Cryopreservation and Thawing of Hematopoietic Stem Cells CD34 Induced Apoptosis Through Caspases Pathway Activation: Key Role of Granulocytes

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ABSTRACT

Introduction: Cell damage inescapably occurs during both the freezing and the thawing graft process for autologous HSC transplantation. In order to estimate HSC injury, a quality control is performed including: i) CD34⁺ quantification; ii) % of CD34⁺ viability and iii) evaluation of HSC functional ability to form colony “CFU-GM”. Apoptosis involves complex pathways such as caspase enzymes. Here, we assess the extent of apoptosis caspase-dependent before and after cryoconservation of CD34⁺, using a Fluorescent labeled Inhibitor of Caspases “FLICA”. **Methods:** Caspases pathway activation status was evaluated in 46 patients (Multiple Myeloma (n=24), Lymphoma (n=22)), by flow cytometry, using a 7AAD/FLICA staining test, in CD34⁺, CD3⁺, CD14⁺ and CD56⁺ cells. Viable 7AAD⁻/FLICA⁺ cells were then correlated with various parameters. **Results:** We showed a significant caspases pathway activation, with 23% CD34⁺/7AAD⁻/FLICA⁺ cells after thawing, compared with the 2% described in fresh CD34⁺ cells ($p < 0.0001$). Moreover, caspases pathway was significantly activated in thawing CD3⁺, CD56⁺ and CD14⁺ cells. We also report a significant correlation between the rate of CD34⁺/7AAD⁻/FLICA⁺ cells and post thawing granulocytes count ($p = 0.042$) and their potent to be differentiated into CFU-GM ($p = 0.004$) **Discussion:** Our results show substantial cell death, induced by the increase of caspases pathway activation, secondary to the thawing process, and across all study cell types. This observation may affect the immune response quality during recipient aplasia, without detecting a clinical impact. Moreover, caspases pathway activation through CD3⁺ and CD56⁺ subpopulations could modify the therapeutic result of donor lymphocytes infusion (DLI).

Introduction

Cryopreservation is the only reliable form of long-term storage of viable hematopoietic stem cells (HSC). This process is increasingly used to conserve peripheral blood stem cells (PBSC) HSC CD34⁺, between collection and infusion through an autologous HSC transplantation. Traditional protocols rely on supplementation of the freeze medium with penetrating cryoprotectants, such as dimethyl sulfoxide (DMSO)¹. Nevertheless, cell damage inescapably occurs during both the freeze and the thaw process². In order to estimate HSC injury, currently before each freezing and after each thawing, a quality control is performed including *a minima*: i) CD34⁺ quantification ; ii) estimation of the percentage of PBSC CD34⁺ viability, via 7-aminoactinomycin-D (7AAD) staining and iii) evaluation of HSC functional ability to form colony “CFU-GM” (Colony Forming Unit – Granulocyte Macrophage). However, although impact on quality graft is estimated, cellular mechanisms, cause of observed cell death and their impact on the future of transplantation, remains unevaluated.

Apoptosis, or programmed cell death is an evolutionary conserved active form of cell death. The central component of this process is a proteolytic system involving a family of proteases called caspases, also known as cysteine aspartate-specific proteases that are triggered in response to intrinsic or extrinsic pro-apoptotic signals. During apoptosis, several important proteins such as Bcl-2 family members, cytochrome c, and apoptosis protease activation factor-1 (Apaf-1) occur around caspases activation^{3,4}. Initiation of apoptosis pathways may be required for cellular differentiation, be the result of cellular injury, or occur as a consequence of cryopreservation damage⁵.

Evaluation of apoptosis Cord Blood (CB) CD34⁺ cells after cryopreservation suggested a post thawing reduction of CB CD34⁺ viable cells⁶. Moreover, increased apoptosis and reduced clonogenic capacity (CFU-GM) in cryopreserved/thawed PBSC CD34⁺ have been reported based on the reactivity of early apoptotic nonspecific markers: Syto16,

Annexin-V and 7AAD^{7,8}. *Stroh and al.* showed that blockage of the caspase-activated apoptotic pathway (by the caspase inhibitor zVAD-fmk) following cryopreservation and thawing of mobilized PBSC, enhanced CFU-GM and burst-forming unit erythroid (BFU-E) colony formation, suggesting that apoptosis plays an important role in cryoinjury cells⁹.

We have conducted further assays to assess the extent of apoptosis occurred in cryopreservation process of autologous PBSC CD34⁺ using another marker of early apoptosis: FLICA. Activated caspase enzymes cleave proteins by recognizing a 3 or 4 amino acid sequence. Caspases activation may be detected by an *in vitro* flow cytometer method; using FLICA® probes i.e. non-cytotoxic Fluorescent Labeled Inhibitors of Caspases that covalently bind only to active caspase enzymes, through the 3 or 4 amino acid sequence lost¹⁰. We evaluate precise percent of apoptotic cells dependent on caspases activation pathways in viable cells (7-AAD⁻/FLICA⁺) before freezing and after thawing, and **assessed** results to the fate of the autograft.

Methods

Patients and collection

This study was conducted using samples from 46 autografted patients, between June 2013 and March 2017, with cryopreserved PBSC provided by clinical hematology department of CHU Amiens-Picardie. All patients gave written informed consent for the transplant and collection of anonymous data for research purposes.

The graft was constituted after mobilization with recombinant human granulocyte-stimulating factor (G-CSF - Filgrastim®) administrated subcutaneously after a single daily dose of 10µg/kg after various chemotherapy regimens. PBSC collections were performed with a cell collector (Cobe Spectra, Terumo BCT, Zaventem, Belgium) and started as soon as the circulating CD34⁺ cells in the peripheral blood exceeded 10 cells /microliters. Leukapheresis procedures were continued for 1 to 2 more consecutive days until the targeted yield of 4 x 10⁶ CD34⁺ per kilogram of body weight and per transplantation were reached.

Peripheral blood was collected using bags containing citrate phosphate dextrose (ACD) anticoagulant and stored at 1-6°C before and during the courier delivery.

Cryopreservation and thawing of PBSC

Autologous collection cells were stored overnight at 4°C. Following the process of volume reduction or increasing, 20% of dimethyl sulfoxide DMSO (CryoSure-DMSO, WAK Chemie, Steinbach, Germany) diluted in Hydroxy Ethyl Amidon HEA (Fresenius kabi, France), was added to an equal volume of the apheresis product to ensure a final concentration of 10% DMSO. Samples were cryopreserved in freezing bags (CryoMACS® Freezing Bag750, Miltenyi Biotech, Munich, Germany) with a final concentration per bag equal or less to 300.10⁺⁸ cells. The cells were cooled at 1°C per minute to -40°C with compensation for heat of surfusion and then 8°C per minute to -140°C using a rate-controlled freezer (Freezal

product number 106, Air Liquide, Bussy Saint Georges, France). Cryopreserved samples were rapidly removed from freezer and maintained in the vapor phase of liquid nitrogen at -180°C until reinfusion.

After being removed from the nitrogen tank, the graft was rapidly incubed at 39°C during 6 min in a dry water bath Plasmatherm Barkey (Sebac, France). It was then centrifuged with a Bio-wash solution (Glucose 0.2% and NaCl 0.9%, Haemonetics, Suisse) containing 10% of ACD-A anticoagulant (Macopharma, France) and finally resuspended in a 5% glucose solution (B. Braun, France) before reinfused.

Cell viability (CD45⁺/7AAD⁻), total nucleated cells (TNCS), granulocytes, CD34⁺ cells, were performed routinely using Stem-Kit™ Reagents (Beckman Coulter, France) and the Flow Cytometer Systems (Navios, Beckman Coulter, France) for each product before freezing and after thawing. Moreover, myeloid progenitor colonies were evaluated with methylcellulose-based culture system (MethoCult™ H4535 enriched without EPO, StemCell Technologies, Grenoble, France). Cells were plated in duplicate, in 6-wells cell culture plates at a concentration of 300 CD34⁺ pre freezing cells and 500 CD34⁺ post thawing cells per well. Clonogenicity was estimated based on counted CFU-GM number after 14 days of culture in a 37°C humidified (>95%) incubator with ambient 5% CO₂. The clonogenicity percentage was presented as ratio calculated as CFU-GM number/cultured CD34 number. Finally, a sterility testing was performed at two step (before freezing and after thawing) in which 500µl of pre freezing solution (fresh cells/DMSO) or thawed cell was inoculated and incubated for 10 days, in Aerobic and Anaerobic Culture vials (BD Bactec™ PLUS, Becton Dickinson, USA).

Caspases activity measurement and phenotype analysis

We evaluated caspase-dependent apoptosis by using a FLICA® polycaspases detection kit (FAM-FLICA® Caspase Assay Kits, Immunochemistry Technologies, Bloomington, MN, USA) following the customer instructions. FLICA® test, by unbound pro-caspases or inactive forms of the enzymes, leads to measure the intracellular process of apoptosis caused by activated caspases, and to eliminate false positive apoptosis detected with other currently used apoptotic markers. A caspase enzyme cannot cleave the FLICA® inhibitor probe, instead, both forms an irreversible covalent bond and becomes inhibited from further enzymatic activity. To distinguish necrosis and late apoptosis, we counter-stain labeled cells with 7AAD stain (Beckman Coulter, France). To validate and determine a reproducible method for obtaining a positive control by triggering caspase activity, we tested the presence of 7AAD⁻/FLICA⁺ cells by induced apoptosis with 1μM Camptothecin (Sigma Aldrich, France).

Briefly, 4x10⁶ cells were stained with FLICA® (1/10) in a total volume of 300μl and incubated for 1 hour at 37°C. Cells were agitated every 20 minutes before washes by PBS before cell surface staining. Cells were analyzed using flow cytometer (Navios, Beckman Coulter, France), and apoptotic cells are highlighted by 7AAD⁻/FLICA⁺ (with intracellular green fluorescent signal).

To complete the panel, following antibodies were used: APCAF700-conjugated antibody anti-CD34, APC-conjugated anti-CD56, PC7-conjugated anti-CD3, ECD-conjugated anti-CD45, PE-conjugated anti-CD14 and matched isotypes IgG1 all purchased from Beckmann Coulter (Beckman Coulter, France).

Statistical analysis

The data were expressed as the mean $\pm$ standard deviation. Significant differences were tested for significance by Mann Whitney Test for paired observations using GraphPad Prism 5.0, software. $p < 0.05$ was considered statistically significant.

Results

Patient's and graft characteristics

Clinical characteristics of the 46 patients grafted between June 2013 and March 2017 are reported in Table 1. The median age was 62 ± 2 years. Sex ratio (M/F) was 32/14. At the beginning of the conditioning regimen, all patients were in remission of either lymphoma (L, n=22 included 17 non-Hodgkin's lymphoma vs 5 Hodgkin lymphoma) and multiple myeloma (MM, n=24). All characteristics of previous protocols of chemotherapies and mobilization regimen were described in Table 1. We followed the engraftment kinetics after transplantation with an absolute PNN count greater than 0.5 G/L. Patients recovered PNN counts at a mean of 15 days (± 1) after autograft infusion without significant difference between patients' lymphoma vs multiple myeloma. At the collection step, cells viability **CD45⁺/7AAD⁻** (98% vs 96%, $p = 0.023$) and CD14⁺ absolute cells count ($2430 \cdot 10^8$ vs $1626 \cdot 10^8$, $p = 0.02$) were both decreased in MM patients. All other parameters analyzed were not significantly different between patients' lymphoma vs patients' myeloma. Focus on graft reinfused step, we described again a low CD14⁺ absolute cells count ($1930 \cdot 10^8$ vs $674 \cdot 10^8$, $p < 0.001$) in thawing graft of MM patients. Moreover, TNCs absolute count follow this trend ($4.3 \cdot 10^8/\text{kg}$ vs $2.5 \cdot 10^8/\text{kg}$, $p = 0.007$). Cell viability **CD45⁺/7AAD⁻** in post thaw graft was not significantly different between two groups, as well as, the whole parameters tested.

Apoptosis is induced in thawing PBSC CD34⁺ and in other cells subpopulations by caspases pathways activation

We used a Fluorescent Labeled Inhibitor of Caspases FLICA for caspases activation measurement. We checked the caspases activation in fresh CD34⁺, CD3⁺, CD14⁺ and CD56⁺ cells and in the same subtypes thawing cells from patients whose clinical features and protocols used are summarized in Table 2.

In all patients, the FLICA staining (7AAD⁻/FLICA⁺) showed a significant ($p < 0.0001$) apoptosis induction by caspases pathway in 23% (23.5 ± 2.1) of CD34⁺ cells compared to less than 2% (1.9 ± 0.4) only in fresh CD34⁺ stained cells - Figure 1A.

The high activation of caspases pathway is not limited to CD34⁺ in thawed grafts. Indeed, the percent of 7AAD⁻/FLICA⁺ cells is 20, 34 and 7% (19.7 ± 2.7 , 34.1 ± 3.4 and 6.4 ± 1.0 , respectively), obtained before thawing in CD3⁺, CD56⁺ and CD14⁺ cells compared to 3, 7 and less than 1% (2.5 ± 0.5 , 6.8 ± 2.0 and 0.3 ± 0.05 , respectively), obtained in fresh cells - Figure 1A.

Caspases-dependent apoptosis was not differently induced in thawing cells according to patients' diseases

We analyzed the caspases activation in thawing CD34⁺ according to the treated pathology. Our results show that in cells from patients with MM, CD34⁺ cells have not significantly (17.6 ± 2.6), different level of caspases activation after thawing than in patients with lymphoma (23.8 ± 3.4 , $p = 0.56$). This observation was also verified for CD3⁺, CD56⁺ and CD14⁺/7AAD⁻/FLICA⁺ cells (22.2 ± 3.8 vs 15.9 ± 3.7 , $p = 0.47$; 34.8 ± 6.1 vs 32.0 ± 3.9 , $p = 0.42$; 9.6 ± 1.3 vs 5.5 ± 1.4 , $p = 0.41$ respectively (L vs MM)) - Table 2. As to note, the weak CD14⁺ absolute count cells in patients MM was not due to increase of apoptotic process in this cell subtypes, under homogeneous level of 7AAD⁻/FLICA⁺ cells in both MM and lymphoma patients.

Caspases-dependent apoptosis in CD34⁺ was differently induced in thawing cells according to the level of granulocytes in graft

To assess the impact on CD34⁺ cells caspases activation of granulocytes, we investigate the influence of granulocytes count on the level of CD34⁺/7AAD⁻/FLICA⁺ cells at graft

reinfusion step. We observed correlation under these two parameters ($p = 0.042$), granulocytes increased causing increased of caspases-dependent apoptosis in CD34⁺ cells - Figure 1B.

Induction of caspase-dependent apoptosis in CD34⁺ after thawing affects clonogenicity

We measured the number of CFU-GM colonies obtained in different cultures of CD34⁺ cells after thawing and correlated to the rate of caspases activation levels. In whole patient, an inverse correlation was obtained between the rate of CD34⁺/7AAD⁻/FLICA⁺ cells and their potent to be differentiated into CFU-GM ($p = 0.004$) - Figure 1C.

Induction of caspase-dependent apoptosis in CD34⁺ after thawing does not affect the clinical course of auto-grafted patients

In addition, we compared the length of clinical aplasia according to level of CD34⁺/7AAD⁻/FLICA⁺ cells in reinfused graft. A non-significant correlation was obtained between these two parameters, either in L or MM patients ($p = 0.21$, data not shown). Moreover, neither CD3⁺/7AAD⁻/FLICA⁺ cells, nor CD14⁺/7AAD⁻/FLICA⁺ cells, nor CD56⁺/7AAD⁻/FLICA⁺ cells, were influencing the length of neutrophil recovery after autologous HSC transplantation (data not shown).

Discussion

To clarify the implication of priming apoptosis in HSC sub-population on graft quality, we conducted study to estimate the percentage of cells with precocious apoptosis, triggered by the specific caspases-pathway. Based on FLICA characteristics kit, our method could distinguish between necrotic cells and/or late apoptosis cells which are 7AAD⁺/FLICA⁻, viable cells 7AAD⁻/FLICA⁻ and early priming-apoptosis cells 7AAD⁻/FLICA⁺¹¹. Our results show that in CD34⁺ cells, the apoptosis caspases-dependent is, significantly increased after thawing. Many previous studies¹²⁻¹⁵ have already explore cell viability and apoptosis effect on potential graft with a non-specific marker of early apoptotic cells: Annexin V, a Ca²⁺-dependent phospholipid-binding protein with high affinity for phospholipid phosphatidylserine (PS) which, in early apoptotic cells, was translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment¹⁶. Concerning cryopreserved CB unit, discrepancies were objective between different studies: CD34⁺/7AAD⁻/Annexin V⁺ post thawing cells level ranging from 5.3% up to 40%^{13,14}. About studies being interested by autologous hematopoietic cells collected by apheresis, CD34⁺/7AAD⁻/Annexin V⁺ post thawing cells levels described were 7% to 15%^{12,15}. Many explications can be given to explain our results. First, we suppose that the caspase-mediated cleavage of the flippase is essential to externalize irreversibly PS at the cell surface¹⁷. Our detection method by FLICA test explore the effect of early apoptotic MNC, by using FLICA assay, an *in vitro* method that provide whole cell detection of only caspase activity in early apoptotic cells¹⁰ and detected earlier apoptotic events then Annexin V staining. It should be noted that different gene families such as caspases, inhibitor of apoptosis proteins, B cell lymphoma (Bcl)-2 family of genes, tumor necrosis factor (TNF) receptor gene superfamily, or p53 gene are involved and/or collaborate in the process of apoptosis¹⁸. Secondly, variability of cryopreservation methods is known to influence, among

others, apoptosis and necrosis in CD34⁺ cells^{19,20}. In our study, increase of caspases-dependent apoptosis is not restricted to CD34⁺ cells but affects other cell sub-populations contained in the apheresis: lymphocytes CD3⁺, monocytes CD14⁺ and natural killer CD56⁺ cells. To our knowledge, it is the first time that a study distinguishes the various impacts of apoptosis on different cryopreserved sub-populations of MonoNuclear Cells (MNCs). Previous only relate the level of global apoptotic MNCs, based on Annexin V staining, which is ranging from 5.4 to 32%^{12,13,15}.

By focusing on parameters potentially affecting the rate of CD34⁺ cells caspases activation, we find an impact on post thawing graft level of granulocytes whereas patients diseases do not seem to have of influence. A significant negative correlation was previously determined by Winter et al., between pre-freeze percent granulocyte content and post-thaw percent viability of TNCs, not specifically targeting CD34⁺ cells²¹. Those observations might be related to degranulation of granulocytes probably occurs during thawing. In fact, we know that granulocytes are more vulnerable than CD34⁺ cells during cryopreservation process with DMSO²² and that granulations include in their cytoplasm, contain many toxic mediators for others cells/tissues, such as elastase, myeloperoxidase, cathepsins, defensins, ...²³ About the influence of treated pathology type on caspases-dependent apoptosis of CD34⁺ cells, it seems to us interesting to investigate from part of presence of pathology-dependent factors (cytokines, malignant cellular clone ...) which may influence CD34⁺ sensibility to apoptotic mechanisms.

Concerning the slopes of hematopoietic reconstitution, we interestingly demonstrate that majored apoptosis observed in CD34⁺ cells affect clonogenicity without impact the quality of the immune response during the aplasia period. Our results joined observations of *Stroh and al.*, who showed that CFU-GM were enhanced with the use of zVAD-fmk, a caspase inhibitor, following cryopreservation and thawing of mobilized PBSC⁹. Similar

results were described in NOD/SCID mouse transplanted with expanded cord blood (CB) CD34⁺. Higher clonogenicity and improved in vivo engraftment were showed with CB CD34⁺ cells expanded with cytokines in the presence, among others, of zVAD-fmk, compared with expanded cells without this inhibitor of caspases²⁴.

In considering, the level of 7AAD⁻/FLICA⁺ cells in others subpopulations studied (CD3⁺, CD14⁺, CD56⁺), we did not find an impact on the delay of clinical aplasia. *Wu et al.* performed a case-control study, in 326 lymphoma patients who received an autologous HSC transplantation. Their results, contrary in ours, suggest that reinfusion of grafts containing increased levels of early apoptotic cells is associated with prolonged primary delay of neutrophil recovery after autologous HSC transplantation, particularly in patients with increased numbers of early apoptotic MNCs (MonoNuclear Cells 7AAD⁻/Annexin V⁺) in their post-thaw graft. Moreover, in these cases patients, reduced number of CFU-GM was observed, compared to patient's group control¹².

Our results show substantial cell death, induced by the increase of caspases pathway activation, secondary to the thawing process, and across all study cell types. To our knowledge, our study is the first to detail early caspases activation in various MNCs types after cryopreservation process. This observation may affect the immune response quality during recipient aplasia, without detecting a clinical impact. Moreover, caspases pathway activation through CD3⁺ and CD56⁺ subpopulations could modify the therapeutic result of donor lymphocytes infusion (DLI) cryopreserved, which could be investigated in future studies.

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Legends of Figures and tables

Table 1: Clinical patient's characteristics

	Lymphoma	Multiple Myeloma
Patients, n	22	24
Age (years) Mean (range)	63 (18-79)	60 (43-67)
Sex, n (%)		
Male	16 (73)	16 (67)
Female	6 (27)	8 (33)
Disease, n (%)		
Multiple Myeloma		24 (100)
B cell Lymphoma	7 (32)	
Hodgkin	5 (23)	
Cerebral Lymphoma	4 (18)	
Mantle cell Lymphoma	1 (4)	
Others	5 (23)	
Mobilization regimen, n (%)		
Chemotherapy + G-CSF	18 (82)	16 (67)
G-CSF alone	4 (18)	8 (33)
Mozobil n (%)		
Yes	5 (23)	1 (4)
No	17 (77)	23 (96)
Previous cycles of chemotherapies		
Number of cycles	7 +/- 4	4 +/- 2
Multiple Myeloma n (%)		
VTD		16 (67)
VCD		5 (21)
VRD		3 (12)
VMP		2 (8)
Others		2 (8)
Lymphoma, n (%)		
R-DHAP	10 (45)	
R-CHOP	9 (41)	
R-ARA	5 (23)	
Others	14 (64)	
Conditionning regimen, n (%)		
BEAM	18 (82)	
Melphalan		24 (100)
Thiotepa + chemotherapy	4 (18)	

Abbreviations: G-CSF, granulocyte-colony-stimulating factor; VTD, bortezomib-thalidomide-dexamethasone; VCD, bortezomib-cyclophosphamide-dexamethasone; VRD, **bortezomib-lenalidomide-dexamethasone**; VMP, *bortezomib*-melphalan-prednisone; R-DHAP, rituximab-dexamethasone-cytarabine-cisplatin; R-CHOP, rituximab-cyclophosphamide-doxorubicine-vincristine-prednisone; R-ARA, rituximab-cytarabine; BEAM, carmustine-etoposide-cytarabine-melphalan

Table 2: Biological features and parameters of post autograft recovery at the step of collection and graft reinfusion in lymphoma and multiple myeloma patients

	Collection			Graft reinfusion		
	Lymphoma	Multiple Myeloma	P value	Lymphoma	Multiple Myeloma	P value
TNCs (10^9)	49.9 +/- 7.3	51.1 +/- 5.5	ns (p=0.72)	24.7 +/- 3.9	16.6 +/- 2.6	ns (p=0.06)
TNCs (10^8 /kg)	7.4 +/- 0.9	6.3 +/- 0.7	ns (p=0.32)	4.3 +/- 0.5	2.5 +/- 0.3	** (p=0.007)
Granulocytes (%)	23 +/- 3.9	27.3 +/- 3.4	ns (p=0.13)	1.6 +/- 1	2.3 +/- 1.4	ns (p=0.31)
CD34+/7AAD- cells (10^6)	407 +/- 70.7	506.8 +/- 85.4	ns (p=0.12)	273.1 +/- 50.9	265.5 +/- 27.6	ns (p=0.66)
CD34+/7AAD- cells (10^6 /kg)	4.9 +/- 1.0	6.8 +/- 0.9	ns (p=0.22)	3.1 +/- 0.8	3.5 +/- 0.3	ns (p=0.31)
CD34+/7AAD-/FLICA+ cells (%)	1,9 +/- 0.9	1.3 +/- 0.3	ns (p=0.16)	23.8 +/- 3.4	17.6 +/- 2.6	ns (p=0.56)
CD3+/7AAD- cells (10^7)	743.2 +/- 144.2	930.9 +/- 184.1	ns (p=0.33)	721.4 +/- 142.9	560.3 +/- 152.1	ns (p=0.56)
CD3+/7AAD-/FLICA+ cells (%)	2.8 +/- 0.7	1.65 +/- 0.6	ns (p=0.58)	22.2 +/- 3.8	15.9 +/- 3.7	ns (p=0.47)
CD56+/7AAD- cells (10^6)	32.7 +/- 9.1	52.2 +/- 28.3	ns (p=0.12)	43.5 +/- 37.2	19.9 +/- 16.9	ns (p=0.41)
CD56+/7AAD-/FLICA+ cells (%)	8.5 +/- 3.7	5.3 +/- 1.7	ns (p=0.14)	34.85 +/- 6.09	32.0 +/- 3.9	ns (p=0.42)
CD14+/7AAD- cells (10^6)	2430.4 +/- 354.1	1626.3 +/- 180.8	* (p=0.02)	1930.3 +/- 265.5	674.4 +/- 128.7	*** (p=0.0007)
CD14+/7AAD-/FLICA+ cells (%)	0.3 +/- 0.1	0.3 +/- 0.1	ns (p=0.85)	9.6 +/- 1.3	5.5 +/- 1.4	ns (p=0.41)
Viability (CD45+/7AAD- cells) (%)	98.2 +/- 0.42	96.01 +/- 0.39	* (p=0.023)	59.2 +/- 3.8	54.3 +/- 2.3	ns (p=0.36)
Recovery of TNCs (%)				48.4 +/- 2.7	45.6 +/- 2.3	ns (p=0.79)
CFU-GM (10^4 /kg)	118.7 +/- 18.5	114.1 +/- 30.6	ns (p=0.35)	20,2 +/- 6.5	32.3 +/- 4.5	ns (p=0.45)
Clonogenicity (%)				7.3 +/- 1	9.2 +/- 1	ns (p=0.37)
Recovery of CD34+ cells (%)				65.3 +/- 3.9	67.1 +/- 2.8	ns (p=0.61)
Engraftment, days				15.5 +/- 0.8	15 +/- 0.6	ns (p=0.50)
Period of graft cryopreservation, days				103.0 +/- 24.5	40.0 +/- 75.7	ns (p=0.06)

Abbreviations: TNCs, Total Nucleated Cells; 7AAD, 7-aminoactinomycin D; FLICA, Fluorescent Labeled Inhibitors of Caspases; CFU-GM, Colony Forming Unit – Granulocyte Macrophage; ns, non-significant; *, p<0.05; **, p<0.01; ***, p<0.001

Figure 1: A) Comparison of caspases activation in fresh and thawing CD34⁺, CD3⁺, CD14⁺ and CD56⁺ cells; B) Correlation between CD34⁺/7AAD⁻/FLICA⁺ cells and granulocytes quantity **at the reinfusion graft step**; C) Correlation between CD34⁺/7AAD⁻/FLICA⁺ cells and their potent to be differentiated into CFU-GM - *, p<0.05; **, p<0.01; ***, p<0.001 –