Evaluation of ice recrystallization inhibitors

for preserving the post-thaw function of iPSC-derived natural killer cells

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Abstract

Human induced pluripotent stem cell (iPSC)-derived natural killer cells (iNKs) are promising candidates for scalable, allogeneic "off-the-shelf" cancer immunotherapies. However, effective cryopreservation is required to support their manufacture, storage, distribution, and clinical use, and freeze-thaw processes can adversely affect NK-cell recovery and function. In this study, we evaluated whether supplementation of CryoStor® CS10 with a proprietary small-molecule ice recrystallization inhibitor (IRI) influences the post-thaw properties of iNKs following storage in liquid nitrogen for 1, 3, and 5 months. IRIs are a class of cryoprotective compounds that inhibit the growth of larger ice crystals at the expense of smaller ice crystals during recrystallization and act through a mechanism distinct from conventional colligative cryoprotectants such as DMSO. Immediate post-thaw viability and recovery were evaluated descriptively, while cytotoxic function against MDA-MB-231 breast cancer cells was assessed in three independent experiments.

IRI supplementation did not result in an apparent detrimental effect on post-thaw viability or recovery. Although cryopreserved iNKs exhibited reduced cytotoxic activity compared with fresh, non-cryopreserved cells, iNKs cryopreserved with IRI-supplemented CS10 exhibited significantly greater cytotoxic activity than cells cryopreserved in CS10 alone after 3 and 5 months of storage. These preliminary findings suggest that supplementation of conventional cryopreservation media with IRIs may help preserve iNK cytotoxic function following extended cryogenic storage. Additional studies incorporating greater biological replication and broader functional endpoints will be needed to validate these findings.

Methods

At the end of iNK differentiation, 1 × 10^6 iNK cells were cryopreserved in either CryoStor® CS10 or CryoStor CS10 supplemented with a proprietary small-molecule ice recrystallization inhibitor (CS10 IRI).

Frozen vials were placed in a Thermo Scientific Mr. Frosty™ freezing container and stored at -80°C overnight. Vials were transferred to liquid nitrogen the following day and stored for 1, 3, or 5 months.

For thawing, frozen vials were placed in a 37°C water bath for 2–3 minutes and immediately transferred to 15 mL tubes containing 5 mL of iNK medium. Cells were centrifuged at 300 × g for 5 minutes, the supernatant was aspirated, and the cells were resuspended in 1 mL of iNK medium.

Cell recovery, viability, and cytotoxic function were assessed immediately after thawing without an intervening post-thaw recovery or culture period. Cell number was determined using a DeNovix CellDrop™ automated cell counter. Post-thaw recovery was calculated as the number of cells recovered after thawing relative to the number of cells frozen. Viability was determined by flow cytometry using an anti-CD56 antibody and ethidium homodimer.

For Figures 1 and 2, viability and recovery measurements represent a single independent cryopreservation experiment at each storage time point (n = 1). These data are therefore presented descriptively and were not subjected to statistical analysis.

Post-thaw cytotoxic function was assessed against MDA-MB-231 breast cancer cells at an effector-to-target ratio of 1:1. Fresh, non-cryopreserved iNK cells that had not been exposed to either CryoStor CS10 or CS10 IRI were included as a reference control. Cytotoxicity measurements were carried out in triplicate (n = 3). Statistical comparisons between the CryoStor CS10 and CS10 IRI cryopreservation conditions were performed using Welch's t-test, with $P \leq 0.05$ considered statistically significant.

Results

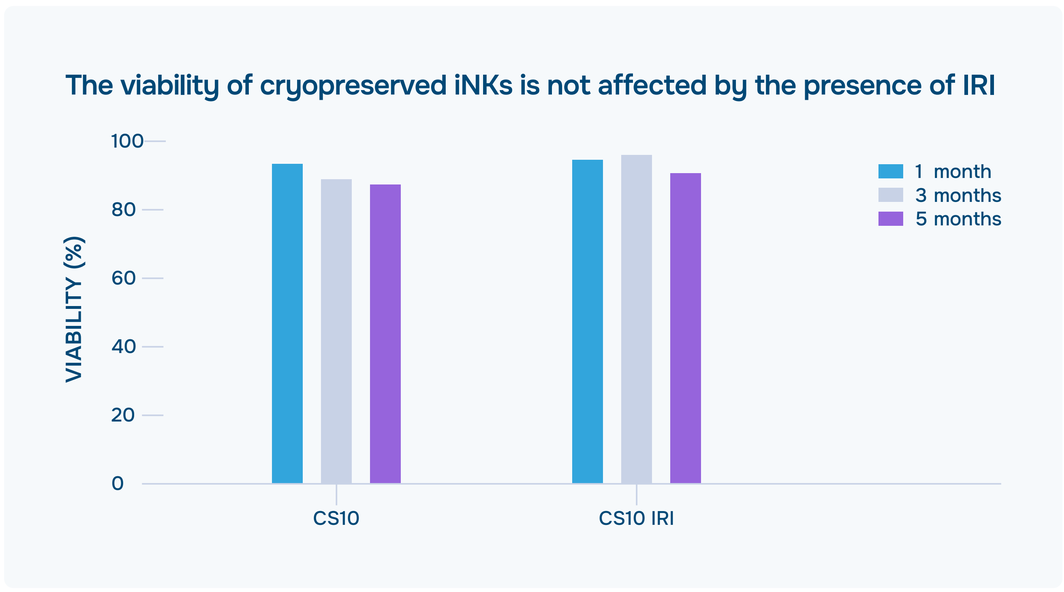

Figure 1. Percentage post-thaw viability of iNKs cryopreserved in CryoStor CS10 (CS10) or IRI-supplemented CryoStor CS10 (CS10 IRI) following 1, 3, and 5 months of cryogenic storage. Measurements were performed immediately after thawing. Data represent one independent experiment at each storage time point (n = 1) and are presented descriptively; no statistical analysis was performed.

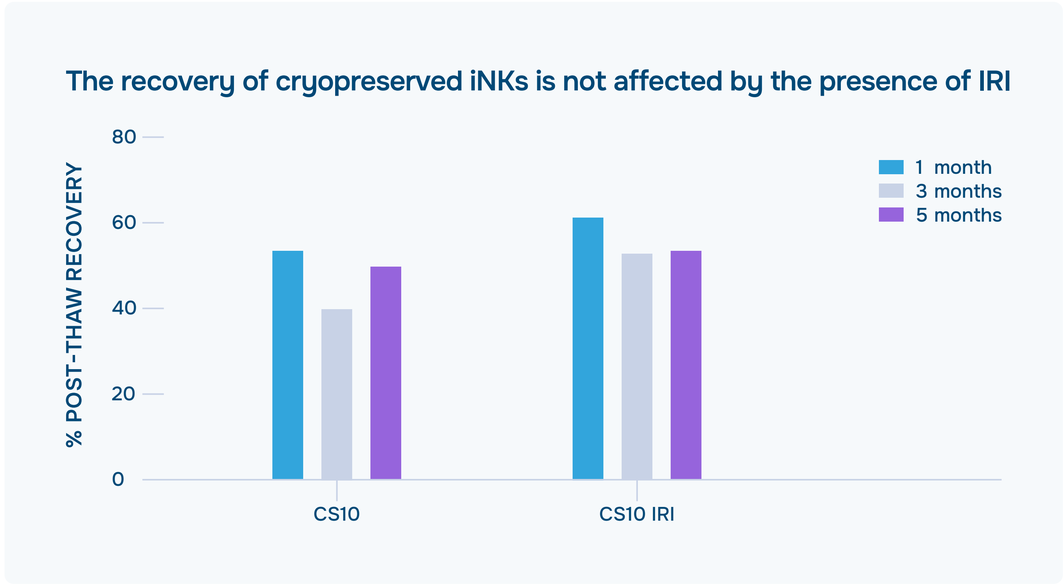

Figure 2. Percentage post-thaw recovery of iNKs cryopreserved in CryoStor CS10 (CS10) or IRI-supplemented CryoStor CS10 (CS10 IRI) following 1, 3, and 5 months of cryogenic storage. Recovery was calculated as the number of cells recovered immediately after thaw divided by the number of cells frozen. Data represent one independent experiment at each storage time point (n = 1) and are presented descriptively; no statistical analysis was performed.

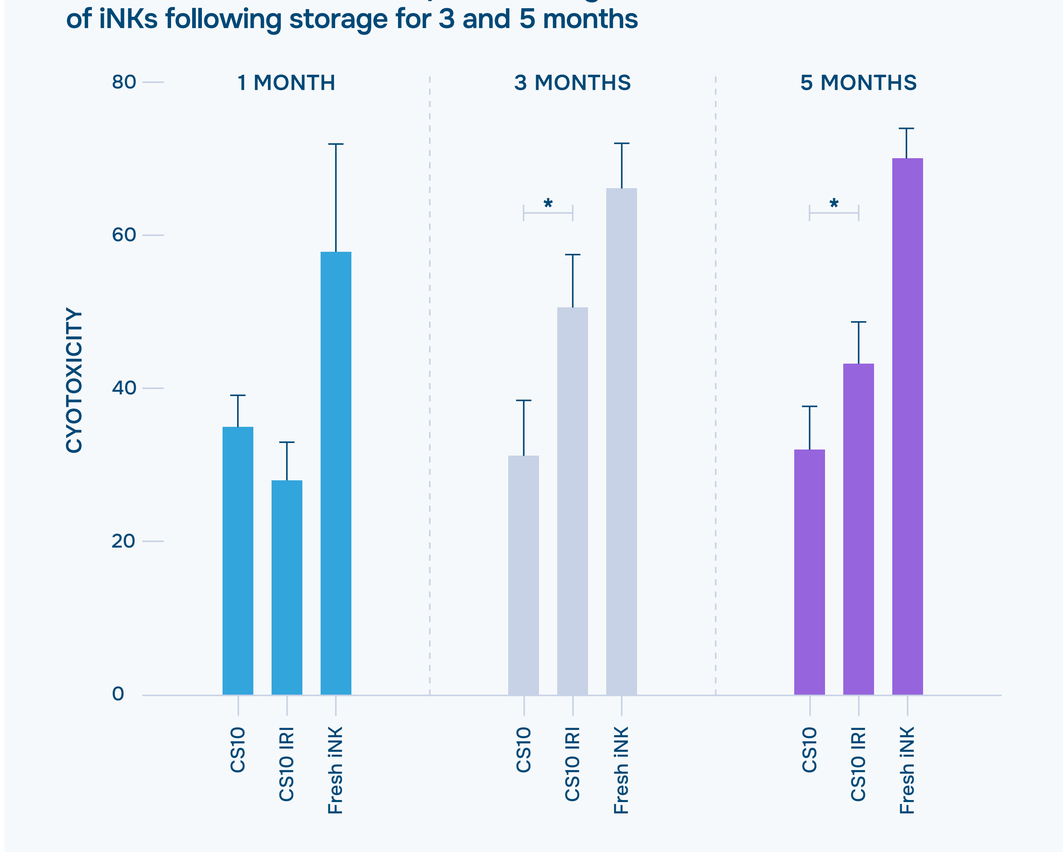

Figure 3. Mean percentage cytotoxicity against MDA-MB-231 breast cancer cells for fresh, non-cryopreserved iNK cells and iNK cells cryopreserved in CryoStor CS10 (CS10) or IRI-supplemented CryoStor CS10 (CS10 IRI). Cytotoxicity was evaluated immediately following thaw at an effector-to-target ratio of 1:1. Data represent three independent experiments (n = 3). Statistical comparisons between CS10 and CS10 IRI were performed using Welch's t-test. *$P \leq 0.05$.

Conclusions

The development of scalable, allogeneic iPSC-derived NK-cell therapies requires cryopreservation strategies that preserve not only cell survival but also functional potency following storage and thawing. **In this preliminary study, supplementation of CryoStor CS10 with a proprietary small-molecule IRI did not adversely affect immediate post-thaw viability or recovery and was associated with significantly greater cytotoxic activity against MDA-MB-231 breast cancer cells relative to CS10 alone after 3 and 5 months of cryogenic storage.**

These findings do not establish that IRI supplementation completely prevents cryopreservation-associated loss of iNK function, and the biological significance of the observed differences requires validation in larger studies incorporating additional functional endpoints. Nevertheless, the results support continued investigation of ice recrystallization inhibition as a complementary strategy for preserving the post-thaw functional properties of iPSC-derived NK cells and potentially improving the cryopreservation of off-the-shelf cellular therapies.