Updated Title: Process Optimization for CD3+ T-Cell Formulation and Cryopreservation

Alireza Abazari[1], Aby J. Mathew[1]

[1]BioLife Solutions, Inc., Bothell, WA

BIOLIFE SOLUTIONS™
cell and gene therapy tools

INTRODUCTION

Cell-based therapies require specialized handling to ensure their successful transport from the manufacturing facility to the patient. To provide logistical flexibility, many current commercialization models employ a frozen cell product that can be delivered to the clinic and stored, then thawed and infused into patients on demand. In manufacturing of such therapies, cryopreservation critical process parameters overlap with manufacturing process immediately prior to (starting material) and after the end of the cell culture processing (formulation of product). As such, the efficiency and efficacy of the final therapeutic dose, as well as the manufacturing process, are directly affected by cryopreservation. Hence, thorough understanding of the impact of cryopreservation on cells is vital for successful commercial manufacturing of cellular therapies. In this study, we investigated the impact of some of the process parameters (CPPs) on post-thaw viability and post-activation expansion and cytokine secretion in human pan CD3+ T cells, to assess criticality of those parameters for cryopreservation process. Those parameters included cryomedia formulation, DMSO content, post-thaw stability prior to dilution/wash, and activation timeline. We used viability as assessed by membrane integrity, proliferation rate, and INFγ secretion upon activation to characterize the cells and the process. Our results suggest that, Biopreservation Best Practices for cryomedia formulation, including elimination of protein/serum from the formulation step can be achieved with enhanced efficacy using the intracellular-like CryoStor (BioLife Solutions, Inc., WA) formulation.

METHODS

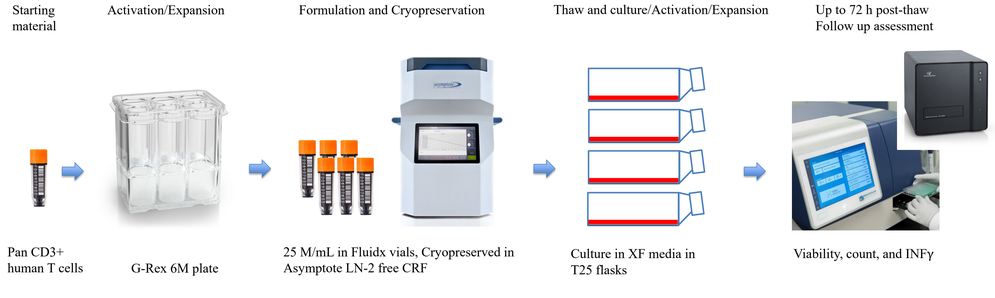
Cell Culture: Cryopreserved, negatively-selected, human pan CD3+ T cells were purchased from HemaCare (Northridge, CA). Once thawed, the cells were plated at 5M/well in a G-Rex 6M special 6-well plate format (WilsonWolf, Saint Paul, MN) supplied with 100 mL/well fresh xeno-free T-cell culture medium (STEMCELL Technologies, Vancouver, BC) supplemented with 10^6 IU/mL IL-2 (STEMCELL Technologies). The cells were activated using 1 mL/100 mL anti-CD3/CD28/CD2 T-cell activation agent (STEMCELL Technologies) 1 hour post-thaw, and the plate was placed in the incubator at 37°C and 5% CO_2. Fresh IL-2 was supplied to each well at 10^6 IU/mL IL-2 every 3 days. After 10 days, the plate was removed from the incubator, and cells were harvested for formulation and cryopreservation.

Cryopreservation Media: Four different cryopreservation media, CryoStor CS5, CryoStor CS10, and 2 common home-brew formulations were used to cryopreserve T cells. The 2 home-brew formulations were 5% w/v recombinant human serum albumin (rHSA, InVitria, CO) and 10% v/v DMSO (BloodStor 100, BioLife Solutions, WA) in either Normosol-R or PlasmaLyte-.

Cryopreservation: The cell-containing media were removed from flasks and collected in 15 mL centrifuge tubes. A sample of each flasks was removed to assess count and viability prior to freezing. After pelleting, T cell pellets were resuspended in cryopreservation media 25M/mL, and distributed at 1 mL volumes in 2 ml FluidX cryovials (Brooks Life Sciences, MA). The cells were incubated at 2-8°C for 15 min (n=5 per condition). Cryovials were then transferred to an Asymptote LN2-free controlled-rate freezer (GE), and were cooled down at a rate of -1°C/min, with nucleation manually performed at -10°C. After reaching -70°C, the vials were transferred to LN_2 storage for a minimum of 24 h.

Thawing and Assessment: Samples were thawed in 37°C water bath, then resuspended 1:10 in pre-warmed XF T cell culture media supplemented with 10^6 IU/mL IL-2. A sample was collected at immediately post-thaw for viability and count measurements. Each sample was plated in 2 wells of a regular 6-well plate, one well as resting control and one for assessing activation, at 5M cell/5 mL XF media/well. Activation post-thaw was performed by adding 200 uL of anti-CD3/CD28/CD2 activation reagent (STEMCELL). At this point, the 6-well plates were transferred to the incubator at 37°C and 5% CO_2. For studying the impact of rest before activation, a group of samples, cryopreserved in CryoStor CS5, were activated after 24 h rest in culture post-thaw. For studying the impact of DMSO concentration, two groups were compared, cryopreserved in CryoStor CS5 vs. CryoStor CS10, with 5% and 10% v/v DMSO respectively. For post-thaw processing considerations, a group of samples were allowed to rest at room temperature on the bench for 1 h immediately after thaw and before further processing. For all samples, at 24 h and 72 h post-thaw, a small volume from each well was removed to assess viability, count, and for measuring INFγ secretion. Viability and count measurement were performed using VIA-1 cassettes on ChemoMetec NC-3000. INFγ was measured using ELISA plates (STEMCELL Technologies) per manufacturer's protocol.

Statistical Analysis: For all measurements, data represent the mean of 3-11 independent experiments and are normalized to pre-freeze conditions. Groups were analyzed for statistical significance via two-way ANOVA with Tukey correction and $p<0.05$ set *a priori*. Error bars represent standard deviation (SD). Cell cycle experiments were analyzed separately, using the identical statistical method.



RESULTS

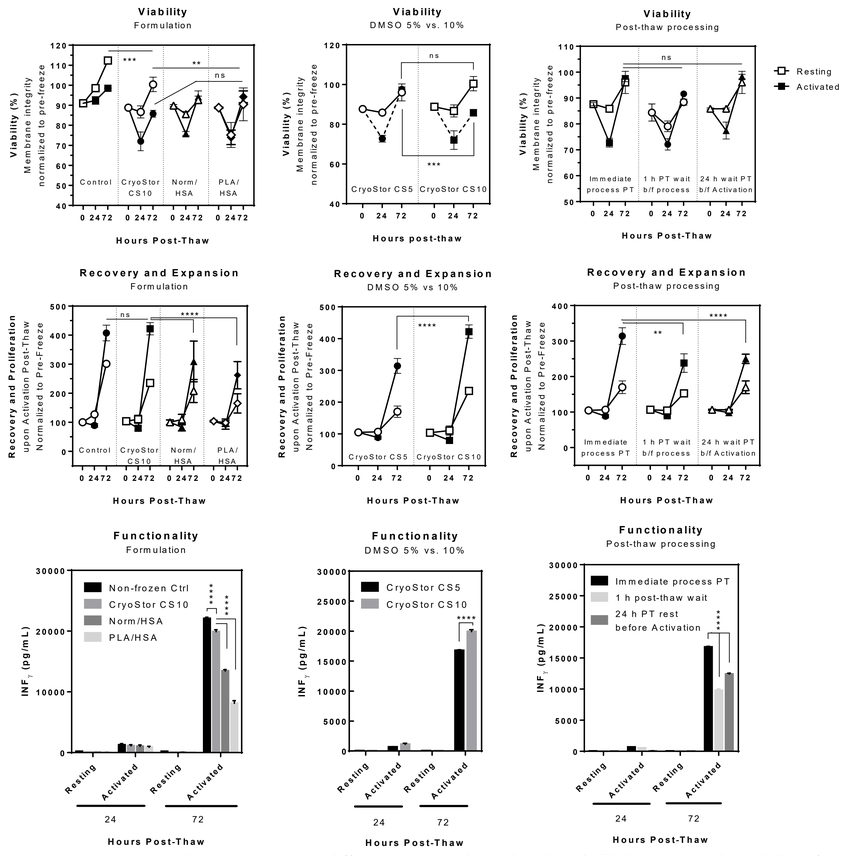


Figure 1: Post-thaw viability of cryopreserved human T cells, normalized to pre-freeze. (Left) Comparison of the performance of 3 different formulations with similar DMSO content (10% v/v). **(Middle)** Comparison of the impact of DMSO concentration in CryoStor formulations with 5% and 10% DMSO. **(Right)** Comparison of 3 different scenarios for activation of T cells post-thaw: (1) Immediate post-thaw processing and activation, (2) post-thaw wait (1 h) before further processing, and (3) activation 24 h after processing and rest in culture. White markers denote Control condition, and black (filled) markers denote activated samples. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$)

Figure 2: Post-thaw recovery and expansion potential of cryopreserved human T cells, normalized to pre-freeze. (Left) Comparison of the performance of 3 different formulations with similar DMSO content (10% v/v). **(Middle)** Comparison of the impact of DMSO concentration in CryoStor formulations with 5% and 10% DMSO. **(Right)** Comparison of 3 different scenarios for activation of T cells post-thaw: (1) Immediate post-thaw processing and activation, (2) post-thaw wait (1 h) before further processing, and (3) activation 24 h after processing and rest in culture. White markers denote Control condition, and black (filled) markers denote activated samples. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$)

Figure 3: Post-thaw activation of cryopreserved human T cells, assessed by quantifying INF γ. (Left) Comparison of the performance of 3 different formulations with similar DMSO content (10% v/v). **(Middle)** Comparison of the impact of DMSO concentration in CryoStor formulations with 5% and 10% DMSO. **(Right)** Comparison of 3 different scenarios for activation of T cells post-thaw: (1) Immediate post-thaw processing and activation, (2) post-thaw wait (1 h) before further processing, and (3) activation 24 h after processing and rest in culture. White markers denote Control condition, and black (filled) markers denote activated samples. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$)

(1) Impact of formulation: Comparing across 3 different groups with similar 10% v/v DMSO content, the viability of CS10 groups recovered faster the other two cryomedia, and came close to, but statistically lower than, the non-frozen control. Once activated, a drop in the viability of the T cells is observed in all groups. At 72 h post-thaw, the viability of CS10 group was the closest to a non-frozen control and was statistically higher ($p<0.01$) than the other two cryomedia. Across the board, minimal differences in viability post-thaw and post-activation can be observed with the 3 different formulations with 10% DMSO. However, significant differences between the groups can be observed in expansion and INFγ secretion at 72 h post-thaw. Cells cryopreserved in CryoStor CS10 expanded in number within a 72 h period to the same level as a non-frozen control, and significantly higher than those cryopreserved in Normosol/HSA and PLA/HSA media. Similarly for INFγ, the secreted levels were significantly higher for the CS10 groups compared to the other groups ($p<0.0001$) and was only slightly less than the non-frozen control ($p<0.0001$).

(2) Impact of DMSO content: Increase in the DMSO concentration of the formulation was studied by comparing the efficacy of CryoStor CS5 and CS10 formulations, with 5% and 10%v/v DMSO respectively. As seen in Fig. 1, the viability of the CS10 group at 72 h post-thaw is lower than the CS5 after activation. However, the expansion rate of cells in CS10 group were significantly higher ($p<0.0001$) than in CS5, as well as INFγ secretion ($p<0.0001$). In addition, it must be mentioned that, in terms of viability and expansion potential, the performance of the cells cryopreserved in lower DMSO concentration (CryoStor CS5 with 5% v/v) was on par with the performance of home-brew formulations with 10% v/v DMSO. Cells cryopreserved in CS5 secreted more INFγ compared to PLA-based home-brew formulations containing 10% v/v DMSO ($p<0.0001$), but less than Normosol-based home-brew with 10% DMSO ($p<0.0001$) [statistics not shown on the figures].

(3) Impact of post-thaw processing: As observed in these results, that activation leads to loss of viability in a short window post-thaw. As such it was speculated that a post-thaw resting period for the cells, in this case 24 h, prior to activation may be beneficial in terms of reducing cell loss and increasing expansion rate. In addition, stability post-thaw is a point of risk/concern in cell therapy manufacturing and practice, where it is recommended to know how long the cells remain stable post-thaw, prior to washing or patient administration. The results in this study suggest that, up to 1 h wait post-thaw at room temperature (20-25°C) prior to processing, had significant impact on expansion capacity of the cells as well as INFγ secretion compared to the samples that were processed immediately post-thaw. What is interesting to observe is that viability remained statistically unchanged for the 1 h wait group, and as such, viability alone is not a sufficient measure for assessing the impact of such process parameters on the quality of the T cells. In addition, it was observed that resting for 24 h in culture post-thaw and prior to activation did not necessarily reduce the post-activation cell viability loss (Fig. 1).

CONCLUSIONS

- Intracellular-like CryoStor appears to be a more robust formulation for cryopreservation of human CD3+ T cells than the conventional home-brew cocktails. Human CD3+ T cells cryopreserved in the intracellular-like, serum-free, protein-free formulation of CryoStor CS10 displayed enhanced, or at least equivalent, quality and functionality post-thaw, compared to 2 other tested formulations, namely Normosol-R + 5% w/v rHSA + 10% v/v DMSO, and PlasmaLyte-A + 5% w/v rHSA + 10% v/v DMSO. In addition, performance of the cells cryopreserved in lower DMSO concentration (5% v/v) was on par with the performance of home-brew formulations with 10% v/v DMSO, suggesting a potential way to reduce DMSO content in the final product by employing an intracellular-like base formulation such as in CryoStor.
- In CryoStor formulations, a higher DMSO concentration (10% v/v) appeared to be more effective in preserving human T cells than lower (5% v/v) DMSO concentration.
- Given a similar timeline post-thaw, expansion rate and INFγ secretion for the resting culture were significantly lower than the immediate activation scenario, suggesting that for optimizing manufacturing timeline, immediate post-thaw activation of T cells may be more beneficial.