International Society for Cellular Therapy ISCT

Cytotherapy (2009) Vol. 11, No. 6, 749–760

informa healthcare

Use of different DMSO concentrations for cryopreservation of autologous peripheral blood stem cell grafts does not have any major impact on levels of leukocyte- and platelet-derived soluble mediators

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Background aims

Infusion of stem cell autografts can be associated with adverse effects. Necrotic normal leukocytes, cytokines or intracellular mediators released from leukocytes and platelets or the cryoprotectant dimethyl sulfoxide (DMSO) may contribute to this. Cryopreservation using 5% instead of 10% DMSO improves CD34$^+$ cell viability and therefore we investigated whether using less DMSO had favorable outcomes on leukocyte viability and levels of various soluble mediators in the graft supernatant.

Methods

Peripheral blood autografts were harvested by 20 apheresis procedures in 16 cancer patients, and autograft samples were cryopreserved with 2%, 4%, 5% and 10% DMSO and stored for 5–6 years. After thawing, the viability of neutrophils and lymphocytes was analyzed by flow cytometry and supernatant levels of soluble mediators were determined by enzyme-linked immunosorbent assay (ELISA) analyzes.

Results

The highest viability of both neutrophils and lymphocytes was detected with 4% and 5% DMSO, whereas decreased viability was observed with 2% and 10% DMSO. Low or undetectable levels of leukocyte-derived intereukin (IL)-6 and tumor necrosis factor (TNF)-α and CXCL8, high levels of platelet-derived CCL5 and CXCL4, and high levels of monocyte-derived soluble CD14 were measured independent of the DMSO concentration, except for slightly increased CXCL8 and decreased CXCL4 levels with 2% DMSO. Perforin levels showed a significant inverse correlation with the DMSO concentration.

Conclusions

The use of different DMSO concentrations affects the viability of normal leukocytes in autologous peripheral blood stem cell grafts, but has only minor effects on supernatant levels of leukocyte- and platelet-derived soluble mediators.

Keywords

autotransplantation, cytokines, leukocytes, mobilized peripheral blood stem cells, platelets.

Introduction

Mild adverse reactions, such as transient nausea, vomiting, headache, flushing, chest tightness, hypotension, bradycardia and abdominal cramps, are common during and early after infusion of cryopreserved autologous stem cell grafts [1–3]. More serious reactions have also been described, including hypertension, arrhythmias, cardiac arrest, anaphylaxis, respiratory arrest, neurologic complications and even multi-organ failure [4–7]. Most studies have shown an overall frequency of adverse

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DOI: 10.3109/14653240902980443

events corresponding to 30–50% but the large majority of these reactions are mild.

Infusion of the cryoprotectant dimethyl sulfoxide (DMSO), granulocytes and high patient age are implicated as causes of adverse events [8–10]. Accordingly, a decreased frequency of adverse events is observed for patients receiving autografts with reduced amounts of DMSO [11–15]. Such reductions can be achieved either by DMSO depletion through washing of the autografts or by using 5% instead of 10% DMSO [11–15]. However, approximately 20% of patients show adverse events even when receiving autografts with reduced DMSO concentrations [14,15], an observation suggesting that the adverse reactions may also be caused by other mechanisms.

Autologous stem cell grafts are contaminated by a relatively large number of normal peripheral blood cells. These cells are usually not removed before autograft infusion, because $CD34^+$ enrichment with removal of circulating malignant cells or normal cells does not have any major impact on the risk of post-transplant cancer progression or relapse [16]. Furthermore, infusion of normal leukocytes seems to be important for early lymphocyte reconstitution and may thereby reduce the relapse risk [17–19]. Consequently, autografts are usually infused without further manipulation.

Adverse reactions during or after infusion of autologous stem cell grafts could also be caused by cytokines secreted by contaminating normal blood cells (e.g. platelets, monocytes and neutrophils) that become activated during preparation or *ex vivo* handling of the grafts. This would be similar to the observations for transfusion of platelet and packed red cell concentrates, where leukocyte-derived cytokines are probably responsible for most of the non-hemolytic transfusion reactions [20]. In particular levels of endogenous pyrogens such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α increase during storage of platelet concentrates and these mediators are implicated in febrile reactions [21]. Similar mechanisms may be relevant for autografts where both leukocyte- and platelet-derived cytokines can be detected in the graft supernatant [22]. Furthermore, the viability of peripheral blood progenitor cells (PBPC) depends on the DMSO concentration used for cryopreservation, i.e. the use of 5% DMSO instead of the conventional 10% improves $CD34^+$ cell viability [23], and this may also be true for normal graft leukocytes. Intracellular mediators and membrane molecules will then be released into the graft supernatant by apoptotic or necrotic normal leukocytes and these mediators may subsequently be involved in the development of adverse reactions.

The aim of the present study was to investigate (i) the effects of various DMSO concentrations on the viability of normal leukocytes in autologous stem cell grafts, and (ii) the graft supernatant levels of cytokines and intracellular mediators derived from normal blood cells and how these levels are affected by the use of different DMSO concentrations for cryopreservation.

Methods

Patients, stem cell collection and autotransplantation

The study was approved by the regional ethics committee. Sixteen consecutive patients planned for autologous stem cell transplantation (ASCT) were included after informed consent. All 16 patients received initial chemotherapy, and at the time of harvesting all patients were classified as responders to this treatment. Stem cells were mobilized by initial chemotherapy followed by granulocyte–colony-stimulating factor (G-CSF). Twenty leukapheresis products from these patients were investigated, including two separate products from four patients. The patient and graft characteristics are specified in Table I. Harvesting, cryopreservation and thawing of the grafts were performed as described previously [24]. Software version 5.1 of the Cobe Spectra cell separator (Cobe Laboratories, Gloucester, UK) was used to collect PBPC, and large-volume apheresis processing four times the patients' total blood volume was performed. ACD-A (Baxter FA, Lessin, Belgium) at a blood to citrate ratio of 18:1 was used for anticoagulation, together with 2500 IU heparin (Leo Pharma AS, Oslo, Norway) that was added to each 500 mL ACD-A solution. Moreover, ACD equivalent to 10% of the estimated harvest volume was added initially to the collection bag.

All samples used in the *in vitro* part of the study were stored for 5–6 years in the vapor phase of liquid nitrogen before being examined. We investigated all 20 autografts, but only 11 patients were transplanted with grafts prepared with either 5% or 10% DMSO. Five patients, all with testicular cancer, were harvested in case of no response to conventional therapy, and later they were all in complete remission without needing autotransplantation.

Table I. Clinical characteristics of the 16 patients included in the study and biologic characteristics of the 20 harvested autologous peripheral blood stem cell grafts.

Parameter	Values
Patient characteristics[a]	
Age (years)	45 (22–61)
Gender (men/women)	12/4
Total blood volume (L)	4.8 (3.8–6)
Diagnosis (*n*)	
Multiple myeloma	7
Non-Hodgkin's lymphoma	2
Testicular carcinoma	5
Hodgkin's disease	1
Ovarian carcinoma	1
Total numbers of apheresis to reach at least 2×10^6 CD34+ cells/kg[b] and patients	
One apheresis	Seven patients
Two apheresis	Seven patients
Four apheresis	Two patients
Autograft characteristics[c]	
Total number of harvested $CD34^+$ cells$\times10^6$/kg	8.1 (4.2–20.7)
Concentration of total WBC before freezing 10^9/mL	180 (127–726)
Number of infused $CD34^+$ cells$\times10^6$/kg	3.8 (2.2–8.5)
Graft volume infused (mL)	105 (67–285)
Storage time before autograft infusion (days)	32 (25–116)

[a]Presented as the median value with the variation range in parentheses. Data on blood volume were available only for 13 patients.
[b]Two leukapheresis products were included for four unselected patients out of the 16 patients; three of these four patients were harvested twice and the last patient was harvested four times.
[c]$CD34^+$ cells and WBC counts are given for all 16 patients. Eleven patients were transplanted; the graft volume was measured only for 10 of them, whereas the number of infused $CD34^+$ cells and storage time are given for 11 patients.

The transplanted patients received the autografts within 4 months of harvesting.

Preparation of test samples with various DMSO concentrations

Test samples were handled parallel to autografts as both were prepared to cryopreservation by the same method; cells were either concentrated or diluted in autologous plasma in order to obtain a final total nucleated cell (TNC) concentration of $1–2\times10^8$ cells/mL. For the test samples, cell suspensions of 1568, 1536, 1520 and 1440 µL were transferred to test tubes and kept on ice for up to 5 min. Afterwards, 32, 64, 80 and 160 µL DMSO were slowly added to reach a final volume of 1600 µL and DMSO concentrations of 2%, 4%, 5% and 10%, respectively. In order to eliminate time variables for the exposure of cells to DMSO at room temperature before freezing, DMSO was added randomly to the different test tubes. Autografts concentrated or diluted in autologous plasma, on the other hand, were mixed with DMSO to obtain a final concentration of 5% or 10% DMSO. DMSO was the only cryoprotectant used. The test tubes were placed into controlled-rate freezing together with the grafts (Planer Cryo 10 series III; Planer Ltd, Sunbury on Thames, UK), and when the temperature reached −160°C both the grafts and samples were placed into the vapor phase of liquid nitrogen for storage.

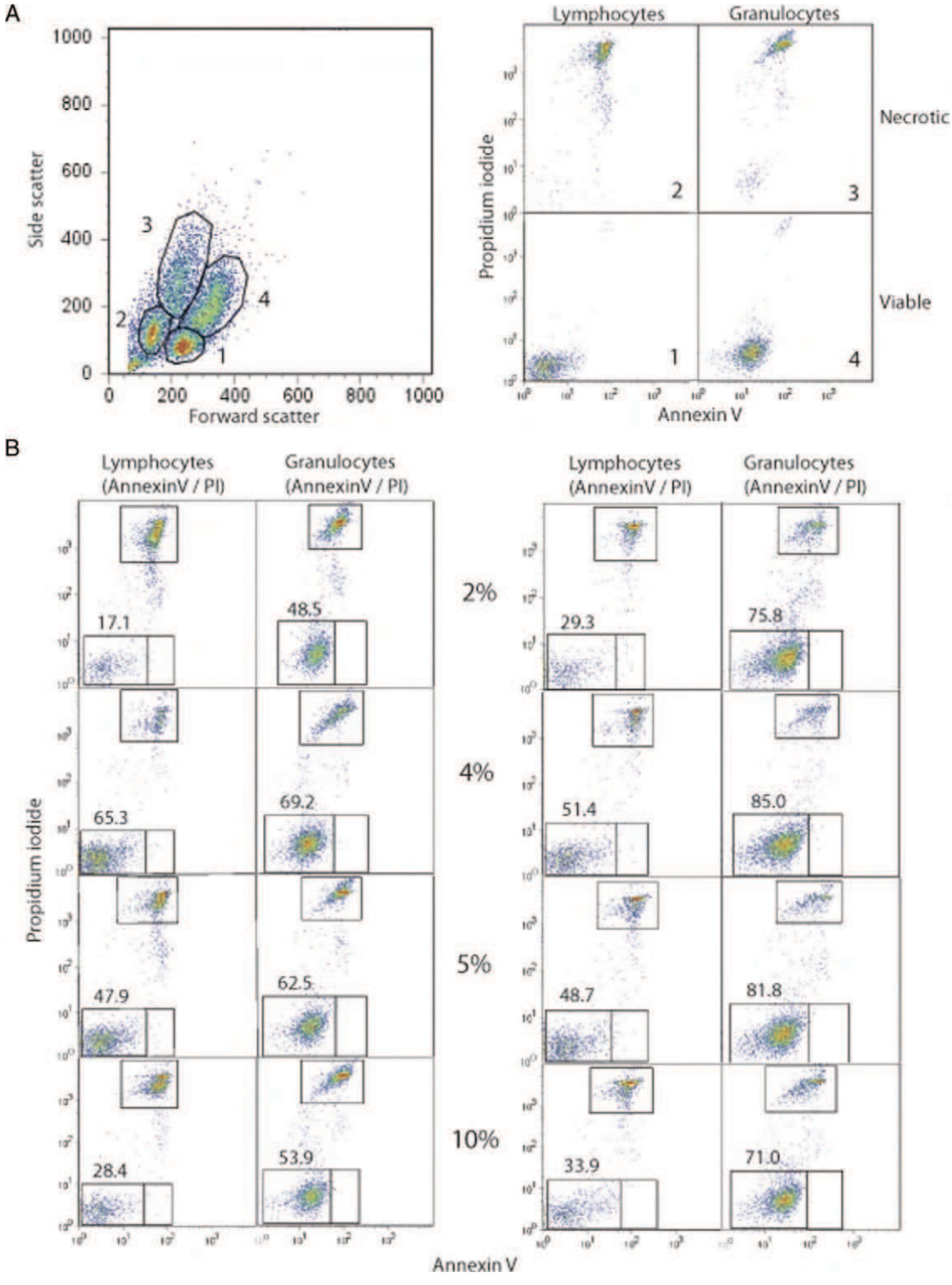
Analysis of leukocyte viability

All samples were transferred to 1 mL RPMI immediately after thawing and washed once with freshly prepared 1% bovine serum albumin (BSA)/phosphate-buffered saline (PBS) and kept on ice during viability testing (Apoptest™– FITC kit; NeXins Research, Kattendijke, the Netherlands). This method has been described previously and characterized in detail [25]. After collecting 10 000 events from duplicate samples using a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA), data were analyzed with the software package FlowJo (TriStar Inc., Ashland, OR, USA). Cytograms based on the forward versus right-angle light scatter were used to eliminate aggregates and debris (Figure 1). The viable, apoptotic and necrotic cells were determined by flow cytometric analysis of Annexin V (AV)–FITC and propidium iodide (PI) staining. AV^- PI^- cells were defined as viable, AV^+ PI^- as apoptotic and AV^+ PI^+ as necrotic.

Total leukocyte measurements and leukocyte recovery

The total leukocytes in the graft samples were measured using flow cytometry and a hematopoietic progenitor cell (HPC) enumeration kit containing CD45–fluorescein isothiocyanate (FITC)/CD34–phycoerythrin (PE), isotype control (CD45–FITC–IsoClonic control–PE reagent), stem-count microbeads/fluorospheres, 7-AAD viability dye and lysing solution (The Stem-Kit, Coulter Immunotech, Marseille, France) [26]. The technique used for both fresh and thawed samples was a single-platform flow cytometry method founded on ISHAGE guidelines [27]. The frozen samples with 2%, 4%, 5%

and 10% DMSO concentrations were diluted immediately to 1:20 after thawing and analyzed in duplicate together with isotype controls following the manufacturer's instructions (Coulter Immunotech). Using a histogram of CD45 versus side scatter, all the $CD45^+$ events were displayed. $CD45^+$ leukocytes were gated, eliminating platelets, erythrocytes, other debris and aggregates. The number of $CD45^+$ events (i.e. leukocyte numbers) was determined on the statistic printout related to this gate.



Bcl-2, Bax and active caspase 3

Bcl-2, Bax and caspase 3 levels were determined as described previously [28]. Washed cells were fixed in 4% paraformaldehyde (PFA)/PBS for 10 min at room temperature before they were washed with cold PBS and permeabilized with 100% ice-cold methanol for 10 min. Afterward, the samples were transferred to –80°C and stored until analysis. The samples were then washed twice with cold PBS and labeled for 1 h in the dark at room temperature with antibodies (Bcl-2–FITC, Bax–PE and active caspase 3–PE; Becton Dickinson, Erembodegem, Belgium) dissolved in binding buffer (5% BSA/PBS). Samples were then washed three times with cold PBS before fixation with 1% PFA/PBS and analysis by flow cytometry.

Analyzes of soluble mediators in graft supernatants

The levels of IL-6, CCL5, CXCL4, CXCL8, TNF-α, sCD14 (R&D Systems, Abingdon, UK) and perforin (Abcam, Cambridge, UK) were determined by enzyme-linked immunosorbent assay (ELISA) analyzes. All assays were performed in duplicate strictly according to the manufacturers' instructions. The minimal detectable levels were 3 pg/mL for IL-6, 2 pg/mL for CCL5, 40 ng/mL (corresponding to 40 IU/mL) for CXCL4, 3 pg/mL for CXCL8, 10 pg/mL for TNF-α, 125 pg/mL for sCD14 and 40 pg/mL for perforin. Lacate dehydrogenase (LDH) measurements were performed using a Roche diagnostics assay (Roche, Mannheim, Germany).

Statistical analyzes

The statistical analyzes were performed with a standard software package (SPSS 15.0, SPSS, Chicago, IL). Most parameters displayed a skewed distribution and therefore the non-parametric Wilcoxon's two-tailed test for paired observations was used. Kendall's tau-b test was used for correlation analysis. *P*-values were considered statistically significant when <0.05.

Results

Leukocyte recovery

The total number of nucleated cells after thawing was determined for all samples, and these results are summarized in Table II. The cell numbers did not differ significantly between samples cryopreserved with different DMSO concentrations. We also calculated the percentage leukocyte recovery for all four DMSO concentrations; only minor, although statistically significant, differences in percentage total leukocyte recovery were detected (Table II). Furthermore, the percentage recovery exceeded 100% for several samples, and in an additional analysis all recovery values exceeding 100% were set to 100% in the statistical analyzes (data not shown). This was done to avoid an underestimation of cell loss due to the variation in cell number estimation, but again only minor differences up to 2% were detected between the DMSO concentrations. Thus, based on the absolute post-thaw leukocyte numbers (no significant differences) and the percentage recovery calculations (small although significant differences) we concluded that high leukocyte recovery was detected for all four DMSO concentrations without any major differences between DMSO 2%, 4%, 5% and 10%.

Leukocyte viability in cryopreserved autologous PBPC grafts

We examined granulocyte viability after graft preservation with various DMSO concentrations (2%, 4%, 5% and 10%). Light scatter analyzes by flow cytometry

Figure 1. *(A, upper part) Examination of leukocyte viability after thawing for autologous peripheral blood progenitor grafts; the results for one representative patient when using 5% DMSO for the preservation. (Left part) Four different leukocyte populations could be identified by light scatter analysis (FSC/SSC), two populations with small and non-granular cells (lymphocytes = 1 and 2) and two populations with large, granular cells (granulocytes = 3 and 4). A separate monocyte population could not be identified. (Right part) The cells were analyzed with an AV/PI assay. It can be seen that the first and larger one of two lymphocyte populations consisted of viable cells (1) whilst apoptotic/necrotic lymphocytes were localized in the second population (2). The same was true for the two granulocyte populations (4 and 3, respectively). (B, lower part) Examination of leukocyte viability after thawing of autologous peripheral blood progenitor grafts; the results for two representative patients (left and right panels, respectively) when using 2%, 4%, 5% and 10% DMSO for preservation. The total lymphocyte and granulocyte populations were examined, and for each sample three different cell subsets were identified: viable (lower left boxes), a minor subset of apoptotic cells (lower right) and a subset of necrotic cells (upper). The percentage of viable cells is indicated in the figure.*

Table II. Total leukocyte recovery and granulocyte characteristics for cryopreserved PBPC autografts; studies of viability and regulation of apoptosis[a].

		2% DMSO	4% DMSO	P-value	5% DMSO	10% DMSO	P-value
Pre-freeze total leukocyte number 4.97×10^8/kg (1.34–6.84)[b]							
Post-thaw total leukocyte number ($\times10^8$/kg)[b]		5.4 (2.5–11.0)	5.3 (2.5–10.2)	0.962	5.2 (2.4–10.0)	4.9 (2.5–9.0)	0.254
Post-thaw leukocyte recovery (%)[b]		112 (73–136)	107 (67–131)	0.048	108 (71–133)	98 (67–123)	0.02
Granulocyte viability	Viable cells (%)	62 (32–87)	71 (39–90)	<0.0005	73 (45–86)	68 (11–83)	0.001
	Apoptotic cells (%)	0.1 (0–3)	0.1 (0–5)	0.013	0.1 (0–2)	0.2 (0–0.5)	0.969
	Necrotic cells (%)	31 (5–64)	22 (2–53)	0.001	21 (8–50)	28 (12–72)	<0.0005
Regulation of apoptosis in granulocytes	Bcl-2$^+$ cells (%)	19.85 (3.3–97.6)	34.95 (2.3–99.6)	0.005	37.3 (1.4–99.4)	46.55 (0.9–99.9)	0.271
	Bax$^+$ cells (MFI)	81.9 (25.7–580)	74.8 (24–559)	0.502	89.4 (31–630)	89.1 (29–532)	0.852
	Caspase 3$^+$ cells (MFI)	19.05 (8.1–50.6)	21.7 (6.8–41.4)	0.457	18.8 (9.5–39.3)	21.8 (9.6–33.8)	0.940

[a]All results are presented as the median and variation range when testing 20 autografts derived from 16 cancer patients. The table shows the comparison of 2% versus 4% DMSO and 5% versus 10% DMSO. The P*-values for comparisons between 4% and 5% were all not significant (i.e.* $P>0.05$*).*

[b]The total leukocyte number was determined for all autografts before addition of DMSO (pre-freeze number) and immediately after thawing for all four DMSO concentrations. The absolute post-thaw numbers are presented together with the post-thaw leukocyte recovery.

allowed identification of viable and necrotic/apoptotic granulocytes and lymphocytes, as illustrated by the results from a typical experiment presented in Figure 1A, and percentages of viable and apoptotic/necrotic cells could then be analyzed as illustrated in Figure 1B. The overall results are presented in Table II. Firstly, the viability showed a wide variation for all concentrations but was highest and exceeded 39% viable cells for all grafts when using 4% or 5% DMSO. Comparison of 4% and 5% DMSO showed no significant differences in (i) frequencies of apoptotic and necrotic cells and (ii) expression of anti-apoptotic bcl-2, pro-apoptotic bax and the downstream mediator activated caspase 3. Secondly, comparison of neutrophils after cryopreservation in 2% and 4% DMSO revealed significantly decreased neutrophil viability for 2% DMSO. This was associated with increased percentages of both apoptotic and necrotic neutrophils. Significantly decreased intracellular levels of anti-apoptotic bcl-2 were observed with 2% DMSO when comparing percentage positive cells ($P=0.005$; Table II) but the difference was not significant when comparing mean fluorescence intensity (MFI) values. Finally, comparing DMSO 5% and 10% showed a significantly decreased neutrophil viability for 10% DMSO; this was associated with an increased amount of necrotic cells but no differences were seen in intracellular bcl-2, bax and activated caspase 3.

The majority of contaminating normal leukocytes in the PBPC autografts were granulocytes, and as expected there was a strong positive correlation between total white blood cell (WBC) viability and granulocyte viability for all DMSO concentrations ($r=0.89$–0.95, $P<0.0005$).

In addition to the granulocyte majority there was relatively high numbers of lymphocytes in the grafts, whereas a separate monocyte population could not be identified with the forward/side scatter analyzes. The granulocyte viability (see Table II for percentages of granulocyte viability) was higher than the lymphocyte viability for all DMSO concentrations ($P\leqslant0.001$). In contrast to the neutrophils, the lymphocyte viability was slightly higher for DMSO 4% compared with 5% (median 23% versus 20% viable lymphocytes; $P=0.025$). Finally, similar to the neutrophils, the lymphocyte viability was significantly decreased for DMSO 2% versus 4% (median 11% versus 23% viable lymphocytes; $P<0.005$) and DMSO 10% versus 5% (median 12% versus 20% viable lymphocytes; $P<0.005$).

Graft supernatant levels of the intracellular mediators perforin and LDH

Neutrophil granulocytes, cytotoxic T lymphocytes and natural killer (NK) cells contain high levels of perforin [29]. We therefore compared perforin levels in graft supernatants when using different DMSO concentrations for cryopreservation. Perforin levels did not differ between DMSO 4% and 5% (Figure 2 and Table III), whereas statistically significant increases were observed for DMSO 2% versus 4% and DMSO 5% versus 10% (Table III). Thus there seemed to be an inverse correlation between the DMSO concentration used and perforin level in the graft supernatant. We observed a correlation of borderline statistical significance ($r=0.382$, $P=0.032$, $n=17$) between perforin levels and granulocyte viability only for 2% DMSO, but no correlations were detected between perforin levels and lymphocyte viability.

LDH is an enzyme that is detected in most cells [30]. Increased supernatant LDH levels were detected for DMSO 10% compared with 5% (Figure 2 and Table III), whereas the levels did not differ significantly when comparing DMSO 4% versus 5% or 2% versus 4%. We found no significant correlations between LDH levels and granulocyte/lymphocyte viability.

Levels of monocyte-derived sCD14 and leukocyte-derived cytokines in graft supernatants

Despite relatively low numbers of monocytes in the autografts, high levels of monocyte-derived sCD14 were detected for all grafts (Table III). However, the levels did not differ between various DMSO concentrations.

TNF-α is released both by activated T cells and monocytes/macrophages [31]. TNF-α levels were undetectable in the supernatants independent of the DMSO concentration used for cryopreservation. IL-6, which is released by the same cells, was also detected at relatively low levels in the supernatants for all the DMSO concentrations (Table III). On the other hand, CXCL8 is released by monocytes and these levels were slightly increased for DMSO 2% versus 4% (median levels 27.8 versus 24.7 pg/mL; $P=0.031$; Table III). These CXCL8 levels were relatively low compared with *in vitro* cultured cells [32].

Levels of platelet-derived cytokines in PBPC autografts

Activated platelets release CCL5/RANTES and CXCL4/PF4 [33], and in our study both mediators were detected in the graft supernatants at relatively high levels. The CCL5 and CXCL4 levels did not differ significantly when comparing autografts prepared with different DMSO concentrations, except for increased CXCL4 levels in grafts preserved with DMSO 2% versus 4% ($P=0.044$; Table III).

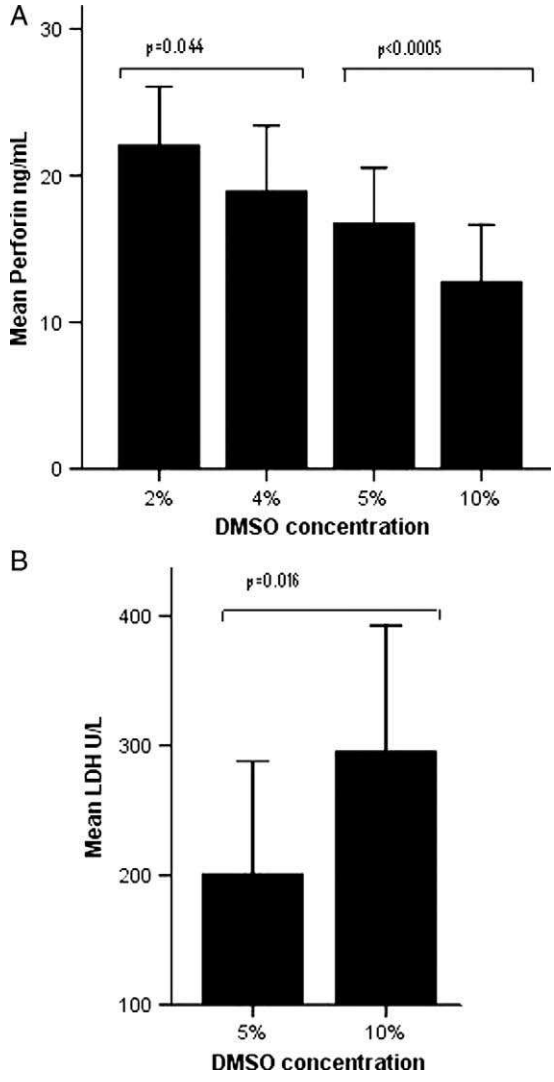


Figure 2. *Perforin and LDH supernatant levels for PBPC autografts cryopreserved with 2%, 4%, 5% and 10% DMSO. Autografts derived from 20 apheresis procedures (16 cancer patients) were cryopreserved in 2%, 4%, 5% and 10% DMSO and stored in the vapor phase of liquid nitrogen. Perforin and LDH levels were determined immediately after thawing. (A) The figure shows the perforin levels (ng/mL) in the supernatants of the autografts. (B) LDH levels (U/L) were determined for all four DMSO concentrations for each autograft, but the figure presents the results only for PBPC grafts stored in 5% and 10% DMSO because the only statistically significant difference was found between these concentrations (see also Table III).*

Table III. Concentrations of intracellular plus leukocyte- and platelet-derived mediators in the supernatants of cryopreserved PBPC autografts[a].

		2% DMSO	4% DMSO	*P*-value	5% DMSO	10% DMSO	*P*-value
Intracellular mediators	Perforin (ng/mL)	24.9 (1.1–34.4)	18.5 (0.9–34.6)	0.044	15.0 (0.8–31.6)	10.8 (0.7–29.5)	<0.0005
	LDH (U/L)	180 (10–480)	140 (10–600)	0.717	140 (10–690)	280 (50–890)	0.016
Monocyte-derived mediators	sCD14 (pg/mL)	1.14×106 (0.7×10^6–2.56×10^6)	1.13×10^6 (0.5×10^6–5.9×10^6)	0.421	1.08×10^6(0.59×10^6–2.39×10^6)	0.9×10^6 (0.38×10^6–2.3×10^6)	0.968
	IL-6 (pg/mL)	1.9 (nd–23.5)	2.3 (nd–30.9)	0.706	2.8 (nd–17.5)	2.4 (nd–14.8)	0.753
	TNF-α (pg/mL)	Undetectable	Undetectable		Undetectable	Undetectable	
	CXCL8 (pg/mL)	27.8 (3–199.5)	24.7 (3–220.2)	0.031	31.07 (3–135.4)	19.4 (3–113.6)	0.248
Platelet-derived mediators	CXCL4 (IU/mL)	3340 (650–21240)	3374 (480–15780)	0.044	3395 (758–13680)	2962 (447–19500)	0.881
	CCL5 (ng/mL)	169.5 (49.3–478.8)	150.3 (45.1–506.4)	0.526	156.6 (52.0–498.0)	171.9 (69.6–518.4)	0.709

[a]All results are presented as the median and variation range when testing 20 autografts derived from 16 cancer patients. The table shows the comparison of 2% versus 4% DMSO and 5% versus 10% DMSO. The P*-values for comparisons between 4% and 5% are all not significant (i.e.* $P>0.05$*). nd, not detectable.*

There were no strong correlations between CCL5 and CXCL4 levels ($r=0.26$–0.46) in autografts cryopreserved with various DMSO concentrations except for 4% DMSO ($r=0.516$, $P=0.001$). No strong correlations were revealed between graft platelet counts and CCL5 levels ($r=0.23$–0.44). The *r*-values were generally higher when investigating correlations between graft platelet counts and CXCL4 levels ($r=0.40$–0.53) and reached statistical significance for DMSO 2% ($P=0.011$), 4% ($P=0.003$), 5% ($P=0.013$) and 10% ($P=0.026$). These relatively weak correlations with platelet counts suggested that the levels of soluble mediators derived from platelets depend not only on the platelet counts but probably also on the activation process during *ex vivo* handling of the autografts and possibly the binding of mediators to graft cells.

Comparison of graft cell viability, mediator levels and clinical outcome

Eleven patients were autotransplanted. Two of them had adverse reactions that were probably related to infusion of DMSO: one patient experienced nausea and the other felt unwell and also had nausea. Furthermore, neutrophil engraftment with a neutrophil peripheral blood count $\geq0.5\times10^9$/L occurred after a median of 10 days (range 8–18), platelet engraftment with a platelet count $\geq20\times10^9$/L after 10 days (range 7–16) and a platelet count $\geq50\times10^9$/L after 14 days (range 10–19). Thus the low frequency of side-effects and early engraftment suggested that our patients were representative of autotransplanted cancer patients.

Discussion

Previous studies have demonstrated that autografts cryopreserved with 5% DMSO show higher $CD34^+$ cell viability than grafts preserved in the conventional 10% DMSO [23], but it is not known whether this lower concentration will alter normal blood cell viability or levels of soluble mediators in graft supernatants. Autologous stem cell grafts contain high amounts of normal leukocytes and platelets that can release soluble mediators during *ex vivo* handling of the grafts [22,34,35]. In addition, one would expect the graft supernatant to contain membrane molecules and intracellular mediators released by dead cells. In the present study, we therefore investigated leukocyte viability and graft supernatant levels of soluble

mediators derived from platelets and various leukocyte subsets.

All sample vials were stored for 5–6 years in the vapor phase of liquid nitrogen before being analyzed. This long storage time was chosen because a second transplantation with the original autograft is now used in relapse treatment [36,37]. Despite this relatively long storage time, a high total WBC recovery was observed. Therefore our results suggest that the observations are also representative of short-term stored autografts. Furthermore, we included 16 patients and 11 of them were later autotransplanted and showed relatively low frequencies of side-effects and time until hematopoietic engraftment, similar to other studies [14,15,38,39]. Taken together these observations strongly suggest that our patients and their autografts were representative of autotransplanted patients in general, and the high cell recovery indicated that the vapor phase of liquid nitrogen is a stable storage environment.

We analyzed total leukocyte numbers pre-freeze and post-thaw by a highly standardized flow cytometric method. Post-thaw leukocyte numbers were generally higher than values estimated pre-freeze (Table II). Similar discrepancies have also been described by other investigators, who have reported up to 200% post-thaw recovery in a large study of more than 900 peripheral blood autografts [13]. A cell recovery (both for $CD45^+$ and $CD34^+$ cells) exceeding 100% has been described for other procedures involving *ex vivo* handling of peripheral blood stem cell grafts [40,41]. Thus such variations seem to be well-known and are probably caused by differences in aggregation of graft leukocytes. We detected no significant differences in total leukocyte numbers between the various DMSO concentrations, an observation suggesting that different cell recovery had only a minor influence on our results. The percentage leukocyte recovery was also calculated, and only relatively small differences were detected when (i) comparing calculated values and (ii) all recovery values exceeding 100% were set equal to 100% in the statistical comparisons. Based on the results for all three analytical approaches, we conclude that there were no major differences in total leukocyte recovery between the four DMSO concentrations.

The major part of the leukocytes in the autografts consisted of granulocytes that generally showed a relatively high viability, although differences were observed depending on the DMSO concentration used for cryopreservation. The granulocytes also constituted the major part of the viable leukocytes, whereas lymphocytes constituted a relatively larger fraction of the necrotic cells. Granulocytes thus seemed to be better preserved than lymphocytes for all DMSO concentrations tested. Our present study describes relatively low lymphocyte viability in the autograft samples, whereas previous reports have described high viability of normal peripheral blood mononuclear cells (PBMC) that contain mainly lymphocytes when cryopreserved in 10% DMSO [42]. However, this may be because of differences in cell concentration and freezing solution. In the autografts the lymphocytes were cryopreserved at a relatively high cell concentration and together with a large fraction of granulocytes, whereas in the studies of normal PBMC the cells were cryopreserved in culture medium supplemented with a relatively high serum concentration and 10% DMSO.

The lowest percentage of viable granulocytes was detected with 2% DMSO, and this was associated with decreased levels of anti-apoptotic bcl-2 and increased numbers of apoptotic neutrophils. Bax is a pro-apoptotic mediator [43] and was expressed at high levels by neutrophils independent of the DMSO concentration. Thus cryopreservation with low DMSO concentrations induces apoptosis possibly through decreased bcl-2 levels, whereas an altered bcl-2/bax balance is less likely as the explanation for decreased viability with 10% DMSO.

Perforin is an intracellular cytolytic protein found in neutrophils, cytotoxic T cells and NK cells [29]. In contrast to granulocyte and lymphocyte viability, perforin levels were highest with 2% DMSO and decreased gradually with increasing DMSO concentrations. Thus the perforin levels did not simply reflect the overall degree of cell death because the viability was also low with 10% DMSO; additional mechanisms, such as concentration-dependent DMSO effects and/or differences in perforin release from various cells as a result of the DMSO effects, may be operative.

LDH release was used as a general marker of cell damage [44]. We observed relatively high LDH levels for DMSO 2% and 10% (the two concentrations with the lowest viability) but LDH levels showed no strong correlations with cell viability. A possible explanation for such a lack of correlation could again be that the effects of various DMSO concentrations on leukocyte subsets differ, and the cell subsets may also show diversity in LDH content.

CD14 is a glycolipid-anchored membrane glycoprotein of monocytes–macrophages and has a soluble (sCD14) and a membrane-bound (mCD14) form [45]. We detected high supernatant levels of sCD14 for all autografts independent of the DMSO concentrations used for cryopreservation. In contrast, TNF-α, IL-6 and CXCL8 levels were either low or undetectable. These three cytokines can be released at relatively high levels by activated monocytes–macrophages. A number of mechanisms may contribute to the discrepancy between high sCD14 levels and low cytokine levels. Firstly, cytokines must be actively secreted, whereas sCD14 can be shed from the plasma membrane as a result of the cryopreservation process, and cryopreserved cells show decreased levels of several membrane molecules because of molecular shedding [46,47]. Secondly, supernatant levels represent a balance between release and binding, and released cytokines may be bound by receptor-expressing leukocytes in the graft. Finally, the release of intracellular proteases may destroy released cytokines.

As expected, the autograft supernatants showed high levels of the platelet-derived mediators CCL5 and CXCL4. The levels of these two chemokines, however, showed no statistically significant correlations; we regard the most likely explanation to be different binding of the two chemokines by receptor-expressing leukocytes in the autografts.

Our present study demonstrates that the supernatants of autologous stem cell grafts contain a wide range of soluble mediators derived from leukocytes and platelets, including cytokines, soluble membrane molecules and intracellular mediators probably derived from apoptotic/necrotic cells. Even though some minor differences were observed, the use of different DMSO concentrations for cryopreservation (2%, 4%, 5% and 10%) did not have any major impact on the supernatant mediator profile.

The procedures for cryopreservation and storage of autologous stem cell grafts vary considerably between transplantation centers [48]. The grafts are frequently stored without $CD34^+$ cell enrichment, i.e. similar to the grafts described in our present study. Windrum *et al*. [48] reported a wide variation of the DMSO concentration used (from 2.2% to 20%), 10% DMSO being the most commonly utilized. We therefore examined 10% DMSO in our present study. In addition, we selected the DMSO concentrations 2%, 4% and 5% because cryopreservation with these concentrations has been characterized in detail and compared with DMSO 10% in a previous study [23]. Furthermore, in several other studies DMSO 5% has been compared with 10% and proved to have better $CD34^+$ cell viability and hematopoietic stem cell survival, less adverse effects and comparable engraftment potential [14,24,49]. In one study 5% DMSO was associated with slower hematologic recovery, but these authors used storage at –80°C instead of storage in nitrogen as we did [50]. DMSO is also used in combination with hydroxyl ethyl starch (HES). Two hematopoietic engraftment studies have compared cryopreservation using HES combined with 5% versus 10% DMSO alone and storage in nitrogen [51,52], and hematopoietic engraftment with the lower DMSO concentration was either equal to or better than DMSO 10%. In the present study another reason for choosing sample vial cryopreservation with 2% and 4% DMSO was to investigate how close these concentrations are to the lower limit of the optimal DMSO concentration range. The present results also support the use of DMSO 5% for cryopreservation; the potential problem of infusing necrotic/apoptotic cells is minimized and for most soluble mediators we observed only minor differences compared with DMSO 10%. We would, however, emphasize that our present clinical data on adverse effects should be interpreted very carefully because there were few patients in the transplanted group. Nevertheless, whether cell viability or soluble mediator levels influence the risk of side-effects has to be investigated further in larger clinical studies.

Acknowledgements

The study received financial support from the Norwegian Cancer Society and grants from the Center for Clinical Research, Helse Vest, and the Solveig and Ove Lundes Foundation.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

1. Kessinger A, Schmit-Pokorny K, Smith D, Armitage J. Cryopreservation and infusion of autologous peripheral blood stem cells. *Bone Marrow Transplant* 1990;5:25–7.
2. Alessandrino EP, Bernasconi P, Caldera D, Colombo A, Bonfichi M, Malcovati L, *et al*. Adverse events occurring during bone marrow or peripheral blood progenitor cell infusion: analysis of 126 cases. *Bone Marrow Transplant* 1999;23:533–7.

3. Donmez A, Tombuloglu M, Gungor A, Soyer N, Saydam G, Cagirgan A. Clinical side effects during peripheral blood progenitor cell infusion. *Transfus Apher Sci* 2007;36:95–101.
4. Nishihara G, Sakemi T, Ikeda Y, Baba N, Shimamoto Y. Multiple organ failure associated with dimethylsulfoxide and hydroxyethyl starch in autologous blood stem cell transplantation. *Nephron* 1996;72:356–57.
5. Bauwens D, Hantson P, Laterre PF, Michaux L, Latinne D, Tourtchaninoff MD, *et al.* Recurrent seizure and sustained encephalopathy associated with dimethylsulfoxide-preserved stem cell infusion. *Leuk Lymphoma* 2005;46:1671–4.
6. Hentschke S, Hentschke M, Hummel K, Salwender HJ, Braumann D, Stang A. Bilateral thalamic infarction after reinfusion of DMSO-preserved autologous stem-cells. *Leuk Lymphoma* 2006;47:2418–20.
7. Chen-Plotkin AS, Vossel KA, Samuels MA, Chen MH. Encephalopathy, stroke, and myocardial infarction with DMSO use in stem cell transplantation. *Neurology* 2007;68:859–61.
8. Calmels B, Lemarié C, Esterni B, Malugani C, Charbonnier A, Coso D, *et al.* Occurrence and severity of adverse events after autologous hematopoietic progenitor cell infusion are related to the amount of granulocytes in the apheresis product. *Transfusion* 2007;47:1268–75.
9. Cordoba R, Arrieta R, Kerguelen A, Hernandez-Navarro F. The occurrence of adverse events during the infusion of autologous peripheral blood stem cells is related to the number of granulocytes in the leukapheresis product. *Bone Marrow Transplant* 2007;40:1063–7.
10. Milone G, Mercurio S, Strano A, Leotta S, Pinto V, Battiato K, *et al.* Adverse events after infusions of cryopreserved hematopoietic stem cells depend on non-mononuclear cells in the infused suspension and patient age. *Cytotherapy* 2007;9: 348–55.
11. Syme R, Bewick M, Stewart D, Porter K, Chadderton T, Glück S. The role of depletion of dimethyl sulfoxide before autografting: on hematologic recovery, side effects, and toxicity. *Biol Blood Marrow Transplant* 2004;10:135–41.
12. Lemarie C, Calmels B, Malenfant C, Arneodo V, Blaise D, Viret F, *et al.* Clinical experience with the delivery of thawed and washed autologous blood cells, with an automated closed fluid management device: CytoMate. *Transfusion* 2005;45:737–42.
13. Foïs E, Desmartin M, Benhamida S, Xavier F, Vanneaux V, Rea D, *et al.* Recovery, viability and clinical toxicity of thawed and washed haematopoietic progenitor cells: analysis of 952 autologous peripheral blood stem cell transplantations. *Bone Marrow Transplant* 2007;40:831–5.
14. Akkök CA, Liseth K, Nesthus I, Løkeland T, Tefre K, Bruserud O, *et al.* Autologous peripheral blood progenitor cells cryopreserved with 5 and 10 percent dimethyl sulfoxide alone give comparable hematopoietic reconstitution after transplantation. *Transfusion* 2008;48:877–83.
15. Akkök CA, Holthe MR, Tangen JM, Østenstad B, Bruserud Ø. Hematopoietic engraftment of dimethyl sulfoxide-depleted autologous peripheral blood progenitor cells. *Transfusion* 2008; 49:354–61.
16. Steward AK, Vescio R, Schiller G, Ballester O, Noga S, Rugo H, *et al.* Purging of autologous peripheral-blood stem cells using CD34 selection does not improve overall or progression-free survival after high-dose chemotherapy for multiple myeloma: results of a multicenter randomized controlled trial. *J Clin Oncol* 2001;19(17):3771–9.
17. Boulassel MR, Herr AL, deB Edwardes MD, Galal A, Lachance S, Laneuville P, *et al.* Early lymphocyte recovery following autologous peripheral stem cell transplantation is associated with better survival in younger patients with lymphoproliferative disorders. *Hematology* 2006;11:165–70.
18. Kim H, Sohn HJ, Kim S, Lee JS, Kim WK, Suh C. Early lymphocyte recovery predicts longer survival after autologous peripheral blood stem cell transplantation in multiple myeloma. *Bone Marrow Transplant* 2006;37:1037–42.
19. Porrata LF, Gertz MA, Inwards DJ, Litzow MR, Lacy MQ, Tefferi A, *et al*. Early lymphocyte recovery predicts superior survival after autologous hematopoietic stem cell transplantation in multiple myeloma or non-Hodgkin lymphoma. *Blood* 2001;98:579–85.
20. Hartwig D, Härtel C, Hennig H, Müller-Steinhardt M, Schlenke P, Klüter H. Evidence for de novo synthesis of cytokines and chemokines in platelet concentrates. *Vox Sanguinis*. 2002;82:182–90.
21. Muylle L, Joos M, Wouters E, De Bock R, Peetermans ME. Increased tumor necrosis factor alpha (TNF alpha), interleukin 1, and interleukin 6 (IL-6) levels in the plasma of stored platelet concentrates: relationship between TNF alpha and IL-6 levels and febrile transfusion reactions. *Transfusion* 1993;33(3):195–9.
22. Foss B, Abrahamsen JF, Bruserud Ø. Peripheral blood progenitor cell grafts contain high levels of platelet-secreted mediators. *Transfusion* 2001;41:1431–7.
23. Liseth K, Abrahamsen JF, Bjørsvik S, Grøttebø K, Bruserud Ø. The viability of cryopreserved PBPC depends on the DMSO concentration and the concentration of nucleated cells in the graft. *Cytotherapy* 2005;7:328–33.
24. Abrahamsen JF, Bakken AM, Bruserud Ø. Cryopreserving human peripheral blood progenitor cells with 5-percent rather than 10-percent DMSO results in less apoptosis and necrosis in $CD34^+$ cells. *Transfusion* 2002;42:1573–80.
25. Ryningen A, Wergeland L, Glenjen N, Gjertsen BT, Bruserud Ø. In vitro crosstalk between fibroblasts and native human acute myelogenous leukemia (AML) blasts via local cytokine networks results in increased proliferation and decreased apoptosis of AML cells as well as increased levels of proangiogenic Interleukin 8. *Leuk Res* 2005;29:185–96.
26. Ryningen A, Ersvaer E, Øyan AM, Kalland KH, Vintermyr OK, Gjertsen BT, *et al.* Stress-induced in vitro apoptosis of native human acute myelogenous leukemia (AML) cells shows a wide variation between patients and is associated with low bcl-2:bax ratio and low levels of heat shock protein 70 and 90. *Leuk Res* 2006;30:1531–40.
27. Abrahamsen JF, Wentzel-Larsen T, Bruserud Ø, Autologous transplantation: the viable transplanted $CD34^+$ cell dose measured post-thaw does not predict engraftment

kinetics better than the total CD34+ cell dose measured pre-freeze in patients that receive more than 2×10^6 $CD34^+$ cells/kg. *Cytotherapy* 2004;4:356–62.
28. Keeney M, Chin-Yee I, Weir K, Popma J, Nayar R, Sutherland DR. Single platform flow cytometric absolute $CD34^+$ cell counts based on the ISHAGE guidelines. International Society of Hematotherapy and Graft Engineering. *Cytometry* 1998;34:61–70.
29. Liu CC, Walsh CM, Young JD. Perforin: structure and function. *Immunology Today* 1995;16:194–201.
30. Kim H, Yoon SC, Lee TY, Jeong D. Discriminative cytotoxicity assessment based on various cellular damages. *Toxicol Lett* 2009;184:13–7.
31. Decker T, Lohmann-Matthes ML, Gifford GE. Cell-associated tumor necrosis factor (TNF) as a killing mechanism of activated cytotoxic macrophages. *J Immunol* 1987;138(3):957–62.
32. Bruserud Ø, Ryningen A, Olsnes AM, Stordrange L, Øyan AM, Kalland KH, *et al.* Subclassification of patients with acute myelogenous leukemia based on chemokine responsiveness and constitutive chemokine release by their leukemic cells. *Haematologica* 2007;92(3):332–41.
33. Boehlen F, Clemetson KJ. Platelet chemokines and their receptors: what is their relevance to platelet storage and transfusion practice? *Transfus Med* 2001;11:403–17.
34. Ikeda K, Ohto H, Nemoto K, Yamamoto G, Kato K, Ogata T, *et al.* Collection of MNCs and progenitor cells by two separators for PBPC transplantation: a randomized crossover trail. *Transfusion* 2003;43:814–9.
35. Ikeda K, Ohto H, Kanno T, Ogata T, Noji H, Ogawa K, *et al.* Automated programs for collection of mononuclear cells and progenitor cells by two separators for peripheral blood progenitor cell transplantation: comparison by a randomized crossover study. *Transfusion* 2007;47:1234–40.
36. Kumar A, Kharfan-Dabaja MA, Glasmacher A, Djulbegovic B. Tandem versus single autologous hematopoietic cell transplantation for the treatment of multiple myeloma: a systematic review and meta-analysis. *J Natl Cancer Inst* 2009;101(2):100–6.
37. Mehta J, Singhal S. Current status of autologous hematopoietic stem cell transplantation in myeloma. *Bone Marrow Transplant* 2008;42(1):S28–34.
38. Lane TA, Bashey A, Carrier E, Holman P, Castro J, Mullen M, *et al.* Improving the efficiency of PBPC collection by pre-apheresis peripheral blood and mid-apheresis product measurements of CD34 cells. *Cytotherapy* 2004;318–27.
39. Frère P, Pereira M, Fillet G, Beguin Y. Infections after CD34-selected or unmanipulated autologous hematopoietic stem cell transplantation. *Eur J Haematol* 2006;76(2):102–8.
40. Calmels B, Houzé P, Hengesse J-C, Ducrot T, Malenfant C, Chabannon C. Preclinical evaluation of an automated closed fluid management device: Cytomate™, for washing out DMSO from hematopoietic stem cell grafts after thawing. *Bone Marrow Transplant* 2003;31:823–8.
41. Rodríguez L, Velasco B, García J, Martín-Henao GA. Evaluation of an automated cell processing device to reduce the dimethyl sulfoxide from hematopoietic grafts after thawing. *Transfusion* 2005;45:1391–7.
42. Pfeffer PF, Hirschberg H. Cell-mediated lympholysis: a one-step microplate technique. In: Ferrone S, Solheim BG, editors. *HLA* typing: Methodology and clinical aspects. CRC Press, Inc, Boca Raton, Florida, USA 1982, issue 2, pages 83–97.
43. Lalier L, Cartron PF, Juin P, Nedelkina S, Manon S, Bechinger B, *et al.* Bax activation and mitochondrial insertion during apoptosis. *Apoptosis* 2007;12(5):887–96.
44. Riley RD, Burchill SA, Abrams KR, Heney D, Sutton AJ, Jones DR, *et al.* A systematic review of molecular and biological markers in tumours of the Ewing's sarcoma family. *European J Cancer* 2003;39(1):19–30.
45. Devitt A, Moffatt OD, Raykundalia C, Capra JD, Simmons DL, Gregory CD, *et al.* Human CD14 mediates recognition and phagocytosis of apoptotic cells. *Nature* 1998;392(6675):505–9.
46. De Boer F, Dräger AM, Van der Wall E, Pinedo HM, Schuurhuis GJ. Changes in L-selectin expression on CD34-positive cells upon cryopreservation of peripheral blood stem cell transplants. *Bone Marrow Transplant* 1998;22:1103–10.
47. Hattori Y, Kato H, Nitta M, Takamoto S. Decrease of L-selectin expression on human CD34+ cells on freeze-thawing and rapid recovery with short-term incubation. *Exp Hematol* 2001;29(1):114–22.
48. Windrum P, Morris TC, Drake MB, Niederwieser D, Ruutu T, EBMT Chronic Leukaemia Working Party Complications Subcommittee. Variation in dimethyl sulfoxide use in stem cell transplantation: a survey of EBMT centres. *Bone Marrow Transplant* 2005;36(7):601–3.
49. Abrahamsen JF, Rusten L, Bakken AM, Bruserud Ø. Better preservation of early hematopoietic progenitor cells when human peripheral blood progenitor cells are cryopreserved with 5 percent dimethylsulfoxide instead of 10 percent dimethylsulfoxide. *Transfusion* 2004;44:785–9.
50. Galmes A, Gutiérrez A, Sampol A, Canaro M, Morey M, Iglesias J, *et al.* Lon-term hematological reconstitution and clinical evaluation of autologous peripheral blood stem cell transplantation after cryopreservation of cells with 5% and 10% dimethylsulfoxide at –80 degrees C in a mechanical freezer. *Haematologica* 2007;92(7):986–9.
51. Rowley SD, Feng Z, Chen L, Holmberg L, Heimfeld S, MacLeod B, *et al.* A randomized phase III clinical trial of autologous blood stem cell transplantation comparing cryopreservation using dimethylsulfoxide vs dimethylsulfoxide with hydroxyethylstarch. *Bone Marrow Transplant* 2003; 31(11):1043–51.
52. Takaue Y, Abe T, Kawano Y, Suzue T, Saito S, Hirao A, *et al.* Comparative analysis of engraftment after cryopreservation of peripheral blood stem cell autografts by controlled- versus uncontrolled-rate methods. *Bone Marrow Transplant* 1994; 13(6):801–4.