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Flow Cytometric Characterization of Hematopoietic Stem and Progenitor Cell Subpopulations in Autologous Peripheral Blood Stem Cell Preparations after Cryopreservation

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Autologous · Cryopreservation · Flow cytometry · Hematopoietic stem cells

Abstract

Introduction: Autologous stem cell transplantation is a successful routine procedure with only a small number of non-engraftment cases, although the time to hematopoietic recovery may vary considerably across patients. While CD34 has been the decisive marker for enumerating hematopoietic stem and progenitor cells (HSPCs) for more than 30 years, the impact of CD34-positive cellular subpopulations in autologous HSPC grafts on hematopoietic reconstitution remains unclear. ***Methods:*** The two-color ISHAGE protocol represents the current gold standard for $CD34^+$ cell enumeration but includes only the number of viable $CD45^+/CD34^+$ cells relative to the body weight of the recipient. We adapted a multicolor flow cytometry marker panel for advanced characterization of CD34 subpopulations in retained samples of autologous peripheral blood stem cell products ($n = 49$), which had been cryostored for a wide range from 4 to 15 years. The flow cytometric analysis included CD10, CD34, CD38, CD45, CD45RA, CD133, and viability staining with 7AAD. The findings were correlated with clinical engraftment data, including reconstitution of leukocytes, neutrophils, and platelets after transplantation (TPL). ***Results:*** We demonstrated that the identification of autologous HSPC subpopulations by flow cytometry after cryopreservation is feasible. Regarding the distribution of HSPC subpopulations, a markedly different pattern was observed in comparison to previously published data obtained using fresh autologous material. Our data revealed the largest ratio of lympho-myeloid progenitors (LMPPs) after freezing and thawing, followed by multipotent progenitors and erythroid-myeloid progenitors. A high ratio of LMPPs, representing an immature stage of differentiation, correlated significantly with early neutrophilic granulocyte and leukocyte engraftment ($p = 0.025$ and $p = 0.003$). Conversely, a large ratio of differentiated cells correlated with late engraftment of neutrophilic granulocytes ($p = 0.024$). Overall, successful engraftment was documented for all patients. ***Conclusion:*** We established an advanced flow cytometry panel to assess the differentiation ability of cryostored autologous peripheral blood stem cell grafts and correlated it with timely hematopoietic reconstitution. This approach represents a

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novel and comprehensive way to identify hematopoietic stem and progenitor subpopulations. It is a feasible way to indicate the engraftment capacity of stem cell products.

Introduction

Autologous hematopoietic stem cell transplantation after high-dose chemotherapy is part of the therapeutic regimen for various hematopoietic diseases, such as multiple myeloma (MM) and B- and T-cell lymphoma [1–3]. Since the late 1980s, mobilized peripheral blood stem cells (PBSCs) have become established as the preferred source for hematopoietic stem cell transplantation, as they are less cumbersome to obtain and associated with faster engraftment than bone marrow (BM) [4, 5].

Approaches for controlling the quality of stem cell products include flow cytometric enumeration of $CD34^+$ cells and the total number of nucleated cells (TNCs/WBCs [6]), as well as assessing viability and conducting colony formation assays (CFAs) [7, 8]. It has been reported previously that the $CD34^+$ cell count and CFA results do not correlate reliably with timely engraftment of neutrophilic granulocytes and platelets after transplantation in most diagnosis-specific groups [9–12]. However, there is a consensus in the literature on the use of 2×10^6 $CD34^+$ cells/kg body weight (bw) as the minimum dose for autologous transplantation after high-dose myeloablative chemotherapy [13]. The extent to which PBSC grafts with a higher $CD34^+$ cell count may result in clinically relevant accelerated hematopoietic engraftment in the context of specific conditions is controversial [14].

Hematopoietic stem cells are routinely identified by a protocol that was delineated by the International Society of Hematotherapy and Graft Engineering (ISHAGE) in 1996 [15]. As CD34 is a marker for heterogeneous subgroups of cells, the composition of hematopoietic subpopulations as progenitor cells of the hematopoietic system [16–19] according to the revised model of hematopoietic differentiation [20, 21] might be the key for obtaining a better understanding of graft quality. Online supplement 1 (for all online suppl. material, see https://doi.org/10.1159/000533624) provides a schematic overview of this concept. Regarding the analysis of cryopreserved material, it remains unclear whether a valid enumeration of $CD34^+$ cells after cryopreservation and thawing is feasible. In fact, $CD34^+$ recovery rates of >100% after cryopreservation have been reported [22–24].

Furthermore, the lack of specific viability testing for the reinfused cells has been criticized, as viability is usually not reevaluated after cryopreservation [22, 25, 26]. We used a novel dual platform protocol to assess post-thaw viability and evaluate CD34 on the first platform and evaluate subpopulations on the second platform. As the timing after thawing may be crucial, we used platform 1 for the standardized process of CD34 enumeration directly after thawing using a procedure similar to the initial measurement prior to cryopreservation (ISHAGE protocol). On platform 2, cells were stained after a recovery phase of 1 h in an incubator with antibodies to enumerate subpopulations.

It has been presumed that hematopoietic stem and progenitor cells (HSPCs) follow dedicated differentiation pathways in which highly immature hematopoietic stem cells divide asymmetrically, and this has been demonstrated via multicolor immunophenotyping by flow cytometry for various stem cell-containing grafts [27]. A varying distribution of subpopulations has been demonstrated for allogeneic and fresh autologous PBSCs, as well as for BM [1, 28]. A comprehensive approach was presented by Dymtrus et al. [28], who, in line with Worel et al. [1], observed a significantly higher ratio of very immature subpopulations in fresh autologous PBSCs than in BM. We modified this protocol to analyze the impact of cryopreservation and the engraftment kinetics of cryopreserved autologous samples.

The goal of this study was to examine the distribution pattern of HSPC subpopulations prior to and after cryopreservation and to correlate the defined HSPC subpopulations with the clinical engraftment of the respective autografts.

Materials and Methods

Patient Selection and Data Collection

We collected clinical data from 49 patients who had undergone autologous stem cell transplantation in the Department of Hematology and Oncology at the University Medical Center Mannheim (UMM) between 2006 and 2018. We assessed clinical characteristics, disease- and transplant-related parameters, and treatment and follow-up data. All patients except one had received their first stem cell transplantation. The majority of the patients were diagnosed with MM or amyloidosis ($n = 32$; 65%). The other indications for autologous transplantation included B-cell non-Hodgkin lymphoma ($n = 11$; 22.5%) and T-cell lymphoma ($n = 3$; 6.25%), as well as other diseases (multiple sclerosis, testicular carcinoma, and acute myeloid leukemia; $n = 3$; 6.25%). On average, the patients received 3.15×10^6 $CD34^+$ cells/kg bw (median 2.59; range 0.52–17.28). The stem cell products had been cryopreserved for an average of 85.5 days (median 43; range 5–769) prior to transplantation. The patient characteristics are shown in Table 1. PBSC retention samples were stored for quality control. Further validation of our setup and strategy was performed by evaluating a series of fresh and cryopreserved allogeneic PBSC samples ($n = 5$). All patients and donors consented to storage and comprehensive analysis of quality control samples when autologous or allogenic stem cells were collected. An Institutional Ethics Committee approved the analysis of the samples as well as the retrospective evaluation of clinical data (Ref. 2019-726N).

Table 1. Patient characteristics and stem cell graft composition

Variables	Mean/*n*	Median	Range
Patients (*n* = 49)			
Age, years	59.6	61	20–74
Gender (female/male)	22/27		
Diagnosis and mobilization regimen			
MM	*n* = 32	65%*	
CAD	12	37.5%**	
Cyclophosphamide	19	59.4%**	
G-CSF (steady-state)	1	3.1%**	
B-NHL	*n* = 11	22.5%*	
(R-)CHOP	1	9.1%**	
(R-)CHOEP	1	9.1%**	
(R-)DHAP	4	36.4%**	
(R-)Ara-C/thiotepa	2	18.2%**	
Dexa-BEAM	1	9.1%**	
Matrix	1	9.1%**	
G-CSF (steady-state)	1	9.1%**	
T-cell lymphoma/others	*n* = 6	12.5%*	
CHOP	2	16.7%**	
DHAP	1	33.2%**	
TAD/HAM	1	16.7%**	
Taxol + ifosfamide	1	16.7%**	
G-CSF (steady-state)	1	16.7%**	
Body weight, kg	82	82	46–139
Graft composition			
Duration of cryostorage, days	85.5	43	5–769
$CD34^+$ cell count prior cryopreservation, $\times 10^6$/kg	3.15	2.59	0.52–17.28
$CD34^+$ cell count post-cryopreservation, $\times 10^6$/kg	2.94	2.33	0.33–9.3
viable $CD34^+$ among single cells, %	2.91	1.76	0.05–29.6
$CD34^+$ subpopulations after cryopreservation			
MPPs, %***	18.02	13.6	0.09–51.5
MPPs, $\times 10^6$/kg bw	0.49	0.32	0.01–2.45
LMPPs, %***	42.27	43	2.18–74.8
LMPPs, $\times 10^6$/kg bw	1.42	0.99	0.01–5.7
MLPs + BLPs, %***	11.44	8.67	0.01–74.16
MLPs + BLPs, $\times 10^6$/kg bw	0.3	0.2	0.01–1.57
late GMPs, %***	9.87	8.11	1.44–32.8
late GMPs, $\times 10^6$/kg bw	0.26	0.14	0.03–1.17
EMPs, %***	18.12	14.30	0.09–63.20
EMPs, $\times 10^6$/kg bw	0.46	0.33	0.01–2.69

MM, multiple myeloma; B-NHL, B-non-Hodgkin lymphoma; MPPs, multipotent progenitors; LMPPs, lympho-myeloid progenitors; MLPs, multi-lymphoid progenitors; BLPs, B-lymphoid progenitors; late GMPs, granulocyte and macrophage progenitors; EMPs, erythroid-myeloid progenitors; (R-)Ara-C, (rituximab-)cytarabine; CAD, cyclophosphamide, adriamycin, dexamethasone; (R-)CHOEP, rituximab, cyclophosphamide, doxorubicin, vincristine, etoposide, prednisone; (R-)CHOP, rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone; Dexa-BEAM, dexamethasone, BCNU (bis-chloroethyl-nitrosourea), etoposide, cytarabine, melphalan; (R-)DHAP, rituximab, dexamethasone, cytarabine, cisplatin; Matrix, rituximab, methotrexate, cytarabine, thiotepa; TAD/HAM, 6-thioguanine, cytarabine, daunorubicin/high-dose cytarabine, mitoxantrone; G-CSF, granulocyte stimulating factor. *Ratio (*n* = 49). **% among diagnosis group (MM, B-NHL, T-cell). ***% among viable CD34+ cells.

PBSC Collection and Cryopreservation

All samples were collected using the Spectra Optia™ (Terumo BCT®, Garching, Germany) leukapheresis machine. For cryopreservation, the collected cells were diluted with autologous plasma and dimethyl sulfoxide (DMSO) and divided into 2–6 cryobags (average: 4). Before July 2011, the final product included 5.5% DMSO, then the procedure was modified and the final product included 7.5% DMSO. After controlled-rate freezing, it was stored in vapor-phase nitrogen at a temperature of ≤−150°C.

For correlation analysis of the CD34 subpopulations and engraftment kinetics, we analyzed the time in days after autologous transplantation until the thresholds of leukocytes >1.0/nL (*n* = 49), neutrophilic granulocytes >0.5/nL (*n* = 41), and platelets >20/nL and >50/nL (*n* = 47 and *n* = 38) were reached.

Flow Cytometry Assay Development

After rapid thawing and washing, blood cell counts were obtained using a Casy Cell Counter TT (OMNI Life Science GmbH & Co. KG, Bremen, Germany). We chose a **dual platform** approach: first, the classical ISHAGE protocol was used for CD34 enumeration directly after thawing using the BD stem cell kit and BD Trucount tubes, and second, to enumerate subpopulations, cells were stained after a recovery phase of 1 h in an incubator with appropriate titrated antibodies (for details, see below). To ensure accurate classification of HSPCs into the subpopulations presented in the lineage tree (online supplement 1), we included the markers CD10, CD38, CD45RA, and CD133, in addition to evaluation of CD34, CD45, and the viability dye 7AAD (all antibodies: BD, Heidelberg, Germany). A FACSCanto™ equipped with three lasers (FACSDiva™ 8 software) and FlowJo™ 10.6 analysis software were used (BD, Heidelberg, Germany). To address the question of which viability dye was most appropriate, we compared trypan blue with 7AAD in previous tests; the latter included in ISHAGE gating. 7AAD turned out to be the most compatible and precise marker for cell viability measurement via FACS.

For ***platform 1***, a part of the sample (100 µL) was used directly after thawing for the standardized process of CD34 enumeration using a BD stem cell kit and BD Trucount tubes; this protocol was procedurally similar to the initial measurement prior to cryopreservation (ISHAGE protocol).

For ***platform 2***, the remaining part of the sample was used for subpopulation analysis and immediately diluted in MACS buffer (PBS, Lonza, Walkersville, USA; BSA and EDTA, Sigma, Darmstadt, Germany). To allow recovery from cryopreservation, the cells were kept for 1 h in long-term bone marrow culture medium (LTBMC: IMDM, FCS, horse serum, penicillin/streptomycin (Thermo Fisher Scientific, Dreieich, Germany), hydrocortisone (Stem Cell Technologies, Cologne, Germany), and L-Glutamine (Sigma, Darmstadt, Germany)) in an incubator at 37°C, with 5% CO_2 and 80% humidity. The cells were resuspended in PBS and adjusted to 1×10^6 cells/100 µL for staining. FcR blockers were added to reduce nonspecific staining. Fluorescence minus one controls were used to define exact gating borders. The stopping gate was a minimum of either 1,000 $CD34^+$ cells or 150,000 WBCs. The gating strategy was used according to Dmytrus et al. [28] (the gating strategy is shown in online supplement 2).

Statistical Analysis

Correlation analyses of subpopulations and engraftment or patient data were performed using Pearson's and Spearman's correlation coefficient. This analysis and the similarly applied Maloney-Rastogi test or *t*- and Wilcoxon tests were performed with SAS (Statistical Analysis System, Version 9.4). p values ≤0.05 were considered statistically significant. Unless otherwise indicated, the data describe the mean, standard deviation, and range in terms of the minimum and maximum.

Visuals

Visuals have been created using BioRender.com (online supplement 1), Microsoft Excel, and PowerPoint.

Results

Handling of Cryopreserved Material

To obtain high sample quality and to reduce cell clumping and loss of cell viability, we ensured the absence of microbial contamination, prevented ice crystal formation and cell dehydration (via a narrow window for the freezing rate), and ensured rapid warming rates, a short thawing time and rapid dilution of DMSO [29]. We achieved the best results with rapid removal of DMSO, the addition of FBS to the resuspended samples, and a short recovery culture in LTBMC medium [30].

$CD34^+$ Cell Enumeration

Calculation of the absolute number of $CD34^+$ cells:

$$\frac{absolute\ number\ of\ live\ CD34+cells}{number\ of\ beads\ counted} \times \frac{total\ number\ of\ beads\ per\ tube}{sample\ volume\ (100\ \mu l)} \times sample\ dilution\ factor = Y \quad (1)$$

$$\frac{Y\ x\ PBSC\ volume\ (\mu L)}{body\ weight\ (kg)} \div 1{,}000 \times dilution\ factor\ prior\ to\ cryopreservation = Z \quad (2)$$

Y = absolute number of $CD34^+$ cells per µl; Z = absolute number of $CD34^+$ cells per kg bw. First, we compared $CD34^+$ counts before (as part of the obligatory quality control) and after cryopreservation (the same ISHAGE protocol was used). For the 49 samples tested, we observed that the $CD34^+$ cell number differed significantly from the initial value before cryopreservation. Fifty percent of our samples had a lower $CD34^+$ cell count and 50% had a higher $CD34^+$ cell count than before cryopreservation (Fig. 1). No correlation was found between the duration of cryostorage and CD34 recovery (p = 0.161). On average, 3.15×10^6 $CD34^+$ cells/kg bw (median 2.59; SD 2.63; range 0.52–17.28) were counted prior to cryopreservation, and 2.94×10^6 live $CD34^+$ cells/kg bw were counted after cryopreservation (SD 2.26; range 0.33–9.3). The average recovery rate was 105% (median 97.7; SD 64%; range 0.05–271). The mean percentage of $CD34^+$ cells among single cells was 5.57% (median 3.13; range 0.09–33.8) after cryopreservation. A procedural correlation was excluded by running the Maloney-Rastogi test and the *t*- and Wilcoxon tests (p = 0.304, p = 0.856).

CD34 Cell and WBC Viability

Second, using the ISHAGE protocol, we determined CD34 cell viability immediately after thawing. As measured by 7AAD dye exclusion by flow cytometry, mean CD34 cell viability was 68.6% (median 75.3; SD 22.69; range 7.74–95), and mean WBC viability was 48.6% (median 53.9; SD 22.8; range 5.6–86.3). WBCs from the same retention samples examined microscopically with trypan blue showed a higher mean viability of 77% (median 82; SD 14; range 35–98).

Notably, in autologous transplants, the viability of WBCs must exceed 70% after freezing and thawing for product release, tested by trypan blue staining. All autografts fulfilled this release criterion. No negative

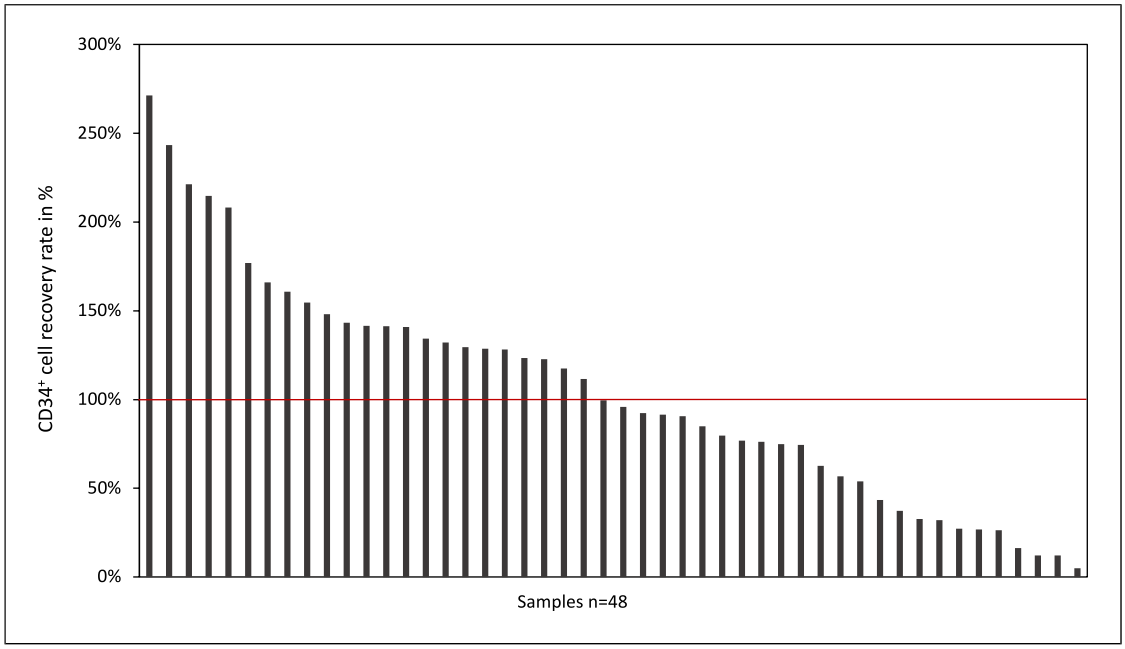

Fig. 1. Recovery rate of $CD34^+$ cells after cryopreservation in percent. The red line represents 100%.

correlation of CD34 cell or WBC viability with the duration of cryostorage was observed, which is in line with previous findings [12]. While the routinely applied trypan blue viability test routinely delivers high values above 70% (with only very few exceptions), the results of viability testing by flow cytometry vary considerably.

Analysis of HSPC Subpopulations

Technically, the first step in gating the CD34 subpopulation was the subdivision according to CD45RA expression. The mean percentage of $CD45RA^+$ cells among cells of the lympho-myeloid branch was 63.8% (SD 19.22), with an absolute number of 1.99×10^6/kg bw (SD 1.77). For $CD45RA^-$ cells, this percentage was 36.2% (SD 19.22), and the absolute number was 0.95×10^6/kg bw (SD 0.77) (online supplement 3).

To identify HSPC subpopulations in cryopreserved autologous HSPC products, we decided to add a short incubation phase first to allow the HSPCs to recover from cryodamage. In contrast to Dmytrus et al. [28], who analyzed fresh and 1-year posttransplant BM preparations, as well as autologous fresh PBSC preparations, and Worel et al. [1] who reported 47% multipotent progenitors (MPPs), 27% erythroid-myeloid progenitors (EMPs), and 22% lympho-myeloid progenitors (LMPPs) (Fig. 2a) in autologous fresh PBSCs, we noted a more heterogeneous composition of HSPC subpopulations: MPPs represented only 18% (SD 13.2), whereas LMPPs represented 42% (SD 19.3) and EMPs represented 18% (SD 12.9). Late granulocyte and macrophage progenitors (GMPs) and multi-lymphoid progenitors (MLPs)/B-lymphoid progenitors (BLPs) represented only 2% each in the Worel study (Fig. 2a) but increased in percentage to 10% (late GMPs) and 12% (MLPs/BLPs) in this study (Fig. 2b). The proportions of MLPs and BLPs are usually summarized in the literature as an indicator of the lineage of differentiation. However, in our study, we found a substantial proportion of MLPs (10.6%; SD 10.77; Fig. 3), whereas the proportion of BLPs was minimal (0.8%; SD 1.23; Fig. 3). All data of $CD34^+$ subpopulations are summarized in Table 1.

This obvious difference in cell composition led us to propose that this was related to the cryopreservation process. To assess this possibility, we performed a matched pair analysis comparing fresh and cryopreserved allogeneic PBSCs. In the fresh material (Fig. 2c), the distribution of subpopulations was highly comparable to the data reported by Worel et al. [1] (Fig. 2a). However, in the cryopreserved/thawed samples (Fig. 2d), the cellular composition resembled the cryopreserved samples shown in Figure 2b: MPPs were dominant in fresh samples but were found to be present in lower numbers in cryopreserved samples, which were dominated by LMPPs (compare to online supplement 1).

Correlation of HSPC Subpopulations with Clinical Data

For statistical analysis of hematopoietic reconstitution of leukocytes, neutrophilic granulocytes, and platelets, clusters were formed ranging from early to average (= normal) and delayed (= late) engraftment times. Timespan was measured in days after autologous transplantation (Fig. 4a–d).

- A high ratio of LMPPs was correlated with early leukocyte and neutrophilic granulocyte engraftment (in days, $p = 0.003$ and $p = 0.025$; in cluster, $p = 0.037$ and $p = 0.048$) (Table 2).

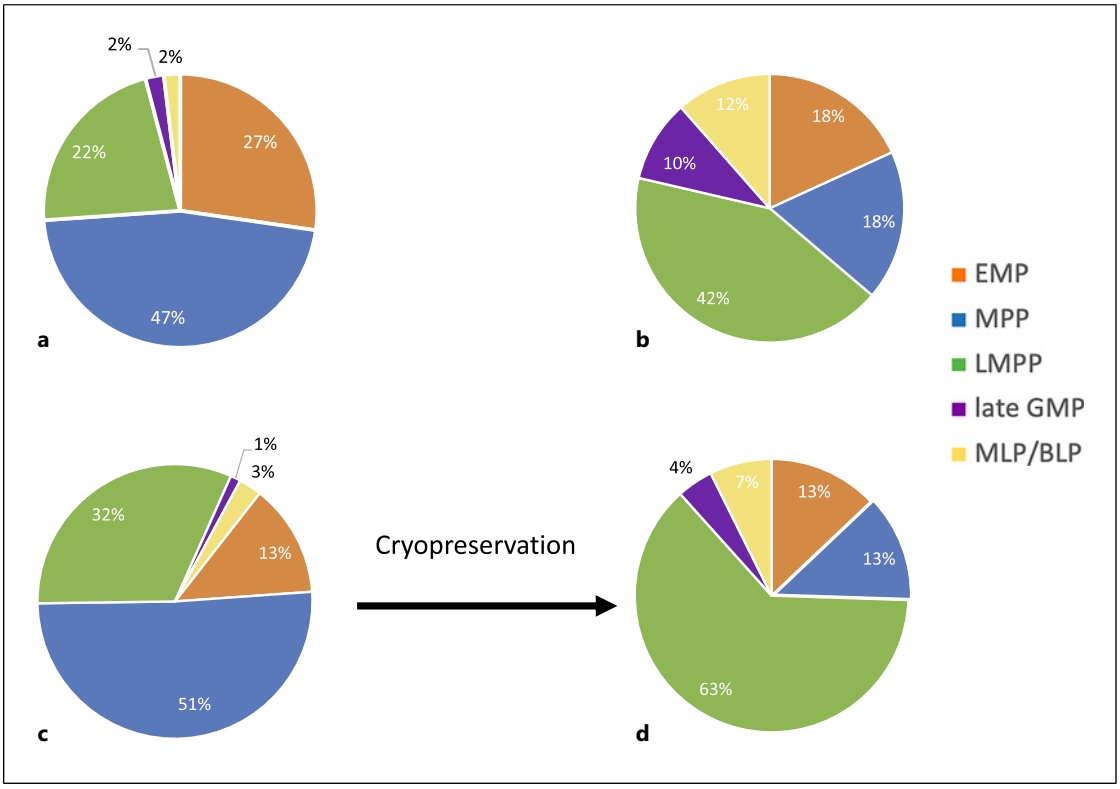

Fig. 2. Distribution of HSPC subpopulations. **a** For comparison: Worel et al. [1] (fresh autologous PBSCs prior to cryopreservation, $n = 40$). **b** Autologous PBSCs after cryopreservation and thawing ($n = 49$). **c** Fresh allogeneic PBSCs evaluated immediately after apheresis ($n = 5$). **d** Allogeneic PBSCs after cryopreservation and thawing ($n = 5$).

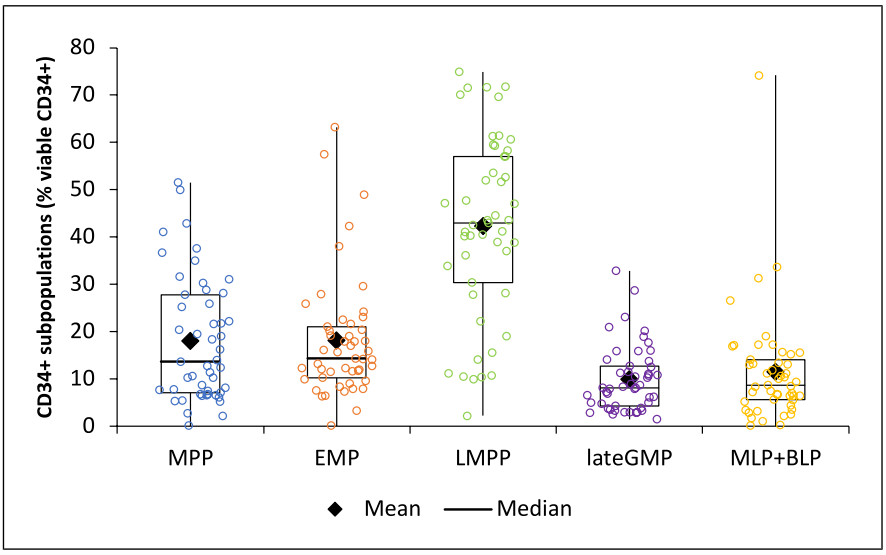

Fig. 3. Distribution of HSPC subpopulations in autologous cryopreserved preparations. All cells were viable and $CD34^+$.

- Conversely, a high ratio of EMPs was correlated with late engraftment of neutrophilic granulocytes (in days, $p = 0.031$) and leukocytes (in cluster, $p < 0.05$) (Table 2).
- A higher ratio of differentiated cells (BLPs) was found in the group of patients with late engraftment of neutrophilic granulocytes (in cluster, $p = 0.024$) (Table 2), whereas a high ratio of mature cells

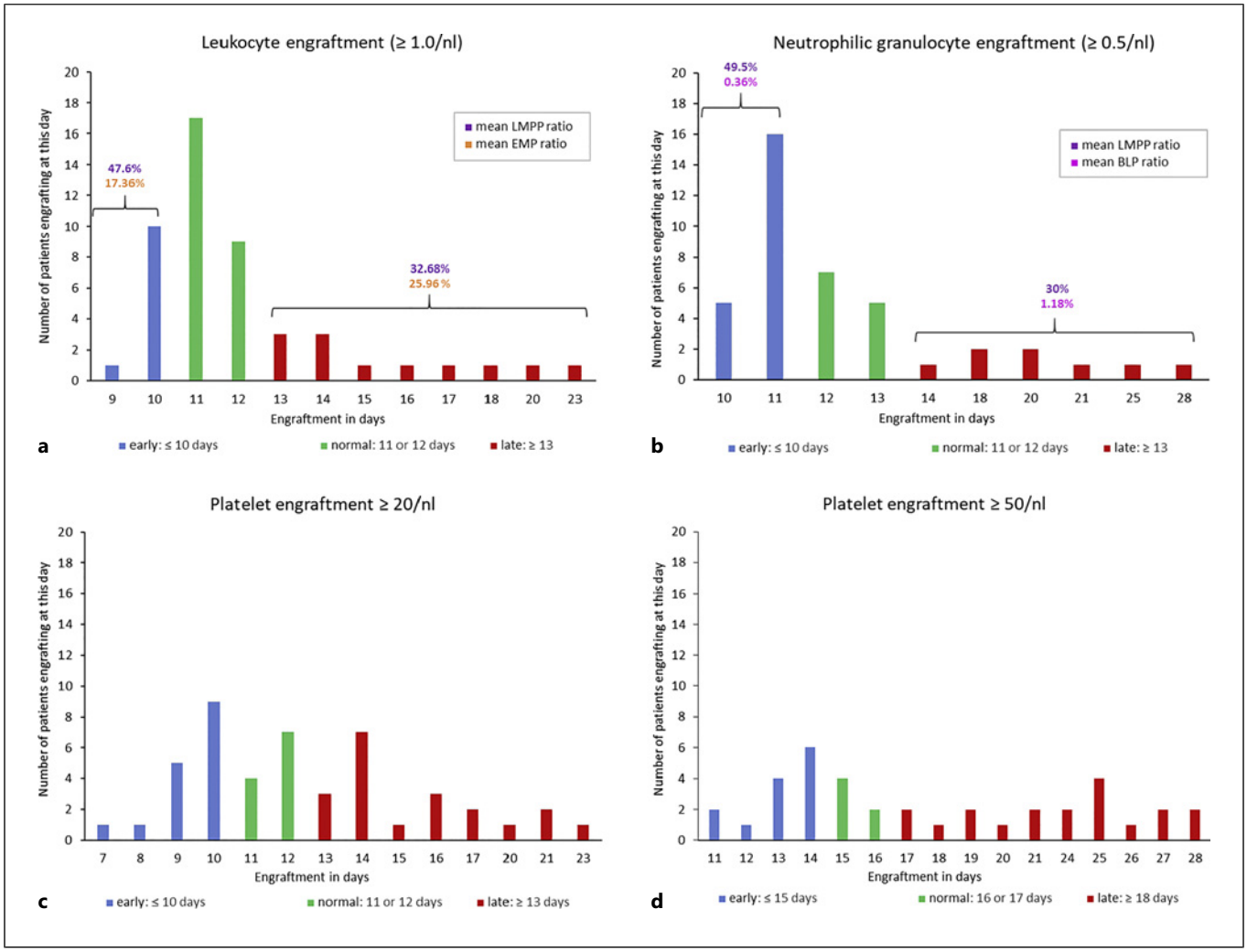

Fig. 4. Engraftment in days summarized for three clusters: early (blue), normal (green), and late (red) hematopoietic recovery after HSCT. Significantly different HSPC subpopulations are shown. **a** Engraftment of leukocytes (>1.0/nL in PB) after HSCT (n = 49). **b** Engraftment of neutrophils (>0.5/nL in PB) after HSCT (n = 41). **c** Engraftment of platelets (>20/nL in PB) after HSCT (n = 47). **d** Engraftment of platelets (>50/nL in PB) after HSCT (n = 38). HSCT, hematopoietic stem cell transplantation.

correlated with a low proportion of immature cells in the respective differentiation pathway (LMPPs and BLPs: p = 0.001, MPPs and late GMPs: p = 0.007).

- Only in the group of 32 MM patients, a correlation between a higher ratio of MPPs and late platelet engraftment ≥50/nL was observed (in cluster, p = 0.045).

Data from the literature indicating that high $CD34^+$ cell counts may correlate with faster platelet engraftment [31] could not be confirmed, with neither the $CD34^+$ cell count prior to cryopreservation (p = 0.47 for >20/nL, p = 0.07 for >50/nL) nor the $CD34^+$ cell count post-cryopreservation (p = 0.94 for >20/nL, p = 0.72 for >50/nL). Regarding patient age, no significant correlation with any of the analyzed parameters was observed, except for later platelet engraftment ≥50/nL (p = 0.032).

Discussion

The main goal of the study was to assess $CD34^+$ cell recovery after cryopreservation in autologous stem cell products and to evaluate the correlation of an adjusted flow cytometry panel for CD34 subpopulations with clinical engraftment data.

CD34 Assessment after Cryopreservation

There is a consensus in the literature that a threshold of 2×10^6 $CD34^+$ cells is the lowest limit for safe engraftment [13, 18]. Usually, the number of $CD34^+$ cells is determined only prior to cryopreservation. Freezing and thawing impose several technical challenges for any analysis of the cellular composition of the graft. This is why, after cryopreservation, most transplants perform

Table 2. Correlation of HSPC subpopulations with clinical data

Variables	Leukocyte engraftment >0.5/nL	Neutrophilic granulocyte engraftment >1.0/nL	Platelet engraftment >20/nL	Platelet engraftment >50/nL
Engraftment in days				
BLPs	n.s.	n.s.	n.s.	n.s.
Late GMPs	n.s.	n.s.	n.s.	n.s.
LMPPs	−0.41 ($p = 0.003$) early engraftment	−0.35 ($p = 0.025$) early engraftment	n.s.	n.s.
MLPs	n.s.	n.s.	n.s.	n.s.
EMPs	n.s.	0.34 ($p = 0.031$) late engraftment	n.s.	n.s.
MPPs	n.s.	n.s.	n.s.	n.s.
Engraftment in cluster				
BLPs	n.s.	0.35 ($p = 0.024$) late engraftment	n.s.	n.s.
Late GMPs	n.s.	n.s.	n.s.	n.s.
LMPPs	−0.3 ($p = 0.037$) early engraftment	−0.31 ($p = 0.048$) early engraftment	n.s.	n.s.
MLPs	n.s.	n.s.	n.s.	n.s.
EMPs	0.28 ($p < 0.05$) late engraftment	n.s.	n.s.	n.s.
MPPs	n.s.	n.s.	n.s.	n.s.

Spearman's correlation coefficient ($n = 49$); n.s., not significant; BLPs, B-lymphoid progenitors; late GMPs, granulocyte and macrophage progenitors; LMPPs, lympho-myeloid progenitors; MLPs, multi-lymphoid progenitors; EMPs, erythroid-myeloid progenitors; MPPs, multipotent progenitors.

only viability testing and CFA assays on a regular basis, with only viability post-thaw being a release criterion. Data in the literature indicate that CD34 enumeration by flow cytometry after freezing and thawing is prone to error [22–24]. In our hands, the mean recovery rate was 105%, with a high variance. For almost half of the samples, we obtained recovery rates exceeding 100%. The results were reproducible in several control assays, making this result unlikely to be considered a random artifact. Possible explanations for the phenomenon of "increasing" $CD34^+$ cell numbers after cryopreservation might include nonspecific binding to mature, post-clonogenic precursor cells or conformational changes in the binding site after freezing and thawing [23].

Viability

In routine clinical practice, the number of $CD34^+$ cells is determined directly after leukapheresis before cryopreservation, and clinical decision-making usually refers to that number. Furthermore, viability testing of WBCs after cryopreservation must exceed a certain threshold (in Germany, 70%), and the CFA must be positive if performed (not mandatory). However, the time span between HSPC collection and the actual transplantation may vary considerably, and a number of factors may change in that time: the bw of the patient, the viability of the cellular product, or the recovery rate of the $CD34^+$ cells. None of these changes are usually taken into consideration.

As proposed by Lee et al. [25], among others, viability should be reassessed after a prolonged period of freezing and thawing because post-thaw viability may reflect the actual composition of the graft more precisely than the initial viability test. Most centers simply use WBC viability to recalculate the number of $CD34^+$ cells. However, this practice may be misleading, as $CD34^+$ cells have higher viability than WBCs (in our study, 68.6 vs. 48.62%). This approach may result in less than 2×10^6 $CD34^+$ viable cells/kg bw after cryopreservation in some cases [22, 25, 26]. However, very few non-engraftment cases are encountered in clinical routine, which is why a recount is not routinely performed or recommended.

In terms of the best viability staining method, routine trypan blue staining and analysis by light microscopy has the following shortcomings: cell viability is equated with cell membrane integrity, and the approach is examiner-dependent and requires experience, as weak staining may be missed [24, 32]. For a more objective approach, we evaluated several viability dyes for flow cytometry: 7AAD, propidium iodide (PI), Viability Stain 510 (all from BD, Heidelberg, Germany), and Sytox Blue (Thermo Fisher Scientific, Dreieich, Germany). In our hands, 7AAD could be best implemented in the abovementioned staining protocol. Notably, testing by trypan blue resulted in higher viability (77% for trypan blue vs. 48.6% for 7AAD), which should be taken into account when

comparing these two methods. At this stage, a general conclusion regarding which test is most suitable cannot be drawn.

Subpopulations after Cryopreservation

A comprehensive assessment of a stem cell product requires more than the number of $CD34^+$ cells. Worel et al. [1] demonstrated a specific distribution pattern of HSPC subpopulations in fresh autologous samples before cryopreservation. In comparison, we found a shift toward subpopulations with a more mature status (from MPPs to LMPPs) after cryopreservation. Considering that these differences in the results may be attributed to the process of cryopreservation, we analyzed 5 allogeneic HSPC samples prior to and after cryopreservation. The composition of subpopulations within the fresh allogeneic samples resembled the data from Worel et al. [1]; however, the cryopreserved allogeneic samples resembled the cryopreserved autologous samples (Fig. 2). We conclude that the proposed flow cytometry protocol is reproducible for both allogenic and autologous stem cell grafts.

A possible explanation for the observed changes between cryopreserved/thawed and fresh samples is the recovery phase in LTBMC medium, which we added after thawing and might induce rapid changes. In addition, different levels of oxidative, cellular, and replicative stress [33] and the influence of already differentiated cells [29] may also account for these changes. Additional hypotheses, which need to be tested, are that MPPs, as the most immature HSPCs, may be prone to DMSO toxicity and that the conditions in vitro might favor the survival of more differentiated subpopulations, resulting in a change in composition (measured by percentage). This is also a possible explanation for why we observed a significantly higher number of MLPs, which are already advanced in the process of differentiation from LMPPs. In routine clinical practice, MLPs, as precursors of B lymphocytes, have no direct engraftment marker for correlation analysis.

To better estimate correlations with MLPs after cryopreservation, the flow panel might be expanded to include B lymphocyte-specific antibodies. To mimic the in vivo situation, placing the cells into a niche-like model after thawing might be an option, and this has already been realized by some groups using mesenchymal stromal cells [34–37].

Our findings are in line with the revised model of early hematopoiesis (first described by Goergens et al. [38]), demonstrating that progenitor cells follow specific pathways of differentiation. Furthermore, in agreement with Dmytrus et al. [28], we found a significantly smaller B-cell progenitor population in autologous PBSCs than in historical data derived from BM.

Correlations with Clinical Data

Considering that compared to published data, the subpopulation composition differed in our cryopreserved autologous samples and considering that similar patterns were observed in a pairwise comparison of allogeneic PBSCs analyzed before and after cryopreservation, we questioned the correlations with engraftment data. We hypothesized that a high ratio of immature cells should result in early recovery, as a larger number of these early progenitors could migrate into the niche and establish sufficient cell lines there. The more mature cells in the transplanted graft could, in turn, act as effector cells in the periphery.

To facilitate statistical analysis, we formed clusters ranging from *early* to *average* (= normal) and *delayed* (= late) engraftment times (Fig. 4a–d), in line with previously published data [14, 39]. We were able to demonstrate that a high ratio of LMPPs (i.e., immature cells) correlated with early leukocyte and neutrophilic granulocyte engraftment. Conversely, following the differentiation lineage, a high ratio of EMPs correlated with later engraftment of neutrophilic granulocytes, which followed the LMPP branch of the pathway. A significantly higher percentage of differentiated cells (i.e., mature BLPs) was assessed in the group of patients with late engraftment of neutrophilic granulocytes, and large numbers of EMPs correlated with late leukocyte engraftment. Taken together, these findings support the notion that graft composition reflects hematopoietic engraftment.

Limitations of the Study and Outlook

Platelet and erythrocyte transfusions given to patients during aplasia might represent confounders that impede the determination of the exact minimum number of these cells. 44 out of 49 patients received at least one platelet concentrate transfusion (mean: 2.4, range: 1–8). Furthermore, 39 out of 49 patients received granulocyte colony-stimulating factor after transplantation, according to best clinical practice. A full blood count including neutrophils was not available for all patients. These patients could not be fully included in this analysis (n = 8 out of 49).

Even though the maximum duration of cryopreservation of HSPC products in our survey was 2.1 years, retention samples were cryopreserved significantly longer. However, previous studies have demonstrated that cryostorage has no clinically relevant negative effect on stem cell quality and potency, even after an extended period of more than 10 years [12, 40].

If the described panel was applied routinely in clinical practice, we projected the additional costs for the user. Given the fact that many parameters (like staff and equipment costs) may vary widely between different laboratories, we estimated the costs for the reagents including antibodies between 20 and 40 EUR per sample. Considering the overall costs for an autologous stem cell graft, this seems a minor matter of expense.

As we conducted a retrospective study, our findings regarding the prediction of engraftment need to be validated prospectively. An issue that remains open is the impact of the subpopulation composition on the incidence of infections, relapse, and overall survival. In addition, the role of different mobilization methods in combination with the application of plerixafor on the composition of HSPC subpopulations should be explored in future studies.

Conclusions and Key Findings

1. The enumeration of HSPC subpopulations in cryopreserved material, both autologous and allogeneic, was possible.
2. The CD34 recovery rate after cryopreservation varied consistently: for half of the samples, the post-cryopreservation $CD34^+$ cell counts were above or below the initial $CD34^+$ cell count.
3. Standardized protocols need to be established regarding the handling of cryopreserved HSPCs in terms of the evaluation of $CD34^+$ cells by flow cytometry, freezing/thawing conditions, and viability testing of $CD34^+$ cells [41].
4. HSPC subpopulations showed distinct distribution patterns before and after cryopreservation, with a shift toward more differentiated stages after cryopreservation.
5. HSPC subpopulations correlated with the time of clinical engraftment: a higher ratio of immature subpopulations (especially LMPPs) in the graft correlated with an earlier recovery of leukocytes and neutrophil granulocytes. Subpopulation analysis may provide additional information in cases with unusual findings (e.g., low viability, low cell numbers, recurrent lack of engraftment) and help identify risk factors for delayed hematopoietic reconstitution.

Statement of Ethics

Patients consented to sample storage and the associated quality control procedures when the specimen was collected. The Institutional Ethics Committee of the Medical Faculty Mannheim at Heidelberg University approved the analysis of the samples as well as the retrospective evaluation of clinical data (Ref. 2019-726N).

Conflict of Interest Statement

Patrick Wuchter: Membership on Advisory Boards for Sanofi-Aventis. All other authors have no conflicts of interest to declare.

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Author Contributions

Anabel Heuer, Karen Bieback, Harald Klüter and Patrick Wuchter conceptualized the study. Anabel Heuer, Svea Löwhagen, Britta Pflästerer, Anke Diehlmann and Stefanie Uhlig performed the experiments and gathered the clinical data. Svetlana Hetjens and Sylvia Büttner performed the biostatistical analyses. Stefan Klein treated the patients and contributed clinical data. All authors proofread and approved the manuscript and provided important intellectual input.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material. Further inquiries can be directed to the corresponding author.

References

1 Worel N, Frank N, Frech C, Fritsch G. Influence of plerixafor on the mobilization of CD34+ cell subpopulations and lymphocyte subtypes. Transfusion. 2017;57(9):2206–15.
2 Kriegsmann K, Schmitt A, Kriegsmann M, Bruckner T, Anyanwu A, Witzens-Harig M, et al. Orchestration of chemomobilization and G-CSF administration for successful hematopoietic stem cell collection. Biol Blood Marrow Transplant. 2018;24(6):1281–8.
3 Kriegsmann K, Wack M, Pavel P, Schmitt A, Kriegsmann M, Bruckner T, et al. Collection, cryostorage, transplantation, and disposal of hematopoietic stem cell products. Biol Blood Marrow Transplant. 2019;25(2):382–90.
4 Reddy RL. Mobilization and collection of peripheral blood progenitor cells for transplantation. Transfus Apher Sci. 2005;32(1):63–72.
5 Schmitz N, Linch DC, Dreger P, Goldstone AH, Boogaerts MA, Ferrant A, et al. Randomised trial of filgrastim-mobilised peripheral blood progenitor cell transplantation versus autologous bone-marrow transplantation in lymphoma patients. Lancet. 1996;347(8998):353–7.
6 Martin PS, Li S, Nikiforow S, Alyea EP 3rd, Antin JH, Armand P, et al. Infused total nucleated cell dose is a better predictor of transplant outcomes than CD34+ cell number in reduced-intensity mobilized peripheral blood allogeneic hematopoietic cell transplantation. Haematologica. 2016;101(4):499–505.
7 Mishra S, Kulkarni U, Mathews N, VR, Ch Nair S, George B, et al. A study to compare Hematopoietic Progenitor Cell count determined on a next-generation automated cell counter with flow cytometric CD34 count in peripheral blood and the harvested peripheral blood stem cell graft from autologous and allogenic donors. Int J Lab Hematol. 2021;43(1):76–83.
8 Peerschke EI, Moung C, Pessin MS, Maslak P. Evaluation of new automated hematopoietic progenitor cell analysis in the clinical management of peripheral blood stem cell collections. Transfusion. 2015;55(8):2001–9.
9 Yang H, Acker JP, Cabuhat M, Letcher B, Larratt L, McGann LE. Association of post-thaw viable CD34+ cells and CFU-GM with time to hematopoietic engraftment. Bone Marrow Transplant. 2005;35(9):881–7.

10 Sutherland HJ, Eaves CJ, Lansdorp PM, Phillips GL, Hogge DE. Kinetics of committed and primitive blood progenitor mobilization after chemotherapy and growth factor treatment and their use in autotransplants. Blood. 1994;83(12):3808–14.
11 Pettengell R, Morgenstern GR, Woll PJ, Chang J, Rowlands M, Young R, et al. Peripheral blood progenitor cell transplantation in lymphoma and leukemia using a single apheresis. Blood. 1993;82(12):3770–7.
12 Lisenko K, Pavel P, Kriegsmann M, Bruckner T, Hillengass J, Goldschmidt H, et al. Storage duration of autologous stem cell preparations has No impact on hematopoietic recovery after transplantation. Biol Blood Marrow Transplant. 2017;23(4):684–90.
13 Mohty M, Hubel K, Kroger N, Aljurf M, Apperley J, Basak GW, et al. Autologous haematopoietic stem cell mobilisation in multiple myeloma and lymphoma patients: a position statement from the European Group for Blood and Marrow Transplantation. Bone Marrow Transplant. 2014;49(7):865–72.
14 Lisenko K, Wuchter P, Hansberg M, Mangatter A, Benner A, Ho AD, et al. Comparison of different stem cell mobilization regimens in AL amyloidosis patients. Biol Blood Marrow Transplant. 2017;23(11):1870–8.
15 Sutherland DR, Anderson L, Keeney M, Nayar R, Chin-Yee I. The ISHAGE guidelines for CD34+ cell determination by flow cytometry. International Society of Hematotherapy and Graft Engineering. J Hematother. 1996;5(3):213–26.
16 D'Arena G, Musto P, Cascavilla N, Di Giorgio G, Zendoli F, Carotenuto M. Human umbilical cord blood: immunophenotypic heterogeneity of CD34+ hematopoietic progenitor cells. Haematologica. 1996;81(5):404–9.
17 Gonzalez-Requejo A, Madero L, Diaz MA, Villa M, Garcia-Escribano C, Balas A, et al. Progenitor cell subsets and engraftment kinetics in children undergoing autologous peripheral blood stem cell transplantation. Br J Haematol. 1998;101(1):104–10.
18 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.
19 Roug AS, Hokland LB, Segel E, Nielsen K, Toft-Petersen M, Van Kooten Niekerk PB, et al. Unraveling stem cell and progenitor subsets in autologous grafts according to methods of mobilization: implications for prediction of hematopoietic recovery. Cytotherapy. 2014;16(3):392–401.
20 Gorgens A, Radtke S, Horn PA, Giebel B. New relationships of human hematopoietic lineages facilitate detection of multipotent hematopoietic stem and progenitor cells. Cell Cycle. 2013;12(22):3478–82.
21 Radtke S, Gorgens A, Kordelas L, Schmidt M, Kimmig KR, Koninger A, et al. CD133 allows elaborated discrimination and quantification of haematopoietic progenitor subsets in human haematopoietic stem cell transplants. Br J Haematol. 2015;169(6):868–78.
22 Sartor M, Antonenas V, Garvin F, Webb M, Bradstock KF. Recovery of viable CD34+ cells from cryopreserved hemopoietic progenitor cell products. Bone Marrow Transplant. 2005;36(3):199–204.
23 Fietz T, Reufi B, Mucke C, Thiel E, Knauf WU. Flow cytometric CD34+ determination in stem cell transplantation: before or after cryopreservation of grafts? J Hematother Stem Cell Res. 2002;11(2):429–35.
24 Humpe A, Beck C, Schoch R, Kneba M, Horst HA. Establishment and optimization of a flow cytometric method for evaluation of viability of CD34+ cells after cryopreservation and comparison with trypan blue exclusion staining. Transfusion. 2005;45(7):1208–13.
25 Lee S, Kim S, Kim H, Baek EJ, Jin H, Kim J, et al. Post-thaw viable CD34(+) cell count is a valuable predictor of haematopoietic stem cell engraftment in autologous peripheral blood stem cell transplantation. Vox Sang. 2008;94(2):146–52.
26 Allan DS, Keeney M, Howson-Jan K, Popma J, Weir K, Bhatia M, et al. Number of viable CD34(+) cells reinfused predicts engraftment in autologous hematopoietic stem cell transplantation. Bone Marrow Transplant. 2002;29(12):967–72.
27 Gorgens A, Ludwig AK, Mollmann M, Krawczyk A, Durig J, Hanenberg H, et al. Multipotent hematopoietic progenitors divide asymmetrically to create progenitors of the lymphomyeloid and erythromyeloid lineages. Stem Cell Reports. 2014;3(6):1058–72.
28 Dmytrus J, Matthes-Martin S, Pichler H, Worel N, Geyeregger R, Frank N, et al. Multicolor immune-phenotyping of CD34 subsets reveals unexpected differences between various stem cell sources. Bone Marrow Transplant. 2016;51(8):1093–100.
29 Uhrig M, Ezquer F, Ezquer M. Improving cell recovery: freezing and thawing optimization of induced pluripotent stem cells. Cells. 2022;11(5):799.
30 Taubert I, Saffrich R, Zepeda-Moreno A, Hellwig I, Eckstein V, Bruckner T, et al. Characterization of hematopoietic stem cell subsets from patients with multiple myeloma after mobilization with plerixafor. Cytotherapy. 2011;13(4):459–66.
31 Remberger M, Torlen J, Ringden O, Engstrom M, Watz E, Uhlin M, et al. Effect of total nucleated and CD34(+) cell dose on outcome after allogeneic hematopoietic stem cell transplantation. Biol Blood Marrow Transplant. 2015;21(5):889–93.
32 Strober W. Trypan blue exclusion test of cell viability. Curr Protoc Immunol. 2015;111:A3.B.1–A3.B.3.
33 Aerts-Kaya F. Strategies to protect hematopoietic stem cells from culture-induced stress conditions. Curr Stem Cell Res Ther. 2021;16(7):755–70.
34 Gorgens A, Murke F, Kordelas L, Giebel B. Lost in Transplantation? Unexpected shift from multipotent to late lymphomyeloid hematopoietic stem and progenitor cells in patients 1 year after hematopoietic stem cell transplantation. Bone Marrow Transplant. 2016;51(8):1073–5.
35 Radtke S, Gorgens A, Liu B, Horn PA, Giebel B. Human mesenchymal and murine stromal cells support human lympho-myeloid progenitor expansion but not maintenance of multipotent haematopoietic stem and progenitor cells. Cell Cycle. 2016;15(4):540–5.
36 Wuchter P, Saffrich R, Giselbrecht S, Nies C, Lorig H, Kolb S, et al. Microcavity arrays as an in vitro model system of the bone marrow niche for hematopoietic stem cells. Cell Tissue Res. 2016;364(3):573–84.
37 Ludwig A, Saffrich R, Eckstein V, Bruckner T, Wagner W, Ho AD, et al. Functional potentials of human hematopoietic progenitor cells are maintained by mesenchymal stromal cells and not impaired by plerixafor. Cytotherapy. 2014;16(1):111–21.
38 Gorgens A, Radtke S, Mollmann M, Cross M, Durig J, Horn PA, et al. Revision of the human hematopoietic tree: granulocyte subtypes derive from distinct hematopoietic lineages. Cell Rep. 2013;3(5):1539–52.
39 Kamel AM, El-Sharkawy N, Mahmoud HK, Khalaf MR, El Haddad A, Fahmy O, et al. Impact of CD34 subsets on engraftment kinetics in allogeneic peripheral blood stem cell transplantation. Bone Marrow Transplant. 2005;35(2):129–36.
40 Lysak D, Brychtova M, Leba M, Čedíková M, Georgiev D, Jindra P, et al. Long-term cryopreservation does not affect quality of peripheral blood stem cell grafts: a comparative study of native, short-term and long-term cryopreserved haematopoietic stem cells. Cell Transplant. 2021;30:9636897211036004.
41 Fritsch G, Frank N, Dmytrus J, Frech C, Pichler H, Witt V, et al. Relevance of flow cytometric enumeration of post-thaw leucocytes: influence of temperature during cell staining on viable cell recovery. Vox Sang. 2016;111(2):187–96.