

INNOVATOR INSIGHT

Evaluation of ice recrystallization inhibitors for preserving the post-thaw function of iPSC-derived natural killer cells

Anna Jezierski and Robert N Ben

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. 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.

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Human induced pluripotent stem cells (iPSCs) provide a renewable and potentially standardized source of cells for regenerative medicine and cell-based therapies. Their capacity for extensive self-renewal and directed differentiation enables the manufacture of large

numbers of defined cell populations from a common starting material. Among these applications, differentiation of iPSCs into natural killer (NK) cells has emerged as a promising approach for developing scalable, allogeneic cancer immunotherapies. Unlike patient-specific autologous

therapies, iPSC-derived NK (iNK) cells have the potential to be manufactured in large batches, characterized in advance, cryogenically stored, and distributed as an ‘off-the-shelf’ therapeutic product [1–3].

NK cells are innate immune effector cells capable of recognizing and eliminating malignant and virus-infected cells without prior antigen sensitization. Their antitumor activity involves multiple mechanisms, including the release of cytotoxic granules containing perforin and granzymes that promote target-cell apoptosis. These properties, together with their potential for allogeneic administration and genetic engineering, have generated considerable interest in NK cells as platforms for cancer cell therapy [4].

The development of an off-the-shelf iNK product, however, requires effective cryopreservation. Manufacturing large batches of cells at a centralized facility provides limited benefit if cells cannot subsequently be stored and transported while maintaining adequate recovery and therapeutic function. Cryopreservation can adversely affect NK-cell viability, phenotype, migration, and cytotoxic activity; and the preservation of functional potency after thawing remains an important consideration in the development of NK-cell therapies [5]. Importantly, maintenance of cell viability following thaw does not necessarily indicate that cytotoxic function has been fully preserved [5,6].

Conventional cryopreservation strategies typically employ controlled cooling in the presence of cryoprotective agents (CPAs), such as dimethyl sulfoxide (DMSO). During slow cooling, extracellular ice formation increases the concentration of solutes in the remaining liquid phase and promotes cellular dehydration, thereby reducing the probability of lethal intracellular ice formation. However, cells remain susceptible to multiple forms of cryoinjury arising during cooling, storage, and warming.

One potentially important source of cryoinjury is ice recrystallization, during which

larger ice crystals grow at the expense of smaller crystals. Conventional CPAs, such as DMSO, provide important protection against freezing injury but do not specifically control ice recrystallization at concentrations normally employed for cellular cryopreservation [7–9]. Ice recrystallization inhibitors (IRIs) are compounds designed to suppress this process. Small-molecule IRIs have been developed as alternatives to naturally occurring antifreeze proteins and glycoproteins and have demonstrated cryoprotective activity in several biological systems [7–10].

Previous work demonstrated that supplementation of conventional cryopreservation media with small-molecule IRIs can improve post-thaw outcomes in iPSCs and iPSC-derived neurons. In particular, IRI supplementation enhanced post-thaw recovery of iPSC-derived neurons and accelerated recovery of neuronal network and synaptic function despite relatively modest effects on immediate post-thaw viability [10]. These observations suggest that post-thaw functional endpoints may reveal cryopreservation-associated effects that are not evident from viability measurements alone.

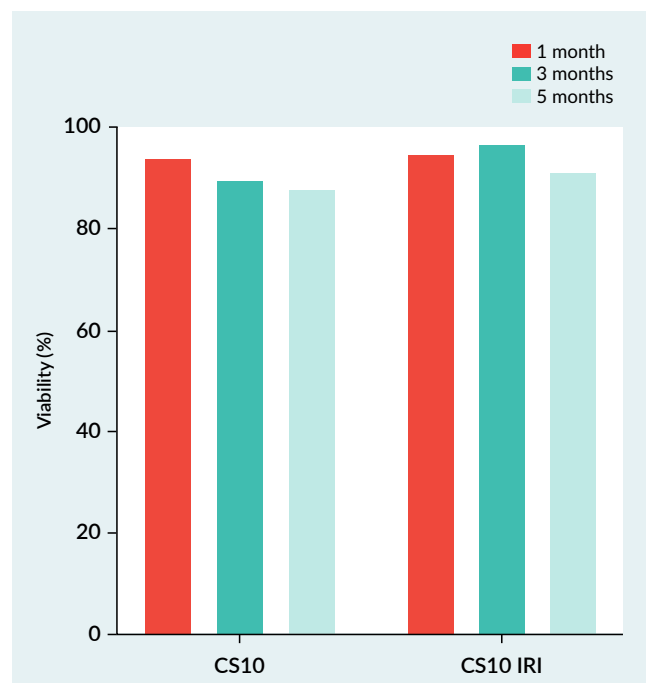
In the present study, we investigated whether supplementation of CryoStor® CS10 with a proprietary small-molecule IRI could improve post-thaw outcomes of iPSC-derived NK cells. Immediate post-thaw viability, recovery, and cytotoxic activity were evaluated following 1, 3, and 5 months of cryogenic storage. The primary objective was to determine whether IRI supplementation could help preserve post-thaw iNK function relative to the conventional CS10 formulation.

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). The chemical identity and concentration of the IRI are

► **FIGURE 1**

Viability of cryopreserved iNKs in the presence or absence of IRI.



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.

proprietary. 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 [7–10].

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 $300 \times g$ 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

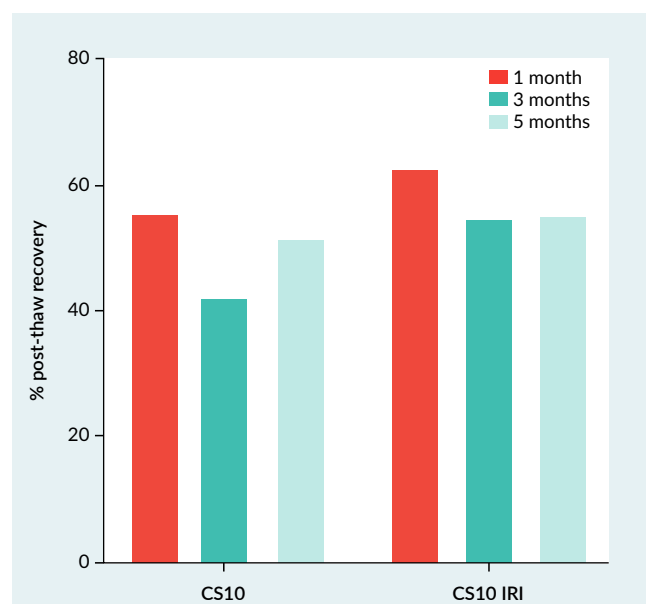
IRI supplementation does not adversely affect immediate post-thaw viability or recovery

The effects of IRI supplementation on immediate post-thaw viability and recovery were evaluated after 1, 3, and 5 months of cryogenic storage. iNK cells were cryopreserved in either CryoStor CS10 or CS10 supplemented with the proprietary IRI.

As shown in **Figure 1**, iNK cells can be cryopreserved and maintain substantial viability following cryopreservation in both CryoStor CS10 and CS10 IRI at each storage time examined. No apparent detrimental effect of IRI supplementation on

► FIGURE 2

Recovery of cryopreserved iNKs in the presence or absence of IRI.



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.

post-thaw viability was observed under the conditions evaluated.

Post-thaw recovery was also observed in both cryopreservation conditions at all storage time points (Figure 2). Although differences between the two formulations were observed, the absence of biological replication precludes conclusions regarding whether IRI supplementation improves recovery. Collectively, the viability and recovery measurements indicate that addition of the IRI did not result in an obvious detrimental effect on these immediate post-thaw parameters.

IRI supplementation is associated with greater post-thaw cytotoxic activity after extended storage

Because preservation of viability does not necessarily indicate preservation of

NK-cell function, cytotoxic activity was assessed against MDA-MB-231 breast cancer cells. Fresh, non-cryopreserved iNK cells were included as a reference for functional activity.

Cryopreservation reduced cytotoxic activity relative to fresh iNK cells, demonstrating that neither cryopreservation formulation completely prevented the functional effects associated with freezing and thawing. After 1 month of storage, there was no statistically significant difference in cytotoxicity between iNK cells cryopreserved in CS10 and CS10 IRI. In contrast, after 3 and 5 months of cryogenic storage, iNK cells cryopreserved in CS10 IRI exhibited significantly greater cytotoxic activity against MDA-MB-231 cells than cells cryopreserved in CS10 alone (Figure 3).

These results indicate that IRI supplementation did not restore cytotoxic activity to the level observed in fresh cells but was associated with greater immediate post-thaw cytotoxic function relative to conventional CS10 following the longer storage intervals evaluated.

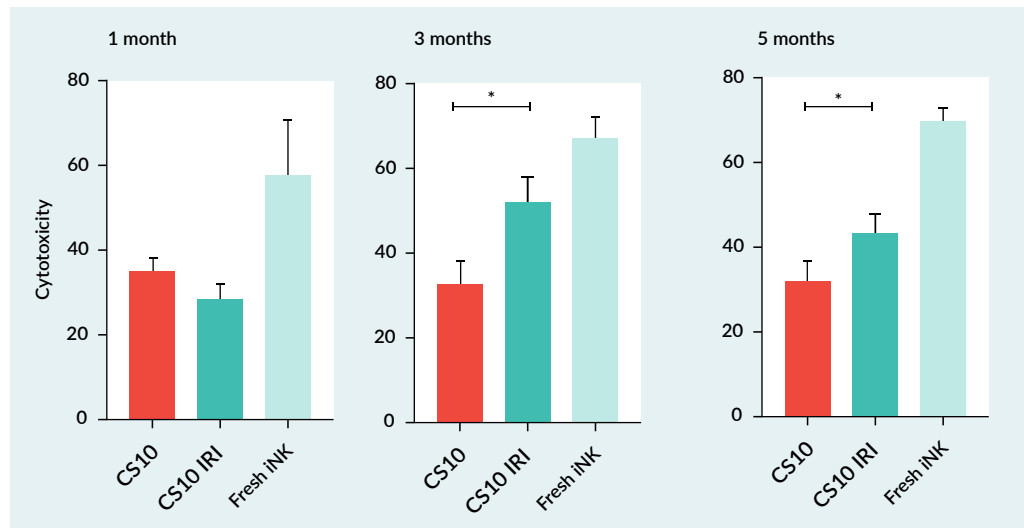
DISCUSSION

Effective cryopreservation is an important component of the manufacturing and distribution strategy for allogeneic cell therapies. For iPSC-derived NK cells, the ability to manufacture large, standardized batches and store them for subsequent administration is central to the proposed off-the-shelf model [1–4]. Consequently, preservation of functional potency following thaw is at least as important as achieving acceptable immediate post-thaw viability and recovery.

The present study provides preliminary evidence that supplementation of CryoStor CS10 with a small-molecule IRI may help preserve the immediate post-thaw cytotoxic function of iNK cells following extended cryogenic storage. Viability and recovery were broadly maintained in both

► **FIGURE 3**

Cytotoxic function of iNKs following cryopreservation in the presence or absence of IRI.



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 ≤ 0.05.

CS10 and CS10 IRI, and no obvious detrimental effect of IRI supplementation was observed. However, because these endpoints were evaluated in a single independent experiment at each storage time, the data should be interpreted descriptively and do not establish an improvement in viability or recovery.

A different pattern was observed for functional activity. Cytotoxicity experiments performed in three independent experiments demonstrated significantly greater activity against MDA-MB-231 breast cancer cells in the CS10 IRI condition relative to CS10 alone after 3 and 5 months of storage. This observation may be relevant because cryopreservation-associated impairment of NK-cell function can occur even when immediate post-thaw viability remains comparatively well preserved [5,6]. Thus, viability and recovery measurements alone may not fully describe the effect of cryopreservation on the functional quality of an NK-cell product.

The findings are also consistent with previous studies of IRIs in other biological systems. Small-molecule IRIs have been shown to reduce cryoinjury associated with uncontrolled ice recrystallization, and previous work with iPSC-derived neurons demonstrated that IRI supplementation could improve functional recovery without necessarily producing correspondingly large changes in immediate post-thaw viability [7-10]. Together, these observations support further investigation of ice recrystallization as a target for improving the functional cryopreservation of differentiated cell products.

Several limitations of the present study should be emphasized. First, viability and recovery measurements were obtained from a single independent experiment at each storage interval and therefore cannot establish statistically significant differences between formulations. Second, post-thaw function was evaluated using a single functional endpoint – cytotoxicity

against the MDA-MB-231 breast cancer cell line. Although this assay provides a direct measure of an important NK-cell effector function, it does not establish preservation of the broader functional phenotype of the cells. Future studies should include additional biological replicates and functional endpoints such as cytotoxicity against additional target cell lines, degranulation, cytokine production, expression of relevant NK-cell surface markers, and assessment of perforin and granzyme-mediated effector function.

Importantly, IRI supplementation did not restore cytotoxicity to the level observed in fresh, non-cryopreserved cells. The present results should therefore be interpreted as preliminary evidence that IRI supplementation may partially preserve post-thaw cytotoxic function rather than as evidence that the effects of cryopreservation have been eliminated.

Further work involving larger datasets, additional iNK preparations, broader functional characterization, and clinically relevant manufacturing and storage conditions will be required to determine whether IRI supplementation provides a reproducible and therapeutically meaningful advantage for iNK cryopreservation.

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.

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AUTHORSHIP & CONFLICT OF INTEREST

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