THESIS

# VALIDATING THE EFFICACY OF NOVEL CRYOPRESERVATION SOLUTIONS AND TECHNIQUES FOR APPLICATIONS IN CELL THERAPY

Submitted by

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# ABSTRACT


## VALIDATING THE EFFICACY OF NOVEL CRYOPRESERVATION SOLUTIONS AND TECHNIQUES FOR APPLICATIONS IN CELL THERAPY

This study evaluates the efficacy of various cryopreservation solutions in maintaining post-thaw viability and functionality of cell types commonly used in cell therapy. Additionally, it explores the potential benefits of incorporating ADAM-17 inhibitors for maintaining primary human natural killer cell function after cryopreservation and an alternative freezing technique, vitrification, on the survival and function of primary human T cells. Human pluripotent stem cells (hPSCs), primary T cells, and primary natural killer (NK) cells were cryopreserved using both commercially available and novel cryoprotective solutions. Post-thaw assessments included evaluations of short- and long-term viability, phenotypic stability, and functional capacity, tailored to each cell type. Novel cryoprotectants, including CaseCryo DMSO and CaseCryo NON-DMSO provided by CaseBioscience met or exceeded the performance of commercial solutions across these metrics. hPSCs showed the highest long term survival when cryopreserved in CaseCryo DMSO compared to other solutions with a maintenance of normal physiology as shown by differentiation assays. T cells showed similar rates of survival and surface marker expression when cryopreserved in CaseCryo DMSO and CaseCryo NON-DMSO as compared to the currently available industry standard solution, however they showed slightly improved visible morphology after exposure to both the CaseCryo solutions compared to the control. NK cells similarly showed no significant difference in expansion rates and surface marker expression, however donor to donor variability seemed to effect these experiments to a greater

degree than the others. Among the ADAM-17 inhibitors tested, GW280264X was most effective in preserving CD16 expression in cryopreserved NK cells. Furthermore, vitrification of T cells emerged as a promising alternative to conventional passive freezing, offering potential advantages in cost and processing time for cell therapy manufacturing.

# ACKNOWLEDGEMENTS

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**<3**

TABLE OF CONTENTS

# INTRODUCTION

As an increased number of cell therapies become FDA approved, more widely available, and more commonly prescribed in the modern medical field, the need for effective storage of these treatments becomes more critical. Cryopreservation, the process of storing cells at temperatures well below freezing, is the most effective method of preserving therapies during the necessary transportation and storage between manufacturing and patient administration. These cells are expected to maintain a consistent and high post thaw viability and functionality to ensure proper medical use for the intended indications, as variations in either can impact the efficiency and efficacy of the therapy. The solutions used during the cryopreservation process to protect the cells in these harsh conditions not only affect the cells themselves, but also the patients as the therapy is often directly injected without a wash step and can have broader physiological effects. Thus, it is imperative the solutions used are effective in maintaining the health and activity of the cells while frozen as well as being formulated with the health and safety of the patient in mind.

CaseBioscience has formulated two novel solutions they believe promote better cell health and functionality post thaw as well as being formulated with ingredients generally regarded as “safer” than standard cryoprotectants. This body of work seeks to evaluate these claims as well as to propose additional additives and alternative methods to further enhance the quality of these CaseCryo solutions and cryopreservation workflow as a whole. Three types of human cells commonly used in cell therapy (Pluripotent Stem Cells (PSCs), T- cells, and Natural Killer cells) were chosen to be frozen down using the CaseCryo solutions along with commercially available counterparts serving as a baseline control.

Among the most publicly recognized applications of cell therapy is the use of stem cells in regenerative medicine. Human stem cells, induced pluripotent stem cells (iPSCs) in particular, are establishing themselves as foundational to contemporary biomedical research. They are incredibly versatile, not only being heavily utilized as emerging cell therapies but additionally playing essential roles in studying developmental processes, for disease modeling, and in drug development[1]. As of mid 2024, nearly 60 different iPSC derived therapies are currently in trial, with the number of new trials increasing each year[2].

T cells and NK cells are the most common cells used in immunotherapy (the treatment of cancer using either the patient's own or donor immune cells)[3]. With the evolution of genetic engineering immunotherapy research has increased exponentially; since the first FDA approved immunotherapy using CAR-T cells was approved in 2017, over several thousand clinical trials using T cells and NK cells have begun worldwide. Using these systems, along with pluripotent stem cells, gives real world weight to the following experiments, as the objective of this body of work is to assess the quality of the solutions developed by CaseBioscience as cryoprotective solutions for cell therapy applications.

## BACKGROUND

### *Cell Therapy*

While blood transfusions and hematopoietic stem cell (bone marrow) transplantations have a long history, new advances in genetic engineering and stem cells have led to a recent re-emergence of cell therapy as a powerful medicinal practice for the treatment or prevention of disease. Pioneered after the regenerative properties of stem cells and the exploitability of immune cell's natural functions were discovered, the field has broadened to include a wide variety of treatments and cell types and continues to grow exponentially[4]. Cell therapy is generally divided into two principal categories: regenerative medicine, which focuses on tissue repair and functional restoration, and immunotherapy, which targets the immune system to combat diseases such as cancer (Figure 1). Both categories are comprised of therapies based in similar cell types. The former is dominated by stem cells (both embryonic and induced pluripotent) as well as fibroblasts, keratinocytes, chondrocytes and other somatic cell types recognized for their capacity to promote tissue repair and regeneration. For example, pluripotent stem cells have been successfully differentiated into dopaminergic neurons for the treatment of Parkinson's disease[5], ventricular cardiomyocytes for heart failure[6], and pancreatic islet cells for type 1 diabetes[7]. In each case, the introduction of exogenous cells is intended to promote the regeneration of functional tissue, addressing diseases characterized by the loss or dysfunction of specific cell populations. Moreover, the self-renewing properties of progenitor cells facilitate scalable and sustainable production of these therapies over time[4]. Immune cell therapies have been developed mainly for the treatment of cancer using genetically modified T-Cells cells over the last couple of decades. Although these therapies have shown clinical success

and great promise for future development, challenges remain with immune cell based therapies as discussed below.

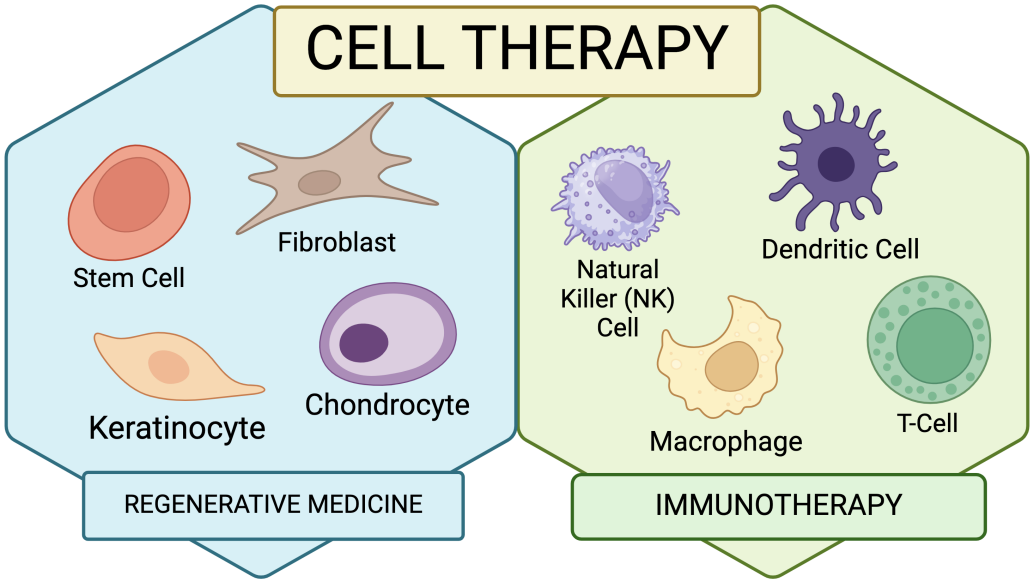


***Figure 1. Overview of Cell Therapy***

Cell therapy can be divided into two distinct factions based on use and cell type. Regenerative medicine (left) focuses on tissue repair and functional restoration and is often derived from stem cells, fibroblasts, keratinocytes, and chondrocytes. Immunotherapy (right) largely utilizes the immune system to combat malignancies and is based on the natural function of T cells, NK cells, dendritic cells, and macrophages.

### *Immunotherapy*

Immunotherapy encompasses treatments that leverage the natural functions of immune cells. These approaches involved utilizing either the patient's own immune system or externally derived immune cells to target and eliminate pathological cells, most commonly in the context of cancer. Immunotherapeutic strategies include the administration of small molecules, such as checkpoint inhibitors, which activate the patient's endogenous immune response[8], as well as the use of engineered immune cells,

such as chimeric antigen receptor T-cell (CAR-T) and chimeric antigen receptor natural killer (CAR-NK) cell therapies.

CAR based immunotherapies constitute the largest portion of the immunotherapy field with over 700 CAR-T therapies in trial as of 2022[9] and a growing number of CAR-NK therapies in development. These approaches involve the genetic modification of T cells or NK cells to express CARs that are specifically designed to enhance immune recognition and cytotoxic activity against target cells. CARs are synthetic receptors that combine an extracellular antigen receptor domain, typically derived from an antibody, with intercellular co-stimulatory and signaling domains. These engineered receptors enable immune cells to bypass conventional stimulatory pathways and cellular checkpoints, allowing for a more rapid and targeted response to malignant cells[4]. For example B cells whose malignancies are implicated in lymphomas and leukemias, are known to express high levels of the surface marker of CD19. CARs incorporating an anti-CD19 binding domain have demonstrated substantial clinical success in the treatment of B cell malignancies and autoimmune diseases[10]. As a whole, CAR-based therapies have demonstrated efficacy against hematologic cancers, which are often resistant to conventional treatments.

T Cells were initially the primary platform for CAR based immunotherapies; however the field is increasingly exploring NK cells as an alternative. While highly effective, CAR-T therapies are often associated with significant negative side effects, including cytokine release syndrome (CRS) and graft vs host disease (GvHD). In contrast, CAR-NK therapies have shown a notably lower incidence of these complications[11]. Additionally, CAR-T therapies have generally been manufactured in an autologous fashion; using cells derived

from the individual patient. CAR-NKs can be produced allogenically using donor cells offering advantages in scalability, cost-effectiveness, and broader patient accessibility.

*Autologous vs Allogenic*

Cell therapies, regardless of purpose or cell type, are generally classified into two categories: autologous and allogenic. These categories reference the origin of the cells used in manufacturing the therapy. Autologous therapies are derived from cells taken from the patient the treatment is intended for, whereas allogenic therapies are generated from cells taken from a healthy donor (Figure 2). Each approach offers distinct advantages and limitations, and either method can be more common depending on the disease being treated and the specific therapeutic modality[12].

Being derived from the patient's own cells, autologous therapies have a lower likelihood of eliciting an immune response or rejection of the therapy by the patient. Moreover, they can be tailored to the specific genetic and pathological context of the individual patient, allowing for a personalized treatment approach. However, these therapies typically involve complex, patient-specific manufacturing processes that are time-intensive and costly. The logistical burden of isolating, engineering, and re-administering cells for each individual also limits their scalability and rapid deployment in urgent clinical settings[13].

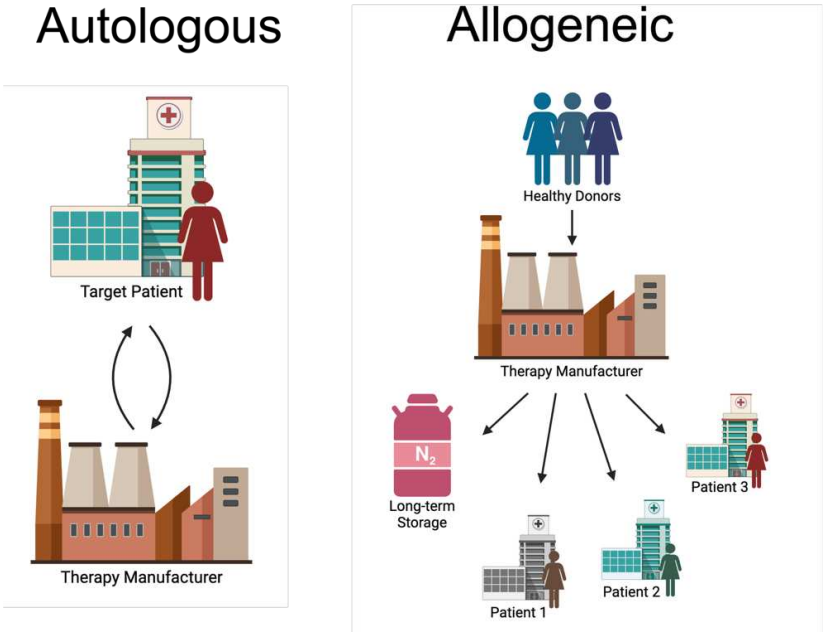


*Figure 2. Autologous Versus Allogeneic Cell Therapy Manufacturing Workflows*

Autologous (left) workflows follow a personalized path where cells are taken directly from the patient they are intended for, engineered for treatment, and administered back into their rightful owner. Cryopreservation occurs often during the transport of cells between the site of collection/administration and the site of manufacturing, as well as during storage at either location to adhere to timing of treatments (for example, CAR-T therapy is often administered in 3 separate doses over a period of weeks). Allogeneic (right) workflows sources cells from healthy donors that are then engineered and administered to patients. Cryopreservation occurs when transporting starting material from the site of collection to the site of engineering as well as transportation from the site of engineering to the site of administration. Allogeneic therapies are often cryopreserved for longer periods of time to fit the "off the shelf" model of preparing large batches ahead of time to be ready for injection when as needed.

In contrast, allogeneic therapies are produced from donor-derived cells and can be manufactured at larger scales, reducing both production costs and time to treatment. These therapies are well suited to the "off the shelf" model the field is exploring: premade cellular products that are cryopreserved and readily available for immediate clinical use. Being premade in larger batches enables more rigorous and standardized quality control and testing, potentially improving the overall safety, consistency, and efficacy of the therapeutic[14]. However, patients treated with non-self cells have an increased risk of immune complications, such as graft vs host disease (GvHD). This often necessitates the

administration of immunosuppressant agents during treatment, leaving patients more susceptible to opportunistic infections and other complications.

NK cells are being actively explored in multiple clinical trials as an allogenic immunotherapy as they are associated with fewer immune complications than T cell transplantation[15]. This is due in part to NK transplantation not requiring human leukocyte antigen (HLA) compatibility. HLAs, also known as the major histocompatibility complex (MHC) can be separated into 3 classes and their expression helps the body to reject foreign tissues[16]. MHC Class I expressed on the surface of nearly all nucleated cells and are able to bind endogenously derived antigens, whereas MCH Class II expressed only on a subset of immune cells that includes T cells but does not include NK cells and are able to bind exogenously derived antigens. Because of this, NK cells are able to recognize the loss of MHC Class I molecules ("missing self") seen often in malignancies and virally infected cells, but are unable to recognize and exert cytotoxicity against cells presenting MHC Class II molecules (identification as "other")[16]. This greatly decreases the risk of immune complications in NK derived allogenic therapies as opposed to T cell derived allogenic therapies; implanted T cells may identify their host as "other" and mount an attack on cells outside the specified targets if pre-treatment HLA matching is inadequate.

*Cryopreservation in Cell Therapy*

Cryopreservation, the process of preserving biological samples at ultra-low temperatures, is a critical component in the both the autologous and allogenic cell therapy manufacturing workflow[17]. It is employed during both transportation and in short- and long-

term storage. Cells may undergo several freeze/thaw cycles between the initial harvesting from the donor and final administration to the patient. Thus, robust and reliable cryopreservation techniques are imperative for ensuring the commercial viability, clinical efficacy, and overall success of cell-based therapies[17].

Currently, cell therapies are cryopreserved using either a custom formulated ("homebrewed") cryosolution[18], or a singular commercially available cryosolution that is generally accepted in the field for use in cell therapies[19]. In nearly all formulations, the main cryopreservation agent utilized is dimethyl sulfoxide (DMSO). DMSO, a highly penetrative, hygroscopic compound, protects cells during the cryopreservation process by dissuading intercellular ice crystal formation and isolating ice nucleation points. This preserves the integrity of both the outer cell membrane as well as sub compartments and organelles within the cells themselves. Despite its protective properties, DMSO is also linked to negative impacts on both cell viability and post-thaw functionality[20].

Following manufacturing and cryostorage, cell therapies are transported frozen to the clinical administration site and thawed shortly before being directly infused into the patient[19,18]. As a result, the cryosolution in which the cells are suspended is also infused directly into the patient. Although the volume administered is generally small, often limited to 5-10% of the total infusion volume, even low concentrations of DMSO have been linked to adverse clinical effects including cardiovascular, hepatic, and gastrointestinal symptoms[21]. Additionally, DMSO exposure can compromise the therapeutic cells themselves, potentially diminishing their efficacy or persistence post-infusion[17]. While dilution or removal of the cryoprotective solution is recommended by manufacturers, such

practices are often impractical in clinical settings, where time constraints and sterility requirements limit the feasibility of post-thaw manipulation[17]. These challenges underscore the need for the development of alternative, DMSO-free cryopreservation solutions that can maintain cell integrity without compromising safety or clinical workflow.

*Cryopreservation*

Cryopreservation is the process of preserving biological samples at extremely low temperatures, typically in the range of –80°C to –196°C. While the precise duration for which cells can be stored under these conditions remains a topic of debate, it is widely accepted that, when properly performed, cryopreservation can maintain cellular integrity anywhere from weeks to decades[22]. Cell therapies, particularly allogeneic products, rely heavily on cryopreservation for both transportation and short- and long-term storage.

The primary challenge in effective cryopreservation is mitigating damage caused by intracellular ice formation (Figure 3). Ice forming in the cytoplasm of a cell is considered cytotoxic; there is potential for membrane disruption of both intracellular compartments and the outer cell membrane itself as well as damage to the cell's DNA[23]. Intracellular ice formation is commonly addressed by adding cryoprotective agents (CPAs) such as desiccants to the cryopreservation solution or by increasing the cryopreservation solution's osmolarity higher than the cell's internal environment; both of these approaches encourage the outward flow of water from the cell, reducing the volume of intracellular water available to form damaging ice crystals[24]. However, excessive dehydration can itself be detrimental to

cell viability and function. Thus, it is critical to balance water efflux sufficiently to prevent ice formation without inducing cytotoxic levels of dehydration[24].

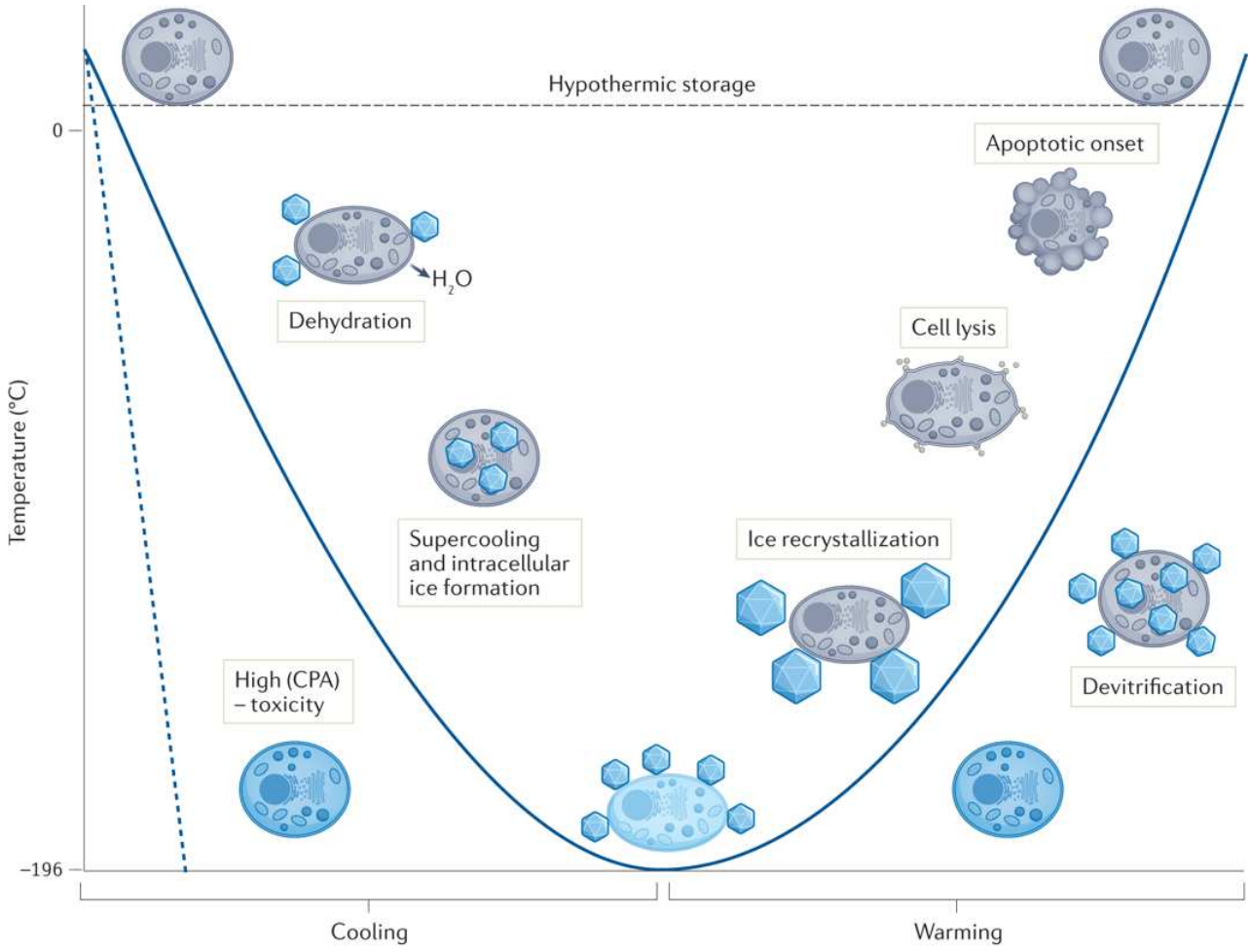


*Figure 3. Potential mechanisms of cellular damage during cryopreservation*

Several challenges occur when cells are cooled for cryogenic storage, as well during rewarming. During cooling, the formation of extracellular ice crystals leads to an osmotic imbalance across the cell membrane and subsequent cell dehydration. In the absence of extracellular ice, samples might supercool below the equilibrium freezing point, leading to an increased chance of fatal intracellular ice formation. Fast cooling rates and the addition of high concentrations of cryoprotective agents (CPA) can achieve vitrification, an amorphous, ice-free state. However, high concentrations of CPA can be toxic to cells. During warming, ice recrystallization can occur, where ice crystals grow and cause mechanical damage and cell lysis. Vitrified samples may become unstable and devitrify, leading to further ice growth. Finally, cryopreservation can induce apoptosis, leading to delayed cell death post-thaw. Note that these are all extremes, do not necessarily occur simultaneously and can be partially mitigated by the addition of cryoprotective agents. Ice crystals are not to drawn to scale for illustrative purposes. Reprinted from "Chemical approaches to cryopreservation" by K.A. Murray, *Nat Rev Chem., Volume 6(8),* Pages 579-593, Copyright 2022 by Springer Nature Limited[25]

Timing, of course, also plays a crucial role in this process. Cells exposed for prolonged periods to low concentrations of cryoprotective agents may experience similar damage as cells exposed for shorter durations to highly concentrated solutions. The field has largely adopted the "slow cooling" method in an attempt to both standardize and optimize this balance between solution potency and exposure time[26] (Figure 4). Slow cooling involves introducing cryoprotective solution to cells at or just below room temperature, and lowering the temperature in a controlled manner (typically decreasing by 1°C per minute) until the solution falls below the range that ice formation occurs (around -130°C)[27]. This can be done using a control rate freezer, which changes the internal temperature of the freezer (and by extension, the sample temperature) at a programmable rate, or using an insulating device around the samples before placing them in a standard -80°C freezer[26].

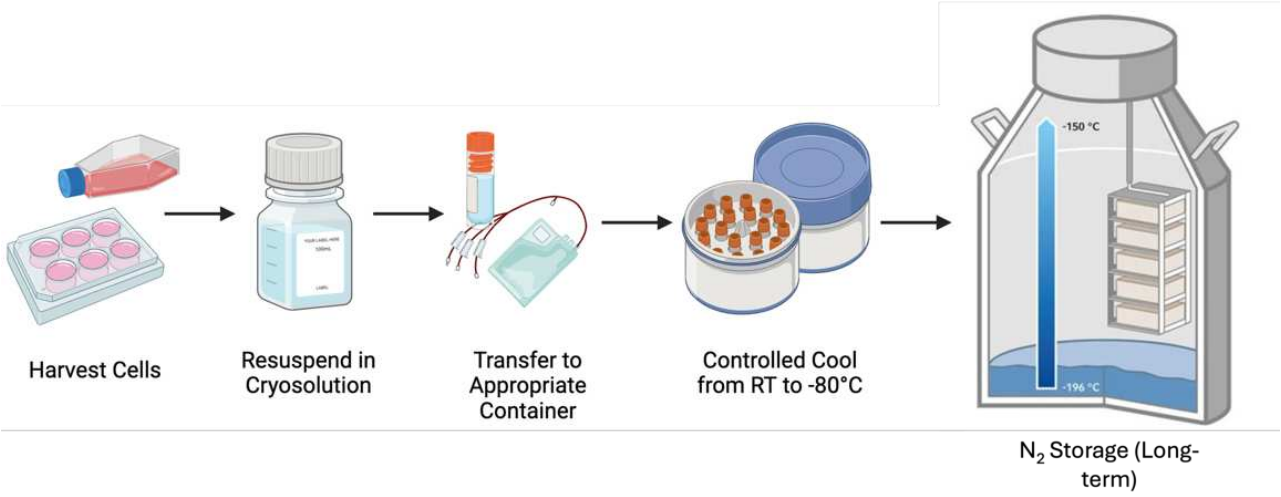


*Figure 4. Standard Slow Cooling Cryopreservation Workflow*

The slow cooling workflow beings with harvesting cells (either from culture as pictured or from a donor), removing the fluid or medium they are suspended in, resuspending them in a cryopreservation solution, transferrin them to an appropriate container (often 2mL cryovials for small scale projects and bags up to 5L for large scale manufacturing), then decreasing the temperature of the sample at a rate of -1°C/min. This controlled rate is achieved either by insulating the samples with ethanol or a specific solid substance (as pictured) or by placing them in a programmable freezer that changes internal temperature according to specifications. When the samples reach -80°C they are moved into either vapor or liquid phase nitrogen (-150°C to -196°C) for long term storage.

*Vitrification*

Vitrification, sometimes referred to as “fast freezing” or “rapid cooling”, is an alternative technique to slow cooling. While only recently employed in the cell therapy space, this method has been heavily utilized in the assisted reproductive technology (ART) field for over 30 years[28]. In an ART context, sperm, embryos, and oocytes are often cryopreserved for storage and/or further testing. In an effort to save time and funds, ART clinics will vitrify these biological samples by placing the device containing these cells directly into liquid phase nitrogen to achieve the ultra-fast cooling rates exceeding -2,000°C/min[29]. This technique is an attempt to completely bypass the ice crystal formation phase and force the intracellular water directly into its glass state (a non-crystalline solid phase achieved by an extraordinarily rapid decrease in temperature)[27], eliminating any chance of damage caused by intracellular ice formation (Figure 5).

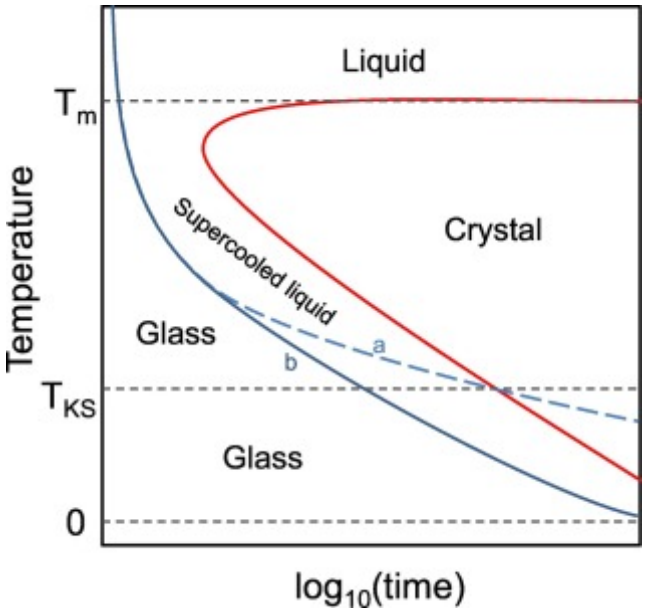


***Figure 5. Phase Diagram Displaying the Glass State of Solid Matter***

Note, failure to decrease temperature at a fast enough rate can allow the crystal form of water to form. Adapted from “The glassy state of matter: Its definition and ultimate fate” by Edgar D. Zanotto, 2017, *Journal of Non-Crystalline Solids, Volume 471*, Pages 490-495. Copyright 2017 by Elsevier B.V[30]

The cryopreservation solutions used in vitrification have some key differences than the ones used during standard slow cooling. While often containing a similar base formulation, solutions used in vitrification usually contain a higher amount of cryoprotective agent (ie, DMSO) than slow cooling cryopreservation solutions: the concentration of cryoprotectant is generally around 40% v/v for vitrification versus the 5%-10% v/v concentration used for slow cooling[28]. Due to the higher cryopreservation agent concentrations, vitrification solutions are considered more cytotoxic and are handled under different protocols, such as "stepping in" the product by exposing it to increasingly more concentrated solutions from its base media to the vitrification solution in order to reduce osmotic shock as well as reduce the amount of time the sample is in contact with the highest concentration of cryoprotectants.

*Negative effects on cell health and functionality post thaw*

The process of cryopreservation is known to have many adverse effects on living cells. Decreased viability post thaw is often seen due to injury to the cells via osmotic imbalances, intracellular ice formation, and cell dehydration[17]. Additionally, changes in structure and function have been visually noted in cells immediately after thawing[20]. Long term effects may also persist, such as alterations in histone-DNA binding patterns[31], inappropriate chromosomal alterations[32], changes in plasma membrane fluidity that affects transport systems, changes in microtubule assembly that affects cell adhesion and the cytoskeleton as a whole, and delayed activation of apoptotic pathways[20].

In addition to the extreme temperature changes, exposure to ingredients in the cryopreservation solutions themselves has been shown to negatively affect a wide array of cell types. Exposure to DMSO, which comprises 5%-10% v/v of most cryopreservation solutions, has been implicated in a variety of short- and long-term detrimental effects to therapies as well as the patients being treated. Cells treated with a 0.1% DMSO solution (ten times more dilute than the industry standard cryopreservation solution for use in cell therapy) have exhibited significant changes in their epigenetic landscape including differential expression of over 2000 genes, changes to DNA methylation, and a dysregulation of microRNAs pertinent to a variety of biological functions[33]. DMSO exposure has also been implicated in the spontaneous differentiation of cells, a particular hinderance to stem cell based therapies[34].

On a more macroscale, during various clinical trials for CAR-T based therapies, 30%-60% of patients injected with cell therapies in a DMSO based cryopreservation solution experience negative effects such as fatigue, nausea, fever, hyper/hypotension, edema, musculoskeletal pain, and encephalopathy[18]. Furthermore, roughly 30% of patients injected with cell therapies in a DMSO based cryopreservation solution experience what are considered “serious adverse reactions”: gastrointestinal hemorrhage, muscular weakness, pulmonary embolism, and sepsis[19]. Other adverse effects, such as anaphylaxis, have also been noted in patients exposed to DMSO[18]. These effects are seen in patients across many different cell therapy types, pointing to the DMSO rather than the injected therapy themselves as the culprit.

*Cryopreservation induced phenotypic change of Natural Killer cells*

Along with the broad negative effects of cryopreservation on cells, often specific cell types will face specific challenges with being cryopreserved. One of the main cell types utilized in this body of work are Natural Killer (NK) cells, known for their ability to identify and eliminate infected or cancerous cells without prior sensitization to specific antigens[35]. Using an activating surface receptor Fcγ receptor III (CD16) to bind to IgG antibodies attached to target cells, NK cells are able to form a transient immune synapse and release cytotoxic granules to terminate the malignancy[36].

Broadly, NK cells can be divided into two distinct phenotypes based on a separate surface neural cell adhesion molecule (NCAM/CD56) expression: $CD56^{bright}$ and $CD56^{dim}$. CD56 expression is generally inversely correlated with CD16 expression with the $CD56^{bright}$ subpopulation exhibiting low levels of CD16 whereas the $CD56^{dim}$ subpopulation exhibits higher levels of CD16. These subpopulations can also be described as active ($CD56^{dim}/CD16^{++}$) or immature ($CD56^{bright}/CD16^{neg}$) with active NK cells being the prominent arbitrators of killing infected and malignant target cells[37] (Figure 6). NK cells are able to reversibly switch between these states in response to external stimuli[38]. High CD16 expression by a population of NK cells is necessary for both natural and antibody dependent killing to be efficiently carried out.

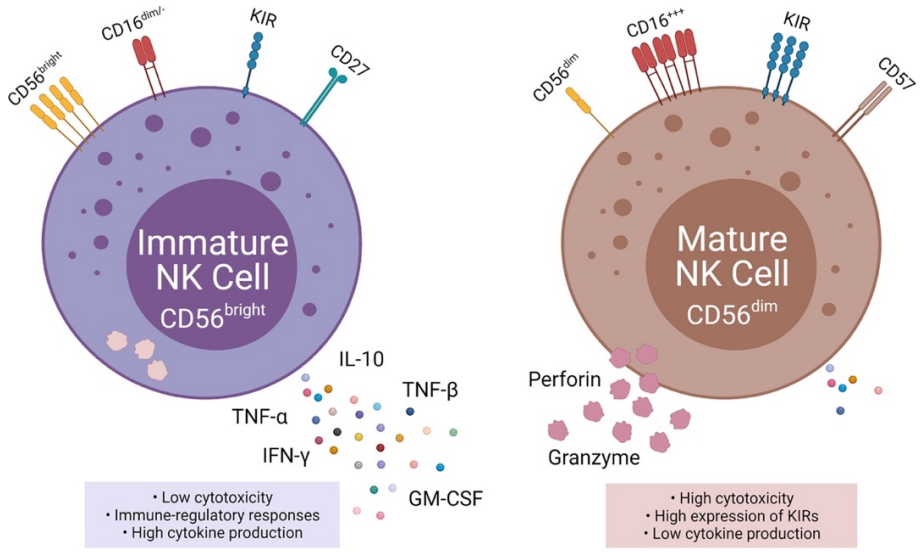


***Figure 6. Phenotypic and functional properties of immature (left) and mature (right) NK cells***

Immature NK cells express $CD56^{bright}$, absent, or $CD16^{dim}$, low KIR, and CD27 as well as exhibit low cytotoxicity, but high cytokine production. Mature NK cells, in contrast, express $CD56^{dim}$, high CD16, high KIRs, and CD57 as well as exhibit high cytotoxicity and low cytokine production. Adapted from "Current Perspectives on "Off-The-Shelf" Allogeneic NK and CAR-NK Cell Therapies" by E.L. Heipertz, *Front Immunol.*, *Volume 12*, Copyright 2021 by Heipertz, Zynda, Stav-Noraas, Hungler, Boucher, Kaur and Vemuri

While many factors play a role in prevalence of NK subpopulation frequencies, active NK cells generally account for roughly 90% of the circulating population in humans[39]. After cryopreservation, this fraction can fall to as low as 30% of the total NK population[40] due to the active population shifting back into an inactive state. This phenotypic shift greatly impacts the functionality of NK cells and has negative implications for the feasibility of an engineered NK product being used in allogenic therapies that rely heavily on cryopreservation during the manufacturing process. This phenotypic change is thought to be due to an activation of matrix metalloproteases, specifically a disintegrin and metalloprotease-17 (ADAM17), in response to the stress of cryopreservation[41].

*Role of ADAM17 in CD16 regulation and serial killing*

A disintegrin and metalloprotease-17 (ADAM17), a member of the adamalysin subfamily of the metzincin metalloproteinase superfamily, plays the primary role in regulating CD16 surface expression[42]. It acts as a pair of extracellular scissors, cleaving CD16 proximal to the cell membrane rapidly once activated. This function serves to decrease binding avidity to target cells after degranulation, the release of cytotoxic granules from the NK cell at the target cell, allowing the NK cell to move on from the dying target to a new victim. Proper cleavage of the immune synapse is essential for sequential (or serial) killing; NK cell's ability to attack multiple infected or malignant targets[43]. While ADAM17 knockout lines have been generated for potential use in NK based therapies in an effort to mitigate cryopreservation induced loss of CD16, any evaluation of these lines serial killing ability were not reported[44]. The most multifaceted benefits may come from transiently inhibiting ADAM17 during the cryopreservation process yet allowing it to regain full functionality after the final thawing step.

*Goals of this thesis*

Given the critical importance of cryopreservation in cell therapy and the limitations of the currently available DMSO based cryopreservation solutions, the goals of this thesis were to evaluate novel cryopreservation solutions for use in cell therapy applications. Using three of the most common cell types found in cell therapy, the novel solutions provided by CaseBioscience were evaluated on their ability to preserve both cell viability and functionality through the cryopreservation process. ADAM17 inhibitors were explored as an additive to increase post thaw functionality in NK cells by preserving the active ($CD56^{dim}/CD16^{++}$) phenotype.

## RESULTS AND DISCUSSION

### *hPSCs*

After some preliminary testing done on human embryonic kidney cells, pluripotent stem cells were chosen as the first test model to validate the efficacy of CaseBioscience's cryopreservation solutions. First, the toxicity of various known cryoprotectants were evaluated to confirm trends seen in previous HEK experiments. H1 embryonic stem cells (ESCs) were allowed to sit in solutions comprised of varying amounts of known cryoprotective agents diluted in a neutral buffer for 30 minutes at room temperature. The metabolic activity of the cells was then measured via an alamarBlue assay, and the toxicity of each solution was determined to be equivalent to the inverse of metabolic activity. The results were graphed and analyzed for significance (Figure 7). No significant differences were found in cell survival between the six solutions tested except no cells seemed to survive the solution containing 20% Glycerol (Figure 7A). Based on this and similar assays, a novel DMSO-free ("NON-DMSO" ) cryopreservation solution was developed along with a DMSO containing cryopreservation solution with a similar base formulation for comparison. The toxicity of these two solutions were compared to two solutions currently on the market using the same alamarBlue assay (Figure 7B). The cells incubated in CaseCryo DMSO, along with both NON-DMSO solutions, saw significantly less cell death after 30 minutes at room temperature. This temperature and time point were chosen because they were deemed the most translationally applicable to standard cryopreservation workflows.

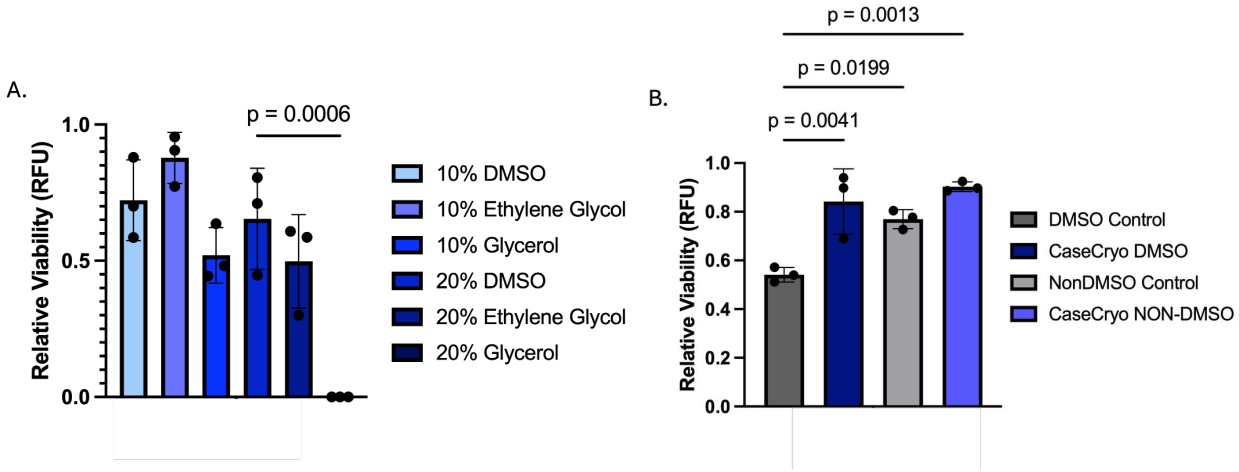


*Figure 7. alamarBlue Assay Results*

H1 embryonic stem cells (ESCs) were incubated at room temperature (25°C) for 30 minutes in **(A)** solutions containing either 10% or 20% v/v cryoprotectant in a proprietary base solution of salts, essential and non-essential amino acids, and inert small molecules and **(B)** CaseCryo solutions along with industry standard control solutions. Results are graphed in relative fluorescence units (RFU) based on the fluorescence intensity of resorufin detected in the sample after a 4-hour incubation period. A higher intensity was interpreted to indicate higher metabolic activity of the sample as well as higher viability in the cell population, indicating a more minor cytotoxic effect from the solution versus samples with lower fluorescence intensities of resorufin. Chosen statistical test is One-way ANOVA.

To evaluate the efficacy of these CaseCryo solutions in maintaining cell health, H1 ESCs were cryopreserved using both CaseCryo DMSO and CaseCryo NON-DMSO along with a control, industry standard DMSO containing cryopreservation solution. Post thaw viability was assessed immediately after freezing, and there was found to be no significant difference between the three solutions in this aspect, along with all three solutions maintaining a relatively high post thaw viability when compared to the field (Figure 8B). This suggests that there are no major short term detrimental effects on cell health brought about by any of the solutions. The cells were then plated at matching densities and allowed to expand for a four day culture period with images being taken each day to monitor expansion (Figure 8A). The cells frozen down in CaseCryo DMSO saw a significantly higher fold expansion (calculated by normalizing the ending live cell number to the plated live cell

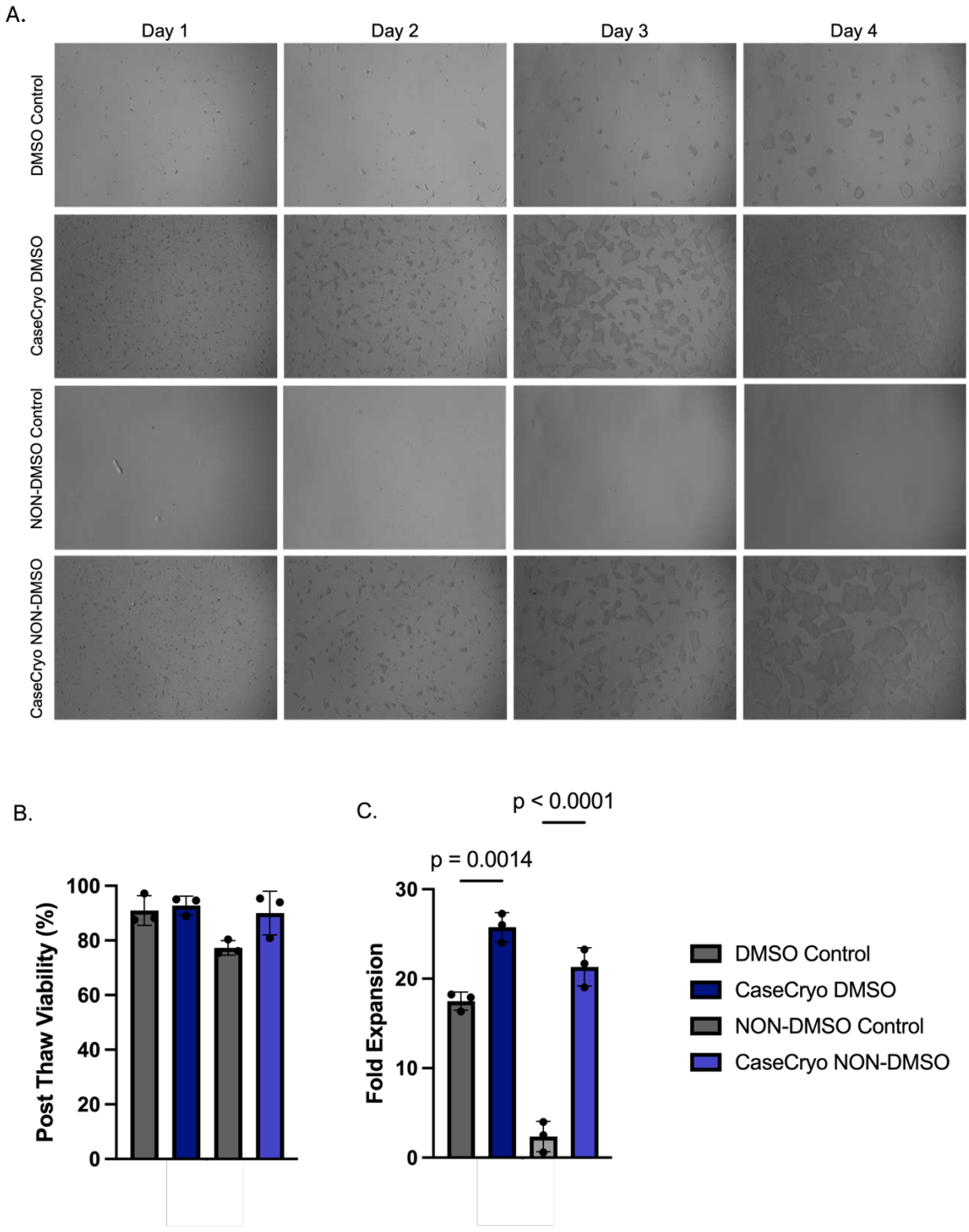


*Figure 8. Post Thaw Viability, Culture, and Expansion of hPSCs*

H1 embryonic stem cells (ESCs) were cryopreserved using both CaseCryo DMSO and NON-DMSO as well as both an industry standard DMSO based and DMSO-free cryopreservation solution serving as a control. The cells were thawed and immediately assed for viability using an AO/DAPI co-stain **(B)** then plated in 6 well tissue culture dishes and monitored over a 4 day culture period. The images **(A)** taken of each culture were used to quantify confluency of the cultures. The images shown in this figure are representative and have been modified for enhanced visualization. Unmodified images were used during quantification. Confluency measurements from Day 4 were compared to confluency measurements from Day 1 to calculate the fold expansion of each culture **(C)**.

number) than the industry standard DMSO control condition as well as between the NON-DMSO control and the CaseCryo NON-DMSO condition, however no significant difference was found between the CaseCryo NON-DMSO condition and the DMSO control condition (Figure 8C). While other factors come into play when assessing cell health, the higher rate of expansion seen may suggest that the cells frozen down in the CaseCryo solutions are healthier than cells frozen down in the industry standard control solution counterparts and may suffer less long-term detrimental effects.

Beyond both short- and long-term cell health, the functionality of the ESCs in terms of maintaining pluripotency was also assessed. This was achieved qualitatively through a series of differentiations and immunocytochemistry (ICC) imaging protocols. First, cells frozen down in CaseCryo DMSO and CaseCryo NON-DMSO were thawed and cultured for a 4-day period. After the initial culture, the cells were then passaged onto glass plates and differentiated into each of the three lineages: mesoderm, endoderm, and ectoderm. The differentiated cells were then fixed and stained for the appropriate markers for each lineage and imaged to confirm the presence of said markers (Figure 9A). The presence of Otx2/Sox17/Brachyury in each lineage signifies proper differentiation and suggests that "stemness" was properly maintained through the cryopreservation process. The appropriate markers were seen in both the cells frozen down in CaseCryo DMSO and CaseCryo NON-DMSO which suggests that both solutions properly maintain the cells' ability to differentiate. Additionally, WTC11 induced pluripotent stem cells (iPSCs) were frozen down in CaseCryo DMSO and CaseCryo NON-DMSO and examined for functionality post thaw. While also a type of pluripotent stem cell, WTC11s have been modified to easily differentiate into

neurons and were chosen to test the solutions ability to preserve more specific differentiation functionality in pluripotent stem cells. Similarly to the trilineage differentiation, the differentiated cultures were assessed qualitatively by the presence of neural progenitor marker phalloidin and the proper assembly of neurofilament (Figure 9B). Both were seen appropriate in differentiated cultures that had been frozen in CaseCryo DMSO and CaseCryo NON-DMSO, adding to the claim that the CaseCryo solutions are able to preserve pluripotent stem cell functionality through the cryopreservation process. Finally, cells were stained for pluripotency marker Oct3/4 post thaw, and again qualitatively assessed. A co-staining was done with DAPI and F-actin to assess colony phenotype (Figure 9C). In both CaseCryo DMSO and CaseCryo NON-DMSO conditions it was determined that Oct3/4 was expressed in the appropriate amount and the colonies displayed the appropriate morphology. All this appears to support the claim that both CaseCryo solutions maintain functionality and health of pluripotent stem cells during the cryopreservation process.

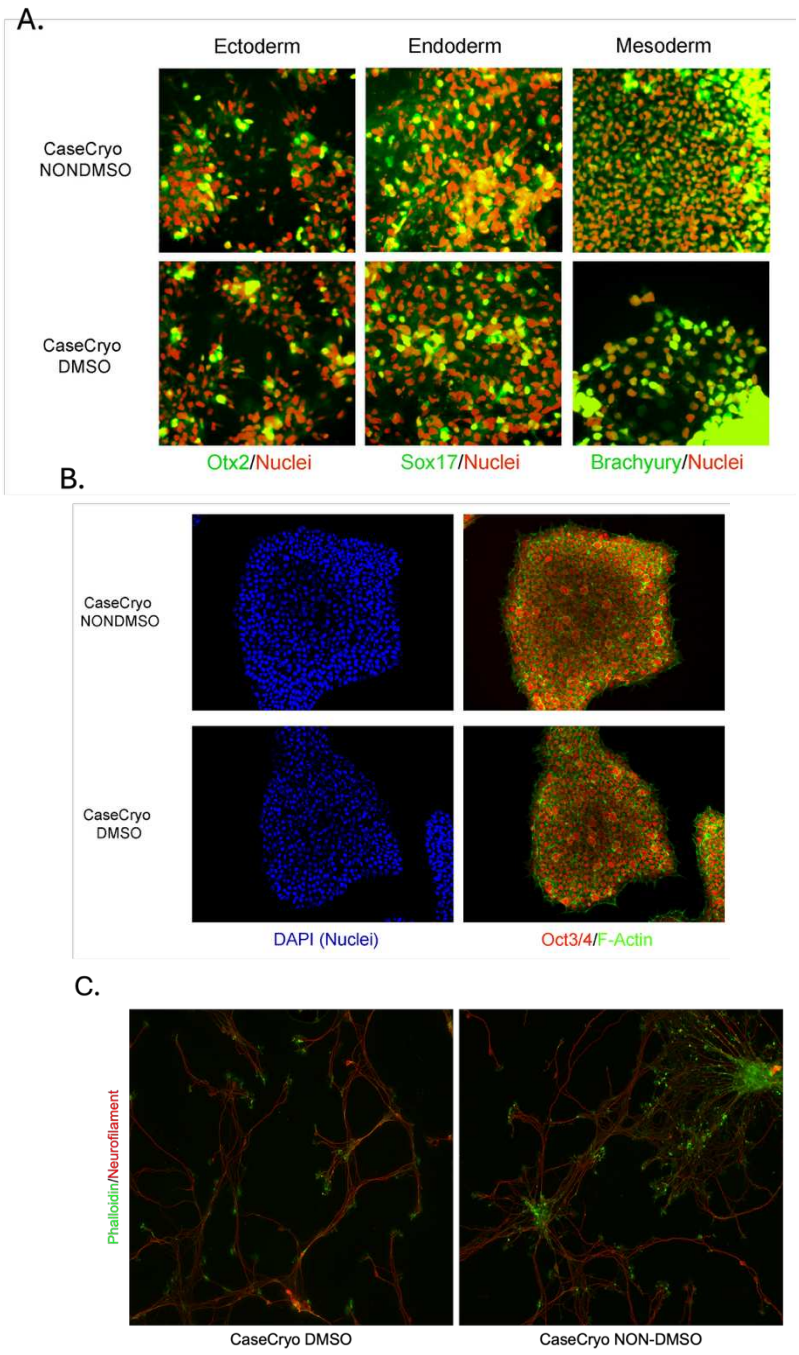


*Figure 9. Fluorescence Microscopy of Pluripotent and Differentiated Stem Cells*

H1 ESCs were cultured and differentiated into mesoderm, endoderm, and ectoderm progenitors **(A)**, fixed and stained for the appropriate marker for their lineage. WTC-11 induced pluripotent stem cells (iPSCs) were differentiated into neural progenitors **(C)** and fixed and stained for neurofilament and phalloidin. Undifferentiated ESCs were cultured and stained for appropriate markers and imaged using fluorescence microscopy **(B)**.

*T Cells*

The CaseCryo base formulation was specifically created with pluripotent stem cells in mind, however after the success seen in maintaining post thaw health and functionality of that cell type it was wondered if the same results would be seen in other cell types. Human T-Cells were chosen as the next test cell line, as they are both common in the cell therapy field and considered more robust than other peripheral blood mononuclear cells (PBMCs) such as other lymphocytes, monocytes, and dendritic cells.

The efficacy of the CaseCryo solutions at maintaining cell health was again evaluated by examining both the post thaw viability, visual observation, and fold expansion (Figure 10). CaseCryo DMSO and CaseCryo NON-DMSO were compared to both a commercially available DMSO based and DMSO-free cryopreservation solution. The T cells were cultured for a 10-day period before determining fold expansion based on the starting number of cells. As suspension cultures, confluency was not needed to determine expansion. Instead, cells were counted at the end of the culture period using an automated cell counter in an identical manner as determining post thaw viability. Brightfield images of each culture were still taken for visual assessment (Figure 10A). No significant difference was found between any of the post thaw viabilities or fold expansion of cells frozen in the four solutions (Figures 10B, 10C). These experiments were done in triplicate, using T-cells isolated from three separate donors. There is often great donor to donor variability in PBMCs, with a variety of outside factors contributing to the health and functional characteristics of the cells isolated from each person[45]. This could stand as an explanation for the heavily overlapping error bars in these T-cell experiments, and if so, would be remedied by repeating these with a higher number of donors to mitigate the drift in data.

To assess the post-thaw functionality of T-cells after being cryopreserved with the CaseCryo solutions, the cells were stained for very basic surface markers and examined via flow cytometry for proper expression of CD3 and absence of CD56 (Figure 11B). Both the CaseCryo conditions as well as the DMSO control condition were found to be generally positive for CD3 expression and generally negative for CD56 expression indicating no major phenotypic shifts occurred during contact with the novel solutions. As a secondary measure of cell functionality, brightfield images were taken of each culture to examine colony

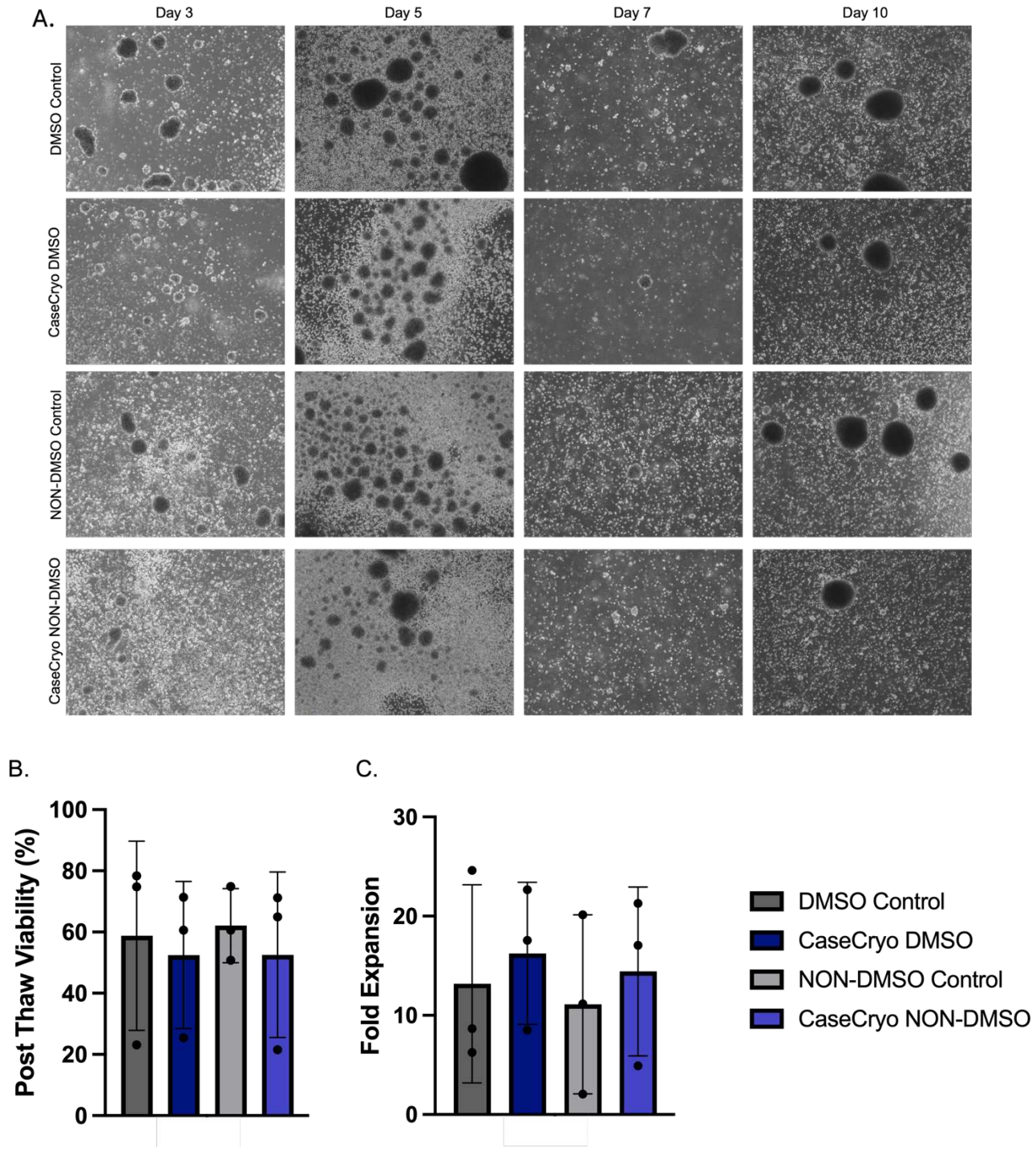


*Figure 10. Post Thaw Viability, Culture, and Expansion of T Cells*

Primary T Cells were cryopreserved using both CaseCryo DMSO and NON-DMSO as well as industry standard DMSO based and DMSO free cryopreservation solutions serving as a controls. The cells were thawed and immediately assed for viability using an AO/DAPI co-stain **(B)** then plated and monitored over a 10 day culture period. The images **(A)** taken of each culture were used to visually assess culture health. The images shown in this figure are representative. The cells were harvested after 10 days in culture and counted using the same AO/DAPI co-stain. The Day 10 counts were compared to the Day 1 counts to calculate the fold expansion of each culture **(C)**.

phenotype (Figure 11A). Generally, a healthy and active colony exists in a clump-like state whereas suboptimal health and inactivity is demonstrated by a majority of cells being in single cell suspension. The cells from the novel CaseCryo solutions were observed to have equal if not more promising colony phenotypes across the length of culture, suggesting equal if not better cell health during post thaw expansion.

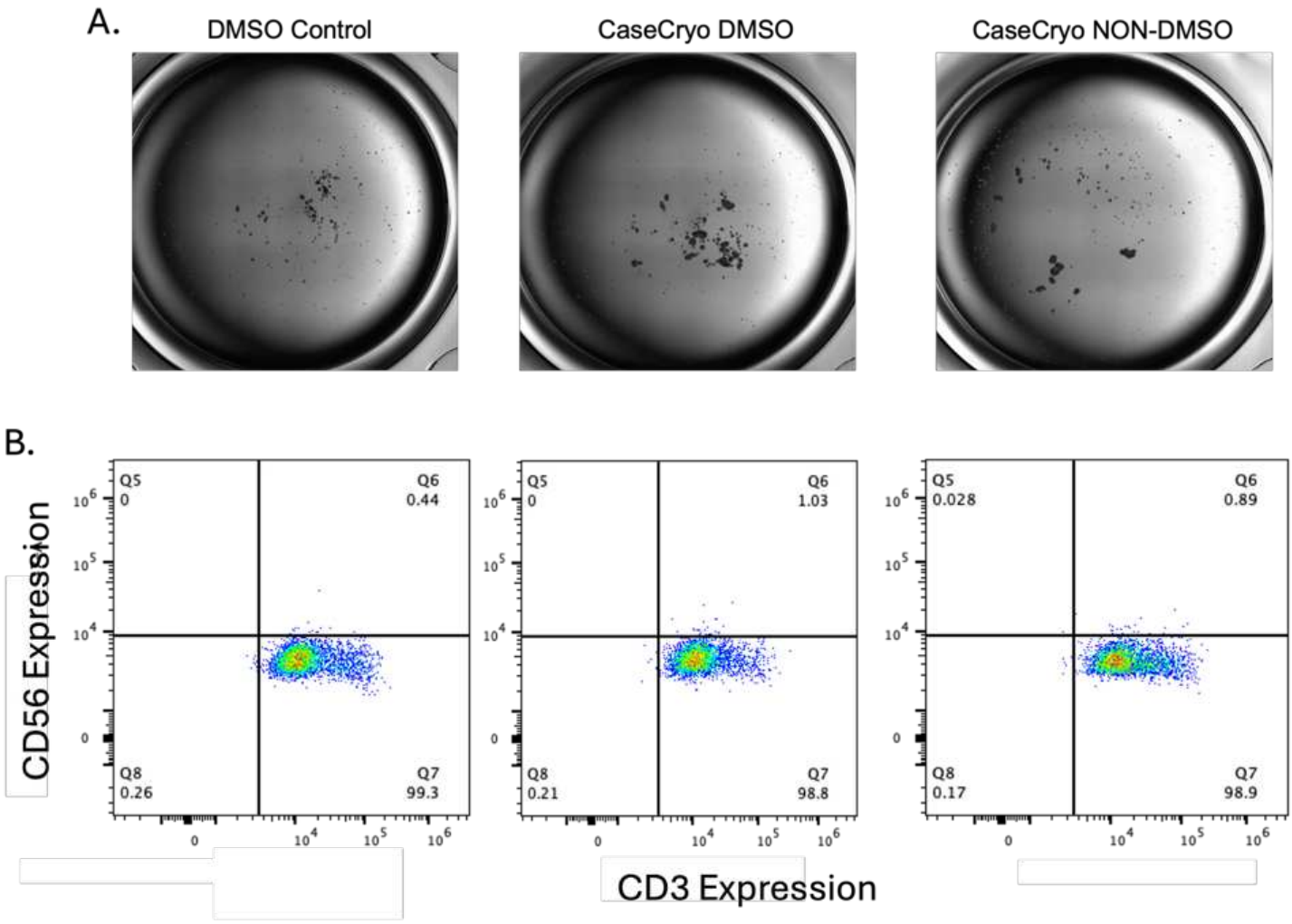


***Figure 11. Functional Assessment of T Cells***

Brightfield images of T cell cultures were taken on Day 10 to assess the frequency of "clump" phenotype **(A)**. Cells were stained for surface markers CD56 and CD3 and quantified via spectral flow cytometry **(B)**.

*Natural Killer Cells*

The final cell type tested using the novel CaseCryo cryopreservation media was Natural Killer (NK) cells. Another PBMC, NKs are being examined as a potent alternative to T-cells for the use of immunotherapies due to their allogeneic potential, however they lack the robustness seen in T-cells during the cryopreservation process and are often greatly affected both in health and functionality after being cryopreserved. These cells were chosen because of their importance to new allogeneic immune cell therapies and because they represent astringent test of efficacy for both CaseCryo DMSO and CaseCryo NON-DMSO.

Similarly to the previous two cell types, the solutions were first tested for their ability to promote a high post thaw viability and expansion rate in cryopreserved NK cells (Figure 12). CaseCryo DMSO and CaseCryo NON-DMSO were tested in triplicate against two different controls: an industry standard DMSO based cryopreservation solution as well as an industry standard containing an alternative cryopreservation agent ("Non-DMSO"). Post thaw viability measurements were taken as noted above (Figure 12A), and the cells were cultured for a 14-day period before being imaged for the prevalence of healthy "clump" phenotype (Figure 12C) and again counted to determine fold expansion (Figure 12B). In this cell type, the post thaw viabilities measured seemed to indicate that the CaseCryo DMSO solution underperformed as opposed to its control, whereas no significant difference was seen between the DMSO-free solutions. As with all primary PBMCs, NK cells are affected by donor-to-donor variability and this is demonstrated strongly in the fold expansion measurements; similarly to the T cell experiments, each repetition utilized a different donor. While there seems to be a trend in the data when examined by eye, the statistical analysis notes no significant difference between the fold expansion of the CaseCryo solutions and

their commercially available control counterparts. It should be noted that these sample sizes are small; additional replicates may elucidate a statistically significant effect.

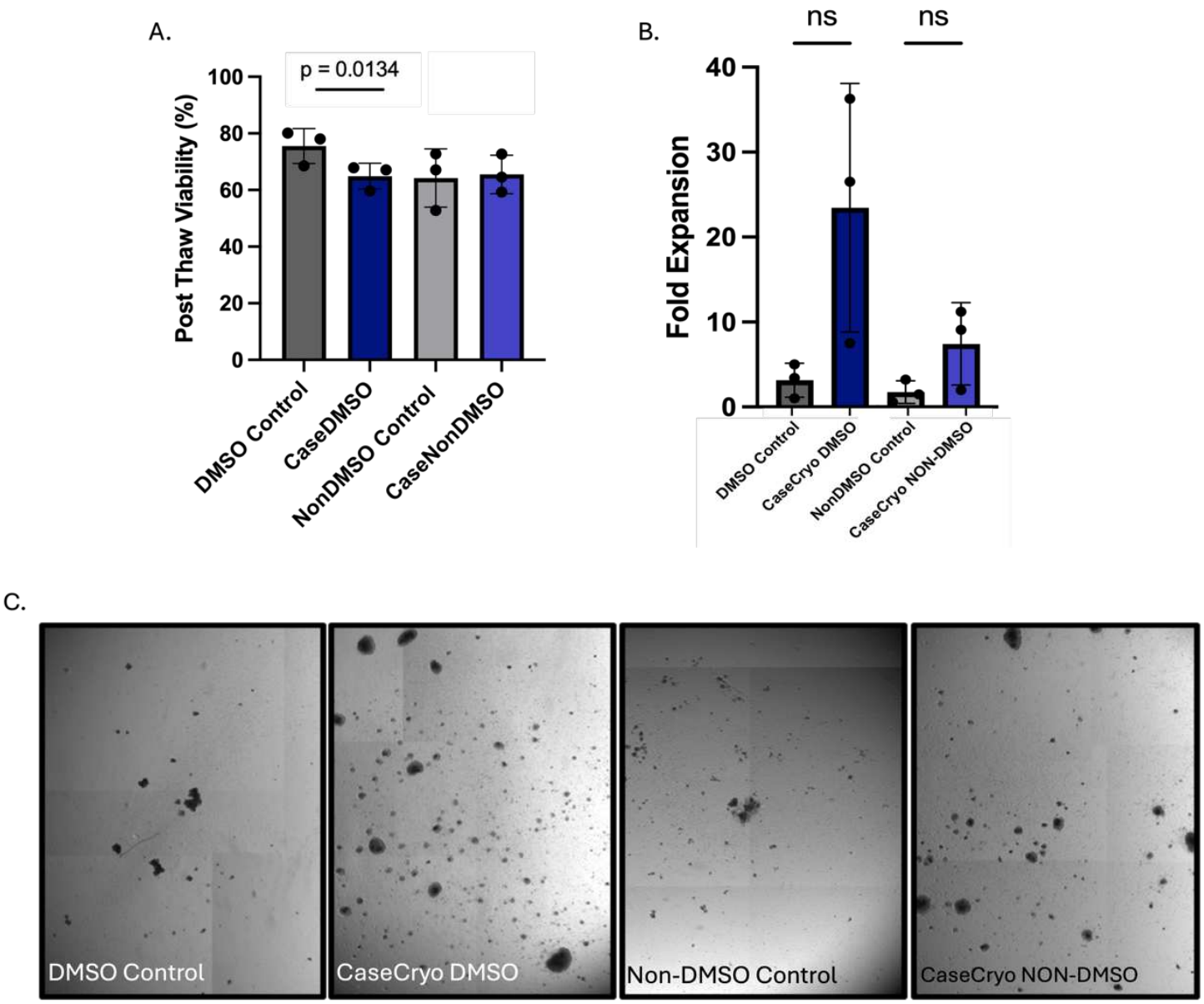


*Figure 12. Post Thaw Viability, Culture, and Expansion of NK Cells*

Primary NK cells were cryopreserved using both CaseCryo DMSO and NON-DMSO as well as both an industry standard DMSO based and DMSO-free cryopreservation solution as controls. The cells were thawed and immediately assessed for viability using an AO/DAPI co-stain **(A)** then plated and monitored over a 14-day culture period. The images **(C)** taken of each culture were used to visually assess culture health. The images shown in this figure are representative. The cells were harvested after 10 days in culture and counted using the same AO/DAPI co-stain. The Day 14 counts were compared to the Day 1 counts to calculate the fold expansion of each culture **(B)**.

Post thaw functionality of NK cells is perhaps the largest hurdle in cryopreservation of this cell type, as there is a well noted change in phenotype from active to naïve as classified by a decrease in surface expression of CD16. Also known as FCγRC3a, CD16 plays a key role in NK cells antibody dependent killing and is necessary for proper functionality. CD16 expression of NK cells frozen in CaseCryo DMSO, CaseCryo NON-DMSO, and an industry standard DMSO based cryopreservation solution was measured post thaw using spectral flow cytometry (Figure 13A). The median fluorescence intensity (MFI) of CD16 signal was compared visually (Figure 13B) as well as quantified (Figure 13C) and examined for any downregulation, with a decrease in MFI being correlated to a decrease in surface expression. No difference in expression was seen between the CaseCryo DMSO and commercially available DMSO based solution, but a slight decrease in expression was noted in cells frozen in the CaseCryo NON-DMSO solution. More replicates of this experiment should be done to enable statistical analysis so that proper conclusions may be drawn.

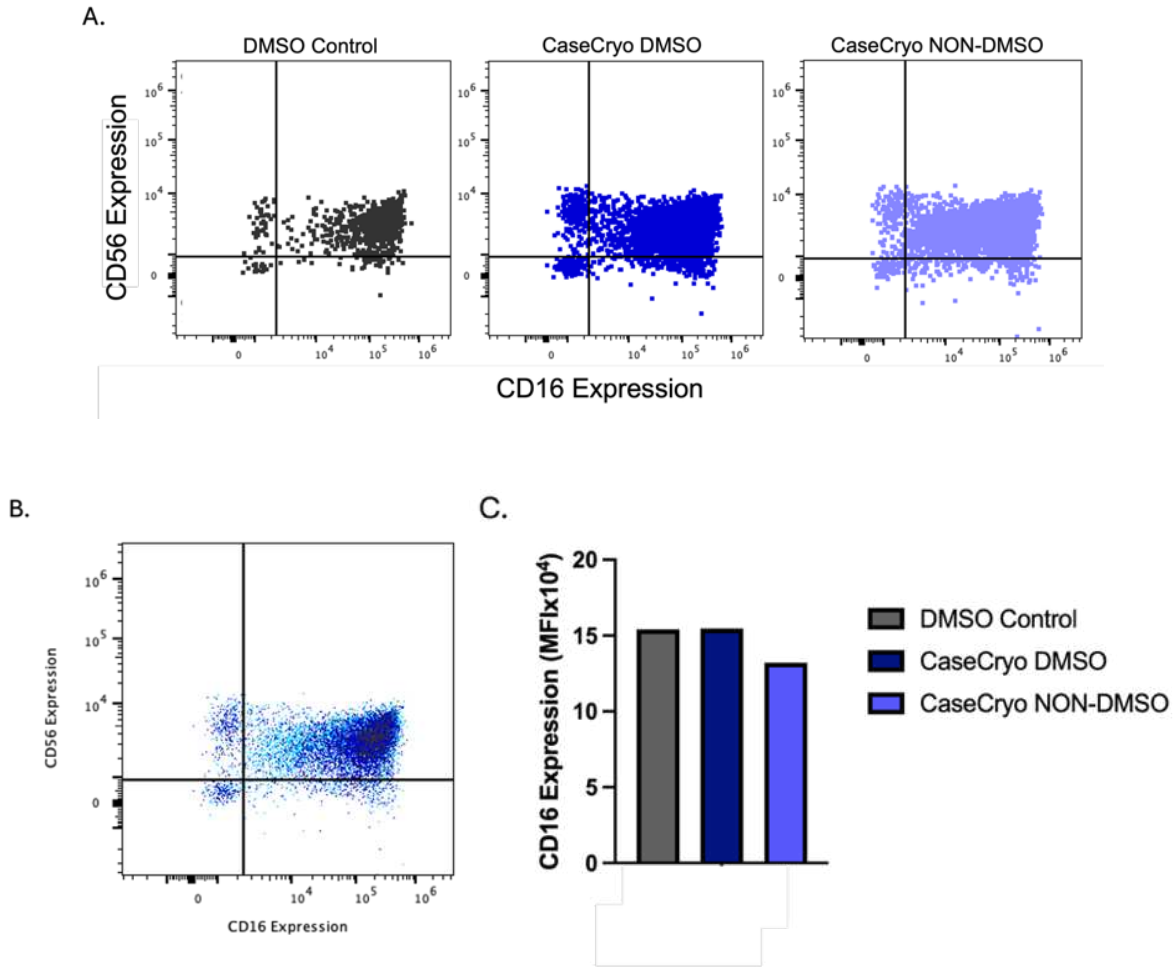


***Figure 13. CD16 Surface Expression in NK Cells Post Thaw***

CD16 surface expression was measured in post thaw primary NK cells using spectral flow cytometry and graphed against CD56 surface expression **(A)**. The results from the three conditions were overlayed **(B)** and quantified **(C)** to identify differences in CD16 expression in NK cells frozen in CaseCryo DMSO versus CaseCryo NON-DMSO versus the industry standard DMSO based cryopreservation solution.

*Cryopreserving NK Cells with the addition of ADAM17 inhibitors*

The loss of CD16 surface expression is thought to be due to the overactivation of a disintegrin/metalloprotease called ADAM17[41]. Under normal conditions, ADAM17 helps regulate the serial killing ability of NK cells. The binding of CD16 to antibody-opsonized target cell is irreversible and the receptor must be cleaved for the NK cells to move on from the dead cell to another live target[42]. Therefore, it would not be feasible from a treatment

standpoint to knockout or permanently inhibit the function of ADAM17. However, there is a class of transient metalloprotease inhibitors with a specificity for ADAM17. This next set of data examines how the addition of two types of this small molecule into the cryopreservation workflow impacts expression of CD16 in NK cells post thaw. TNF-alpha protease inhibit I (TAPI-1) is a general inhibitor of matrix metalloproteases (MMPs) as well as ADAM-17 and was used in the following experiments at three different concentrations, 0.5μM, 1μM, and 2μM, chosen based on previous research in the field[46]. G28264X is a more specific inhibitor blocking ADAM17 and similar MMP ADAM10 with little off target effects seen. This compound was also used at three different concentrations, 0.5μM, 1μM, and 2μM, chosen based on previous research in the field[47].

First, it was ensured that the addition of these compounds did not negatively affect the baseline ability of the cryoprotective solutions to preserve cell health. In a similar manner to prior experiments, post thaw viability (Figure 14A) and fold expansion (Figure 14B) over a 14-day culture period were measured and compared across a range of concentrations of GW280264X and TAPI-1 using CaseCryo DMSO as a base and the industry standard. Brightfield images were taken on Day 14 to assess prevalence of the healthy “clump” phenotype (Figure 14C). While no definitive conclusions can be reasonably drawn from an experiment of n=1, it can be noted that there does not appear to be any great impact on the short- or long-term health of NK cells with the addition of these small molecules into the cryopreservation media.

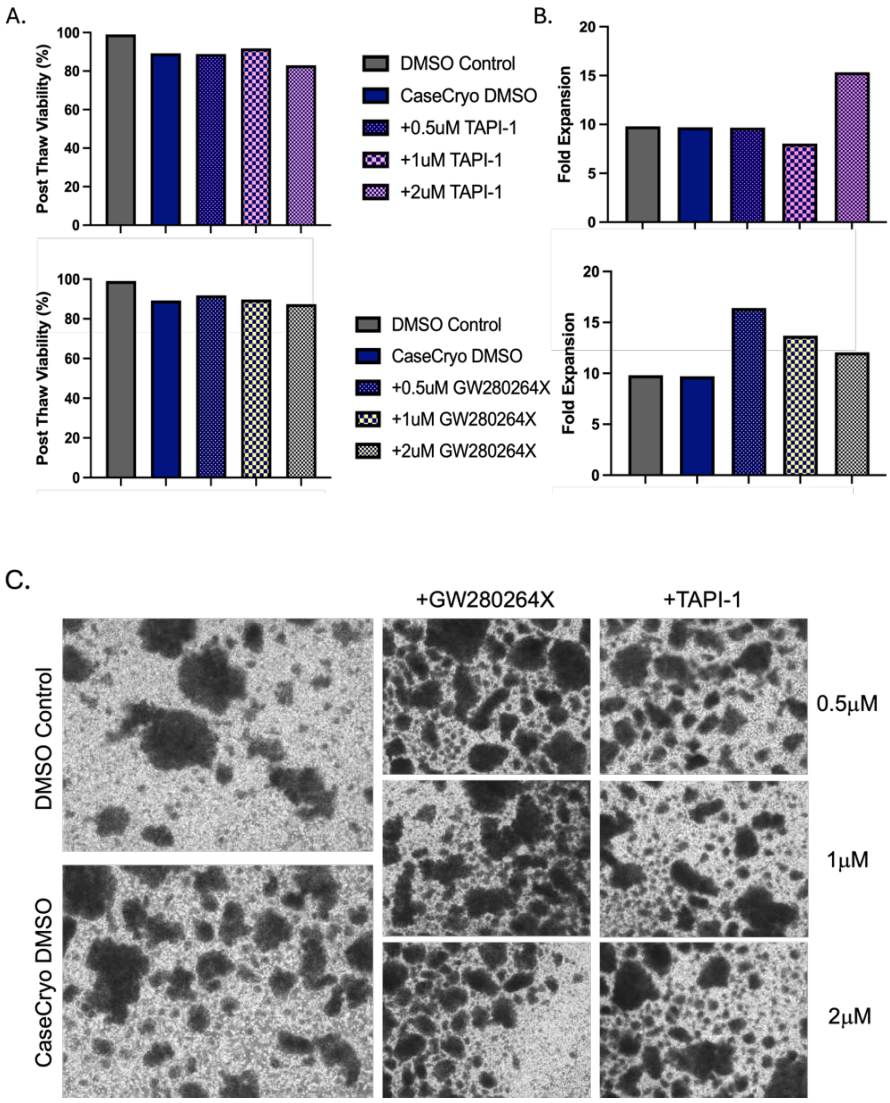


*Figure 14. Post Thaw Viability and Expansion of NK Cells After Exposure to ADAM17 Inhibitors*

Primary NK cells were cryopreserved using CaseCryo DMSO with the addition of either TAPI-1 or GW280264X at a range of concentrations as well as an industry standard DMSO based cryopreservation solution serving as a control. The cells were thawed and immediately assessed for viability using an AO/DAPI co-stain **(A)** then plated and monitored over a 14 day culture period. The images **(C)** taken of each culture were used to visually assess culture health. The images shown in this figure are representative. The cells were harvested after 10 days in culture and counted using the same AO/DAPI co-stain. The Day 14 counts were compared to the Day 1 counts to calculate the fold expansion of each culture **(B).**

Next, flow cytometry was performed to examine surface expression of CD16 post thaw to assess the efficacy of both ADAM17 inhibitors at a range of concentrations. TAPI-1 and GW28-264X were added to CaseCryo DMSO at concentrations of 0.5μM, 1μM, and 2μM, and the

MFI of CD16 was assessed in NK cells cryopreserved using both standard CaseCryo DMSO as well as the solutions with added small molecules in a similar protocol as prior (Figure 15A). While no sound conclusions can be drawn from an experiment of n=1, there appears to be an indication that the addition of GW280264X to the cryopreservation media does rescue CD16 expression, whereas the addition of TAPI-1 appears to have minimal effect as compared to the base CaseCryo DMSO cryopreservation formula.

Next, the surface expression of ADAM17 was examined to see if the inhibitors induced any unwanted decrease in expression (Figure 15B). This was also examined using MFI recorded with spectral flow cytometry and the results were graphed as compared to a RAJI control (a cell type known to express little to no ADAM17). Fortunately, there does not appear to be any meaningful change in ADAM17 surface expression in cells exposed to any tested concentration of the GW280264X compound. Lastly, it was tested whether adding a pretreatment of ADAM17 inhibitors to the cryopreservation workflow had any greater positive effect on surface CD16 expression post thaw versus simply adding the compounds to the cryopreservation solution itself. GW280264X was added at a concentration of 1μM to the culture of NK cells at a range of timepoints from 24 hours to 30 minutes before the initiation of the cryopreservation workflow. The MFI of CD16 as measured by spectral flow cytometry post thaw was examined for these pretreated NK cells versus a control of completely untreated cells (CaseCryo DMSO) and cells exposed to GW280264X in the cryopreservation solution only (Figure 15C). Replicates must be performed to corroborate this; there appears to be no significant advantage to pretreating NK cells with an ADAM17 inhibitor prior to cryopreservation versus having the inhibitor in the cryopreservation

solution alone. This is promising data for the scalability of NK cell cryopreservation, as adding an extra step such as pretreatment only adds friction to an already precarious and laborious process.

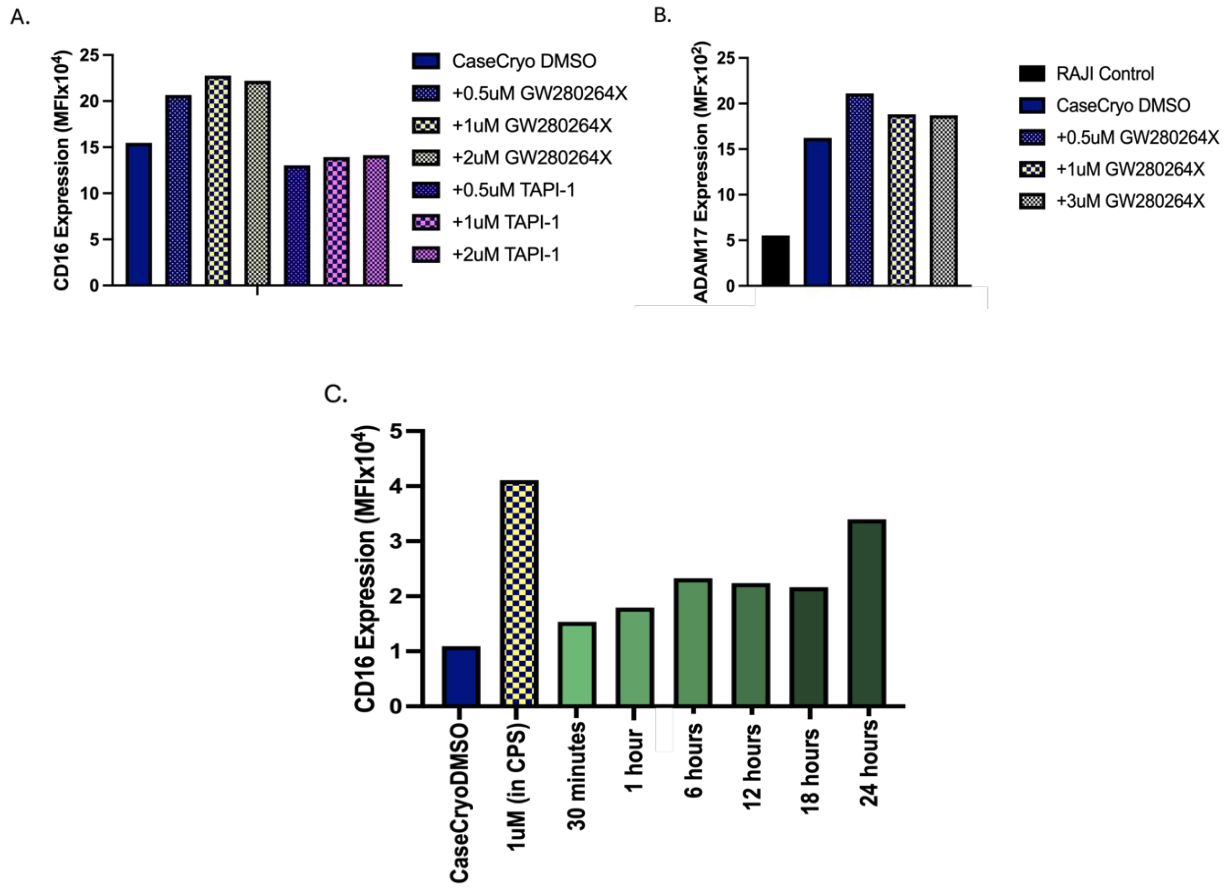


***Figure 15. Surface Expression of CD16 and ADAM17 Post Thaw in NK Cells Treated with Small Molecules***

Primary NK cells were cryopreserved using CaseCryo DMSO with the addition of varying amounts of GW280264X and TAPI-1. The MFI of CD16 was measured using spectral flow cytometry **(A)** as well as the MFI of ADAM17 as compared to another cell type known to express little to no ADAM17 **(B)**. Primary NK cells were pretreated with ADAM17 by adding 1μM directly into the culture media 24 hours to 30 minutes before initiation of the cryopreservation process. Surface expression of CD16 was examined by means of MFI post thaw comparing untreated NK cells to NK cells pretreated for varying lengths of time **(C)**.

### *Vitrification of T Cells*

In addition to the cryopreservation media, aspects of the cryopreservation workflow have been explored in this body of work for feasible alternatives to increase both viability and functionality to cryopreserved cells post thaw. Vitrification, or the act of "fast freezing"

samples, is commonly implemented in the assisted reproductive technologies (ART) field but has not yet been applied to the similar workflows within the cell therapy space. Vitrification of T cells was attempted using modified versions of the CaseCryo solutions. Three different test solutions were made by substituting and/or increasing the concentrations of cryoprotective agents in the CaseCryo NON-DMSO solution. These solutions are henceforth referred to as “Vit Solution 1”, “Vit Solution 2”, and “Vit Solution 3” and were evaluated on their ability to preserve the both the short- and long-term health of T cells as compared to samples cryopreserved in a standard “slow cool” manner as well as vitrified using the industry standard DMSO based cryopreservation solution. The samples were loaded into 500µL straws before being directly submerged into liquid nitrogen to achieve vitrification. The cells were then thawed following the same method as the “slow cooling” cryopreservation workflow and the experiment was repeated for a total of triplicates. Viability measurements were taken immediately post thaw (Figure 16A). No significant difference was seen in the post thaw viabilities between any of the vitrification solutions nor the two controls (the slow cooled sample and the sample vitrified using the industry standard DMSO based cryopreservation solution). The T cells were then cultured for a 10-day period before determining fold expansion based on the starting number of cells (Figure 16B). Surprisingly, the vitrified T cells displayed a significantly higher fold expansion than either control, including the “slow cooled” samples. The higher rate of expansion seen may suggest that the vitrified T cells are healthier than cells frozen down following the industry standard “slow cooling” protocol and may suffer less long-term detrimental effects. This is mildly corroborated by the brightfield images of each condition

taken at the end of the culture period (Figure 16C). By visual assessment, the vitrified T cells display an equivalent if not increased amount of the healthy "clump" phenotype as compared to both the "slow cool" control and industry standard DMSO solution control.

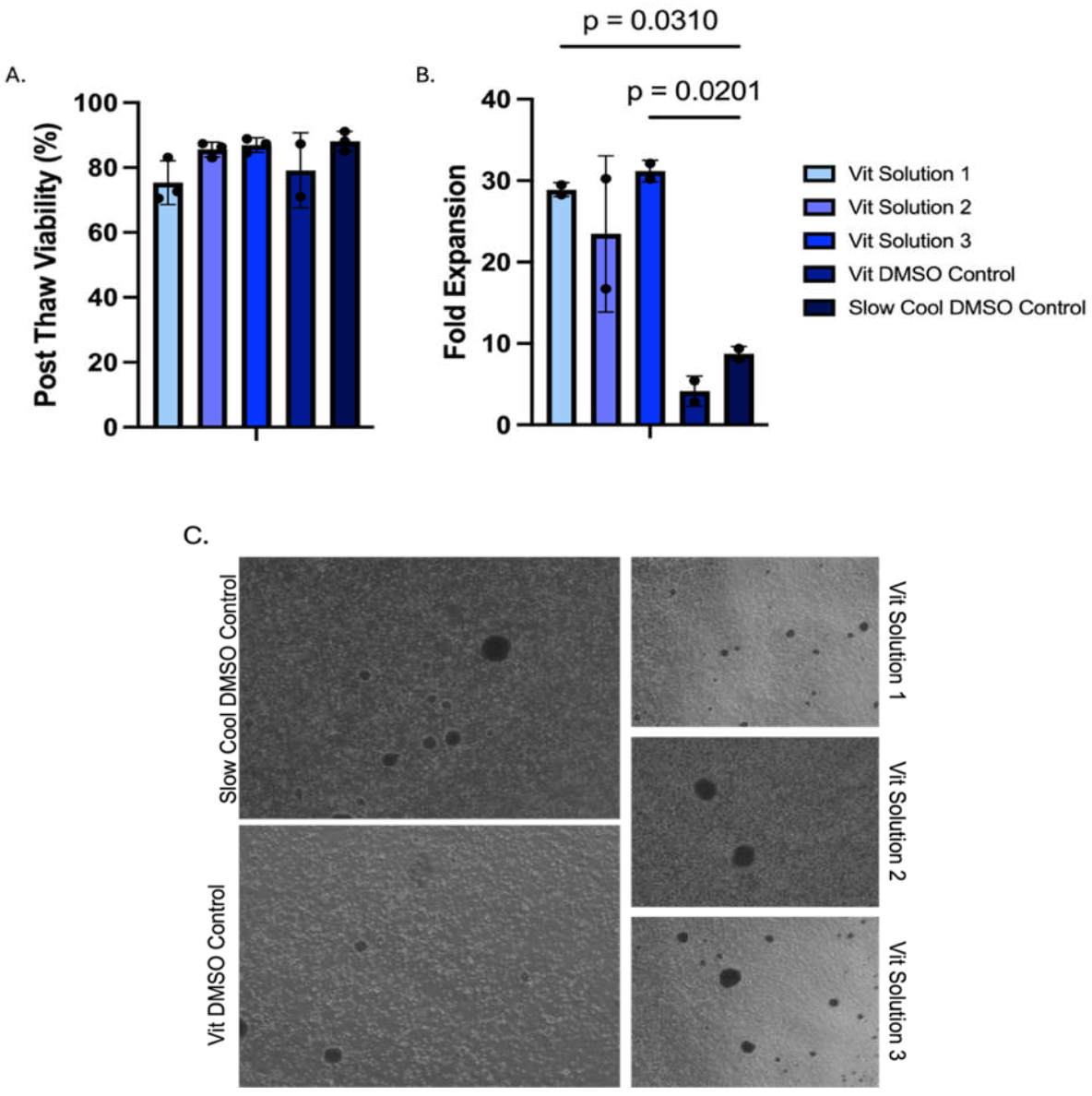


***Figure 16. Post Thaw Viability, Expansion, and Morphology of Vitrified T Cells***

Primary T Cells were vitrified in three different vitrification solutions or an industry standard DMSO based cryopreservation solution. A subset of T cells was slow cooled to serve as a control. The cells were thawed and immediately assessed for viability using an AO/DAPI co-stain **(A)** then plated and monitored over a 10 day culture period. The images **(C)** taken of each culture were used to visually assess culture health. The images shown in this figure are representative. The cells were harvested after 10 days in culture and counted using the same AO/DAPI co-stain. The Day 10 counts were compared to the Day 1 counts to calculate the fold expansion of each culture **(B)**.

# FUTURE EXPERIMENTATION AND CONCLUDING REMARKS

The field of Cell and Gene Therapy (CGT) holds immense promise for transforming medicine utilizing engineered T cells, NK-cells, and cells derived from pluripotent stem cells (PSCs). The success of cell therapy depends on the integrity and quality of these engineered cells which require effective growth, enrichment, storage, and implantation processes. Highly effective cryopreservation protocols are critical to mitigate cellular stress and ensure the long-term viability of cell therapies during storage. Unfortunately, current cryopreservation methods are hampered by the use of outdated techniques and toxic agents, such as dimethyl sulfoxide (DMSO), which diminish cell viability and functionality as well as compromise patient safety and long-term outcomes. While some newer cryoprotective solutions with alleged lower toxicity have been introduced in the scientific literature, the few commercially available DMSO-free solutions have poor performance with stem cells and immune cells.

In this work, novel DMSO and DMSO free cryopreservation solutions formulated by CaseBioscience were tested in three key cell types crucial for emerging cell therapies: PSCs, T cells, and NK-Cells. The results show that CaseCryo DMSO has improved performance as evaluated by post thaw viability and functionality of the cells, in all three cell types compared to the leading commercially available DMSO solution and CaseCryo NON-DMSO appears to meet the standards set by the leading commercially available DMSO solution. Although for T-cells it appears the CaseCryo solutions offer no improvement over existing solutions, long term survival trends higher for NK cells using CaseCryo DMSO. Since NKs offer an path to meet the need for allogenic cancer cell therapies, we focused on

experiments that may help improve NK cell function following cryopreservation. The diminished cell function of cryopreserved NK cells is at least partially due to the loss of CD16 expression which could be due to an overactivation of surface metalloprotease ADAM-17. The inclusion of ADAM-17 inhibitors, particularly GW280264X, appear to increase CD16 expression which may translate into a higher active population of NK cells versus inactive.

Of the data presented in this body of work several warrant, if not demand, further investigation. First, several experiments must be rounded out before this project may be considered finished. As is standard for science, all experiments should be repeated to achieve at minimum n=3. For experiments using T or NK cells, replicates should be performed with cells taken from a minimum of 6 different donors to minimize the skewing of trends by donor-to-donor variety. Three is generally accepted as the minimum number of donors, however some projects utilized as many as 25-50 unique samples to produce trustworthy conclusions.

Additionally, the functionality of immune cells post thaw should be investigated to a deeper level than surface marker staining. Killing assays, or the mediated co-culture of immune (effector) cells with labeled target cells, are an excellent way to support the flow cytometry analysis performed as marker expression often correlates with but is not a complete surrogate for killing ability. Assays such as luciferase based killing assays can quantify the amount of target cells successfully eliminated over set periods of time by measuring decrease in fluorescence signal given off by the target cells as they are attacked and destroyed. These assays can be performed using both T cells and NK cells with slight

modifications to the protocol. The luciferase assay is performed using a plate reader; however, these types of experiments can also be done using live cell imaging. Specialized microscopes such as an Incucyte can take time-lapse images tracking either whole cultures or individual effector cells as they navigate the serial killing process, giving in-depth insights to cellular activity post thaw. Initial experiments were performed using both these methods, however further optimization of each protocol needs to be done before this data can be shown or used to generate conclusions.

In regard to the pluripotent stem cell experiments, hindsight has revealed that confluency images are not the most optimal way of tracking expansion over a culture period. There is great variability in image quality and brightness not only from day to day but based on where the well is positioned on the tissue culture plate as well. This variability makes automation of this process incredibly difficult and to perform analysis of thousands of images by hand nearly importunes error. Additionally, adherent cell culture expansion is limited by the size of the culture dish to a greater degree than suspension cultures. If cultures get too confluent, their rate of expansion will slow or cease as they fill up all the available space on the bottom of the well. It was avoided as much as possible however this factor could skew expansion measurements as well. To address the first issue, cells can be dissociated and harvested at the end of the culture period. This allows them to be counted in the same automated manner as the post thaw viably measurements and may provide a more accurate final count. This method would not allow for growth to be tracked over time as the daily images do, so ideally it would be performed supplementary to the established protocol.

The results of the vitrification experiments, while promising, beget many further paths of experimentation. First off, while the vitrified T cells appear to be healthier than cells frozen down following the industry standard "slow cooling" protocol based on their significantly increased rates of expansion, their functionality remains untested. A robust flow cytometry panel incorporating not only CD3 and CD56 tags, but other activation and exhaustion markers should be performed to give an in-depth insight into population phenotypes and potential functionality of the cells as compared to their slow cool counterparts. Additionally, killing assays (either live cell imaging or plate reader based) should be performed alongside the in-depth flow cytometry panel to round out the functionality assessment of vitrified T cells. The biggest challenge with utilizing vitrification in cell therapy workflows will be scalability. The protocol used in these experiments handled 500μL of sample at a time, whereas current CAR-T manufacturing processes have working volumes of up to 5L[48]. At volumes of 1mL and above, the challenge of equally cooling the sample and solution hinders effective vitrification. Uneven cooling allows for ice nucleation points to spread quickly through the sample which is incredibly detrimental to the cells' viability. Straws are not feasible at the working volumes of cell therapy manufacturing, which is why the idea of bags is being explored currently by both our group and others. Bags may offer a high enough surface area to volume ratio to avoid ice crystal formation during the vitrification process. It remains to be seen if they are as effective as the straw method, or if a more creative solution is needed.

In summary, both CaseCryo DMSO and CaseCryo NON-DMSO appear effective at maintaining cell health, viability, and functionality post thaw to varying degrees across

three cell types commonly found in the cell therapy space. In some cases, CaseCryo DMSO significantly outperformed CaseCryo NON-DMSO, however both generally met or exceeded the expectations set by the current industry standard solutions. Targeting molecular pathways, such as temporary inhibition of ADAM17 in NK cells, appears to improve cell function post thaw and provides promising groundwork for future studies. Lastly, vitrification may be a viable alternative to the current slow freezing process if the hurdle of scalability can be summited.

# MATERIALS AND METHODS

## *hPSC Cell Culture*

Human Pluripotent Stem Cells (hPSCs) were maintained in ExCellerate™ iPSC Expansion Medium (R&D Systems) with 100 units/mL HyClone Antibiotic Antimycotic (Cytiva) on plates coated with 0.1mg/mL Cultrex™ UltiMatrix RGF BME (R&D Systems). hPSCs were passaged at 70% confluency (roughly every 4 days) after dissociation into a single cell suspension using ACCUMAX™ (STEMCELL Technologies). All hPSCs were cultured at 37°C in 5% $CO_2$ with daily medium changes.

## *T Cell Culture*

Peripheral blood mononuclear cells (PBMCs) from healthy human donors were isolated from fresh human peripheral blood leukopaks (STEMCELL Technologies). T cells (characterized by CD56-CD3+ expression) were isolated from the PBMC population using the EasySep Human T cell Isolation Kit (STEMCELL Technologies).

Feeder-free T-cells were maintained in RPMI-1640 (Gibco) supplemented with Glutamax™ (Gibco), 10% fetal bovine serum (Atlas Biologicals), and 2ng/mL of IL-2 (R&D Systems). Fresh medium was added every other day to maintain 1e6 cells/mL of medium. All PBMCs were cultured at 37°C in 5% $CO_2$.

## *NK Cell Culture*

PBMCs from healthy human donors were isolated from fresh human peripheral blood leukopaks (STEMCELL Technologies). NK cells (characterized by CD56+CD3-

expression) were isolated from the PBMC population using the EasySep Human NK Cell Isolation Kit (STEMCELL Technologies).

Feeder-free NK cells were maintained in Immunocult NK Expansion Medium (STEMCELL Technologies) supplemented with 10ng/mL of IL-21, IL-18,and IL-15, 2ng/mL of IL-2, and 10ng/mL of IL-12 (R&D Systems) added on day 0 only. All PBMCs were cultured at 37°C in 5% $CO_2$ with medium being doubled by volume every two days.

Feeder expanded NK cells were cultured in CTS AIM VTM (ThermoFisher) with 5% CTS Immune cell SR (ThermoFisher), and 100 IU/mL IL-2 (PeproTech), and co-cultured with X-irradiated (100 Gray) feeder cells (K562 expressing membrane-bound IL-21 and 41BB-L). All PBMCs were cultured at 37°C in 5% $CO_2$ with medium being doubled by volume every two days.

*Cryopreservation*

hPSCs were harvested at approximately 70% confluency via dissociation into a single cell suspension using ACCUMAX™ (STEMCELL Technologies). Cells were washed with CaseBase® Washing Medium (CaseBioscience) and split into equal volumes and cell numbers into th e appropriate number of conical tubes before being centrifuged (Beckman Coulter) at 80xg for 6 minutes.

NK and T cells were harvested on indicated days and suspended in CaseBase® Washing Medium (CaseBioscience) equal to 3x the original volume of the sample. The cell solution was then split equally into 15mL conical tubes (Falcon) equivalent to the number of

cryopreservation solution conditions. The conical were then centrifuged at 100xg for 6 minutes.

For all cell types, all supernatant was aspirated off without disturbing the cell pellet, and the cells were resuspended in the appropriate cryopreservation solution at 1e6 cells/mL, unless otherwise indicated. The cell solution was then distributed at 1mL of cell solution per cryovial (Corning) unless otherwise noted and placed into a Corning® CoolCell® Container at -80°C overnight. After approximately 16 hours, the vials were transferred to liquid phase $N_2$ for long term storage.

For thawing all cell types, the cryovials were removed from the liquid phase $N_2$ and immediately placed in a 37°C water bath for 2 minutes. After removal, 0.5mL CaseBase® Washing Medium (CaseBioscience) was added dropwise to each vial over 30s and allowed to rest for an additional 30s. 1mL CaseBase® Washing Medium was then added dropwise to each vial over 30s and the mixture was triturated gently to encourage homogeneity before resting an additional 30s. The contents of the cryovial were then transferred dropwise over 30s to a 15mL conical tube (Falcon) already containing 1mL CaseBase® Washing Medium. The cell solution was allowed to rest for an additional 30s before the total volume was increased dropwise to 6mL with CaseBase® Washing Medium. The volume was then increased to 10mL per conical dropwise with DMEM/F12 medium (ThermoFisher) before the cell solution was centrifuged for 6 minutes at 60xg. All but 0.5mL of the supernatant was removed, without disturbing the cell pellet, and 2.5mL of the appropriate cell medium was added before gently resuspending the cell pellet. Cells were then counted and plated according to type.

*Cell Counting and Confluency Quantification*

Cell counts for hPSCs were achieved using either hand counting via hemocytometer and Trypan Blue (ThermoFisher) or using the NucleoCounter® NC-202™ (Chemometec) and associated reagents. Cell counts for PBMCs were achieved using the NucleoCounter® NC-202™ (Chemometec) and associated reagents or Trypan Blue (ThermoFisher) and the Countess 3 (ThermoFisher) and associated reagents.

Confluency measurements for hPSCs were determined by using a BZ-X710 (Keyence) to take nine images of each well in a 3x3 manner covering the entirety of the well. Images were taken daily, roughly 24 hours after the previous day's images, or 24 hours after plating for the day 1 set of images. Images were imported into FIJI[49] and colony edges were identified using the Phantast plugin[50]. Phantast was then used to calculate the ratio of colony area to plate area, and resulting numbers were interpreted as percentage of confluency. The nine confluency measurements corresponding to the nine images taken of each well were averaged to achieve an overall confluency measurement for that day.

*Resazurin Based Cytotoxicity Assays*

Cells were seeded and cultured to confluency on a 96 well plate. At 0 hours, the existing medium was aspirated and replaced with 180uL per well of various test conditions and controls. The cells were then incubated at 37°C in 5% $CO_2$ for the noted amount of time. 4 hours before the assay's endpoint, 20uL of resazurin (alamarBlue™, ThermoFisher) was added per well and allowed to develop at 37°C in 5% $CO_2$ for the remainder of the assay (4

hours). Resazurin was not added to one well of each condition to correct for autofluorescence of both the cells and test medium. The plate was then read in endpoint mode using a CLARIOstar Plus microplate reader (BMG LABTECH) at an excitation/emission of 560/590nm. The resulting measurements in relative florescence intensity (RFI) indicate metabolic activity, with higher metabolic activity being interpreted as a higher amount of living cells.

*hSC Differentiation and Immunocytochemistry*

The Human Pluripotent Stem Cell Functional Identification Kit (R&D Systems) was used to differentiate hPSCs into endoderm, ectoderm, and mesoderm along with staining for different differentiation markers. Antibodies used to identify the differentiation state of hPSCs include the pluripotency marker Oct3/4 (Catalogue #967150, R&D Systems), the ectoderm marker Otx2 (Catalogue #AF1979, R&D Systems), the endoderm marker Sox17 (Catalogue #AF1924, R&D Systems), and the mesoderm marker Brachyury (Catalogue #AF2085, R&D Systems). The kit was followed as written using reagents provided. Cells were imaged using a BZ-X710 (Keyence).

WTC-11 induced pluripotent stem cells were cultured to roughly 30% confluency before switching the culture medium to N3 medium comprised of: DMEM-F12 (Gibco), B27 (R&D Systems), N2 supplement (R&D Systems), insulin (Thermo Fisher), and 2µg/mL doxycycline (Thermo Fisher). Medium exchange occurred daily with N3 medium for 8 days, when cells were stained for neuronal markers phalloidin (Catalogue #T7471, Thermo

Fisher) and neurofilament (SMI31 801601, BioLegend) following the staining protocol from above and imaged. Cells were imaged using a BZ-X710 (Keyence).

*Antibodies and Flow Cytometry*

The following antibodies and dyes were used for flow cytometry: Alexa Fluor 647- or Alexa Fluor 700-conjugated anti-CD56 (clone 2524C, R&D Systems), BV421-conjugated anti-CD16 (clone 3G8, BioLegend), PE-conjugated anti-TACE/ADAM17 (clone 111633, R&D Systems), PE-conjugated anti-NKp46 (clone 195314, R&D Systems), APC-conjugated anti-CD3 (clone UCHT1, R&D Systems), FITC-conjugated Live-or-Dye™ 488/515 (Biotium).

Flow cytometry was performed on a Cytek 3-laser Aurora flow cytometer (Cytek) or alternatively on a CytoFLEX S flow cytometer 514 (Beckman Coulter). All data were analyzed using FlowJo version 10.10.0 software (FlowJo LLC).

*Statistics*

Whenever possible, the determination of statistical significance was performed using Student T-Tests when comparing two conditions or Analysis of Variance (ANOVA) with Tukey's posthoc test when comparing multiple conditions. A p-value of $\leq 0.05$ was considered significant. Graphpad Prism was used to perform statistical analysis.

## REFERENCES


1. Shi, Y., Inoue, H., Wu, J. C. & Yamanaka, S. Induced pluripotent stem cell technology: a decade of progress. *Nat. Rev. Drug Discov.* **16**, 115–130 (2017).
2. emilie-plazannet. Overview of iPSC Clinical Trials Landscape. *Treefrog Therapeutics* https://treefrog.fr/2024/10/14/overview-of-ipsc-clinical-trials-landscape/ (2024).
3. Waldman, A. D., Fritz, J. M. & Lenardo, M. J. A guide to cancer immunotherapy: from T cell basic science to clinical practice. *Nat. Rev. Immunol.* **20**, 651–668 (2020).
4. El-Kadiry, A. E.-H., Rafei, M. & Shammaa, R. Cell Therapy: Types, Regulation, and Clinical Benefits. *Front. Med.* **8**, 756029 (2021).
5. Tabar, V. *et al.* Phase I trial of hES cell-derived dopaminergic neurons for Parkinson's disease. *Nature* **641**, 978–983 (2025).
6. Funakoshi, S. *et al.* Generation of mature compact ventricular cardiomyocytes from human pluripotent stem cells. *Nat. Commun.* **12**, 3155 (2021).
7. Butler, P. C. & Gale, E. A. M. Reversing type 1 diabetes with stem cell–derived islets: a step closer to the dream? *J. Clin. Invest.* **132**, e158305.
8. Arafat Hossain, M. A comprehensive review of immune checkpoint inhibitors for cancer treatment. *Int. Immunopharmacol.* **143**, 113365 (2024).
9. Sterner, R. C. & Sterner, R. M. CAR-T cell therapy: current limitations and potential strategies. *Blood Cancer J.* **11**, 69 (2021).
10. Müller, F. *et al.* CD19 CAR T-Cell Therapy in Autoimmune Disease — A Case Series with Follow-up. *N. Engl. J. Med.* **390**, 687–700 (2024).

11. Peng, L., Sferruzza, G., Yang, L., Zhou, L. & Chen, S. CAR-T and CAR-NK as cellular cancer immunotherapy for solid tumors. *Cell. Mol. Immunol.* **21**, 1089–1108 (2024).

12. Pike, R., Motlagh, D. & Hanley, P. Autologous versus allogeneic: the future of manufacturing and standardization in cell therapies. *Cell Gene Ther. Insights* **08**, 1083–1095 (2022).

13. Emborg, M. E. *et al.* Advantages and challenges of using allogeneic vs. autologous sources for neuronal cell replacement in Parkinson's disease: Insights from non-human primate studies. *Brain Res. Bull.* **224**, 111297 (2025).

14. Matsa, E. & Holt, A. Making the switch from autologous to allogeneic cell therapy. *Cell Gene Ther. Insights* **08**, 935–943 (2022).

15. Peng, L., Sferruzza, G., Yang, L., Zhou, L. & Chen, S. CAR-T and CAR-NK as cellular cancer immunotherapy for solid tumors. *Cell. Mol. Immunol.* **21**, 1089–1108 (2024).

16. Choo, S. Y. The HLA System: Genetics, Immunology, Clinical Testing, and Clinical Implications. *Yonsei Med. J.* **48**, 11–23 (2007).

17. Cottle, C. *et al.* Impact of Cryopreservation and Freeze-Thawing on Therapeutic Properties of Mesenchymal Stromal/Stem Cells and Other Common Cellular Therapeutics. *Curr. Stem Cell Rep.* **8**, 72–92 (2022).

18. Research, C. for B. E. and. KYMRIAH. *FDA* (2024).

19. Research, C. for B. E. and. BREYANZI (lisocabtagene maraleucel). *FDA* (2025).

20. Awan, M. *et al.* Dimethyl Sulfoxide: A Central Player Since the Dawn of Cryobiology, is Efficacy Balanced by Toxicity? *Regen. Med.* **15**, 1463–1491 (2020).

21. Kollerup Madsen, B., Hilscher, M., Zetner, D. & Rosenberg, J. Adverse reactions of dimethyl sulfoxide in humans: a systematic review. *F1000Research* **7**, 1746 (2019).

22. Malinin, T. I., Pegg, D. E., Perry, V. P. & Brodine, C. E. Long-term storage of bone marrow cells at liquid nitrogen and dry ice temperatures. *Cryobiology* **7**, 65–69 (1970).

23. Poisson, J. S., Acker, J. P., Briard, J. G., Meyer, J. E. & Ben, R. N. Modulating Intracellular Ice Growth with Cell-Permeating Small-Molecule Ice Recrystallization Inhibitors. *Langmuir* **35**, 7452–7458 (2019).

24. Elliott, J. A. W. Intracellular Ice Formation: The Enigmatic Role of Cell-Cell Junctions. *Biophys. J.* **105**, 1935–1936 (2013).

25. Murray, K. A. & Gibson, M. I. Chemical approaches to cryopreservation. *Nat. Rev. Chem.* **6**, 579–593 (2022).

26. Djuwantono, T. *et al.* A comparison of cryopreservation methods: Slow-cooling vs. rapid-cooling based on cell viability, oxidative stress, apoptosis, and CD34+ enumeration of human umbilical cord blood mononucleated cells. *BMC Res. Notes* **4**, 371 (2011).

27. Amann-Winkel, K. *et al.* Water's second glass transition. *Proc. Natl. Acad. Sci.* **110**, 17720–17725 (2013).

28. Chen, H., Zhang, L., Meng, L., Liang, L. & Zhang, C. Advantages of vitrification preservation in assisted reproduction and potential influences on imprinted genes. *Clin. Epigenetics* **14**, 141 (2022).

29. Kuleshova, L. L. & Lopata, A. Vitrification can be more favorable than slow cooling. *Fertil. Steril.* **78**, 449–454 (2002).

30. Zanotto, E. D. & Mauro, J. C. The glassy state of matter: Its definition and ultimate fate. *J. Non-Cryst. Solids* **471**, 490–495 (2017).

31. Flores, E. *et al.* Freezing-thawing induces alterations in histone H1-DNA binding and the breaking of protein-DNA disulfide bonds in boar sperm. *Theriogenology* **76**, 1450–1464 (2011).

32. Jenkins, E. C., Ye, L. & Silverman, W. P. Does the cryogenic freezing process cause shorter telomeres? *Cryobiology* **65**, 72–73 (2012).

33. Verheijen, M. *et al.* DMSO induces drastic changes in human cellular processes and epigenetic landscape in vitro. *Sci. Rep.* **9**, 4641 (2019).

34. Jaiswal, A. N. & Vagga, A. Cryopreservation: A Review Article. *Cureus* **14**, e31564.

35. Mace, E. M. Human natural killer cells: form, function, and development. *J. Allergy Clin. Immunol.* **151**, 371–385 (2023).

36. Kim, J., Phan, M.-T. T., Hwang, I., Park, J. & Cho, D. Comparison of the different anti-CD16 antibody clones in the activation and expansion of peripheral blood NK cells. *Sci. Rep.* **13**, 9493 (2023).

37. Heipertz, E. L. *et al.* Current Perspectives on 'Off-The-Shelf' Allogeneic NK and CAR-NK Cell Therapies. *Front. Immunol.* **12**, 732135 (2021).

38. Imširović, V., Wensveen, F. M., Polić, B. & Jelenčić, V. Maintaining the Balance: Regulation of NK Cell Activity. *Cells* **13**, 1464 (2024).

39. Cooper, M. A., Fehniger, T. A. & Caligiuri, M. A. The biology of human natural killer-cell subsets. *Trends Immunol.* **22**, 633–640 (2001).

40. Mark, C. *et al.* Cryopreservation impairs 3-D migration and cytotoxicity of natural killer cells. *Nat. Commun.* **11**, 5224 (2020).

41. Grzywacz, B., Kataria, N. & Verneris, M. R. CD56dimCD16+ NK cells downregulate CD16 following target cell induced activation of matrix metalloproteinases. *Leukemia* **21**, 356–359 (2007).

42. Wu, J., Mishra, H. K. & Walcheck, B. Role of ADAM17 as a regulatory checkpoint of CD16A in NK cells and as a potential target for cancer immunotherapy. *J. Leukoc. Biol.* **105**, 1297–1303 (2019).

43. Srpan, K. *et al.* Shedding of CD16 disassembles the NK cell immune synapse and boosts serial engagement of target cells. *J. Cell Biol.* **217**, 3267–3283 (2018).

44. Pomeroy, E. J. *et al.* A Genetically Engineered Primary Human Natural Killer Cell Platform for Cancer Immunotherapy. *Mol. Ther.* **28**, 52–63 (2020).

45. National Academies of Sciences, E. *et al.* Addressing Variability in Donor Tissues and Cells. in *Exploring Sources of Variability Related to the Clinical Translation of Regenerative Engineering Products: Proceedings of a Workshop* (National Academies Press (US), 2019).

46. Effects of ADAM10 and ADAM17 Inhibitors on Natural Killer Cell Expansion and Antibody-dependent Cellular Cytotoxicity Against Breast Cancer Cells In Vitro. *Anticancer Res.* **37**, (2017).

47. Lin, H. *et al.* Characterization and comparative analysis of multifunctional natural killer cell engagers during antitumor responses. *Cell Rep. Med.* **6**, 102117 (2025).

48. Levine, B. L., Miskin, J., Wonnacott, K. & Keir, C. Global Manufacturing of CAR T Cell Therapy. *Mol. Ther. Methods Clin. Dev.* **4**, 92–101 (2016).

49. Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat. Methods* **9**, 676–682 (2012).

50. Jaccard, N. *et al.* Automated method for the rapid and precise estimation of adherent cell culture characteristics from phase contrast microscopy images. *Biotechnol. Bioeng.* **111**, 504–517 (2014).