The Impact of feeding regimen and cell cycle on post-thaw recovery in a human T-cell model

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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. Most commonly, cryopreservation is employed at both the beginning (starting material) and end (product formulation) of the manufacturing process. As such, the efficiency and efficacy of the final therapeutic dose, as well as the manufacturing process, are directly affected by cryopreservation. However, post-thaw viability and expansion of many cell types dramatically vary, resulting in variable specifications and sometimes "out-of-spec" products. Hence, thorough understanding of the cryopreservation process and its parameters on the manufacturing process and product quality is vital for successful commercial manufacturing of cellular therapies. In a previous study, we observed significant loss of viability post-thaw in cells that had media exchange many days before harvest for formulation. As such, in this study, we investigated the impact of some of the culture process parameters (CPPs) on post-thaw viability and proliferation in a Jurkat T cell model, to assess criticality of those parameters in manufacturing process. In specific, we looked at feeding regimen, cell cycle, media volume, and air/liquid contact surface area. We further scrutinized the impact of cell cycle in follow up experiments, to dissociate the impact of feeding and cell cycle on post-thaw performance. Our results suggest that, feeding regimen was the single-most impactful parameter affecting the post-thaw performance of the cells that were cryopreserved otherwise identically using intracellular-like CryoStor freeze media. There was a correlation observed between the distance of the cells from air/liquid interface, which suggests further scrutiny of this parameter and is consistent with previous reports. Cell cycle in the Jurkat T cell model appeared to have no significant impact on the post-thaw recovery of Jurkat T cells. Based on this preliminary work, further studies into what components of the culture process can impact cryopreservation efficacy post-thaw are warranted.

METHODS

Cell Culture: The Jurkat (Clone E6-1) human acute T-cell leukemia (ATCC, VA) was cultured in complete growth medium (CGM) comprised of RPMI 1640 (Lonza, MD) supplemented with 10% v/v fetal bovine serum (FBS, Atlas Biologicals, CO).

Cryopreservation Media: CryoStor CS5, containing 5% DMSO, was used to cryopreserve all samples in this study.

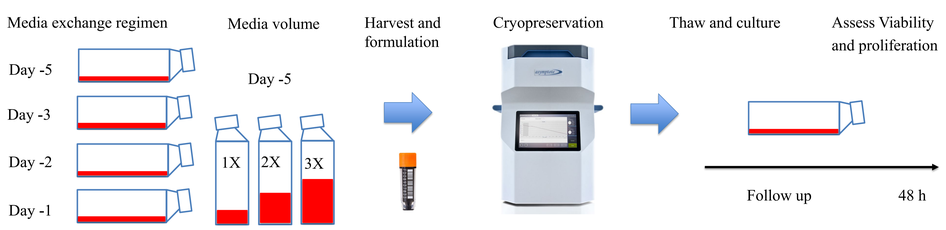
Impact of Cell Cycle: To assess the impact of cell cycle on the efficacy of cryopreservation process, cells were pharmacologically arrested at growth phase G0/G1 by incubation with 10 uM Ribociclib (Sigma, MO) for 24 h. The cells were incubated in 4 separate flasks with identical starting cell density ($5x10^5$/mL). The timing of incubation with Ribociclib was planned to have 3 distinct group representing 24 h, 10 h, and 0 h post-Ribocilib wash at the time of preparation for cryopreservation, each of which was assessed for cell cycle prior to freeze down. Cell cycle assessment was performed on NucleoCounter NC-3000 (ChemoMetec, Denmark) per manufacturer's protocol. In Brief, ~$1x10^6$ were removed from culture and washed once with PBS. After centrifugation and decanting, the cells were resuspended in 250uL of lysis buffer supplemented with DAPI (provided by ChemoMetec) and incubated for 15 min at 37°C. Then, 250uL of stabilizing media was added to the cell suspension, and 10 uL of the suspension was pipetted onto an A8 chip for assessment using cell cycle analysis protocol on NC-3000. All experimental groups were cryopreserved using the standard set of freezing parameters listed in Table below.

Impact of Feeding regimen: 7 groups of Jurkat cells were plated in 7 T25 flasks (Corning, NY), at $2.5x10^5$/flask. Flasks 1 to 4 contained 10 mL of fresh CGM. After plating, the flasks were placed in flat configuration inside the incubator. The media in Flasks 2, 3, and 4 were exchanged with fresh media on days 3, 4 and 5 after plating. The media in flask 1 remained unchanged until cryopreserved. Flasks 5, 6 and 7 contained same numbers of cells with 10, 20 and 30 mL of fresh CGM, and were placed inside the incubator in upright position. The media in flasks 5 to 7 also remained unchanged until removed for cryopreservation (Day 5 after plating).

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, Jurkat T cell pellets were suspended in CryoStor CS5 at a density of 1 M/mL, and then pipetted into 2 ml FluidX cryovials (Brooks Life Sciences, MA), and 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 and nucleated at -10°C. After reaching -70°C, the vials were transferred to LN_2 storage for a minimum of 24 h. Samples were thawed in a 37°C water bath, and were resuspended in CGM (1:10 dilution), and transferred to the incubator at 37°C and 5% CO_2.

Viability & Expansion: Cell viability and count was evaluated based on membrane integrity immediately and at 24 h and 48 h post-thaw, using VIA-1 cassettes on a NucleoCounter NC-3000 imaging cytometer. For cell cycle experiments, the cells were followed up for 48 h post-thaw.

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

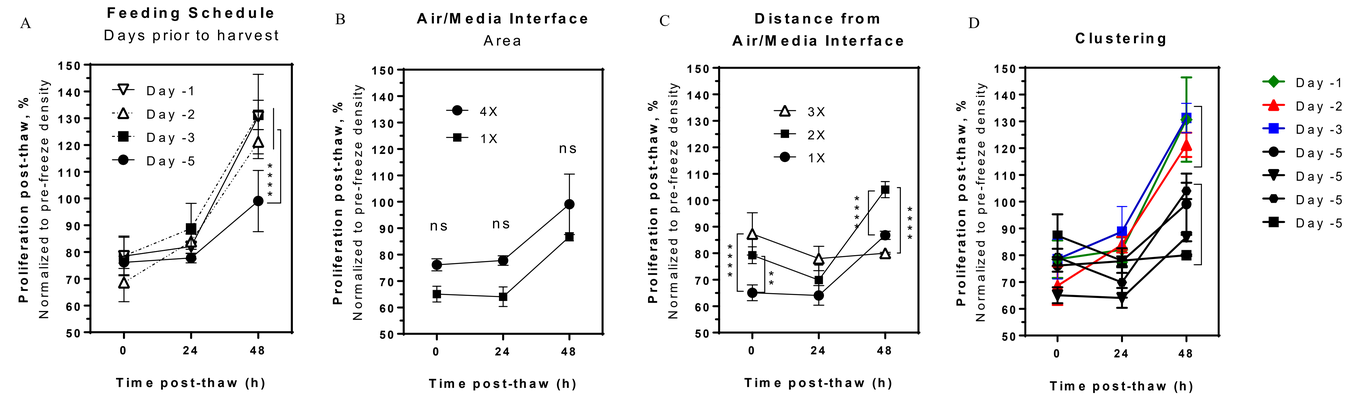


Figure 1: Post-thaw viable recovery and proliferation of Jurkat T cells, assessed at immediately, 24h, and 48h post-thaw. (A) Jurkat T-cells were plated at Day -5, and had media exchange either at Day -5, Day -3, Day -2, or Day -1 prior to harvest and cryo. All cells were cryopreserved in CryoStor CS5®. (B) Jurkat T-cells were plated in T-25 flasks and were placed in upright or flat position inside the incubator, with 4X surface area available for oxygen exchange in the flat group. (C) Jurkat T-cells were placed in T-25 flasks with 10 mL, 20 mL, and 30 mL CGM, resulting in 1X, 2X and 3X distance of the cells from the air/liquid interface. (D) All results clustered together and denoted only by their feeding regimen (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$). Error bars denote SD.

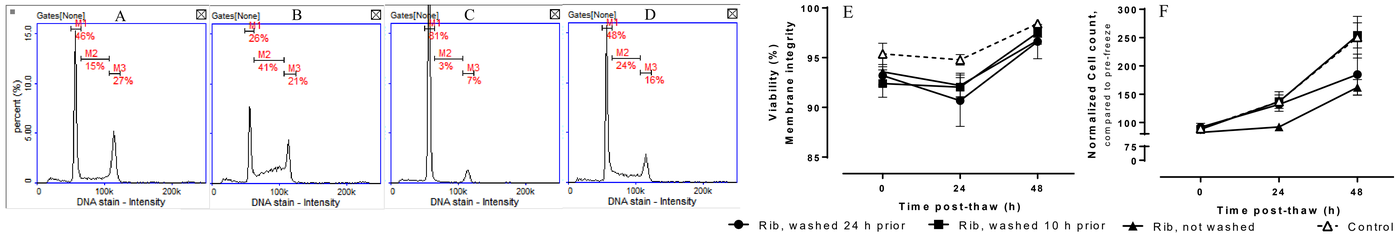


Figure 2: (Left) Cell cycle analysis for Jurkat T-cells incubated with Ribociclib for 24 h, then washed at 24 h (A), 10 h (B), or 0 h (C) prior to cryopreservation. Cell cycle analysis for culture control (D). **Viability (E), and normalized cell count (F)** for respective groups in A to D. The clear differences between the groups in terms of % of the cells in each G0/1, S, and G2/M phases did not amount to significant post-thaw differences in viability or proliferation of each group.

(1) Feeding regimen: We previously reported that Jurkat T-cells cultured without media exchange for an extended period of time displayed hampered functionality post-thaw as assessed by proliferation rate. In this study, we staggered a set of Jurkat T-cell culture with different feeding regimen prior to freeze to further investigate the previous observation. All Jurkat T-cells in this study appeared to be healthy according to the very high viability measurements assessed by membrane integrity prior to harvest. Nonetheless, the culture with the longest streak of starvation (media exchange at Day -5) appeared to suffer most after cryopreservation (Fig. 1A).The cultures with media exchange at least on Day -3 appeared to show no significant difference from fresh control. However, the air/liquid interface available for oxygen exchange appeared to have no significant impact on the post-thaw proliferation rate (Fig.1B). Increasing distance of the cells from the air/liquid interface was detrimental to proliferation rate up to 48h post-thaw, with those cells closest to air/liquid interface demonstrating faster proliferation rate post-thaw (Fig. 1C). When clustering all results together, it appears that the most pronounced CPP in this study was the timing of culture feeding prior to harvest and cryopreservation. Despite appearing as a healthy culture prior to cryopreservation (with 95+% viability), the 5-day old culture was significantly more susceptible to cryopreservation-induced stresses in terms of proliferation capacity post-thaw, regardless of the other culture conditions investigated in this study, i.e. volume of the media and distance from air/liquid interface.

(2) Cell cycle: By incubating with Ribociclib for sufficiently long periods (>24 h), it is possible to arrest the growth cycle of Jurkat T-cells at G0/G1 phase. Cultures incubated with Rib for less times exhibit different distributions of cell cycle (Fig. 2A-2D). Post-thaw analysis of Jurkat T-cells cryopreserved at different stages of growth showed that there was no significant difference among the groups, and suggest that cell cycle may not have a considerable effect on post-thaw viability, recovery, and proliferation rate of these cells. The observed difference between the proliferation rate of the culture that was arrested in G0/G1 phase right before cryopreservation (Fig. 2F, Rib, not washed) could be explained by the fact that those cells were at G0/G1 phase and took up to 24 hour to progress to G2/M phase and proliferate.

CONCLUSIONS

- In Jurkat T-cell model, feeding regimen and the timing of harvest for cryopreservation can have a significant impact on the outcome of cryopreservation process. As such, it is suggested that feeding regimen is considered as a critical process parameter for cryopreservation process development studies. Further investigation must be conducted for specific processes and cell types to validate these results in different cells.
- While analysis of the cell cycle in the 3-day fed culture suggested cell cycle distribution was shifted toward G0/G1 phase, further experiments suggested that cell cycle per se did not have a significant impact on viability and recovery of the cells post-thaw.
- These results also suggest that viability as assessed by membrane integrity is not a sufficient measure of cell/process health, either during culture or post-thaw, and therefore, cells must be scrutinized and characterized with other measures as well to ensure safety, potency, and efficacy of the process and the product.