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Preservation of cell-based immunotherapies for clinical trials

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Abstract

In the unique supply chain of cellular therapies, preservation is important to keep the cell product viable. Many factors in cryopreservation affect the outcome of a cell therapy: 1) formulation and introduction of a freezing medium, 2) cooling rate, 3) storage conditions, 4) thawing conditions, and 5) post-thaw processing. This article surveys clinical trials of cellular immunotherapy that utilized cryopreserved regulatory, chimeric antigen receptor or gamma delta T cells; dendritic cells; or natural killer cells. Several observations are summarized from the given information. The aforementioned cell types have been similarly frozen in media containing 5–10% DMSO with plasma, serum or human serum albumin. Two common freezing methods are an insulated freezing container such as Nalgene Mr. Frosty and a controlled-rate freezer at a cooling rate of −1 °C/min. ~37 °C water bath has been universally used for thawing. Post-thaw processing of cryopreserved cells varied greatly: some studies infused the cells immediately upon thawing; some diluted the cells in a carrier solution of varying formulation before infusion; some washed cells to remove cryoprotective agents; others recultured cells to recover cell viability or functionality lost due to cryopreservation. Emerging approaches to preserving cellular immunotherapies are also described. DMSO-free formulations of the freezing media have demonstrated improved preservation of cell viability in T lymphocytes and of cytotoxic function in NK cells. Saccharides are a common type of molecule used as an alternative cryoprotective agent to DMSO. Improving methods of preservation will be critical to growth in the clinical use of cellular immunotherapies.

Keywords

cell therapies; cryopreservation; dendritic cells; NK cells; T cells

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Disclosure of Interest

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Introduction

The supply chain of cellular therapy is unique and distinct from that of other medical therapies. Cells are harvested from a living donor typically in a clinical location and then sent to a processing facility for selection, expansion, genetic modification, etc. before they are returned to a clinical location for administration (Figure 1). The cells must remain viable and functional along this complex supply chain. Ineffective methods of preservation limit growth in the use of cell therapies and contribute significantly to their cost. Effective methods of cryopreservation permit coordination of the therapy with patient care and completion of safety and quality control testing.

This paper reviews the common methods of preservation for cell-based immunotherapies used for clinical trials. The survey was conducted using publicly available databases: the WHO International Clinical Trials Registry Platform, the U.S. National Library of Medicine Clinical Trials Registry, the E.U. Clinical Trials Register, the (Japan) UMIN Clinical Trials Registry and the (Republic of Korea) Clinical Research Information Service. Certain cell therapy trials did not provide enough information to determine the state of the cells being administered to patients (fresh or cryopreserved). Cell types covered here are T lymphocytes, including chimeric antigen receptor (CAR) T cells, T regulatory cells (Tregs), and gamma delta ($\gamma\delta$) T cells, dendritic cells (DCs), and natural killer (NK) cells. Mesenchymal stromal cells (MSCs) are another cell type involved in a large number of clinical trials, where they are employed for their immunomodulatory effect. Recent reviews have already described methods of preserving MSCs[1,2], and thus this cell type has been omitted.

Factors that influence preservation of cellular therapies

The most common descriptions of cell preservation for clinical trials of cell therapies include the cryopreservation solution and the cooling rate. This section will provide a brief overview of the critical factors that can influence post-thaw recovery of a cellular therapy. More details on these processes can be found in a recent book[3]. Several factors affect the outcome of cellular therapy: 1) formulation and introduction of a freezing medium, 2) cooling rate, 3) storage conditions, 4) thawing conditions, and 5) post-thaw processing.

Formulation and introduction of a freezing medium:

Cells that are cryopreserved must be suspended in a specialized solution containing agents that help the cells survive the stresses of freezing and thawing (e.g. cryoprotective agents, CPAs). The most commonly used cryoprotective agents are dimethyl sulfoxide (DMSO)[4] and glycerol. Cryopreservation solutions are typically hypertonic, and cell losses can result from introduction of the solution (osmotic stress)[5]. Certain CPAs (i.e. DMSO) are toxic to cells, and cell losses are observed as a function of time of exposure (biochemical toxicity).

As a result, the introduction process must be optimized to minimize cell losses from osmotic stress and biochemical toxicity. The window of manufacturing both pre-freeze and post-thaw can be influenced by biochemical toxicity. For example, duration in which cells are exposed to DMSO-containing cryopreservation solutions is limited to 30 minutes pre-freeze and

post-thaw[6]. This restriction complicates the workflow considerably at both the site of manufacture and the site of use. DMSO-free multi-osmolyte solutions have effectively preserved MSCs[7] and T lymphocytes[8] and have provided improved cell stability. Therefore, these CPAs have prolonged the window of manufacturing both pre-freeze and post-thaw.

Cooling rate:

The rate of change of temperature with time from the freezing temperature of the solution to roughly −50 °C is the cooling rate of interest during freezing. Studies have demonstrated that for a wide range of cell types, the post-thaw recovery is strongly influenced by the cooling rate[9]. The prevalence of controlled rate freezing for cell therapy products demonstrates the importance of the cooling rate on post-thaw survival.

Storage conditions:

Once the freezing process is complete, samples are placed into storage. Cell therapy products may be stored for days, weeks or even decades. The scientific basis for selection of a storage temperature is beyond the scope of this review article, but more information on this issue can be found in Hubel *et al.* [10]. Best practices for storage suggest that cell therapy products be stored at temperatures < −150 °C[3]. The stability of a cell therapy product in cryogenic storage is of interest. As with any biospecimen, transient warming of a frozen sample, which occurs when the storage unit is accessed to remove or add samples, is a potential cause of degradation[11]. Proper training or the use of robotic sample retrieval technologies can reduce the potential for degradation of the samples in storage.

Thawing conditions:

Samples are not used in the frozen state and are only useful after thawing. Therefore, the same temperature range traversed during cooling has to be traversed during warming. Standard practice has been to immerse frozen cellular products in a 37 °C water bath and swirl the product to enhance the thawing rate. Recently, controlled thawing devices have been developed that do not use a water bath, reduce the potential for contamination and improve the consistency of the thawing process. Not surprisingly, the proper thawing rate is influenced by the cooling rate used[9]. The thawing process has been studied far less than the cooling process, and a recent study suggests that warming rates are not critical for slowly cooled samples[12].

Post-thaw processing:

Cells that are frozen and thawed may be infused directly, diluted, or washed and suspended in a carrier solution. Cryopreservation solutions are typically hypertonic, and post-thaw wash solutions are typically designed to reduce the osmotic stress associated with dilution or washing, as cells are more sensitive to expansion than contraction[13]. The product infused into the patient reflects the nature of post-thaw processing (diluted, washed or non-manipulated cells).

As described above, a variety of factors influence the post-thaw recovery of cells. In describing the freezing process used for a clinical trial, the most common method is to

describe the composition of the cryopreservation solution and the cooling rate, and the review given below will use that common notation. Including a brief description of the post-thaw processing, so that it is clear the nature of the product being infused into the patient, is also helpful.

Cryopreserved T lymphocytes in clinical trials

Regulatory T-cell therapy

Hematopoietic stem cell transplantation (HSCT) has been implemented successfully for the treatment of hematological malignancies since the late 1950s[14]. However, the incidence of graft-versus-host disease (GvHD) following allogeneic stem cell transplantation is associated with various adverse effects in patients and results in a decrease in survival. Regulatory T cells (Tregs) as a subpopulation of lymphocytes have been explored to mitigate the severity of GvHD due to their capabilities of modulating immune responses and maintaining immunological self-tolerance and immune homeostasis[15,16]. In addition to adoptive transfer of Tregs as a prophylaxis against acute GvHD[17,18] in the setting of HSCT, Tregs have also attracted much research interest in the field of organ transplantation and autoimmune disease treatment[19].

The majority of current immunotherapies rely on the enrichment of Tregs derived from cord blood or peripheral blood and subsequent infusion of fresh Tregs to patients. Only a few clinical studies used cryopreserved Tregs with documentation of the freezing medium and protocols (see Table 1). Some studies only utilized cryopreserved cells when necessary due to patient difficulties and delay[20,21]. 10% DMSO in intravenous infusion solution with a varying concentration of human serum albumin (HSA) was most frequently used as the freezing medium in these studies. Cryopreserved cells were further stored in liquid nitrogen (LN). The freezing method and thawing method were not mentioned in most studies. After thawing, Tregs were either diluted with dextran and HSA solution before infusion or directly infused to patients. In one study, non-expanded Tregs were cryopreserved and after thawing, cells were washed into culture media and expanded for 7 days for a second infusion of Tregs[22]. In another study, cryopreserved Tregs were found to maintain their phenotype and suppressive function after being stored for 3 months in the vapor phase of LN[23].

CAR T-cell therapy

Chimeric antigen receptor (CAR) T-cell therapy is another novel immunotherapy in which T cells from patients are genetically modified followed by ex vivo expansion and infusion [24].

Two immunotherapies based on CD19-targeted CAR T cells have been approved by the FDA to treat two types of hematologic cancers: acute lymphoblastic leukemia (KYMRIAH) and diffuse large B-cell lymphoma (YESCARTA and KYMRIAH)[25]. Other B-cell antigens such as CD20, CD22, and κ light chain have also been targeted in clinical studies for the treatment of different types of lymphoma and leukemia, and studies using CAR T cells targeting B-cell maturation antigen have revealed very promising clinical outcomes for the treatment of multiple myeloma[26]. In addition to hematologic cancers, T cells have also

been genetically modified to target antigens of solid tumors such as prostate cancer[27] and malignant pleural mesothelioma[28,29], although the successful development of CAR T-cell therapy for solid tumors has its unique challenges[25].

Several clinical studies have used cryopreserved CAR T cells, some of which reported the details of freezing medium and protocols (see Table 1). Similar to Tregs, CAR T cells were most frequently frozen in 10% DMSO in intravenous infusion solution with a varying concentration of HSA. Plasma-Lyte A was the most common intravenous infusion solution used in these studies. Some studies have lowered the concentration of DMSO in the freezing medium from 10% to 7.5% or 5% [30,31]. In addition to DMSO, some clinical trials also included dextran and dextrose in the freezing medium[30]. The use of dextran could prevent aggregation of DNA and proteins from lysed cells as well as swelling of cells during thawing. Similar to Tregs, the majority of CAR T-cell therapy clinical studies did not clearly state the freezing and thawing methods. A few studies mentioned using a controlled-rate freezer to freeze cells[23,32] and a 36 to 38 °C water bath to thaw cryopreserved cells[30,33]. Unlike Tregs, the vast majority of CAR T-cell studies infused the cryopreserved cells immediately upon thawing. It is noteworthy that in two clinical trials[34,35], only a portion of the patients received cryopreserved cells, while the rest of the patients received fresh cells.

Gamma delta (γδ) T-cell therapy

Another small subset of T cells, gamma delta (γδ) T cells, has also been investigated for safety and efficiency in immunotherapy due to its broad functionality including production of cytokines and interaction with infected cells or other immune cells[36]. Unlike most T cells, with T-cell receptors (TCRs) composed of an alpha and a beta chain, γδ T cells express TCRs composed of a gamma and a delta chain, making the activation of γδ T cells MHC-independent. The clinical application of γδ T cells has evolved into two basic methods: stimulation of γδ T cells by drugs in vivo and adoptive transfer of enriched γδ T cells population[37]. For the second method, γδ cells are purified from peripheral blood mononuclear cells (PBMCs), and expanded ex vivo to obtain sufficient cells before infused into patients. As the majority of γδ T-cell clinical trials used fresh cells, information regarding cryopreservation of gamma-delta cells for clinical trials is rare. A research study found that γδ T cells expanded from umbilical cord blood (UCB) and cryopreserved in the complete medium with 10% DMSO maintained tumor-killing capacity showing a potential for clinical application[38].

Cryopreserved dendritic cells in clinical trials

Dendritic cells (DCs), a type of antigen presenting cell, function to process antigens and present them on the cell membrane to T cells. DCs act as a bridge between the innate and adaptive immune systems[39]. DCs emerged in immunotherapy with a first clinical trial in 1996 and gained a first and so far only FDA product approval in 2010. DC-based immunotherapy aims to treat various types of cancer or in some cases[40,41], human immunodeficiency virus (HIV) infection by eliciting and boosting a patient's immunity against diseased cells, and hence they are often called DC vaccines. Production of a DC

therapy frequently involves harvesting monocytes from the patient, differentiating them into mature DCs and then loading the cells with tumor antigens, resulting in a final product[42]. Loading of the cells with disease antigens occurs by a variety of different payload delivery methods: incubation with lysed or apoptotic cancer cells; fusion with live, irradiated cancer cells; and electroporation with disease antigen peptide or its encoding mRNA[42]. Cells at different stages of production may be cryopreserved.

Current methods of cryopreserving DCs that are found in practice of DC-based immunotherapy are listed in Table 2. Most clinical trials cryopreserved fully-processed, mature, antigen-loaded DCs at the end of the workflow. There were few exceptions of cryopreserving intermediate, immature or non-loaded DCs while providing freshly cultured DC vaccines to patients. All clinical trials used at least 5% DMSO as a CPA in the freezing media, most of which were formulated in HSA, serum or plasma. Two of the most common freezing modalities were freezing containers (e.g. Nalgene, Mr. Frosty) and controlled-rate freezers (e.g. Planer), both used with a desired cooling rate of 1 °C/min. Long-term storage temperatures at the end of the freezing process varied slightly within the cryogenic range, from −150 °C (vapor phase LN) to – 196 °C (liquid phase LN). A 37 °C water bath was used by all to thaw cryopreserved DCs. However, post-thaw processing varied among the different trials. CPAs were removed by washing or simply diluted in a carrier solution prior to administration of DC vaccines, and the composition of the carrier solution varied.

While the first dose of each multi-dose treatment commonly was freshly harvested DC vaccine, cryopreservation has been used for the remaining doses. The surveyed cryopreserved DC-based clinical trials were also primarily in phase I and II (except for DCVax®-L[43] in phase III and DEN-STEM recruiting in phase II/III), so the supply chain was shortened by in-house manufacturing and on-site administration. As these DC vaccines progress into later phases of clinical investigation and multi-site studies, centralized manufacturing and distribution will become necessary. The cryopreservation methods of these vaccines will be more stringently challenged in their effectiveness and reproducibility upon the introduction of longer storage times and transportation distances.

Cryopreserved natural killer cells in clinical trials

Natural killer (NK) cells are considered to be a critical part of the innate immune system, given their ability to kill tumor and infected cells without prior exposure to them[44]. Naïve NK cells can undergo activation in the presence of stressed, non-self cells with or without antibodies present. The activation is determined by a balance between responses from the inhibitory receptors and the activating receptors on the NK cell membrane[45]. Activated NK cells can act as effector cells releasing cytokines and chemokines to recruit and activate B and T lymphocytes. They are also cytolytic in a nonspecific manner, releasing cytotoxic granules containing perforin and granzymes that cause the target cells to lyse. It was recently discovered that NK cells have a more direct role in the adaptive immune system than initially assumed. In the presence of certain viruses, NK cells can act as memory cells that recognize viral glycoproteins and contribute to adaptive immunity similarly to the antigen recognition by other immune cells[46].

NK-cell immunotherapies have been used to treat a variety of malignancies, especially leukemias. While initial NK-cell studies focused on the treatment of acute myeloid leukemia, NK cells have also demonstrated effective treatment of solid tumors, including melanoma[47]. Before the emergence of NK cellular therapy, IL-2 was commonly administered to treat cancers. IL-2 stimulates endogenous NK cells and enhances their immunity[48] and, as the mechanism of action became better understood, called attention to NK cells as a potential cellular therapy[47]. Both autologous and allogenic NK cells have been clinically used to treat cancers[49–52].

Current protocols for obtaining, expanding and administering NK cells are variable. NK cells are traditionally isolated from allogenic PBMCs or UCB by removing all or some of the other cell types in the sample[53]. The isolated NK cells may then be expanded to obtain a clinically relevant cell quantity using a variety of growth factors and other activating molecules[54]. Allogeneic haploidentical NK cells have shown efficacy on tumors without inducing rejection or severe side effects in patients[55]. NK cells can also be derived from embryonic or induced pluripotent stem cells[56]. Robust allogeneic cell lines such as NK-92 have been used to develop cellular therapy products and can be more reliable than primary NK cells to achieve consistent product and large-scale production, though efficacy remains to be determined[54,57].

Of over 600 clinical trials involving NK cells in the US registry, less than 15 entries mentioned the use of cryopreserved NK cells. The use of fresh versus cryopreserved cells is likely due to observed poor post-thaw viability and functionality of NK cells[49,50,58,59]. In one study directly comparing fresh NK cells and NK cells that had been frozen, thawed and recultured, it was shown that the cryopreserved cells did not show proliferation in the patients who received the cellular therapy[50]. It is noteworthy that cryopreserved NK cells were shown to have fair viability (70%) but poor cytotoxicity after thawing[60]. The cytotoxicity could be regained by reculturing the cryopreserved cells in media containing IL-2, but significant cell losses were also observed in the post-thaw culture over time. For this reason, most clinical trials have opted for the use of fresh NK cells in their therapies. The use of fresh, non-cryopreserved cells creates a critical problem for NK-cell therapies that administer multiple doses, as infusing fresh cells for all doses would require either multiple, timed collections of donor cells or continuous maintenance of donor cell culture to span the timeframe of the multi-dose treatment. Other clinical trials have opted for using fresh cells for the first infusion and cryopreserved cells for the rest, where it is challenging to provide consistent dosage and efficacy to patients between the fresh and cryopreserved cells[52]. Many of the clinical trials that did use cryopreserved NK cells referenced cell banks but made no mention of freezing medium formulation, freezing method, etc. [49,51,59,61].

The cryopreservation methods that could be found for these clinical trials are summarized in Table 3. All but one of the studies used freezing media containing 10% DMSO. Plasma and serum were also common components of the freezing media. Controlled-rate freezers were used in both studies that specified freezing methods, although cooling rate was not specified. Most studies stored their NK cells in vapor phase LN, and one stored the cells in LN without specifying liquid or vapor phase. All studies used a 37°C water bath for rapid thawing,

although post-thaw processing varied among them. A few groups recultured the cells after thawing, one group washed the cells to remove CPAs, and two groups immediately administered the thawed cells in the freezing medium.

Emerging approaches

Most clinical trials of the aforementioned cell types used freezing media containing 5–10% DMSO. However, DMSO-free freezing media may be an advantageous alternative for clinical applications of cryopreserved cells in the future. One recent T-cell study found that when cryopreserved in a DMSO solution, the post-thaw recovery of $CD3^+CD4^+$ and $CD3^+CD8^+$ subpopulations were lower than that of other subpopulations in leukapheresis products[62]. Another study found that the use of 10% DMSO for cryopreservation resulted in lower post-thaw viability for most lymphocyte subpopulations, especially $CD3^+CD4^+$ and regulatory T cells, as well as growth arrest of $CD3^+$ cells in the G2/M phase[63]. These might account for loss of functional cells in cryopreserved T-cell product for immunotherapy. In comparison, DMSO-free freezing media composed of sugars, sugar alcohols and amino acids have been demonstrated to successfully preserve Jurkat cells as a $CD3^+CD4^+$ T lymphocyte model with high post-thaw viability[8,64] and can potentially be used to cryopreserve other types of T cells. Similar to T cells, while NK cells cryopreserved in DMSO have shown significant functional loss in terms of their cytotoxicity[60], a DMSO-free freezing medium composed of poly-l-lysine, dextran and ectoine has demonstrated improved post-thaw cytotoxicity in cryopreserved NK cells[65]. Unlike T cells or NK cells, DCs have not been the focus of recent advancement in cryopreservation technologies. However, DMSO-free freezing media may improve the immunobiological function of cryopreserved DCs by avoiding DMSO-induced depolymerization of the cytoskeleton[66] essential to the antigen-receptor binding-mediated cell signaling.

As healthy cells collected from donors can be significantly expanded in vitro, allogeneic cell therapies are usually manufactured in large batch, where multiple units of cells are introduced to cryopreservation medium sequentially. In these batches, the CPA exposure time and therefore, post-thaw outcome vary greatly. As a result, the need for DMSO-free freezing medium is urgent, as cells are more stable over time in DMSO-free solutions than in DMSO solutions. In addition to the freezing medium, new technologies of freezing and thawing cells for clinical applications have been developed. For example, LN-free controlled-rate freezers as well as dry thawing devices have been developed, as alternatives to LN-based freezers and water baths to prevent contamination of the cell therapy products and provide more consistent, standardized methods for thawing.

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Abbreviations

CAR	Chimeric Antigen Receptor
Treg	Regulatory T Cell

γδ	Gamma Delta T Cell
DC	Dendritic Cell
NK	Natural Killer Cell
MSC	Mesenchymal Stromal Cell
CPA	Cryoprotective Agent
DMSO	Dimethyl Sulfoxide
HSCT	Hematopoietic Stem Cell Transplantation
GvHD	Graft-Versus-Host Disease
HSA	Human Serum Albumin
LN	Liquid Nitrogen
TCR	T-Cell Receptor
PBMC	Peripheral Blood Mononuclear Cell
UCB	Umbilical Cord Blood

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Highlights

- Preservation technology is essential to the supply chain of cellular therapy.
- Five factors in cryopreservation affect the outcome of a cell therapy.
- DMSO is the most commonly used CPA to freeze cells in cell-based immunotherapy.
- −1 °C/min is the most commonly used cooling rate to freeze cells for immunotherapy.
- Non-DMSO CPAs have demonstrated to be better than DMSO at preserving immune cells.

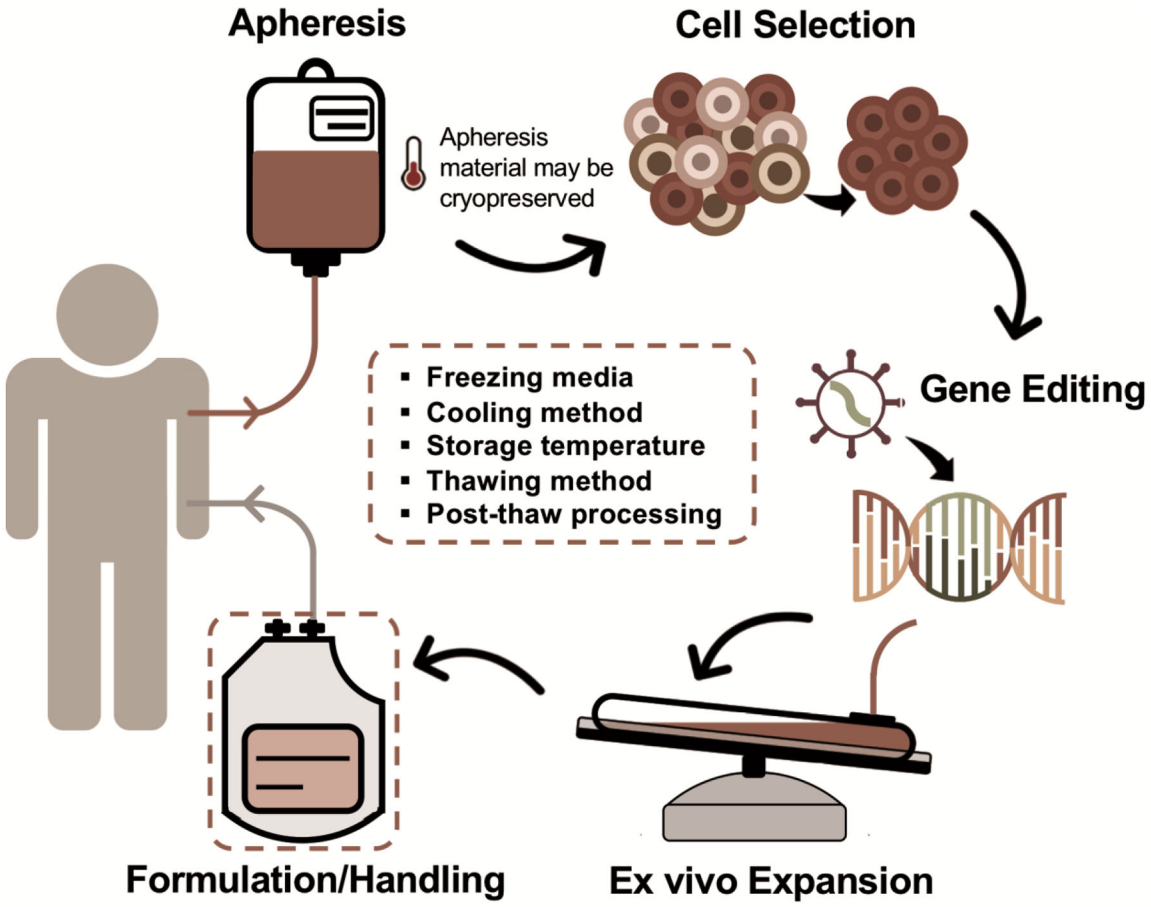

Figure 1.

A schematic of the unique supply chain of cellular immunotherapy where the 5 factors of cryopreservation play central role to keep cells viable. Cells are harvested from a donor (and sometimes cryopreserved at this point), subpopulations of cells selected; they may be genetically modified or antigen-based treatment to express the desired biological properties; expanded in vitro to achieve the desired cell dose, a cryopreservation solution introduced and the cells cryopreserved.

Table 1.

Methods of T-cell preservation for clinical trials

Study ID	Phase	Cell type	Disease	Freezing medium	Freezing method	Storage	Thawing method	Post-thaw processing	Institution	Ref.[1]
	I	Tregs	GvHD prophylaxis	Plasma-Lyte A, 10% DMSO, HSA	(unknown)	(unknown)	(unknown)	Diluted in 5% albumin, 10% dextran 40 and infused	University of Minnesota	[20,21]
EK 206082008	I	Tregs	GvHD prophylaxis	10% DMSO, 200g/L HSA	(unknown)	LN	(unknown)	Diluted and recultured	Technische Universitat Dresden	[22]
	I/II	Tregs	Liver transplant	CryoStor CS10	Controlled-rate freezer	Vapor phase LN	(unknown)	(unknown)	Guy's and St. Thomas' NHS Foundation Trust	[23]
EudraCT2006-004712-44	I/II	Ovalbumin-specific Tregs	Refractory Crohn's disease	(unknown)	(unknown)	Vapor phase LN	(unknown)	Infused immediately upon thawing	Huriez Hospital	[67]*
	I	CD19 CAR T cells	Leukemia, lymphoma	(unknown)	(unknown)	(unknown)	(unknown)	Infused immediately upon thawing	Baylor College of Medicine	[68]*
	I	κ light chain CAR T Cells	Lymphoma, myeloma, leukemia	(unknown)	(unknown)	(unknown)	(unknown)	(unknown)	Baylor College of Medicine	[69]*
	I/II	CD19 CAR T cells	Lymphoma	Cryostor CS5	Nalgene Mr. Frosty	(unknown)	(unknown)	(unknown)	City of Hope Medical Center	[31]
	I	CD19 CAR T cells	Multiple myeloma	31.25% Plasma-Lyte A, 31.25% dextrose, 0.45% NaCl, 7.5% DMSO, 1% dextran 40, 5% HSA	(unknown)	(unknown)	36 to 38 °C water bath	Infused immediately upon thawing	University of Pennsylvania	[30]
	I	CD19 CAR T cells	Leukemia, lymphoma	31.25% Plasma-Lyte A, 31.25% dextrose, 0.45% NaCl, 7.5% DMSO, 1% dextran 40, 5% HSA	(unknown)	(unknown)	36 to 38 °C water bath	Infused immediately upon thawing	University of Pennsylvania	[33,70–72]
	I	CD19 CAR T cells	Leukemia, lymphoma	50% HSA, 40% PlasmaLyte, 10% DMSO	Controlled-rate freezer (Planer)	Vapor phase LN	(unknown)	(unknown)	M.D. Anderson Cancer Center	[32,73,74]
	I	CD20 CAR T cells	Leukemia, lymphoma	Plasma-Lyte A, 5% HSA, 10% DMSO	(unknown)	(unknown)	(unknown)	Infused immediately upon thawing	Fred Hutchinson Cancer Research Center	[35,75]
	I	Her2 CAR T cells	HER2-positive sarcoma	(unknown)	(unknown)	(unknown)	(unknown)	(unknown)	Baylor College of Medicine	[76]*
	I	GD-2 CAR T cells	Neuroblastoma	(unknown)	(unknown)	(unknown)	(unknown)	(unknown)	Baylor College of Medicine	[77]*
	I	Mesothelin CAR T cells	Malignant pleural mesothelioma	(unknown)	(unknown)	(unknown)	(unknown)	Infused immediately upon thawing	University of Pennsylvania	[28,29]*
	I	3Ra2-redirected CD8CAR T Cells	Recurrent glioblastoma	(unknown)	(unknown)	(unknown)	(unknown)	Recultured	City of Hope Medical Center	[78]*
	I	Anti-CEA T cells	Liver metastases	PlasmaLyte, 20% HSA, 10% DMSO, IL2	(unknown)	LN	(unknown)	Infused immediately upon thawing	Roger Williams Medical Center	[79]

1 * denotes clinical trials that used cryopreserved cells but did not provide clear information of the freezing media.

Table 2.

Methods of dendritic cell preservation for clinical trials

Study ID	Phase	Disease	Cell type[2]	Freezing cell concentration	Freezing medium	Freezing method	Storage	Thawing method	Post-thaw processing	Institution	Ref.
	I	Acute myeloid leukemia	DC (R)	1e7 cells/ml	10% DM SO, 2% glucose in serum	Nalgene Mr. Frosty in −80 °C freezer	(unknown)	37 °C water bath	(unknown)	Antwerp University Hospital	[80]
	I	HIV	DC (P)	3e7 cells/ml	10% DM SO, 5% dextrose, 10% Plasma-Lyte A in serum	Controlled-rate freezer	−180 °C vapor phase LN	(unknown)	Diluted 10-fold in saline and infused	Baylor Research Institute	[41]
	I/II	Melanoma	DC (A)	3e7 cells/ml	10% DM SO, 5% dextrose, 10% Plasma-Lyte A in serum	Controlled-rate freezer (Planer), followed by 4–24 hours in −80 °C freezer	LN	(unknown)	Washed, resuspended in Ringer's lactate solution and infused	Baylor Research Institute	[81]
	II	Acute myeloid leukemia	DC (F)	5e6 cells/vial	10% DM SO in plasma	(unknown)	Vapor phase LN	(unknown)	Irradiated at 30cGy and infused	Beth Israel Deaconess Medical Center	[82]
	II	Multiple myeloma	DC (F)	0.5–5e6 cells/vial per cell yield	10% DM SO in plasma	(unknown)	Vapor phase LN	(unknown)	Irradiated at 30cGy and infused	Beth Israel Deaconess Medical Center	[83]
	I	Multiple myeloma	DC (F)	1, 2, 4e6 cells/vial per dose ranging	10% DM SO in plasma	(unknown)	Vapor phase LN	(unknown)	Irradiated at 30cGy and infused	Beth Israel Deaconess Medical Center	[84]
	I/II	Melanoma	DC (L)	1–1.5e6 cells/vial	(unknown)	(unknown)	LN	(unknown)	(unknown)	Cancer Insight, LLC	[85]
	I	Glioblasto ma	DC (P)	1e7 cells/ml	10% DM SO in serum	Nalgene Mr. Frosty in −80 °C freezer	Vapor phase LN	37 °C water bath	Infused immediately upon thawing	Cedars Sinai Medical Center	[86]
	I/II	Chronic myeloid leukemia	DC (P)	2e6 cells/ml	10% DMSO, 40% HSA, 5% FCS in RPMI	Controlled-rate freezer (Consartic) built-in PBMC program	LN	37 °C water bath	Washed, resuspended and recultured	Charite University	[87,88]
	II	Colorectal carcinoma hepatic metastasis	DC (L)	3e7 cells/aliquot	5% DMSO in serum	Programmable step down freezer (Carburos Metalicos)	−120 °C	(unknown)	Resuspended in saline and infused	Clinica Universidad de Navarra, Universidad de Navarra	[89,90]
	II	Metastatic cancer	DC (L)	3e7 cells/aliquot	5% DMSO in serum	Programmable step down freezer (Carburos Metalicos)	−120 °C	(unknown)	Resuspended in saline and infused	Clinica Universidad de Navarra, Universidad de Navarra	[90]
	I/II	Acute myeloid leukemia	DC (A)	(unknown)	10% DMSO (Cryostor CS10)	(unknown)	(unknown)	(unknown)	Infused immediately upon thawing	DCPrime BV	[91]
	I	Metastatic cancer	DC (R)	1–1.4e7 cells/vial	10% DMSO in serum	(unknown)	(unknown)	(unknown)	(unknown)	Duke University	[92]
	I	Multiple sclerosis	DC (P)	1e7 cells/vial	10% DM SO in serum	Nalgene Mr. Frosty in −80 °C freezer	LN	37 °C water bath	Washed with PBS and infused	Fundacio Institut Germans Trias i Pujol	[93]

Study ID	Phase	Disease	Cell type[2]	Freezing cell concentration	Freezing medium	Freezing method	Storage	Thawing method	Post-thaw processing	Institution	Ref.
EudraCT2007-007596-16	II	Breast cancer	DC (P)	minimum 5e6 cells/ vial	10% DM SO, 5% glucose in serum	Controlled-rate freezer (Planer)	(unknown)	(unknown)	Washed, resuspended and loaded with peptides	Herlev Hospital	[94]
	I	Breast cancer	DC (P)	1e7 cells/aliquot	10% DM SO, 5% glucose in serum	Controlled-rate freezer (Planer)	(unknown)	(unknown)	Washed, resuspended and loaded with peptides	Herlev Hospital	[95]
	II	Melanoma	DC (L)	0.5–1.5e7 cells/aliquot	Unknown formulation in saline	Automated freezer	(unknown)	(unknown)	Washed, resuspended in saline and infused	Istituto Scientifico Romagnolo per lo Studio e la cura dei Tumor	[96]
(unknown)	I	Leukemia	DC (A)	1–2e7 cells/ml	10% DM SO in plasma	Controlled-rate freezer (Planer)	−150 °C	(unknown)	Washed, resuspended in saline and infused	Karolinska University Hospital	[97,98]
UMIN000001 024	I	Acute myeloid leukemia	DC (A)	(unknown)	12% hydroxymethyl starch, 10% DMSO, 8% HSA in saline	(unknown)	−150 °C	(unknown)	Mixed with 1 KE OK-432	Kyoto University Hospital	[99]
	I	Ovarian cancer	DC (R) PROCURE®	2.6e7 cells/ml	10% DM SO, 5% glucose in saline	(unknown)	(unknown)	(unknown)	(unknown)	Life Research Technologies GmbH	[100]
	I	Recurrent, progressive malignant glioma	DC (S)	6.67–8e8 cells/vial	10% DMSO	(unknown)	−150 °C vapor phase LN	(unknown)	Recultured with tumor lysate	Masonic Cancer Center, University of Minnesota	[101]
	I/II	HIV	DC (R)	(unknown)	(unknown)	(unknown)	(unknown)	(unknown)	(unknown)	Massachusetts General Hospital	[40]
UMIN000011 423	I	Adult T cell leukemia/ lymphoma	DC (P)	5e6 cells/vial	10% DMSO, 50% serum, 40% RPMI 1640	Bicell biofreezing vessel (Nihon) −1 °C/min to −135 °C	(unknown)	37 °C water bath	Washed and infused	National Hospital Organization Kyushu Cancer Center	[102,103]
	I	Melanoma	DC (P)	0.1–1.5e7 cells/ml	10% DMSO in plasma	Controlled-rate freezer (Planer) −1 °C/min	LN	37 °C water bath	Placed on ice	New York University School of Medicine	[104]
	III	Glioblasto ma	DC (L) DCVax ®-L	0.4, 2, 4e6 cells/ml per dose escalation	(unknown)	(unknown)	(unknown)	(unknown)	(unknown)	Northwest Biotherapeutics	[43,105,106]
	I/II	Melanoma	DC (R)	0.5–2e7 cells/vial	10% DMSO, 50% HSA in CellGro DC medium	Controlled-rate freezer −1 °C/min to −40°C, 1–2°C/min to −80°C	LN	37 °C water bath	Washed, resuspended in PB S and infused	Oslo University Hospital	[107,108]
	I/II	Prostate cancer	DC (R)	2e7 cells/vial	(unknown)	Unknown method to −80 °C	LN	37 °C water bath	Washed, resuspended in PB S and infused	Oslo University Hospital	[109]
	I/II	Acute myeloid leukemia	DC (R)	1–1.25e7 cells/ml	10% DMSO, 5% glucose in HSA	Nalgene Mr. Frosty in −80 °C freezer	Vapor phase LN	(unknown)	(unknown)	Oslo University Hospital	[110]
	II/III	Glioblasto ma	DC (R) DEN-STEM	(unknown)	(unknown)	(unknown)	Vapor phase LN	(unknown)	(unknown)	Oslo University Hospital	[111]

Study ID	Phase	Disease	Cell type[2]	Freezing cell concentration	Freezing medium	Freezing method	Storage	Thawing method	Post-thaw processing	Institution	Ref.
	I	Melanoma	DC (L)	5e7 cells/dose	(unknown)	Nalgene Mr. Frosty in −80 °C freezer	−130 °C	(unknown)	Resuspended in 0.9% NaCl and infused	PrimeVax Immuno-Oncology Inc.	[112]
NTR1086	I	Multiple myeloma	DC (R)	(unknown)	(unknown)	(unknown)	(unknown)	(unknown)	Washed twice with 0.9% NaCl and 5% HSA and infused	Radboud University Nijmegen Medical Centre	[113]
	I	Melanoma	DC (P)	0.3, 1, 3e6 cells/vial per dose ranging	10% DMSO in unknown basal formulation	(unknown)	(unknown)	(unknown)	Loaded with peptides	Radboud University Nijmegen Medical Centre	[114]
KCT0000008	II	Hepatocel lular carcinoma	DC (P) CreaVax-HCC	3e7 cells/vial	5% DMSO in HSA or plasma	Ultralow freezer for >12h	Below −150 °C	(unknown)	Infused immediately	Seoul National University Hospital	[115,116]
KCT0000427	I/II	Hepatocel lular carcinoma	DC (P) CreaVax-HCC	3.3e7 cells/ml	5% DMSO in HSA or plasma	Ultralow freezer for >12h	Below −150 °C	(unknown)	Infused immediately	Seoul National University Hospital	[116,117]
	I/II	Prostate cancer	DC (A)	(unknown)	10% DMSO (CryoStor CS10)	(unknown)	LN	(unknown)	(unknown)	SOTIO a.s.	[118]
(unknown)	I/II	Prostate cancer, renal cell carcinoma	DC (L)	3e6 cells/ml	12% DMSO, 44% FCS, 44% RPMI 1640	Nalgene Mr. Frosty in −70 °C freezer	LN	37 °C water bath	Washed, resuspended and recultured	St. George's Hospital Medical School	[119,120]
	I/II	Melanoma	DC (P)	1e7 cells/ml	10% DMSO, 5% glucose +/− serum	Nalgene Mr. Frosty in −80 °C freezer	Vapor phase LN	(unknown)	Loaded with peptides	University Hospital Erlangen	[121–123]
	I	Melanoma	DC (P)	1e7 cells/ml	10% DMSO, 5% glucose +/− serum	Nalgene Mr. Frosty in −80 °C freezer	Vapor phase LN	(unknown)	Loaded with peptides	University Hospital Erlangen	[122,123]
	I	Type 1 diabetes	DC (R)	1e7 cells/aliquot	(unknown)	(unknown)	(unknown)	(unknown)	Diluted to 1e6 cells/ml in PBS and infused	University of Pittsburgh	[124]
	I	Melanoma	DC (P)	1.5e7 cells/ml	10% DMSO, 40% AIM V, 50% serum	Nalgene Mr. Frosty in −80 °C freezer	LN	37 °C water bath	Diluted in saline and 5% HSA and infused	University of Southern California	[125]

[2](A) denotes DCs pulsed with apoptotic cancer cells; (F) denotes DCs fused with irradiated cancer cells; (L) denotes DCs pulsed with tumor lysate; (P) denotes DCs pulsed with disease antigen peptides; (R) denotes DCs pulsed with disease antigen-encoding mRNAs; (S) denotes DCs pulsed with tumor stem cells.

Table 3.

Methods of natural killer cell preservation for clinical trials

Study ID	Phase	Cell type	Freezing cell concentration	Disease	Freezing medium	Freezing method	Storage	Thawing method	Post-thaw processing	Institution	Ref.
(unknown)	I	NK-92	5e7 cells/mL vial	Advanced, treatment resistant malignancies	10% DMSO, 40% plasma, 50% X-Vivo 10	(unknown)	(unknown)	(unknown)	Recultured	Institute for Transfusion Medicine and Immunohematology, Germany	[126]
(unknown)	I	NK-92	(unknown)	Advanced renal cell cancer and melanoma	(unknown)	(unknown)	(unknown)	(unknown)	Recultured	Rush University Medical Center	[61]
	I	Activated NK	2e7 cells/vial	Refractory or relapsed acute myeloid leukemia	(unknown)	(unknown)	(unknown)	(unknown)	Recultured	University of Pittsburgh	[49]
EudraCT 2005006087–62	I	CD56+ NK	(unknown)	Acute myeloid leukemia	X-Vivo10	(unknown)	Vapor phase LN	37°C water bath	Infused immediately upon thawing	Royal Free Hospital, UCL Medical School	[127]
(unknown)	IIa	Activated NK	<2e8 cells/mL	Lymphoma, metastatic breast cancer	10% DMSO, 20% plasma, 20% Medium 199	Controlled-rate freezer	LN	37°C water bath	Infused immediately upon thawing	University of Pittsburgh	[128]
	II	Expanded NK	2e7 cells/mL	Multiple myeloma	10% DMSO, 40% HBSS, 50% or 25% HSA	Controlled-rate freezer (CryoMed)	Vapor phase LN	(unknown)	(unknown)	University of Arkansas	[50,58]
	I	Invariant NK-T	(unknown)	Malignant melanoma	(unknown)	(unknown)	(Unknown)	(unknown)	(unknown)	Beth Israel Deaconess Medical Center	[51]
	I	mbIL21-NK	(unknown)	Myeloid malignancies	40% PlasmaLyte, 50% serum, 10% DMSO	(unknown)	(unknown)	37°C water bath	Washed, resuspended in 0.5% HSA in PlasmaLyte and infused	University of Texas MD Anderson Cancer Center	[52]
	I	Activated NK	(unknown)	High-risk solid tumors	(unknown)	(unknown)	(unknown)	(unknown)	Recultured	National Cancer Institute	[59]