dose of G-CSF was 10 $\mu\text{g}/\text{kg}$. Apheresis collection was performed according to routine practice at the collection centers. Target CD34 dosage for collection was 5 million to 10 million CD34⁺ cells/kg of recipient body weight.

Transportation and storage conditions

Transportation time was defined as the number of days from apheresis until the graft reached the patient for transplantation. The apheresis product was transported in a sealed, temperature-controlled environment for transportation. The graft was placed in an outer bag to prevent leakage. A rigid outer case was used to insulate the product and protect it from fall damage. The desired temperature during shipping and storage was 4 $^{\circ}\text{C}$. A continuous temperature log was used while the graft was in the transportation container. Grafts arriving late in the evening could be stored at 2 $^{\circ}$ to 6 $^{\circ}\text{C}$ overnight before being transplanted.

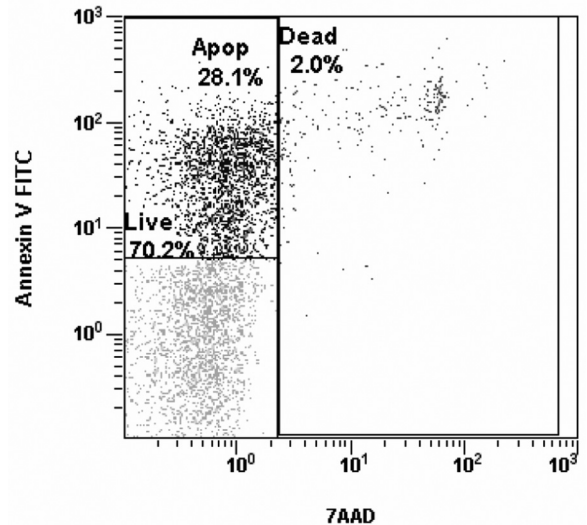


Figure 1. Gating strategy to differentiate viable, apoptotic and necrotic cells in the graft. Lower left gate: Viable cells (Live), Annexin V–negative and 7AAD–negative cells. Upper left gate: Apoptotic cells (Apop), showing various levels of phosphatidylserine binding the Annexin V staining. Right gate: Necrotic cells (Dead), Annexin V–positive or negative and 7AAD–positive, indicating damage to cell membrane integrity.

Flow cytometric analysis

Leukocytes were assessed using an automated blood cell counter (Sysmex XP300, Kobe, Japan). CD34⁺ cell enumeration was performed using a single platform analysis based on the ISHAGE gating strategy [27]. Briefly, 1 million nucleated cells were added to a duplicate of tubes with Trucount beads (BD, Stockholm, Sweden) and incubated for 10 min at room temperature (RT) with 7AAD (Immunotech A07704), CD34-PE-conjugated antibody (HPCA2:8G12 [IgG1], BD) and CD45-FITC-conjugated monoclonal antibody (2D1 [IgG1], BD). Then 1 mL of lysing solution (IOTest3 Lysing Solution; diluted 1:10 with deionized water) was added and incubated for an additional 10 min at RT. Cells were immediately analyzed by flow cytometry (FC500, Beckman Coulter).

Viability was assessed in a 50- μ L sample diluted in 1/20 PBS. Annexin V-FITC, CD3-PE, CD4-PC7 and 7AAD were used in the viability staining. After the addition of Annexin buffer, the sample was incubated for 15 min at RT. Additional 400 μ L Annexin buffer was added before acquiring the sample by flow cytometry (FC500, Beckman Coulter).

Early apoptotic cells are defined as Annexin V-positive and 7AAD-negative cells. Necrotic cells are defined as all 7AAD-positive cells. Graft viability was defined as the percentage of cells in the graft that were double negative for Annexin V and 7AAD. The median graft viability (85.1% viable cells) was used to define the two groups, low and high viability grafts.

Conditioning

Local conditioning regimens have been described previously [23]. Myeloablative conditioning was given to 16 patients and consisted of fractionated total-body irradiation (fTBI), 3 Gy daily for 4 days, combined with cyclophosphamide (Cy), 120 mg/kg (n = 1) or vepesid (n = 6) or busulfan at 4 mg/kg/day for 4 days combined with cyclophosphamide (Cy), 120 mg/kg (n = 3) or fludarabine (n = 5). One patient received treosulfan, 14 g/m² for 3 days combined with thio-tepa and fludarabine. Reduced-intensity conditioning (RIC) was given to 76 patients. RIC included fludarabine, 30 mg/m²/day for 3 to

6 days, combined with busulfan, 4 mg/kg/day for 2 (n = 19) or 3 (n = 3) days, or fTBI (3 Gy/day for 2 days) and Cy 120 mg/kg (n = 15) or treosulfan 14 g/m² for 3 days (n = 37). One patient was given FLAMSA-RIC. Anti-thymocyte globulin (ATG), 2 to 6 mg/kg, was administered depending on the underlying disease and the conditioning regimen (n = 89).

GvHD classification

aGvHD was classified according to the Glucksberg–Seattle criteria [24]. All cases of isolated gastrointestinal GVHD were verified by biopsies. cGVHD was graded according to the National Institutes of Health scoring system [25].

Statistical data

Statistical data were analyzed with R (version 4.0.2, R Core Team 2020, Vienna, Austria). Mann–Whitney *U* test was used to compare lymphocyte contents and patient/donor characteristics. Spearman correlation was used in nonparametric correlations. Categorical parameters were compared using χ^2 test. Survival was calculated with the Kaplan–Meier method and compared with the log-rank test. GvHD risk, TRM and relapse were analyzed with cumulative incidence of competing events and compared with Gray's test [26].

Factors with a *P* value <.40 in univariate analysis were included in the backwards elimination multivariate analysis. Survival was analyzed using Cox regression, and TRM was analyzed with the proportional subdistribution hazard regression model of Fine and Gary. Factors analyzed were patient and donor age, patient and donor sex, ABO mismatch, RIC or MAC, CD34⁺ cell dose per kg, and total nucleated cell (TNC) dose.

Results

In total, 91 patients were included from 2014 to 2017. The median follow-up time was 14 months (range 1 to 44). Median viability of the grafts, defined as Annexin V and 7AAD negative cells, was 85.1% (range 52.8 to 98.9%). Donor/recipient characteristics were

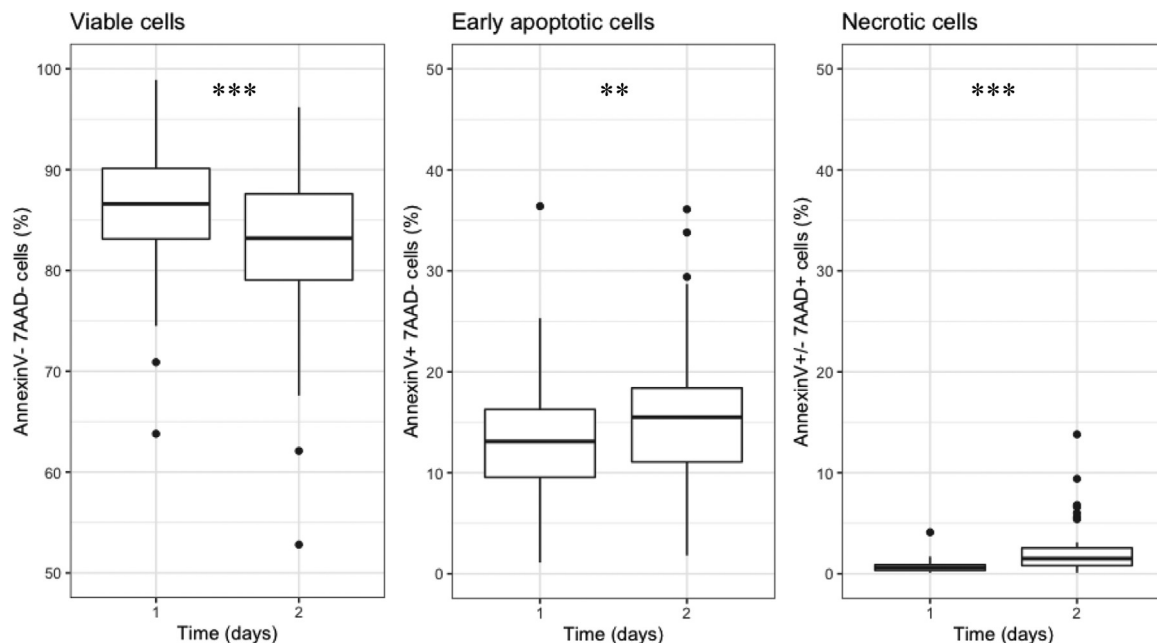


Figure 2. Percentage of graft cells positive for apoptotic or necrotic markers. The box plots are divided based on 1 or 2 days of transportation. Plots from left to right: (a) Viable cells: Annexin V- and 7AAD-negative cells. (b) Early apoptotic cells: Annexin V-positive and 7AAD-negative. (c) Necrotic cells: 7AAD-positive and Annexin V-positive or -negative cells. **P* < .05, ** *P* < .01, *** *P* < .001.

comparable between high and low viability groups (Table 1); no factors differed apart from the graft viability.

Gating strategies for early apoptotic and necrotic cells are shown in Figure 1. Early apoptotic cells constituted 13.5% (range 1.1% to 36.1%) of the grafts, and necrotic cells constituted 0.9% (range 0.1% to 13.8%) of the grafts. The median apoptotic and necrotic cell dose administered to the patients was $144 \times 10^6/\text{kg}$ (range 7 to $580 \times 10^6/\text{kg}$) (supplementary Fig. S1).

A subgroup analysis for CD3⁺ cell viability showed a high correlation with total graft viability ($P = .003$, supplementary Fig. S2a). Median CD3⁺ cell viability was 91.9%, which was higher than overall graft viability ($P < .001$, supplementary Fig. S2b). Eighty-eight patients received 6/6 HLA-matched grafts, and three patients received DR1-mismatched grafts (Table 1). All DR1-mismatched grafts were in the low viability group.

Transportation and graft parameters

Overall, 76 of the grafts came from German centers, 11 from other European centers and four from centers outside of Europe. No difference in graft viability was found when comparing European and non-European collection centers (data not shown). All PBSCs were transported in a sealed box with a temperature log. Graft viability did not correlate with arrival temperature (Table 1). Transportation time had a significant impact on graft viability (Fig. 2). PBSC grafts that had been *ex vivo* for 2 days had lower viability (83.2% [range 52.8% to 96.2%]) than grafts that had been *ex vivo* for only 1 day (86.6% [63.8% to 98.9%]), ($P = .003$).

The median volume of the PBSC grafts was 355 mL (range 120 to 604 mL), and the median leukocyte concentration was $217 \times 10^9/\text{mL}$ (range 107 to $375 \times 10^9/\text{mL}$). These two factors did not influence the content of apoptotic cells in the graft (supplementary Fig. S3).

Clinical outcome

The median time to neutrophil engraftment (absolute neutrophil count $>0.5 \times 10^9/\text{L}$) was 17 days (range 12 to 28 days) for both groups. Platelet engraftment, defined as days to platelet count $>30 \times 10^9/\text{L}$ without transfusion, was 14 days for both groups (range 0 to 94 days). Each group had a single patient suffer from graft failure.

One-year survival was significantly reduced in the low viability group compared with the high viability group (67.4% versus 88.9%, $P = .007$) (Fig. 3). Despite the significant difference in 1-year survival, long-term OS was similar in both groups in univariate analysis (Fig. 3, $P = .2$). Relapse was the most common cause of death in both groups, contributing to both early and late death.

TRM was similar between the two groups (Fig. 4a). Interestingly, five patients in the low viability graft group died from infectious complications/PTLD, and none in the high viability group ($P = .06$) (Table 2). The cumulative incidence of aGvHD grades II to IV (Fig. 4c) and cGvHD (Fig. 4d) was similar between the groups.

We further analyzed the risk of viral reactivation between both groups. The reactivation frequency of cytomegalovirus (CMV), Epstein-Barr virus (EBV) or varicella zoster virus (VZV) was similar (Table 2). Both groups also had the same frequency of bloodstream infection (defined as one positive blood culture for any pathological microbe).

Multivariate analysis

Three Cox regression analyses were performed for the endpoints at which graft viability showed significance or trends in univariate analysis. For 1-year survival, high graft viability showed a significantly lower risk for death in the multivariate analysis (hazard ratio [HR] 0.26, 95% confidence interval [CI] 95% 0.09 to 0.77; $P = .015$)

(Table 3). For long-term OS, HLA-DR1 mismatch was associated with poor survival (HR 6.13, 95% CI 1.40 to 27.03; $P < .016$), whereas graft viability was not significantly correlated with OS. High graft viability was associated with lower TRM (HR 0.16, 95% CI 0.03 to 0.82; $P = .027$).

Discussion

In this retrospective single-center study, longer transportation time was associated with lower graft viability and an increased frequency of apoptotic cells in the PBSC graft (Fig. 2). Low graft viability was associated with lower 1-year survival. We could also show that TRM was significantly associated with graft viability in the multivariate analysis (Fig. 4 and Table 3).

These findings are in line with the clinical experience that poor graft quality influences the post-transplant outcome, in terms of both time to engraftment and survival after transplantation. The immunologic and cellular mechanisms driving these observations were not investigated. Several feasible theories exist on how graft function affects transplantation outcome. Improvements in transportation

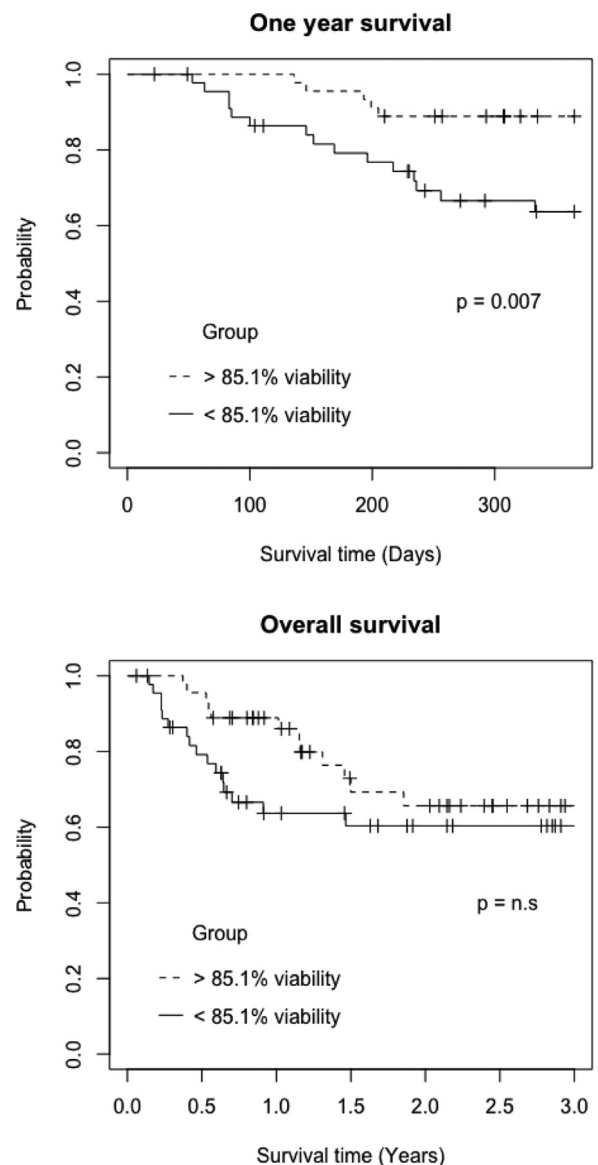


Figure 3. (a and b) One-year survival and overall survival in the low and high viability groups.

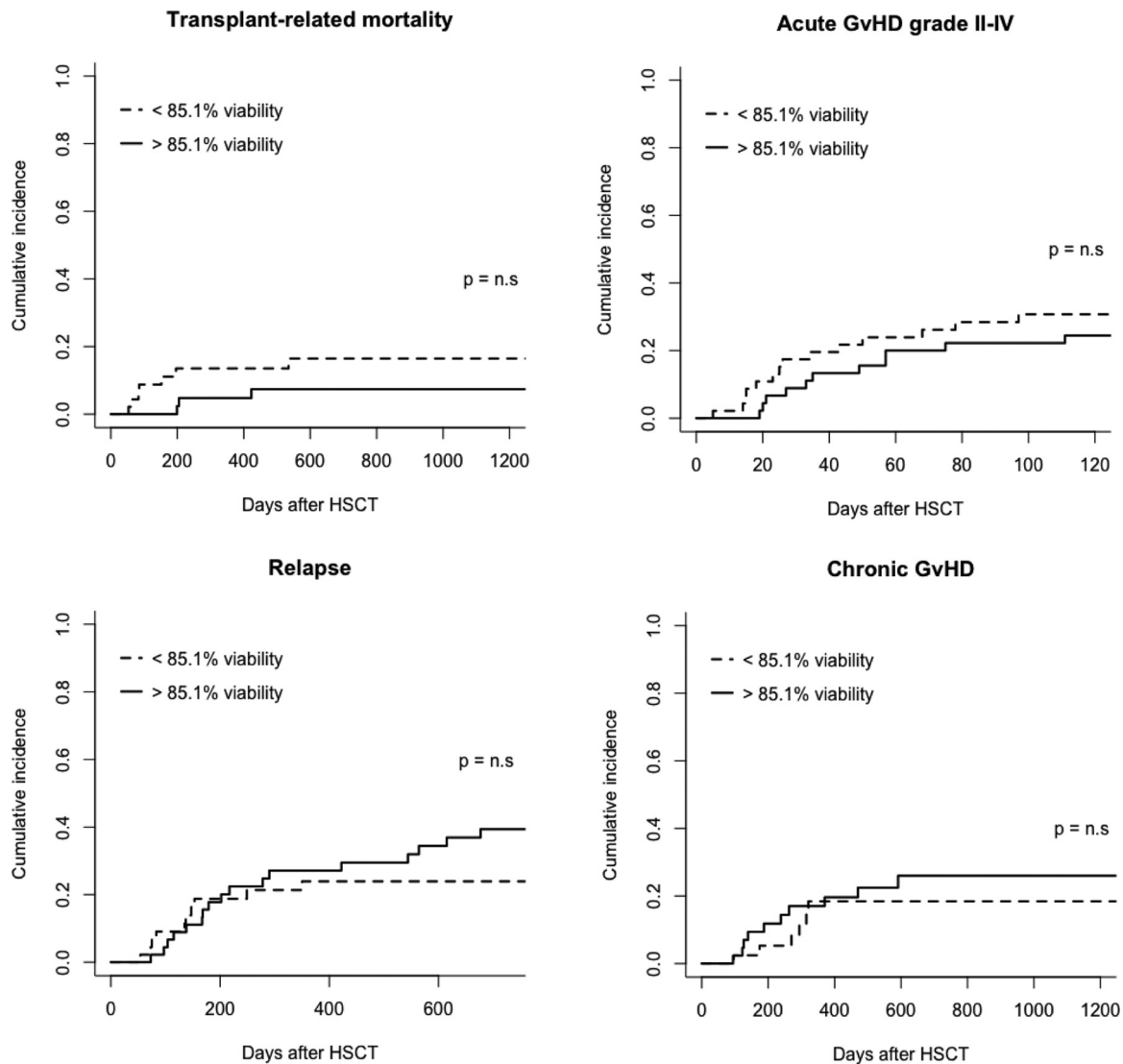


Figure 4. (a) Transplant-related mortality (TRM) illustrated by cumulative incidence. (b) The competitive risk analysis of relapse between high and low viability groups. (c) The cumulative incidence of aGvHD grade II to IV between the two viability groups. (d) The cumulative incidence of cGvHD between the two viability groups.

time and optimized graft handling before transplantation could be important for patient survival.

It is contentious whether shorter transportation time would be beneficial to transplant outcome. One study showed that transportation time did not affect clinical outcomes such as TRM, OS and GvHD with PBSC grafts, whereas another study showed that transportation time did affect survival after bone marrow grafts [27,28]. There are several key differentiators between previous studies and this study. In addition to transportation time, our study measured the frequency of apoptotic cells in the graft. Transportation time affects apoptotic cell content, but it is not known whether all grafts react similarly. Temperature and cell density during transport are two factors known to affect cell viability [11]. The data presented in our study indicate the benefits of having a high frequency of viable cells in the PBSC graft, in which transportation time plays a role (Fig. 2).

To further understand other factors that may contribute to apoptosis in the graft, we analyzed graft arrival temperature, graft volume, and cell concentration. We hypothesized that poor transportation and storage conditions may contribute to higher apoptosis in the grafts. However, we were unable to identify factors that contributed to higher graft apoptosis (Table 1, supplementary Fig. S3). We speculate that it may be a combination of transportation

time, temperature, cell concentration and other factors not accounted for in this study.

The decreased 1-year survival in the low viability group was mainly due to early relapse and infectious TRM. GvHD was an uncommon cause of death in this cohort. In the multivariate analysis, TRM was mostly predicted by TNC in the graft, followed by graft viability ($P = .027$). Surprisingly, five patients died from infections/PTLD in the low viability group compared with none in the high viability group (Table 2). The patients suffered from viral, bacterial and even invasive fungal infections (Table 2). This finding may reflect poor graft function, since all mortalities in infection occurred within the first year. Graft-derived immunity is assumed to be most important during the first few months after transplantation. Finally, although the low viability group had high mortality in the first year, it subsequently stabilized, with few new relapses or deaths occurring after 1 year. This contrasts with the high viability group, in which five patients relapsed after 1 year (Fig. 4b).

HLA mismatch is known to influence transplant outcome and long-term survival [29]. Three patients in the low viability group received a HLA-DR1 mismatch, whereas there was no mismatch in the high viability group. DR1 mismatch was added to the multivariate analysis to examine this confounding factor. For 1-year survival, graft viability remained the strongest predictor, followed by DR1 mismatch. For OS,

Table 2
Clinical outcome

	Low viability (<85.1%); n = 46	High viability (≥85.1%); n = 45	P Value
Engraftment data			
Neutrophils (d)	17 (13 to 27)	17 (12 to 28)	NS
Platelets >30 × 10 ⁹ (d)	13 (0 to 94)	14 (9 to 41)	NS
Graft failure	1	1	NS
GvHD			
aGvHD grade I	11	14	NS
aGvHD grade II to IV	14	11	NS
aGvHD grade III to IV	2	1	NS
cGvHD	17	21	NS
Infectious complications			
CMV	19 (41)	19 (42)	NS
EBV	10 (22)	10 (22)	NS
VZV	2 (4)	5 (11)	NS
BSI	13 (28)	13 (29)	NS
Cause of death			
Relapse	8 (17)	9 (20)	NS
Bacterial sepsis	1 (2)	0	NS
Invasive fungal infection	1 (2)	0	NS
Viral/bacterial pneumonia	1 (2)	0	NS
EBV-PTLD	2 (4)	0	NS
Other	3 (7)	3 (7)	NS

Data are median (range) or n (%) unless noted otherwise. BSI, blood stream infection; PTLD, post-transplant lymphoproliferative disease.

DR1 mismatch was the only remaining significant predictor, followed by recipient age as a borderline significant predictor (Table 3).

By chance, more acute myeloid leukemia (AML) patients received grafts with lower viability (Table 1). This could confound the 1-year survival because of the poor outcome for AML patients after transplantation, especially if they are beyond first remission [30]. Univariate analysis on the influence of the clinical diagnosis on 1-year survival did not show any significance. A separate analysis of AML patients, however, showed that high viability grafts were associated

with better 1-year survival, although this result was not statistically significant (data not shown).

Ninety-six percent of the patients received ATG before transplantation (Table 1). This probably influenced the study outcome significantly. Most of the graft T cells infused into patients who received ATG would enter apoptosis and be cleared rapidly. Phagocytosis of necrotic or apoptotic cells will modify macrophage and DC cell phenotype [31,32]. It has also been shown that the ingestion of ATG-coated cells by macrophages and DCs increases TGF-β and induces an increase in regulatory T cells (Tregs) [33]. However, ATG also depletes Tregs that may reduce the tolerogenic effect generated by ATG. Graft composition, condition regimen and GvHD prophylaxis may all affect APCs and other supporting cells crucial to the immunomodulatory response induced by apoptotic cells. In our study, the main GvHD prophylaxis was cyclosporine A and methotrexate (96% of patients). It has been suggested that sirolimus may promote Treg expansion more than cyclosporine A after apoptotic cell infusion [34]. This is an interesting aspect that we could not evaluate in this study, with only one patient treated with sirolimus.

A subgroup analysis of the CD3⁺ subset in the graft showed significantly higher CD3⁺ cell viability compared with overall graft viability. Despite the known importance of CD3⁺ cells in the graft, no clinical outcome parameter, such as 1-year survival, OS, TRM, GvHD or relapse, correlated with CD3⁺ cell viability in the graft (data not shown). This negative finding does not rule out CD3⁺ cell viability as a factor—it may be the interaction between different immune cell subsets and apoptotic content that is important. One study suggested that donor-derived plasmacytoid dendritic cells (pDCs) are important in the immunosuppressive effects after exposure for apoptotic cells [35]. We lack data on pDCs and several other aspects of the graft composition in this study. Further studies on graft viability should include markers for dendritic cells, NK cells and B cells for a clearer view of apoptotic cells in PBSC grafts. This difference between CD3⁺ viability and overall viability suggests that various subsets respond differently to *ex vivo* transportation situations.

Previous studies have shown that apoptotic donor cells can modify the immune response in transplantation [36,37]. Apoptotic cell therapy has been mostly directed at inducing tolerance and, thus, improving transplantation outcome. It is not known what the optimal

Table 3
Backwards elimination multivariate analysis

Factor	Univariate model Hazard ratio (95% CI)	P Value	Multivariate analysis Hazard Ratio (95% CI)	P Value
1-year survival				
Sex of recipient	1.59 (0.66 to 3.80)	.30		
ABO incompatibility	0.75 (0.45 to 1.25)	.27		
RIC	0.60 (0.22 to 1.67)	.33		
CD34/kg recipient	1.10 (0.97 to 1.24)	.16	1.16 (1.02 to 1.33)	.029*
Graft viability >85.1%	0.27 (0.10 to 0.75)	.01*	0.26 (0.09 to 0.77)	.015*
HLA-DR1 mismatch	6.81 (1.56 to 29.7)	.01*	6.13 (1.30 to 29.41)	.022*
Overall survival				
Age of recipient (y)	0.98 (0.96 to 1.00)	.06	0.98(0.96 to 1.00)	.075
RIC	0.55 (0.23 to 1.30)	.17		
Age of donor (y)	0.98 (0.93 to 1.02)	.33		
TNC	1.04 (0.97 to 1.11)	.30		
CD34/kg recipient	1.07 (0.94 to 1.22)	.27		
Graft viability >85.1%	0.61 (0.29 to 1.30)	.20		
HLA-DR1 mismatch	6.81 (1.56 to 29.7)	.01*	6.13 (1.40 to 27.03)	.016*
TRM				
Sex of recipient	6.49 (1.38 to 30.5)	.02		
Age of donor (y)	0.96 (0.90 to 1.03)	.23		
TNC	1.12 (1.07 to 1.17)	<.001*	1.17 (1.10 to 1.24)	<.001*
CD34/kg recipient	1.17 (1.10 to 1.24)	<.001*		
Graft viability >85.1%	0.38 (0.10 to 1.40)	.14	0.16 (0.03 to 0.82)	.027*
HLA-DR1 mismatch	4.86 (0.48 to 49.1)	.18		

Data are median (range). ABO, ABO blood group incompatibility; RIC, reduced-intensity conditioning. *P < .05.

dose of apoptotic cells is to reach a beneficial effect. However, the absolute number of apoptotic cells infused in our patients were a median of 144 million apoptotic cells/kg. This dose is comparable with the doses administrated by Mevorach *et al.* [16] in the phase I/II study using apoptotic cells as a treatment. However, we did not find any benefit to having high amounts of apoptotic cells in the PBSC graft. The increase in apoptotic cells likely indicates decreased viability. Using markers for early apoptosis, it is possible to modify PBSC collection and transportation factors to find optimal conditions to keep apoptosis in the PBSC graft to a minimum.

This retrospective single-center study is limited by a relatively small study sample. Future larger studies with apoptotic markers would give more confidence to the observations. Another weakness of this study is the lack of stem cell markers and more detailed lymphoid subpopulation markers in relation to apoptotic cells. It would be interesting to investigate whether apoptosis of certain cell subsets in the graft influences the clinical outcome.

Summary

Graft viability measured by Annexin V and 7AAD provides a detailed characterization of PBSC grafts. Even with normal transportation times of 1 to 2 days, apoptosis still occurs to a significant degree. Decreased graft viability was associated with worse clinical outcome. In the multivariate analysis, low graft viability correlated with worse 1-year survival and increased TRM. Infectious complications were a common cause of death in the low graft viability cohort. This suggests that loss of some viable cells in PBSC grafts may affect the early immune reconstitution. It is known that apoptotic cells may provide a more immune-suppressive effect in transplant patients and can induce Treg function, dampening the graft-versus-leukemia effect and resistance to infections. Interestingly, the viability of PBSC grafts did not affect GVHD or engraftment in this study. Further studies are needed to understand the underlying mechanisms. Improving transportation conditions to decrease apoptosis and increase cell viability in the graft are desirable to improve transplantation outcome.

Conflict of interest

None of the authors has any potential financial conflict of interest related to this manuscript.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi: [10.1016/j.jcyt.2021.11.008](https://doi.org/10.1016/j.jcyt.2021.11.008).

Reference

- Passweg JR. Hematopoietic stem cell transplantation in Europe 2014: more than 40 000 transplantations annually. *Bone Marrow Transplantation* 2016(January):1–7.
- Watz E, Remberger M, Ringden O, Ljungman P, Sundin M, Mattsson J, et al. Quality of the hematopoietic stem cell graft affects the clinical outcome of allogeneic stem cell transplantation. *Transfusion* 2015;55(10):2339–50.
- Wang T, Remberger M, Axedorph Nygell U, Sundin M, Björklund A, Mattsson J, et al. Change of apheresis device decreased the incidence of severe acute graft-versus-host disease among patients after allogeneic stem cell transplantation with sibling donors. *Transfusion* 2018;58(6):1442–51.
- Sairafi D, Stikvoort A, Gertow J, Mattsson J, Uhlin M. Donor Cell Composition and Reactivity Predict Risk of Acute Graft-versus-Host Disease after Allogeneic Hematopoietic Stem Cell Transplantation. *Journal of Immunology Research* 2016;2016:1–11.
- Saraceni F, Shem-Tov N, Olivieri A, Nagler A. Mobilized peripheral blood grafts include more than hematopoietic stem cells: the immunological perspective. *Bone Marrow Transplant* 2015;50(7):886–91.
- Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972;26(4):239–57.
- D'Atri LP, Etulain J, Romaniuk MA, Torres O, Negrotto S, Schattner M. The low viability of human CD34+ cells under acidic conditions is improved by exposure to thrombopoietin, stem cell factor, interleukin-3, or increased cyclic adenosine monophosphate levels. *Transfusion* 2011;51(8):1784–95.
- Anthony RS, McKelvie ND, Cunningham AJ, Craig JI, Rogers SY, Parker AC. Flow cytometry using annexin V can detect early apoptosis in peripheral blood stem cell harvests from patients with leukaemia and lymphoma. *Bone Marrow Transplant* 1998;21(5):441–6.
- Antonenas V, Garvin F, Webb M, Sartor M, Bradstock KF, Gottlieb D. Fresh PBSC harvests, but not BM, show temperature-related loss of CD34 viability during storage and transport. *Cytotherapy* 2006;8(2):158–65.
- Kao GS, Kim HT, Daley H, Ritz J, Burger SR, Kelley L, et al. Validation of short-term handling and storage conditions for marrow and peripheral blood stem cell products. *Transfusion* 2011;51(1):137–47.
- Jansen J, Nolan PL, Reeves MI, Morgan JA, Akard LP, Thompson JM, et al. Transportation of peripheral blood progenitor cell products: effect of ambient temperature. *Cytotherapy* 2010;12(7):919–23.
- Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J Immunol Methods* 1995;184(1):39–51.
- Darzynkiewicz Z, Juan G, Li X, Gorczyca W, Murakami T, Traganos F. Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis). *Cytometry* 1997;27(1):1–20.
- Silva MT. Secondary necrosis: the natural outcome of the complete apoptotic program. *FEBS Lett* 2010;584(22):4491–9.
- Krysov DV, Vanden Berghe T, D'Herde K, Vandenabeele P. Apoptosis and necrosis: detection, discrimination and phagocytosis. *Methods* 2008;44(3):205–21.
- Mevorach D, Zuckerman T, Reiner I, Shimoni A, Samuel S, Nagler A, et al. Single infusion of donor mononuclear early apoptotic cells as prophylaxis for graft-versus-host disease in myeloablative HLA-matched allogeneic bone marrow transplantation: a phase I/IIa clinical trial. *Biol Blood Marrow Transplant* 2014;20(1):58–65.
- Wang Z, Larregina AT, Shufesky WJ, Perone MJ, Montecalvo A, Zahorchak AF, et al. Use of the inhibitory effect of apoptotic cells on dendritic cells for graft survival via T-cell deletion and regulatory T cells. *Am J Transplant* 2006;6(6):1297–311.
- Sun E, Gao Y, Chen J, Roberts AI, Wang X, Chen Z, et al. Allograft tolerance induced by donor apoptotic lymphocytes requires phagocytosis in the recipient. *Cell Death Differ* 2004;11(12):1258–64.
- Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J Clin Invest* 1998;101(4):890–8.
- Chen W, Frank ME, Jin W, Wahl SM. TGF-beta released by apoptotic T cells contributes to an immunosuppressive milieu. *Immunity* 2001;14(6):715–25.
- Kleinclauss F, Perruche S, Masson E, de Carvalho Bittencourt M, Biichle S, Remy-Martin JP, et al. Intravenous apoptotic spleen cell infusion induces a TGF-beta-dependent regulatory T-cell expansion. *Cell Death Differ* 2006;13(1):41–52.
- Olerup O, Zetterquist H. HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. *Tissue Antigens* 1992;39(5):225–35.
- Remberger M, Ackefors M, Berglund S, Blennow O, Dahllöf G, Dlugosz A, et al. Improved survival after allogeneic hematopoietic stem cell transplantation in recent years. A single-center study. *Biology of Blood and Marrow Transplantation* 2011;17(11):1688–97.
- Glucksberg H, Storb R, Fefer A, Buckner CD, Neiman PE, Clift RA, et al. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HLA-matched sibling donors. 1974. p. 295–304.
- Filipovich AH, Weisdorf D, Pavletic S, Socie G, Wingard JR, Lee SJ, et al. National Institutes of Health Consensus Development Project on criteria for clinical trials in chronic graft-versus-host disease: I. diagnosis and staging working group report. *Biology of Blood and Marrow Transplantation* 2005;11(12):945–56.
- Fine JP, Gray RJ. Proportional Hazards Model for the Subdistribution of a Competing Risk A Proportional Hazards Model for the Subdistribution of a Competing Risk. *Journal of the American Statistical Association* 1999;1459(April 2013):37–41.
- Lazarus HM, Kan F, Tarima S, Champlin RE, Confer DL, Frey N, et al. Rapid transport and infusion of hematopoietic cells is associated with improved outcome after myeloablative therapy and unrelated donor transplant. *Biol Blood Marrow Transplant* 2009;15(5):589–96.
- Patton WN, Nivison-Smith I, Barty P, Dodds A, Ma D, Shaw PJ, et al. Graft Transit Time Has No Effect on Outcome of Unrelated Donor Hematopoietic Cell Transplants Performed in Australia and New Zealand: A Study from the Australasian Bone Marrow Transplant Recipient Registry. *Biol Blood Marrow Transplant* 2017;23(1):147–52.
- Burt C, Parker A, McQuaker G, Copland M, Brierley C, Little AM, et al. In a 12-allele analysis HLA-DPB1 matching is associated with improved OS in leukaemic and myelodysplastic patients receiving myeloablative T-cell-depleted PBSC from unrelated donors. *Bone Marrow Transplant* 2014;49(5):657–63.

- [30] Jindra P, Raida L, Karas M, Szotkowski T, Lysák D, Hrabětová M, et al. Allogeneic Stem Cell Transplantation in Patients With FLT3-ITD Mutated AML: Transplantation in CR1 Is the Decisive Factor for Good Outcome. *Clin Lymphoma Myeloma Leuk* 2019;19(7):462–9.
- [31] Morelli AE, Larregina AT. Apoptotic cell-based therapies against transplant rejection: role of recipient's dendritic cells. *Apoptosis* 2010;15(9):1083–97.
- [32] Kuwana M, Okazaki Y, Ikeda Y. Splenic macrophages maintain the anti-platelet autoimmune response via uptake of opsonized platelets in patients with immune thrombocytopenic purpura. *J Thromb Haemost* 2009;7(2):322–9.
- [33] Perruche S, Zhang P, Liu Y, Saas P, Bluestone JA, Chen W. CD3-specific antibody-induced immune tolerance involves transforming growth factor-beta from phagocytes digesting apoptotic T cells. *Nat Med* 2008;14(5):528–35.
- [34] Bonnefoy F, Masson E, Perruche S, Marandin A, Borg C, Radlovic A, et al. Sirolimus enhances the effect of apoptotic cell infusion on hematopoietic engraftment and tolerance induction. *Leukemia* 2008;22(7):1430–4.
- [35] Bonnefoy F, Perruche S, Couturier M, Sedrati A, Sun Y, Tiberghien P, et al. Plasmacytoid dendritic cells play a major role in apoptotic leukocyte-induced immune modulation. *J Immunol* 2011;186(10):5696–705.
- [36] Pessach I, Shimoni A, Nagler A. Apoptotic cells in allogeneic hematopoietic stem cell transplantations: "turning trash into gold". *Leuk Lymphoma* 2012;53(11):2130–5.
- [37] Saas P, Daguindau E, Perruche S. Concise Review: Apoptotic Cell-Based Therapies—Rationale, Preclinical Results and Future Clinical Developments. *Stem Cells* 2016;34(6):1464–73.