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DMSO (Me_2SO) concentrations of 1-2% in combination with pentaisomaltose are effective for cryopreservation of T cells

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ABSTRACT

T cell based treatments in the setting of allogenic haematopoietic stem cell transplantation (HSCT) have been used for decades. In addition, the use of chimeric antigen receptor (CAR) T cells has been introduced as a promising cancer immunotherapy. A prerequisite for many of these treatments is the ability to cryopreserve the cells safely and efficiently.

In the present study, we compared freezing media combinations containing pentaisomaltose and 1–2 % DMSO (PIM1 and PIM2, respectively) to 10 % DMSO and commercially available cryosolutions (CS2 and CS10, Cryostor® containing 2 and 10 % DMSO, respectively) for cryopreservation of T cells.

T cells isolated from buffy coats from healthy donors were cryopreserved with different freezing media and analysed for 1) viability immediately post-thaw and the following 24 h, 2) recovery, 3) proliferative potential and 4) migration towards a gradient of SDF-1α.

The results showed that PIM2 was superior to 10 % DMSO and comparable to CS10 when assessing viability. Furthermore, the results indicated that the T cells cryopreserved with 10 % DMSO showed the lowest proliferative potential. The expression levels of CXCR3, CXCR4 and VLA-4 were similar in T cells independent of the freezing media used; however, T cells cryopreserved with PIM2 demonstrated the highest migratory potential.

In summary, the combination of pentaisomaltose and 1–2 % DMSO improves the cryoprotective properties compared to 10 % DMSO while achieving comparable results with CS10 and even showing improved migration towards SDF-1α. Thus, our results show promising potential for pentaisomaltose in combination with low amounts of DMSO for the cryopreservation of T cells.

1. Introduction

T cell-based treatment of malignant diseases has been used for decades in the setting of allogeneic haematopoietic stem cell transplantation (HSCT), but with the promising results of chimeric antigen receptor T cells (CAR-T cells), T cell-based treatment is more relevant than ever [1]. Numerous clinical trials have been testing the effect of CAR-T cells [2], while two commercial products, Yescarta® and Kymriah®, have both already been approved for the treatment of diffuse large cell lymphoma and the latter has also been approved for relapsed/refractory B-cell acute lymphoblastic leukaemia (B-ALL). The production of CAR-T cells involves at least one freeze/thaw procedure; however, in many of the clinical trials and all certified treatments, the cells are cryopreserved twice due to logistical reasons (once after collection and a second time after gene transduction and cell expansion).

In the setting of HSCT, donor lymphocyte infusion (DLI) of cryopreserved cells is used in refract and increasing doses as a treatment for relapse after transplantation [3]. In both settings, the cryopreservation of T cells is a prerequisite for patient treatment.

However, cryopreservation is a stressful event, and thus, a cryoprotective agent (CPA) is necessary to cryopreserve cells safely and efficiently in order to reduce cryo-related cell damage and preserve viability and potency after thawing [4]. Cryoformulations with dimethyl sulfoxide (DMSO) are standard in many laboratories and are used for cryopreservation of most cell types, including T cells. The clinical side effects and undesirable cellular effects of DMSO [5–10] are well known and have led to an increasing demand from health care professionals, authorities and patients for alternative CPAs that can provide safe alternative freezing media alone or in combination with reduced amounts of DMSO.

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Much development has been made to optimize freezing media for T cell products by trying to reduce the amount of DMSO needed [11] by combining existing CPAs such as DMSO and dextran 40 and/or by improving the carrier media [12], but new alternative CPAs have not been introduced for clinical use [4].

Pentaisomaltose, a low-molecular-weight carbohydrate, has recently been described as a promising CPA candidate. Our group has previously shown pentaisomaltose to be an effective CPA for cryopreservation of haematopoietic stem and progenitor cells (HSPCs) from apheresis products [13,14]. Although pentaisomaltose does not seem to be sufficient as the sole CPA for cryopreservation of adipose-derived stromal/stem cells (ASCs) [15], we showed a beneficial effect of combining pentaisomaltose with DMSO and were able to reduce the concentration of DMSO to only 1–2 % for successful cryopreservation.

Based on these encouraging findings, we intended to further explore whether the addition of pentaisomaltose could also be used to reduce the DMSO concentration for the cryopreservation of T cells. Thus, in the present study, we compared a combination of pentaisomaltose and DMSO-based freezing media to the gold standard and in-house freeze media formulation (10 % DMSO) and Cryostor® freeze media containing 2% or 10 % DMSO (CS2 and CS10) for the cryopreservation of T cells. Cryopreserved T cells were characterized post-thaw by assessing their viability, recovery, proliferation and migration.

2. Materials and methods

2.1. Isolation of peripheral T cells

The buffy coats used in this study were obtained with consent from healthy danish blood donors.

PBMCs and lymphocytes from buffy coats of three donors were isolated using Leucosep (Greiner Bio-One) according to the manufacturer's instructions. Isolated PBMCs and lymphocytes were counted using nucleocounter NC-3000 (Chemometec), and 1×10^8 nucleated cells were used for subsequent T cell isolation with Pan T cell isolation kits (biotin-conjugated monoclonal antibodies against CD14, CD15, CD16, CD19, CD34, CD36, CD56, CD123, and CD235a) and large-scale (LS) columns (both from Miltenyi Biotec) according to the manufacturer's instructions. The isolated T cells were counted, and their high purity (>95 %) was verified using Sysmex (XN-1000 haematology analyser) and an anti-45 and anti-CD3 antibody mix followed by subsequent flow cytometry analysis (Navios, Beckman Coulter).

2.2. Cryopreservation

The isolated T cells were aliquoted into Falcon tubes, pelleted by centrifugation (300 *g*, 5 min, 20 °C), and gently resuspended to a final cell concentration of 4×10^6/mL in each of the five freezing media formulations: STD10 (DMSO 10 % + 4% human albumin (HA)), CS2 (Cryostor® 2 preformulated with 2% DMSO and dextran-40, Biolifesolutions), CS10 (Cryostor® 10 preformulated with 10 % DMSO and dextran-40), PIM1 (10 % pentaisomaltose (Pharmacosmos) + 1% DMSO + 4% HA) or PIM2 (10 % pentaisomaltose + 2% DMSO + 4% HA). One millilitre was transferred to 1.8 mL cryotubes (Nunc) (4×10^6 cells/tube, in duplicate) and immediately cryopreserved in a controlled-rate freezer (Kryo 560−16, Planer PLC) using gradient-based cooling (starting temperature of 4 °C, 1 °C/min drop to 0 °C, then a 2 °C/min drop to -45 °C, and finally a 5 °C/min drop to -100 °C). The frozen cryotubes were stored in liquid N_2 (−196 °C) for up to three months before analysis.

2.3. Cell count, viability and recovery

T cells were thawed in a 37 °C water bath for approximately 2 min. The thawed cells were diluted ×10 in complete cell culture media (RPMI1640 (Gibco), supplemented with 5% human serum (Sigma Aldrich), pen/strep and 2 I/E heparin, room temperature) for viability and recovery assessment.

Thawed and diluted T cells were stained with a mix of 4′,6-diamidino-2-phenylindole (DAPI) and acridine orange (Solution 13, Chemometec), and cell count and viability were analysed using an NC-3000 (Chemometec). The obtained cell count and viability were used to calculate recovery ((post-thaw viable cell count/pre-cryopreservation cell count) ×100 %).

2.4. Twenty-four hours post-thaw viability assessment

Cytotoxicity was monitored for 24 h following thawing. The thawed T cells were seeded in 96-well plates (2000 cells/well) in culture expansion media (RPMI 1640 + 10 % human serum (HS), pen/strep and heparin) supplemented with Cytotox red DNA stain (Cytotox red, final concentration of 250 nM, Essen Biosciences). Dead T cell counts post-thaw were monitored and quantified hourly using IncuCyte live cell imaging and analysed using IncuCyte Zoom software (Essen Biosciences) according to the manufacturer's instructions.

2.5. T cell proliferation and adhesion/migration markers

The thawed T cells were pelleted and resuspended in serum-free media (RPMI 1640, pen/strep and heparin) and labelled with 2.5 μM carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) for 10 min at 37 °C. Labelled T cells were washed twice with culture expansion media and seeded (100,000 cells/well in duplicate wells) in 96-well plates (NUNC, Thermo Fisher Scientific) in sterile filtered culture expansion media supplemented with either 500 ng/mL SEB (Staphylococcus enterotoxin B, Sigma Aldrich) or with anti-CD3/anti-CD28 beads (1:2) to stimulate proliferation (Thermo Fisher Scientific). Following four days in culture (37 °C, 5% CO_2), the cells were thoroughly resuspended, and 50 μL of the cell suspension from each duplicate well was transferred to flow tubes for staining.

The cells were stained with 7AAD and a mixture of anti-CD3 (PE), anti-CXCR3 (BV421), anti-CXCR4 (APC) and anti-VLA-4 (PE-Cy7) antibodies (all antibodies from BD Biosciences). Following incubation for 30 min at room temperature, 400 μL of flow buffer (PBS-EDTA with 1% HA) and 20 μL of counting beads (Beckman Coulter) were added, and the cells were analysed by flow cytometry (Navios, Beckman Coulter). The percentage of proliferating T cells and the expression of CXCR3, CVCR4 and VLA-4 markers were evaluated using Kaluza analysis software 2.1 (Beckman Coulter). The relative number of proliferating T cells was calculated as the number of proliferating T cells/counting bead.

2.6. T cell migration assay

The insert (top well) in a clear view 96-well chemotaxis plate was precoated with Protein G (20 μg/mL for 1 h) (Thermo Fisher Scientific) and subsequently with ICAM-1 (5 μg/mL for 2 h). T cells were seeded in the insert (1500 cells/well) in complete culture media, and complete culture media supplemented with SDF-1α (100 ng/mL (Sigma Aldrich)) was added to the reservoir (lower well). The plate was monitored using IncuCyte live cell imaging (Essen Biosciences), obtaining pictures of the insert every hour. T cell numbers were counted using IncuCyte Zoom software, measuring migration as a reduction in the number of counted T cells relative to the start.

2.7. Statistics

One-way ANOVA was used to analyse viability, recovery, proliferation, expression, T cell proliferation data and migratory potential, comparing the means of each type of freezing media. Tukey's multiple comparison test was used when comparing mean values. All statistical analyses were performed with computer software (GraphPad Prism, Version 5.02, GraphPad Software Inc.).

3. Results

3.1. Pentaisomaltose combined with low concentrations of DMSO preserves high viability and recovery

The viability of the T cells measured immediately post-thaw was 69.5 % ± 17.9 (STD10), 90.6 % ± 0.4 (CS2), 92.3 % ± 1.1 (CS10), 86.6 % ± 4.7 (PIM1) and 92.3 % ± 4.3 (PIM2), with significantly higher viability for CS10 and PIM2 than for STD10 ($p = 0.039$ and $p = 0.039$, respectively) (Fig. 1B).

The calculated post-thaw recovery of viable T cells ranged from 52 % to 114 % (Fig. 1A), with PIM2 having the lowest intra-individual variance of less than 10 %. No significant differences were observed when comparing recovery between STD10 (70.0 % ± 17.4), CS2 (77.2 % ± 21.6), CS10 (100.1 % ± 13.2), PIM1 (92.9 % ± 13.3) and PIM2 (100.7 % ± 6.8).

Cells cryopreserved with STD10 displayed an increase of approximately 2-fold in dead T cell count in the first six hours when compared with CS2 ($p < 0.001$), CS10 ($p < 0.001$) and PIM2 ($p < 0.001$). Similarly, an increase in the dead T cell count in the first 6 h, although not as distinct as STD10, was observed for PIM1 compared to CS2 ($p = 0.019$) and CS10 ($p = 0.006$) (Fig. 1B). Samples cryopreserved with CS2, CS10 and PIM2 did not show a noticeable or significant increase in dead cell count.

3.2. Improved proliferation when combining DMSO with pentaisomaltose

T cells from all cryopreservation formulations maintained equivalent percentages of proliferating cells: CS2 (84.2 % ± 9.3), CS10 (85.4 % ± 7.8), PIM1 (89.1 % ± 3.0) and PIM2 (87.9 % ± 7.1), except for STD10 (72.9 % ± 12.5), which had a significantly lower fraction of proliferating cells than PIM2 ($p = 0.039$) (Fig. 2A).

There was a tendency towards a higher relative number of proliferating T cells for CS2 (5.6 ± 1.3), CS10 (5.0 ± 0.1), PIM1 (6.7 ± 0.7) and PIM2 (6.0 ± 1.1) compared with STD10 (2.1 ± 1.6), although only PIM1 was significantly higher than STD10 ($p = 0.018$) (Fig. 2B).

Although not significant, a similar tendency was observed when SEB was used to induce the proliferation of T cells cryopreserved with STD10 (9.3 % ± 7.2), CS2 (25.6 % ± 6.2), CS10 (27.3 % ± 5.4), PIM1 (20.1 % ± 2.7) and PIM2 (22.6 % ± 9.1) (Fig. 2C).

3.3. Increased migration potential when combining 2% DMSO with pentaisomaltose

All T cells expressed CXCR3 and VLA-4 in response to stimulation with both beads and SEB (Fig. 3A). SEB stimulation induced increased levels of CXCR3 and VLA-4 compared with stimulation with beads, whereas no further induction of expression was observed for CXCR4.

T cells cryopreserved with all freezing media were able to transwell migrate towards a gradient of SDF-1α. However, cells cryopreserved with PIM2 displayed a significantly higher migratory potential than T cells cryopreserved with all other cryosolutions: STD10 ($p < 0.001$), CS2 ($p < 0.001$), CS10 ($p < 0.001$) and PIM1 ($p < 0.001$) (Fig. 3B). Cells cryopreserved with PIM1 also showed significantly increased migration compared to STD10 ($p = 0.001$), CS2 ($p < 0.001$) and CS10 ($p < 0.001$).

4. Discussion

Cryopreservation of T cells is an essential part of CAR-T and DLI therapies and a prerequisite for effective production and treatment.

In this study, we show that adding pentaisomaltose to freezing media reduces the DMSO concentration necessary for the efficient cryopreservation of T cells.

T cells cryopreserved with 10 % DMSO alone (STD10) showed lower viability than cells frozen with freezing media combining DMSO and a non-permeating CPA, although this result was only significant for PIM2 and CS10. Others have shown an improvement in T cell viability when reducing the concentration of DMSO from 10 % to 5% by including 6% hydroxyethylstarch (HES) or by using DMSO in combination with dextran 40 (CS10 and CS5) [11]. We did not observe the same effect when comparing CS10 and CS2 or PIM2 and PIM1; however, we cannot rule out an optimum concentration of DMSO somewhere in between 2% and 10 %. Interestingly, the results of the investigated 24 h assessment of cell death post-thaw indicate delayed apoptosis and/or necrosis presumably caused by freeze-related damage. Thus, the actual viability and recovery of cells cryopreserved in STD10 was considerably lower than that estimated immediately post-thaw. The decline in dead T cell count observed after 5–6 hours for STD10 (and PIM1) was likely caused by nuclear degradation following apoptosis and necrosis [16,17] and a fading of the DNA signal, which no longer enabled the detection of the dead cells. The progression of cell death could potentially have implications for the clinical use of T cells. Cryopreserved donor T cells for DLI are administered in increasing doses to balance the achievement of a graft versus leukaemia effect without the induction of graft versus host disease [3]. Considerable progression of cell death can be seriously misleading when treatments are based on the numbers of perceived living cells. Interestingly, although the recovery of CS2 and especially CS10 and PIM2, were not significantly higher than that of STD10, no apparent progression of cell death post-thaw was observed, indicating that these CPA combinations protect the cells from delayed freeze-related damage.

T cells cryopreserved with STD10 had the lowest proliferative potential of all CPA combinations when stimulated with beads or SEB. Furthermore, the cells cryopreserved with STD10 had the lowest number of proliferating T cells. This latter observation could indeed be a consequence of the increased progression of cell death observed. As such, these results help to emphasize the importance of an assay monitoring the progression of cell death, usually not performed in

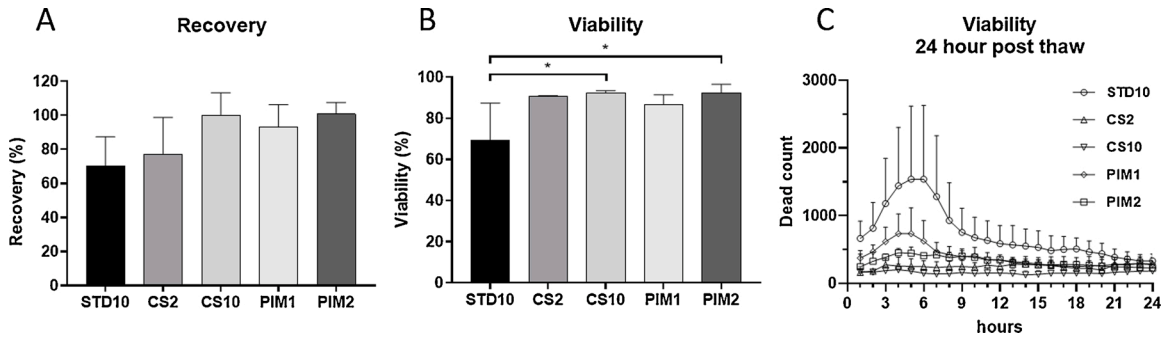

Fig. 1. Viability and recovery of T cells.
T cells were cryopreserved with STD10, CS2, CS10, PIM1 or PIM2, thawed and analysed for A) viability, B) recovery and C) 24 h post-thaw cell viability. Data represent three donors (n = 3).

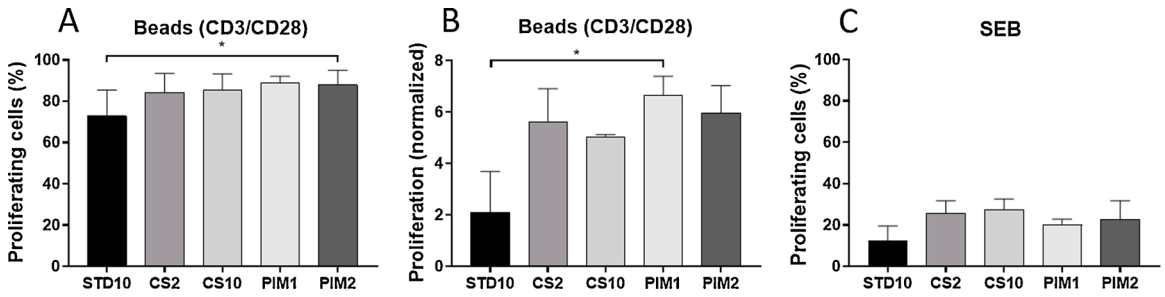

Fig. 2. T cell proliferation.
T cells were cryopreserved with STD10, CS2, CS10, PIM1 or PIM2. T cells were stained with CFSE post-thaw, and proliferation was stimulated with anti-CD3/CD28 Dynabeads. A) Percentage proliferating cells and B) relative number of proliferating cells; or SEB C) percentage proliferating cells. Data represent three donors (n = 3).

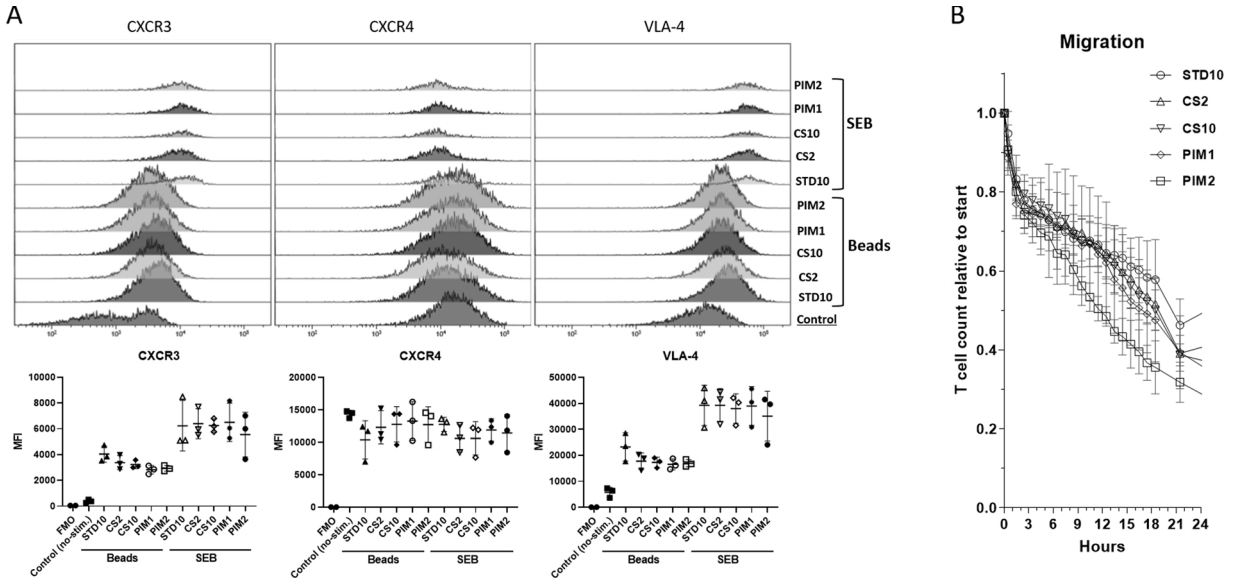

Fig. 3. T cell migration/adhesion.
T cells were cryopreserved with STD10, CS2, CS10, PIM1 or PIM2. Post-thaw, T cells were A) stimulated with anti-CD3/CD28 Dynabeads or SEB and analysed for the expression of CXCR3, CXCR4 and VLA-4 by flow cytometry or B) seeded in Transwell migration plates and allowed to migrate towards a gradient of SDF-1α for 24 h. Data represent three donors (n = 3).

experimental setups assaying frozen-thawed cells, as the undetected loss of cells might lead to biases and possible misinterpretation of experimental data.

The expression of the investigated migration and adhesion markers seemed to be preserved with all tested freezing media. CXCR3 is involved in T cell trafficking and function [18], and VLA-4 is involved in trafficking 17], and both were, in the present study induced by beads or SEB upon activation in T cells cryopreserved with all freezing media. Interestingly, SEB led to higher levels of CXCR3 and VLA-4 than stimulation with anti CD3/CD28 beads. SEB functions as a superantigen by crosslinking the invariant part of the T cell receptor and MHC class II molecules on antigen presenting cells (APCs) 18]. Residual APCs left in the isolated T cell samples are likely responsible for SEB-mediated CXCR3 and VLA-4 induction, as SEB-mediated cross-linking between major histocompatibility complex class II molecules and the T cell receptor is required for T cell activation [20]. Results indicate show that the T cell-APC interaction upon T cell activation is significantly more potent than CD3/CD28-mediated activation alone. This also shows that information may be lost when simplifying the experimental setups.

The chemokine receptor CXCR4 is constitutively expressed in T lymphocytes and involved in regulating migration towards gradients of SDF-1α (CXCL12) [19,21]. Our results show that CXCR4 is not induced by activation by beads or SEB. When assessing the migratory potential towards SDF-1α, T cells frozen with PIM2 displayed a significantly higher fraction of migratory cells than the other tested freezing media. The difference in the observed ability of T cells to migrate might be explained by other cellular functions involved in cell migration, such as signalling through CXCR4 or cytoskeletal rearrangement [22], not investigated in this setup. The fact that the lowest level of migration was observed with STD10 can be partly explained by reduced viability and progression of cell death post-thaw compared with the other CPA combinations tested.

We recently reported the use of PIM for the cryopreservation of ASCs and HSPCs [15,23]. Collectively, the results from these two studies indicate that the cryoprotective efficiency of pentaisomaltose and DMSO is cell type-dependent, e.g., pentaisomaltose seems to be more effective for the cryopreservation of HSPCs compared to ASCs, while 10 % DMSO (STD10) seems to be more effective for the cryopreservation of ASCs [15] than T cells and HSPCs [23]. The results, however, indicate that adding pentaisomaltose to DMSO significantly improves the cryopreservation of T cells and this may also be the case for HSPCs. The differences in viability and recovery observed when using STD10 for ASCs, T cells or HSPCs could partly be caused by a considerably lower membrane permeability of DMSO (P_{DMSO}) in T cells and HSPCs compared to MSCs [24–26].

In conclusion, this study found a beneficial effect of combining

pentaisomaltose with low doses of DMSO for cryopreservation of T cells. The results demonstrate a reduction of the required DMSO concentration to 1–2 % while still achieving better results than 10 % DMSO alone in regard to viability, T cell proliferation and migration towards an SDF-1α gradient, although this study was conducted with relatively few T cell donors. In our study, PIM2 exceeded the cryoprotective capabilities of CS2 with a cryoprotective efficiency comparable to that of CS10. Other functional readouts would be interesting to investigate to further assess T cell potency when cryopreserved with formulations combining DMSO with pentaisomaltose.

Furthermore, based on these results, we recommend quality testing of clinical products to assess the potential progression of cell death and to consider adjusting T cell numbers accordingly for T cell treatments such as DLI and CAR-T cell therapy. This is especially true for laboratories that use freezing media based on DMSO formulated without non-permeating CPAs.

With the increasing focus on lowering the concentration of DMSO in freezing media, these results suggest that 10 % pentaisomaltose provides a means to reduce the effective DMSO concentration to 1–2 %.

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Author contributions

E.K.H. designed the study, analysed the data, and wrote the manuscript. L.M-F. designed the study, performed the experiments, analysed the data, and wrote the manuscript. O.R.B performed the experiments, analysed the data, and wrote the manuscript. A. F–N. designed and the study, analysed the data, and wrote the manuscript. J.D.S. designed the study, performed the experiments, analysed the data, and wrote the manuscript.

Declaration of Competing Interest

None.

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