UNIVERSITY OF CINCINNATI

Date: November 19, 2007

I, _____Kathryn Lee McKay_____,
hereby submit this work as part of the requirements for the degree of:

Master of Science

in:

Transfusion and Transplantation Sciences

It is entitled:

A Cryopreservation Protocol to Retain T Cell Function and Viability for Use in Donor Leukocyte Infusion

This work and its defense approved by:

Chair: Thomas B. Leemhuis, PhD
Jose A. Cancelas, MD
Patricia M. Carey, MD
Brian Susskind, PhD
Susan L. Wilkinson,EdD,MT(ASCP)SBB

A Cryopreservation Protocol to Retain T Cell Function and Viability for Use in Donor Leukocyte Infusion

A thesis submitted to the Division of Research and Advanced Studies of the University of Cincinnati in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

In the Department of Analytical and Diagnostic Sciences of the College of Allied Health Sciences and the University of Cincinnati Academic Health Center

Hoxworth Blood Center,

Committee Chair: Thomas B. Leemhuis, PhD

For the year 2007

By

Kathryn L. McKay, B.S., MT(ASCP)^{CM}

B.S. Ohio University, 2001

ABSTRACT

BACKGROUND: Donor leukocyte infusions (DLI) have recently become more widely used as therapy for the treatment of disease relapse after allogeneic stem cell transplantation. DLIs have been shown to mediate the crucial graft vs. tumor effect needed to reach remission. The white blood cells that make up a DLI, specifically T cell populations, will recognize and destroy cancer cells and will facilitate engraftment by combating residual host T cell populations.[1] The ability to store these therapeutic cells until they are needed is important, but there is inadequate data regarding the analysis of their function after cryopreservation. Routine cryopreservation methods currently consist of protocols designed for stem cell storage, however it may be important to apply different techniques to different cell types, such as T cells. It is vital to the nature of this therapy that T cells remain viable and functional post thaw. STUDY DESIGN AND METHODS: This study was conducted in order to compare two widely used cryopreservation processes, currently used for hematopoietic stem cells, and determine their ability to retain T cell viability and function. Peripheral blood mononuclear cells (PBMCs) from normal donors were cryopreserved in 10% dimethylsulfoxide (DMSO) or 5% DMSO with 3% hetastarch (HES). Fresh vs. frozen/thawed samples were then compared post thaw to determine T cell viability and recovery. To evaluate T cell proliferation, cell cycling ability, and cytokine production, specifically IFN-γ, T cells were mitogen stimulated and evaluated at different time points in culture. RESULTS: No significant differences were found between cryopreservation methods in the post thaw viability and recovery of T cells. In addition, cell cycle distribution was not significantly different between fresh and frozen cells. However, there was a clear delay in the ability of the cells cryopreserved with 10% DMSO to produce IFN-γ. This 10% DMSO method also showed a delay in its ability to support T cell expansion.

CONCLUSIONS: The cells frozen in 5% DMSO and 3% HES did not behave significantly different than fresh cells in terms of cell expansion and IFN-γ production. T cell products cryopreserved for DLI in 10% DMSO showed a disadvantage in their ability to proliferate in vitro, and to produce IFN-γ in response to PHA stimulation.

ACKNOWLEDGEMENTS

I would like to thank each department at Hoxworth for their patience, support and willingness to help whenever and wherever they could. I especially would like to thank Jan Habel and Cathy Beiting for their endless support and for always watching out for me. I truly appreciate the hours of listening and support you have given me over the last year. To Antoinette Birchmore, for her "pep-talks" and advice, not only in my studies, but also in life. To Dr. Jose Cancelas, to whom I am indebted to for acting as a co-advisor on my thesis project. Thank you for taking the time, whether you had it or not, to not only teach me, but to give me the tools to continue to learn. You have challenged me to become a better scientist. To Dr. Tom Leemhuis, for not only serving as my thesis advisor, but for creating the Cellular Therapies program and allowing me the opportunity to be the first student to embark on this journey. I am grateful and excited for my future and for the future of this innovative therapy and feel privileged to be a part of it. To Nicole Worsham, for her countless hours of flow cytometry help and for always taking the time to assist when I had "just one quick question." To Shannon, for being my sounding board and for her endless patience. I would most like to thank my parents Brett and Judy McKay who have never wavered in their support of me pursuing my dreams. I thank them for teaching me about life and about people, making me proud of the person I have become.

TABLE OF CONTENTS

LIST OF ABREVIATIONS

7AAD	7-Amino-Actinomycin-D
APC	Allophycocyanin
BMT	Bone Marrow Transplant
BrdU	Bromodeoxyuridine
CD	Cluster of Differentiation
CFC	Colony Forming Cells
CFSE	Carboxyl Fluorescein Succinimidyl Ester
CML	Chronic Myelogenous Leukemia
DLI	Donor Leukocyte Infusion
DMSO	Dimethyl Sulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
GVHD	Graft vs. Host Disease
GVT	Graft vs. Tumor
HES	Hetastarch
HLA	Human Leukocyte Antigen
HPC	Hematopoietic Progenitor Cells
HSA	Human Serum Albumin
IFN-γ	Interferon gamma
IL-2	Interleukin 2
LSM	Lymphocyte Separation Medium
mAb	Monoclonal Antibody

MAPK	Mitogen-activated protein kinase
MHC	Major Histocompatibility Complex
mL	Milliliter
mM	Millimolar
PBMC	Peripheral Blood Mononuclear Cells
PE	Phycoerythrin
PHA	Phytohemagglutinin
RPM	Revolutions per Minute
U	Units
µg	Micrograms
µl	Microliters
µM	Micromolar

LIST OF FIGURES

INTRODUCTION AND CLINICAL SIGNIFICANCE

High dose chemotherapy followed by allogeneic hematopoietic stem cell transplantation can be an effective therapy for many with otherwise fatal malignancies. Because marrow toxicity is dose limiting for many of these agents, transplantation of hematopoietic stem cells contained in bone marrow or peripheral blood made it possible to administer higher doses of therapy. However, in many patients, small numbers of leukemic cells have the ability to survive and the therapeutic benefit of allogeneic stem cell transplantation is largely related to an associated immune-mediated graft versus malignancy effect.

The initial evidence of the existence of a graft versus tumor (GVT) effect in humans came from studies reporting that relapse rates following allogeneic transplantation were markedly reduced in patients who developed graft versus host disease (GVHD) compared with those who did not.[1] Additional studies revealed that patients who develop acute or chronic GVHD have the lowest risk of relapse when compared to those who develop no clinically evident GVHD. The risk is especially higher in those that receive T cell depleted marrow grafts.[2] Evidence of the GVT effect continued with infusion of donor lymphocytes in hopes of inducing GVHD and an associated anti-tumor effect. Somewhat surprisingly, sustained complete responses were seen in many patients with hematological malignancies.[3] With increased recognition of the potential of an immunotherapeutic approach, clinical research is beginning to focus on development of methods to better exploit the strength of the GVT effect.

Histocompatibility and transplantation

In general, the purpose of an allogeneic stem cell transplant is to replace malignant or genetically defective hematopoietic stem cells with healthy, functional stem cells. Bone marrow or peripheral blood stem cells from one person, the donor, are used for infusion into another, the recipient. Whether a relative or an unrelated donor is used, the cells are immunologically different.[4] T cells are of particular concern when dealing with stem cell transplants because of their ability to recognize both foreign antigens and self antigens. Cells of the immune system express HLA (Human Leukocyte Antigen) molecules on their cell membranes where they present fragments of self and non-self proteins for recognition by antigen-specific T cells. The antigen receptors on each T cell recognize a portion of a self-HLA molecule. In the case of bone marrow transplantation, foreign HLA molecules from the graft (or donor bone marrow/stem cells) serve as targets for T cell receptors in the recipient, initiating a response that can eventually lead to loss of graft function.[5]

Effect of conditioning on engraftment of hematopoietic stem cells

High dose chemotherapy or systemic radiotherapy must be administered to the recipient pretransplant to eradicate host T cells sufficiently to prevent graft rejection. Therefore, before transplant, patients must be “conditioned” with radiation and chemotherapy to deplete their own marrow cells and allow space in the marrow for transplanted cells to establish themselves. This preparative regimen greatly suppresses the host’s immune system permitting successful transplantation. Following a successful

allogeneic transplant, grafted cells will “rescue” the patient from the immunosupressive side effects of chemotherapy. However, allogeneic stem cells are readily rejected by even a minimally immunocompetent host. Even after successful engraftment, two frequent problems remain, GVHD and immunodeficiency.[6]

Induction of immunological tolerance

GVHD occurs when transplanted allogeneic lymphoid cells (T cells) react against alloantigens of the recipient (host). The host is immunocompromised and unable to reject the allogeneic cells in the graft. This can manifest clinically as skin rashes, diarrhea, hepatitis, an increased susceptibility to infection, and in severe cases, death. Significant numbers of T cells reside in the bone marrow, putting BMT patients at risk for developing GVHD, the principal limitation to the success of transplantation. The development of clinically significant GVHD, although associated with a reduced risk of leukemic relapse, leads to poorer overall survival owing to the direct effects of the disease and the consequences of the immunosuppression used to treat it.[6,7]

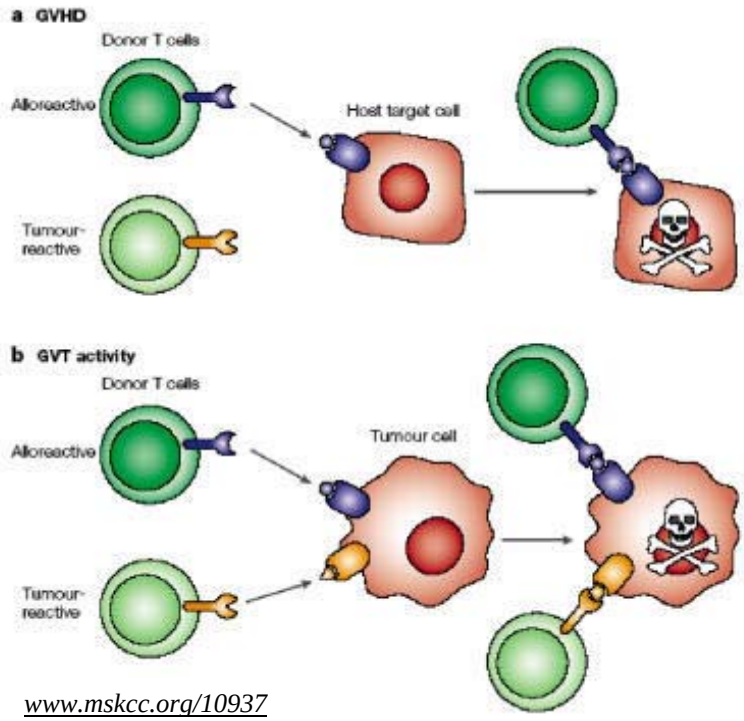
In order to avoid this complication, it is important to match both the donor and recipient in terms of their HLA phenotypes at the allele level. GVHD may represent a life-threatening complication of allogeneic stem cell transplantation. Prolonged, it can produce a chronic GVHD that, in addition, induces hematopoietic failure, severe cellular immunodeficiency, obliterant bronchiolitis and opportunistic infections (e.g., cytomegalovirus).[7]

DLI: a delicate balance between GVT and GVHD

In many myeloablated patients and more frequently in patients submitted to "low-dose conditioning" regimens, small numbers of leukemic cells may have the ability to survive the initial high-dose chemotherapy and generate relapse of the disease. Administration of T cells can be effective in destroying residual leukemic cells by the GVT effect. This process is directed, albeit not exclusively, at the allogeneic MHC molecules present on the recipient's hematopoietic cells, including the leukemic cells. The challenge in using this treatment to improve clinical outcome is to minimize the GVHD that may be mediated by the same donor T cells.[8]

Donor leukocyte infusions (DLI) are the infusion of a transplant donor's leukocytes (white blood cells) that contain cells of the immune system which can recognize and destroy cancer cells. This phenomenon was first described in 1990 by Kolb et al.[9] when "buffy-coat" cells from the original transplant donor were given to three patients with relapsed CML, inducing a cytogenetic remission. The effectiveness of this type of immunotherapeutic treatment has since been studied and confirmed by several groups.[10] DLI are composed of granulocytes, monocytes, and lymphocytes. There is speculation surrounding which of these different types of cells mediate the GVT effect. The exact cell populations that control this mechanism are unknown and require further study. However, literature suggests elimination of T cells from the donor marrow decreases the GVT effect that is critical in treating leukemia. If this is the case, DLI administration can balance T cell numbers to prevent rejection, hence facilitating engraftment, and fight tumor cells.[11] It is the hope that the immunocompetent cells will kill residual malignant cells (foreign) and can be harnessed to induce a GVT effect. The idea is immunological rejection of patient's leukemia cells following transplantation.[6] (See Figure 1)

Figure 1: Mechanism of Graft vs. Host Disease and Graft vs. Tumor



www.mskcc.org/10937

Figure 1. Mechanism of graft versus host disease and graft versus tumor effect is mediated by donor T cells. GVHD develops as donor T cells react by recognizing alloantigens on the host cells. A GVT effect is directed at the alloantigens present on host leukemia cells.

DLI is a common strategy for patients experiencing relapse after a transplant from a related or unrelated donor.[12] It is an immune-mediated therapy for patients in cytogenetic or hematological relapse and may be an alternative to traditional relapse therapies such as salvage chemotherapy and in rare cases, "boost" allogeneic transplants. These therapies may prolong survival and induce complete remission, but side affects cannot always be tolerated by some patients. The main reason for an interest in an immunologic approach like DLI is that current therapies for cancer rely on drugs that kill dividing cells or block cell division and these treatments also have an effect on normal proliferating cells. Extended therapy may be necessary for long term disease control and long-term effects of these

therapies can seriously diminish quality of life because patients can be symptomatic throughout their treatment and even after treatment is completed. DLI may offer a better outlook for prolonged survival than other non-immunological methods.[13,14]

The success of DLI demonstrates the potential to harness the GVT effect of the human immune system for clinical benefit. However, separating the mechanism of the desired GVT effect and GVHD has been a challenge. Although it has been difficult to define precisely the effector cells responsible for GVT induction, donor T cells are likely the primary mediators of this reaction. Drobyski et al[15] used T cell depleted bone marrow for transplantation followed by targeted immunotherapy using DLI in patients with chronic phase CML. Results showed that removal of donor T cells from the original graft compromised the GVT effect and increased the relapse rate causing an overall decrease in survival. Nonetheless, the DLI infusions post-transplant were able to induce remissions in some patients. Similar studies indicate that recipients of T cell depleted marrow experience less GVHD but have a higher incidence of relapse and higher graft failure than patients with unmanipulated marrow for the same disease. For example, Horowitz et al[2] observed increased relapse in patients who received T cell depleted grafts indicating a loss of GVT activity, especially in patients with CML. Studies are being done to determine if the benefits of T cell depletion can be supplemented with DLI to restore GVT activity several weeks or months post transplant. It is clear that a strong correlation exists between DLI and GVHD and exploring the various T cell populations and their functions is critical to understanding graft rejection and leukemic relapse. For example, it is possible that CD8+ cells play a critical role in GVHD, not only in number, but in reactivity. Removal of CD8+ cells from the marrow before infusion (T cell depletion) reduces the risk of GVHD. The removal of CD8+ cells from DLI and enrichment of CD4+ cells may also reduce GVHD. Bunjes et al[16] saw an increase in host-reactive T helper precursors in five patients who responded to DLI and in

another study by Jiang et al[17], two patients treated with DLI for relapsed CML had an increase in cytotoxic T cell precursor activity against cryopreserved host leukemic cells relative to host lymphocytes. These studies provide indirect evidence that GVT and GVHD might be separable.

It appears as though CD4+ cells and CD8+ cells with reactivity to both the host and to leukemic cells exist and may be essential for GVT. Although removal of one type of T cell could prevent GVHD, it could also compromise the GVT effect. In addition, the approximate dose and timing of T cell administration may also contribute to the development of GVHD. Even though GVHD can be induced by DLI as quickly as the day of infusion, it may fail to develop when given as long as 9 months following BMT. It may be that a certain number of T cells are needed to obtain a GVT effect and that this amount may vary between patients depending on several factors including patient disease process, donor-recipient reactivity and compatibility, duration post-transplant and administration of other therapeutic drugs.[18,19] Dazzi et al[20] illustrated that rather than administering one large dose of lymphocytes (called a bulk dose regimen or BDR), the use of smaller escalated dose regimens (EDR) may be more effective. Although this did not affect the likelihood of remission, the smaller dose regimen did decrease the incidence of GVHD.

Manufacturing and cryopreservation of donor leukocyte products for transplantation

DLI are usually collected during the initial bone marrow or peripheral blood stem cell (PBSC) harvest, however, they can be collected at any time as no special medications are given to donors having a leukocyte collection. Mononuclear cells are collected through an apheresis procedure where the cells are removed from the blood and the blood is then

returned to the donor. For most DLI collections, the cells are not manipulated in vitro and based on the CD3+ cell count are given fresh to the patient. However, subsequent doses are often frozen in case the patient should need more cells or should suffer another relapse. If the CD3+ cells need to be removed from the initial graft, small aliquots are made from the CD34 negative cell fraction of the selected product. Once the stem cells are removed for transplant this CD34-/CD3+ cell fraction is separated into smaller CD3+ cell doses according to the patient's weight. The total product is usually divided into aliquots of $1x10^5$ cells per kilogram to $1x10^7$ cells per kilogram. The ability to collect and store these cells until they are needed would be more convenient and would help to avoid complications with donor recruitment and availability. T cells could be stored in pre-designated aliquots based on the dose required for that patient and be readily accessible.[21]

Due to the nature of DLI therapy, cryopreservation plays a crucial role in the long-term storage of these cells. This role is important not only in storage but also in evaluating clinical outcomes. A study by Eckardt et al[22] reported a reduction in GVHD with the use of a cryopreserved stem cell product. However, these data were obtained from a study that was not investigating the impact of cryopreservation on DLI directly. In addition, the increased use of cryopreserved products warrants a need for additional investigation to better clarify the best protocol for freezing and thawing to retain T cell viability and function.[23,24]

Despite numerous studies on the cryopreservation of hematopoietic cells from bone marrow and peripheral blood, very little information is available regarding the effects of cryopreservation on T cells. A cryopreservation composition that is non-toxic and useful for achieving the desired viability rates for cryopreservation of cells is necessary for cellular therapy. In the most commonly used method for cryopreservation of bone marrow or peripheral blood stem cells, mononuclear cells are re-suspended in a cryopreservation

medium containing 10% DMSO and cooled at 1°C/minute. However, previous studies have shown that a lower concentration of DMSO combined with HES can be used for cryopreservation of hematopoietic progenitor cells (HPC), peripheral blood stem cells (PBSCs), bone marrow and selected CD34+ cell products. This 5% DMSO and HES method was found to be equal or superior to 10% DMSO without HES with regard to both viability of thawed CD34+ cells and preservation of mature colony-forming cells (CFCs). Loss of cells that can not be recovered and the infusion of low cell numbers will have a significant influence on the outcome of the treatment.[25,26]

Current protocols do not include the impact of cryopreservation on clinical outcomes for DLI. Data on this subject is limited to bone marrow and peripheral blood stem cell grafts.[27-29] To better address this issue, two methods of cryopreservation used for PBSCs and bone marrow were tested for their ability to retain T cell function and viability after freezing to the same level as a fresh PBMC. Furthermore, it was the aim of this study to test a widely used cryopreservation method and compare it to an alternative method, used for stem cells, to determine if it could also be applied to cryopreservation of DLI products, specifically T cells. The focus was to isolate T cells from normal donors and evaluate the effects of cryopreservation by comparing viability and recovery after cryopreservation. In addition, cells were placed into culture and stimulated to divide in order to assess the ability of the cells to proliferate, progress through the cell cycle and produce IFN-γ in response to mitogenic stimulation.

OBJECTIVE

The need for an optimal method of cryopreservation of therapeutic cells until they are needed remains a crucial, but poorly understood aspect of hematopoietic cell processing.

There is minimal understanding of the impact of cryopreservation on the viability and function of T cells and most studies are limited to bone marrow and peripheral blood stem cells. Furthermore, the ability to store these cells and maintain function and viability post thaw is absolutely necessary for immunotherapeutic treatments, such as DLI.

The objective of this project was to examine T cell viability and function post thaw, using two cryopreservation methods frequently used for hematopoietic stem cells. These methods were compared to fresh products from the same donor and evaluated with in vitro assays. In addition, we evaluated the capability of these cryopreservation methods to maintain a high viable T cell recovery. If recovery of viable T cells is not significantly different than that of fresh cells, dosage adjustments would not be necessary to accommodate for loss of T cells during cryopreservation.

MATERIALS AND METHODS

Donor Material and Sample Preparation

Peripheral Blood Mononuclear Cells (PBMC) from three normal donors were purified from two hundred mL of whole blood using Lymphocyte Separation Medium (LSM) (MP Biomedicals, Aurora, OH) as described by the manufacturer. In brief, PBMC were purified by first centrifuging the whole blood in 50 mL conical tubes (Corning, Corning, NY) at 2900 rpm for 10 minutes and collecting the buffy coat layer. Combined buffy coat layers were diluted with Dulbecco's Phosphate Buffered Saline (DPBS), underlayed with 15 mL LSM, centrifuged at 1900 rpm for 30 minutes at room temperature and the interface layer collected into new 50 mL conical tubes. The cells were washed three times with RPMI 1640 medium, supplemented with 10% Fetal Calf Serum and 2 mM L-glutamine. Automated cell counts were performed using the Beckman Coulter Ac.T 5 diff CP (Beckman Coulter, Fullerton, CA). Three aliquots were made per donor for fresh and frozen (x2) analysis. Two aliquots

were cryopreserved with different media to compare the function and viability of each of the two freezing methods to a fresh product. Fresh aliquots were held overnight with autologous plasma at 4°C **(Fresh)**.

Cryopreservation and Thawing of PBMC

A process intended to mimic the clinical cryopreservation protocol of HSC in bags was designed for cryovials. In brief, two aliquots at approximately 50-80 million cells were diluted in 1 mL of a cold suspension buffer (Cryo A: HSA, DMSO, HES, and Plasmalyte or Cryo B: HSA, DMSO, and Plasmalyte). One mL of the appropriate cryopreservation media previously chilled to 4°C was added slowly to the cell suspension making the final freezing volume 2 mL in a 5 mL cryovial. Final concentrations were as follows: **Cryo A:** 5% DMSO, 3% HES; **Cryo B:** 10% DMSO. The samples were mixed, placed in cryovials on ice, transferred to a controlled rate freezer (Gordinier Model 9010 Controlled Rate Freezing System Gordinier Electronics Inc, Roseville, MI) and frozen at 1°C/minute until they reached a temperature of -20°C, then at 10°C/minute to -75°C. The vials were then placed into a liquid nitrogen freezer (vapor phase) and held overnight at -196°C.

The next morning, two cryovials for Cryo A and Cryo B were thawed at the same time in a 37°C water bath until ice crystals were melted. Each was diluted up to 4 mL with cold RPMI 1640 medium supplemented with 10% FCS and 2mM L-glutamine, transferred to 50 mL conical tubes, diluted up to 30 mL, and washed twice with the same media. Automated cell counts were performed on one aliquot from each freezing process and on the fresh product stored overnight.

Post Thaw Viability and Recovery

To determine the viability and recovery of T cells post thaw, automated cell counts were performed and samples were analyzed by flow cytometry. Lymphocyte surface antigens were identified by the following monoclonal antibodies (mAbs): anti-CD3 (APC), anti-CD4 (PE), and anti-CD8 (FITC); the number of viable cells was determined by staining with 7AAD. Samples were analyzed using FACSCalibur and CellQuest software (Becton-Dickson, San Jose, CA).

T Cell Proliferation

To evaluate T cell proliferation, cell cycling and cytokine production in response to mitogen stimulation, T cells from each donor were stimulated and grown in culture. First PBMCs were placed in 150x25-mm coated tissue culture plates and incubated at 37°C, in 5% CO_2 for 3 hours to deplete monocytes. Nonadherent cells were collected and filtered through a 100 µ filter to remove debris and automated cell counts were performed. Cells were resuspended at 10^6 cells per mL and plated into 24-well round bottom plates containing 1 mL of media consisting of RPMI 1640 with 10%FCS supplemented with 2mM L-glutamine and 100 U/mL IL-2. Phytohemagglutinin (Sigma, St. Louis, MO) at a final concentration of 10 µg/mL was used to stimulate T cells that were incubated at 37°C, in 5% CO_2. To determine T cell expansion, responses were assessed at 24, 48, 72, and 96 hour time points. Separate cell culture plates remaining for subsequent time points were re-stimulated at 24 hour intervals. At each harvest, total cell number and viability were determined using a trypan blue exclusion test (Sigma, St. Louis, MO). Viable CD3+ cell numbers at each time point were calculated by multiplying total viable cell numbers by the %CD3+ cells, obtained by flow cytometry as previously described.

To evaluate cell cycle distribution at each time point, staining was performed with bromodeoxyuridine (BrdU) (BD Pharmingen BrdU flow cytometry kit, San Jose, CA). BrdU is similar in structure to the DNA precursor thymidine and is readily incorporated into newly synthesized DNA as cells progress through the S phase (DNA synthesis) of the cell cycle. Initially, BrdU was added directly to culture media containing PHA stimulated lymphocytes at a final concentration of 20 μM. The treated cells were then incubated at 37°C in 5% CO_2 for 40 minutes. After incubation, cells were stained with anti-CD3 mAb, fixed, permeabilized, to expose the incorporated BrdU, and stained with specific fluorescent anti-BrdU antibody (FITC) and 7AAD according to manufacturer's directions.[30] The stained cells were then analyzed on a FACSCalibur flow cytometer using CellQuest software (Becton-Dickinson, San Jose, CA). The cell histogram was then divided in regions according to cell cycle phases: G0/G1, S, and G2-M.

To determine the number of division cycles through which each cell has progressed (cell division tracking), cells were stained with a carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling technique. CFSE is minimally fluorescent until it is taken up by the cells and intracellular esterases remove 2 acetate groups causing fluorescence. As a result, CFSE binds covalently to proteins and is retained within the cell. With each cell division, the fluorescent intensity per cell division is reduced 50%, thus providing a read-out of the mitotic activity within a specific population of cells. Before placing cells in culture, CFSE stock solution was added to each aliquot for a final concentration of 2 μM. Cells were incubated for 10 minutes in a 37°C water bath, quenched with ice-cold RMPI/10%FCS, incubated for 5 minutes on ice then pelleted by centrifugation. Cells were washed with RPMI/10% FCS by re-suspending the cells a further two times for a total of three washes. After the last wash, cells were re-suspended to 10^6 cells per mL and placed into culture. One aliquot from each

method was analyzed by flow cytometry and used to determine background fluorescence and set the initial parameters in the fourth decade of fluorescence. [31-34]

Cytokine Production

To determine the ability of T cells to produce cytokine after cryopreservation, cell culture supernatants were harvested at the predetermined time points. In brief, culture wells were harvested into 12 mm x 75 mm polypropylene tubes by removing all of the cell culture media and then washing each well one time with 1 mL of 1xDPBS. The tubes were centrifuged at 1500 rpm for 5 minutes. One mL of supernatant was removed and aliquoted into cryovials and placed in a -80°C freezer until testing. INF-γ production was measured by a sandwich enzyme immunoassay technique commercially available as a kit from R&D Systems (Minneapolis, MN). In brief, standards provided in the kit were run in triplicate and then averaged. A standard curve was created by plotting the mean absorbance for each standard against concentration. Culture supernatants were diluted across 24 hour to 96 hour time points (1:2, 1:4, 1:8 and 1:8 respectfully), tested in triplicate and averaged. Sample concentrations were read from the standard curve and then multiplied by the dilution factor. Results are presented in picograms per 10^6 cells and were normalized by dividing the total IFN-γ production in each well (2 mL) by the number of viable T cells per well and multiplying by 10^6 cells.

Statistical Methodology

Data were analyzed using the Student's t test to compare the fresh to each cryopreservation method and then to compare the cryopreservation methods to each other. Statistically significant differences were noted as p-values <0.05.

RESULTS

Post Thaw Viability and Recovery

Viability was determined by flow cytometry immediately post thaw. Note that there was only a slight difference in the viability of the cells frozen with 5% DMSO and 3% HES (Cryo A) to the cells frozen with 10% DMSO (Cryo B), 93.4 ± 2.1% and 92.5 ± 2.7% respectively as compared to the fresh cells after the overnight hold at 93.6 ± 3.2% (Figure 2).

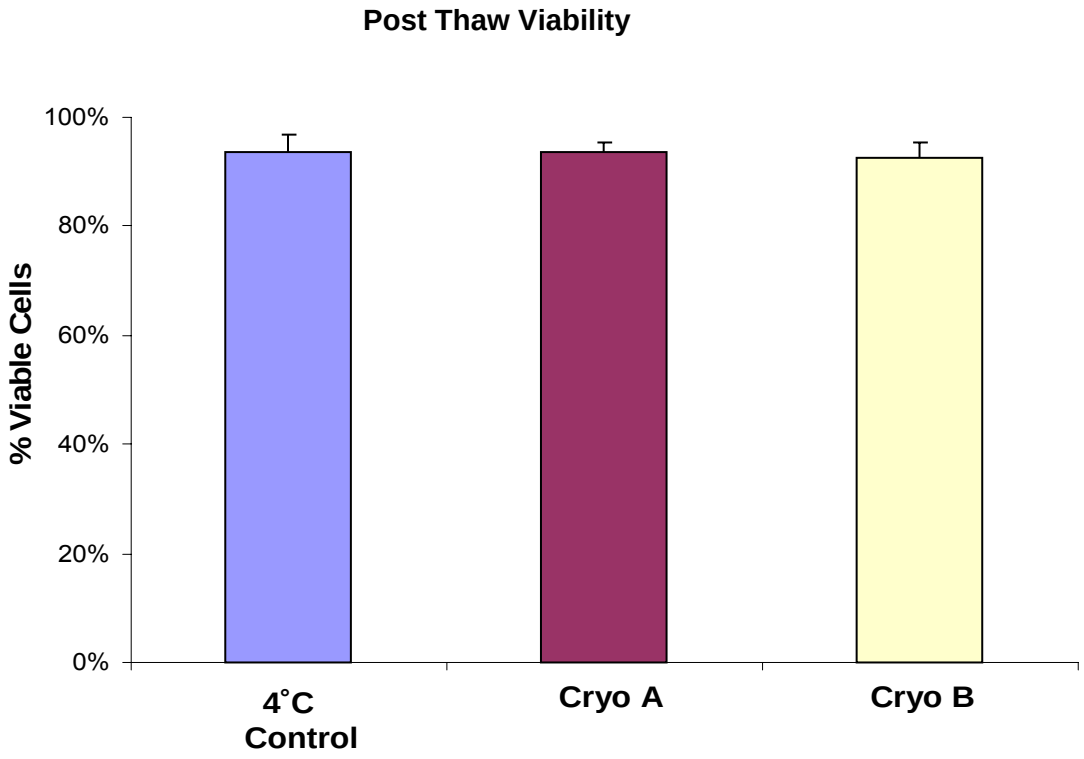


Figure 2. Post thaw viability for the two cryopreservation methods immediately following thaw was similar to the fresh cells which were held in autologous plasma overnight at 4˚C. The conditions tested were: Cryo Method A, 5% DMSO and 3% HES and Cryo Method B, 10% DMSO. Note that cell viabilities were similar in all methods tested (no significant differences as determined with Student's t test). Data are presented as a mean of 3 donors + standard deviation.

Total cell recovery was determined by automated cell counts before and after cryopreservation and after the overnight hold for fresh cells (Figure 3). The mean percent recoveries for cryopreserved cells were 96% for the Fresh Control, 83% for Cryo A and 89% for Cryo B.. The two methods showed no significantly different cell recoveries and they were not significantly different from the recovery of cells in the fresh sample (Cryo A; $p = 0.53$ and Cryo B; $p = 0.73$). There were no significant differences between the post thaw viability and cell recovery between each method demonstrating the ability of these methods to retain viability and cell recovery when compared to fresh cells (4˚C).

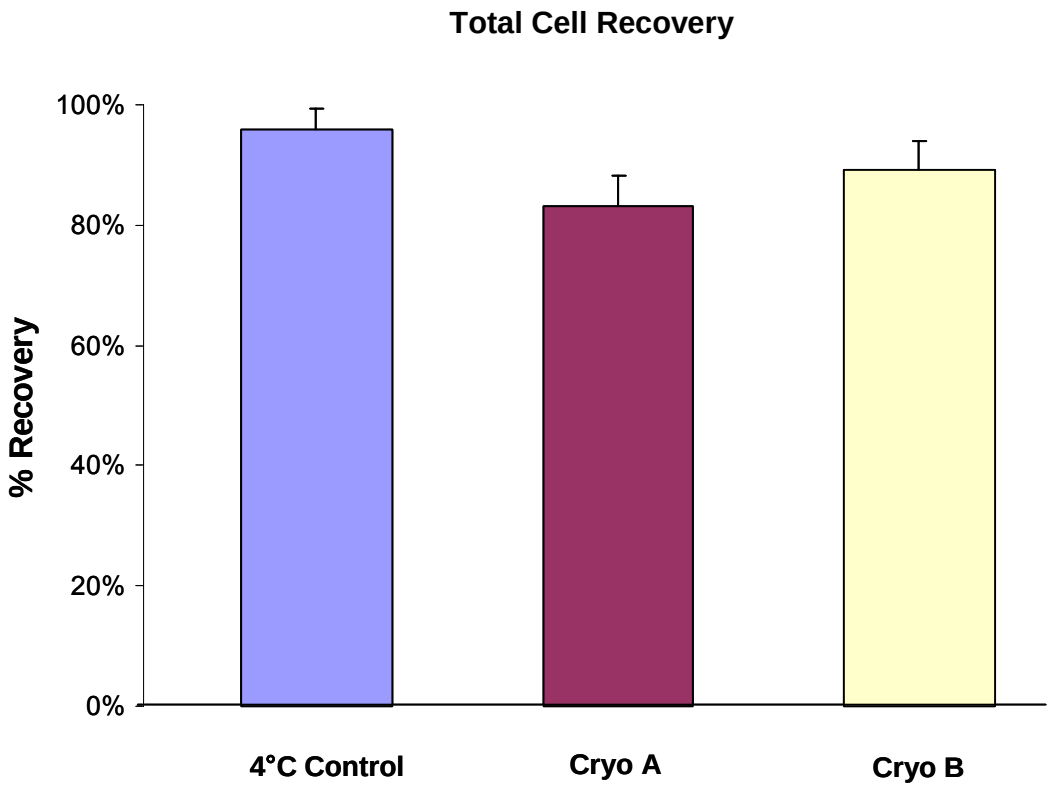


Figure 3. Total cell recovery performed before and after cryopreservation (Cryo A and Cryo B) was similar to the overnight hold (Fresh at 4˚C) method. Cell counts were obtained using the Beckman Coulter Ac.T 5 diff CP (Beckman Coulter, Fullerton, CA). Total cell recoveries are presented as the mean of 3 donors + standard deviations.

Figure 4 shows recovery of T cell subsets that were calculated by multiplying the %CD3+ cells by the automated cell count and then dividing by the starting number of CD3+ cells. The same was done for both CD4+ cells and CD8+ cells. Recovery for CD3+ cells was greater than 90% and for CD4+ cells and CD8+ cells was greater than 85% among all three methods. Percent recoveries among these groups were not statistically significant for T cell subsets as determined by the Student's t test. These data are consistent with previous studies indicating cell surface phenotype is least affected by cryopreservation and thaw procedures.[35]

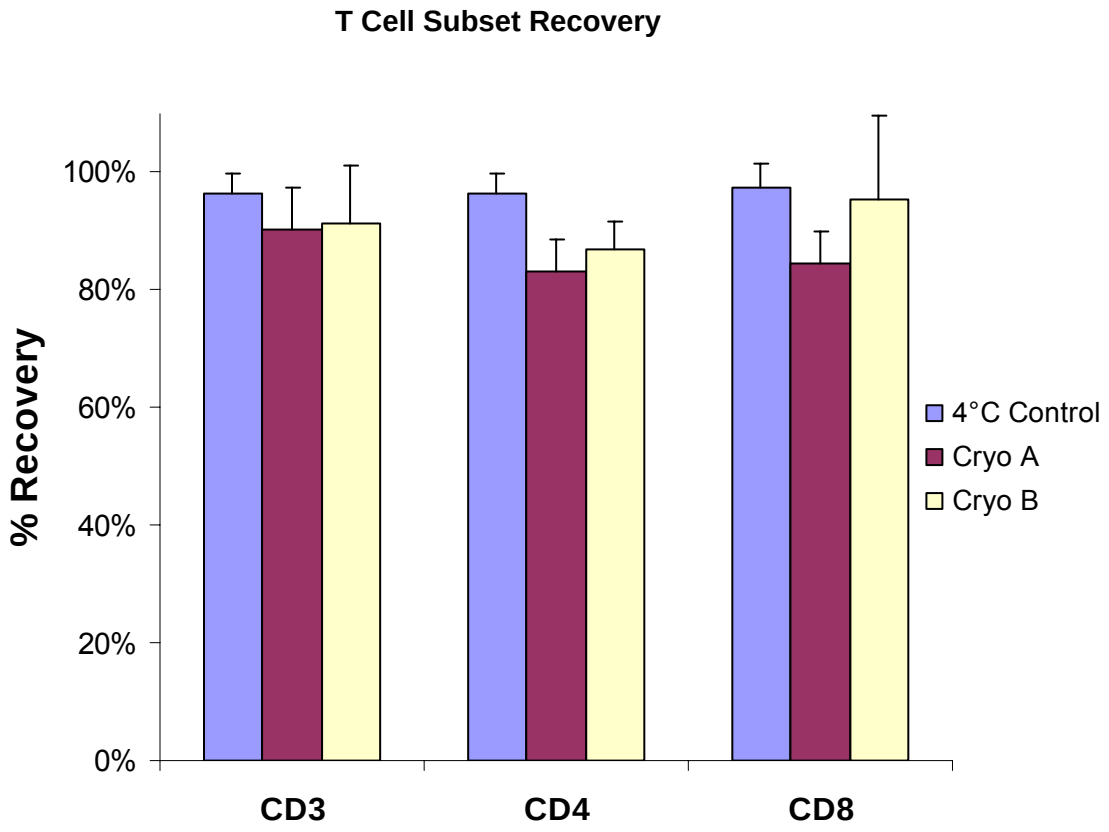


Figure 4. Recovery of T cells and T cell subsets after cryopreservation (Cryo A and Cryo B) and overnight hold at 4°C (Fresh). The % recovery of CD3+, CD4+, and CD8+ cells was determined to be similar in all three methods and no significant differences were noted using the Student's t test. Results are presented as mean of 3 donors + standard deviation.

T Cell Proliferation

To determine the effects of cryopreservation on T cell expansion, cells were placed in culture and stimulated with PHA for four days. At 24, 48, 72, and 96 hours, cells were harvested, counted and assessed for viability. Figure 5 shows the CD3+ cell expansion (fold) for each donor and the lines represent the median CD3+ cell expansion over the four day culture. Cells frozen with 10% DMSO showed inconsistent results among donors indicated by the large variation in fold expansion. At 24, 72, and 96 hours the coefficient of variation for Cryo B method was over 100% (128%, 119%and 141% respectively). In addition, two of the three donors never attained a fold expansion greater than one. It is clear that cells cryopreserved with 5% DMSO and 3% HES retain the ability to proliferate comparable to that of the fresh cells, whereas the cells frozen with 10% DMSO take longer to recover from the freezing process. There is an overall lag in their ability to proliferate as efficiently as the other cryopreserved cells.

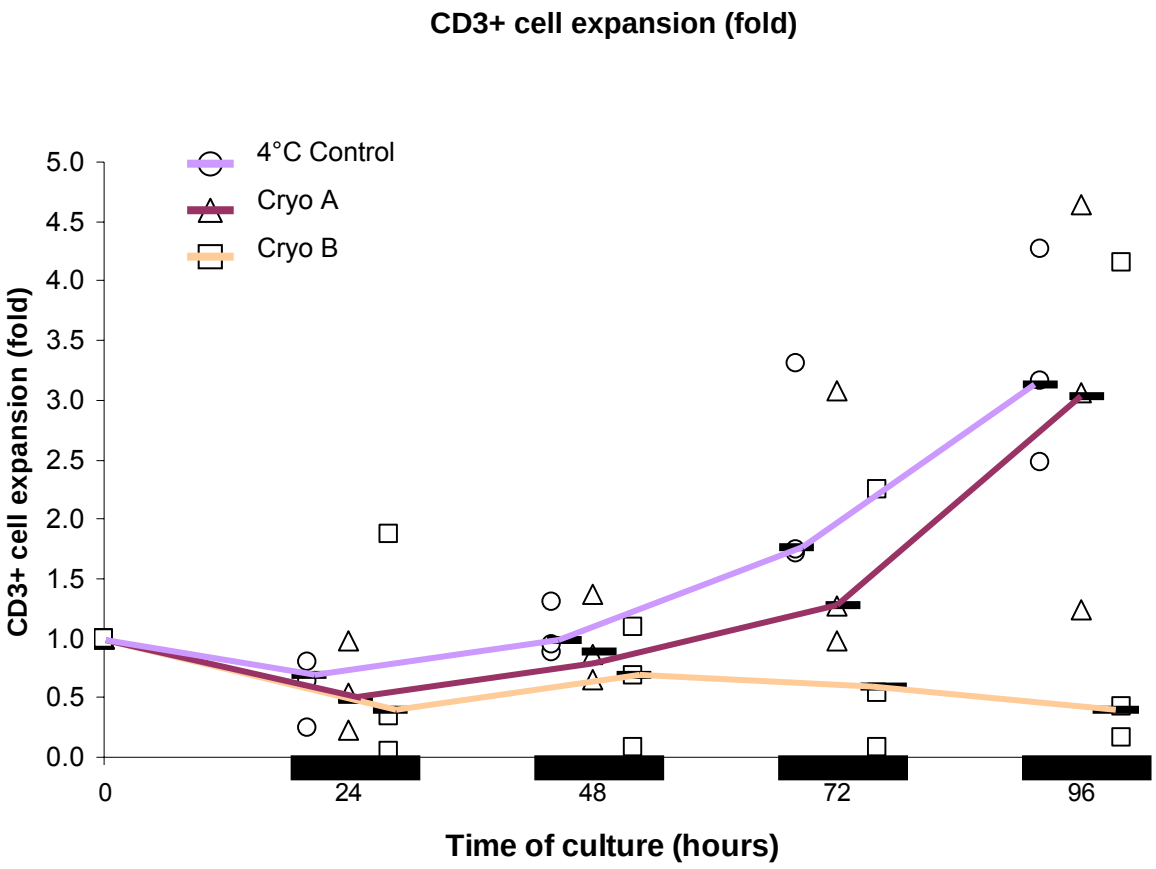
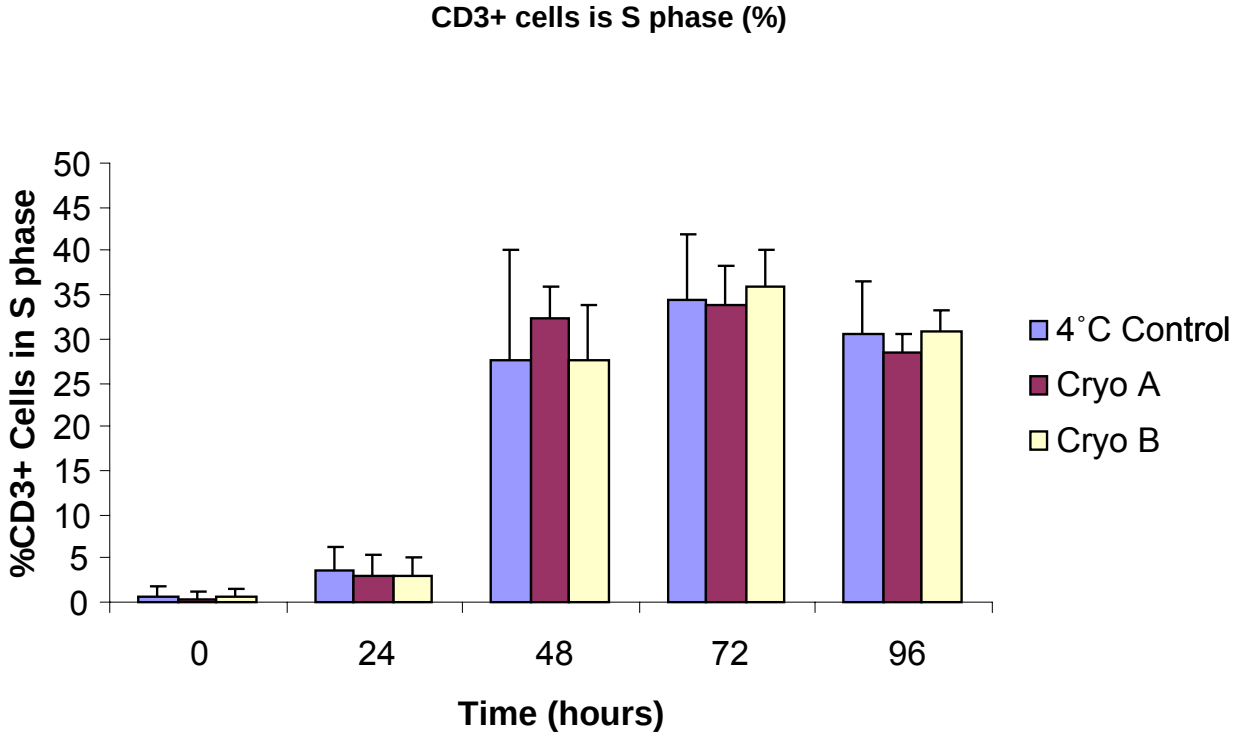


Figure 5. CD3+ cell expansion (fold) of Fresh and Cryo A methods showed a trend to be higher than for the Cryo B method. T cells had been incubated with PHA (10 µg/mL) at 37°C for four days. At 24, 48, 72, and 96 hours, cells were collected, counted and assessed for viability (see Materials and Methods). CD3+ cell counts from each donor are plotted and lines represent the median cell expansion in three replicate samples.

Cell Cycle Distribution

To evaluate T cell cycle distribution after cryopreservation, fresh and frozen cells were stimulated in culture for four days with PHA and then stained with BrdU and analyzed by flow cytometry. The percentage of T cells in the S phase and G2 + M phase were very similar at each time point (Figure 6a and 6b). Compared to the fresh samples, there was no apparent change in cryopreserved cell cycling patterns of T cells after 96 hours, $p > 0.05$, between the three methods.

A



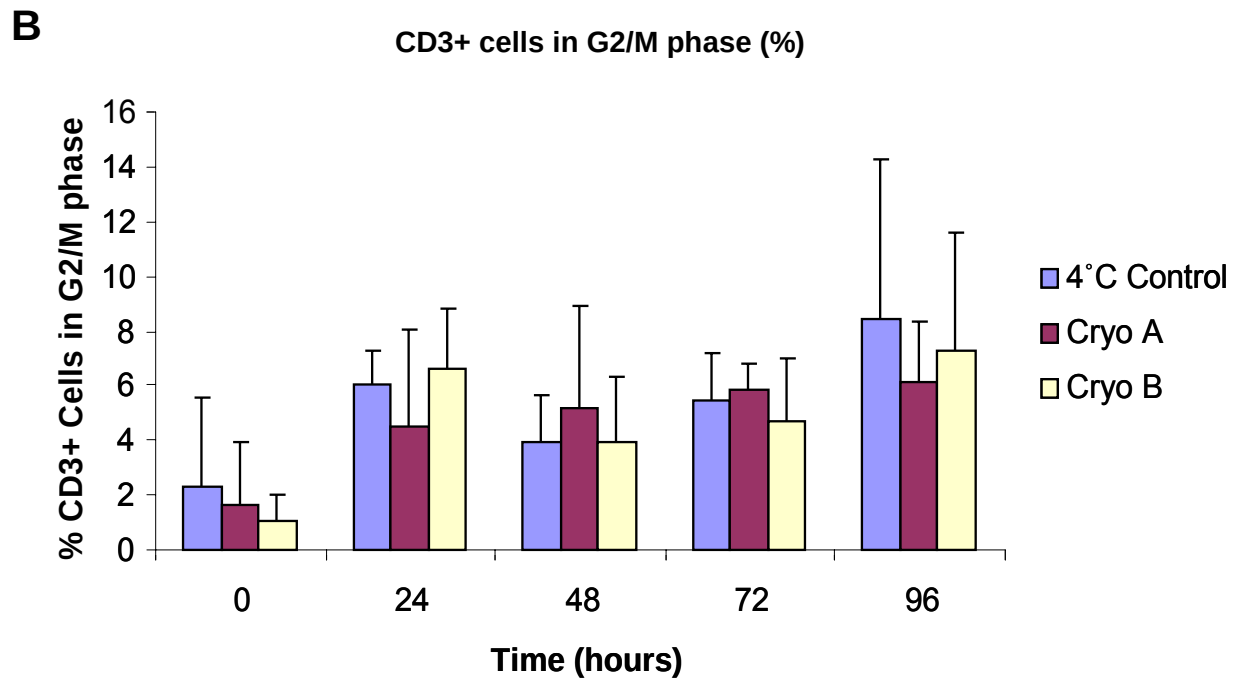


Figure 6. Frequency of T cells in S phase (A) and G2/M phase (B) after thawing and culture for up to 96 hours were similar among all three methods. Fresh (4˚C) and frozen T cells were incubated with PHA (10 µg/mL) at 37˚C for four days and labeled with BrdU (see Materials and Methods). Note that the %CD3+ cells in S phase (6a) and G2/M phase (6b) in each method was not statistically significant at each time point as determined by the Student's t test.

Cell Division Tracking

Viable CD3+ counts were obtained as described above (see Materials and Methods) and all culture wells for both BrdU and CFSE began with 10^6 total cells on day 0. Figure 7 shows the mean viable CD3+ cell count for all donors across four days of culture. On day 0, the mean cell count for all three methods was $7 \pm 0.4 \times 10^5$ and the mean CD3+ cell count at 96 hours was $2 \pm 0.3 \times 10^5$. These data show a clear decrease in viable CD3+ cell numbers from day 0 to the 96 hour time point in the wells containing CFSE, contradicting the CD3+ cell expansion seen

in wells without CFSE (Figure 5). The CFSE appeared to be toxic to the cells at concentrations as low as 2 μM (Figure 7).

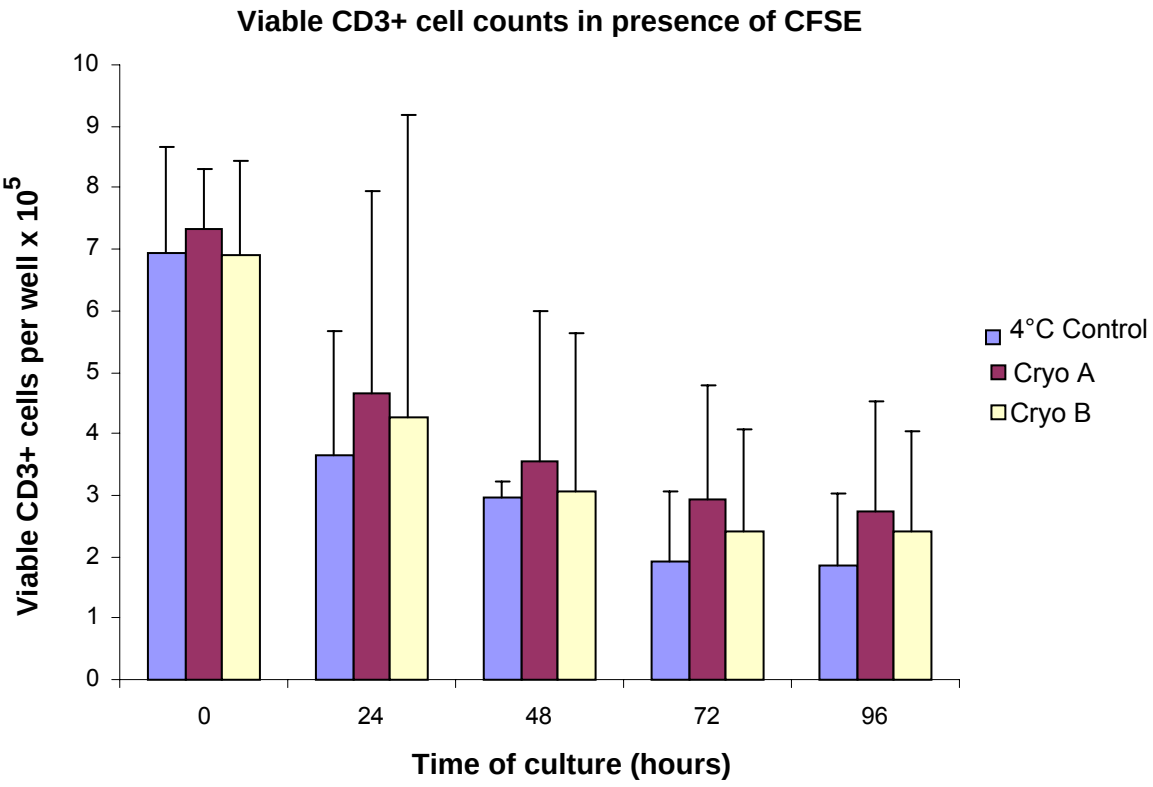


Figure 7. **Kinetics of T cell cultures stained with CFSE denote a toxic effect induced by this dye.** Conditions as shown in Materials and Methods. Results are plotted as a mean of three donors + standard deviation.

To determine the number of division cycles through which each cell has progressed, cells were stained by carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling technique. Flow cytometry data were obtained, but the number of peaks (cell divisions), were not clearly distinguishable (Figure 8). Cells were gated on the viable CD3+ cell population and it appears that both the fresh and cryopreserved cells from both methods did divide in the first 24 hours, mean fluorescent intensity at 298, 323 and 383 respectively, however, by 96 hours, cell counts had dropped and cell division appeared to have stalled. Although the data was

unexpected, it appears that Fresh and Cryo A cells continued to behave in a similar proliferation pattern. The mean channel fluorescence at 24 hours for the fresh and cryo A cells were more closely related (298 and 323) than the cryo B cells (383). In addition, this pattern held at 96 hours with mean channel flurorescence for fresh and cryo A at 180 and 191 and cryo B at 217.

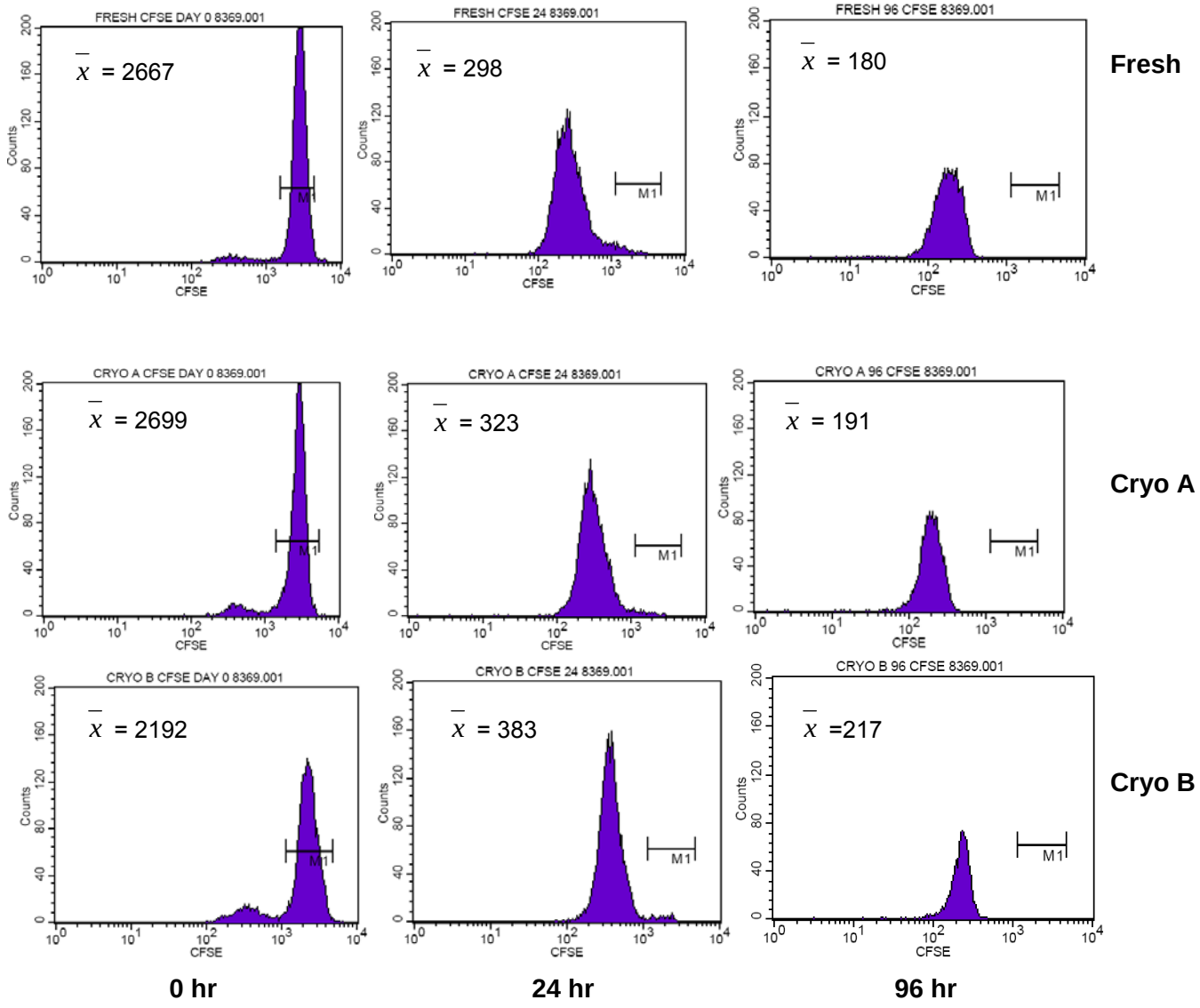


Figure 8. **Example of flow cytometry CFSE data collected at 0 (left panels), 24 (center panels), and 96 hour (right panels) time points for Fresh (upper panels), Cryo A (middle panels) and Cryo B (lower panels).** Means are expressed for each time point including the initial testing on day 0 before the cells were placed into culture.

Cytokine Production

We examined the functional capacity of T cells following the cryopreservation process by assessing their ability to secrete IFN-γ, a potent effector cytokine (Figure 9).[36] These data were normalized to IFN-γ production per million cells. Fresh cells and cells frozen with 5% DMSO and 3% HES (Cryo A) exhibit a similar pattern of IFN-γ levels and were not statistically different across all time points ($p > 0.05$). Cells frozen with 10% DMSO (Cryo B) have a clear delay in IFN-γ producing ability, which was statistically significant ($p<0.05$) at 48 hours when compared to the Fresh cells (4°C).

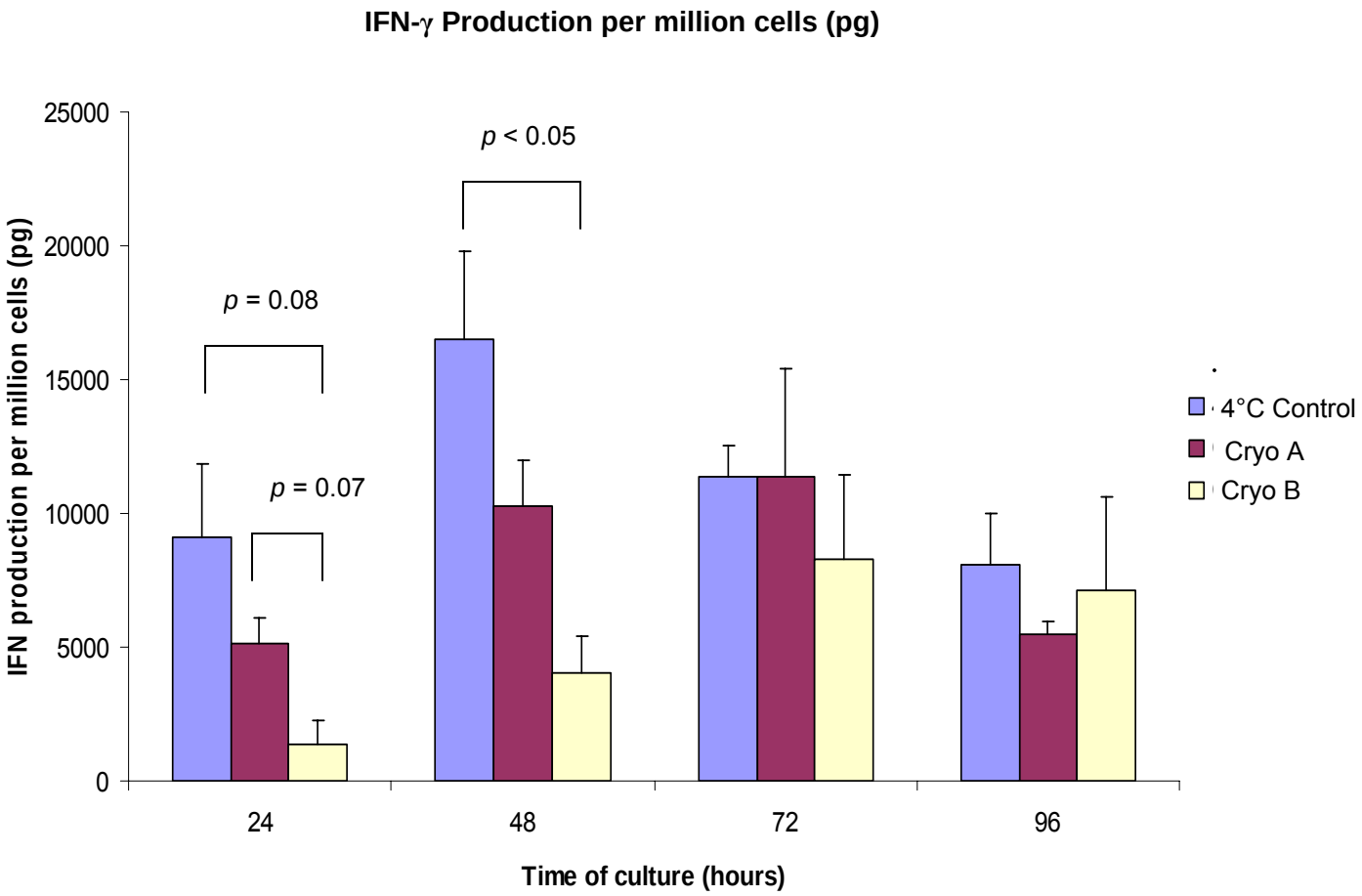


Figure 9. IFN-γ release was significantly decreased in Cryo B method compared to Fresh and Cryo A methods. IFN-γ release was analyzed at each time point (24, 48, 72, and 96 hrs) through four days of culture using ELISA kits from R&D systems. These data were normalized to IFN-γ production per million cells (see Materials and Methods). Note that at 48 hours, cytokine levels between Fresh (4°C) cells and cells frozen in Cryo B method were statistically significant as determined by the Student's t test, $p<0.05$.

DISCUSSION

Increased use of cryopreserved products and the ability to preserve cells for future therapeutic use is a clinically valuable tool. High cell recovery and retention of good cell function are critical factors when developing a cryopreservation program. This study has shown that cryopreserved lymphocytes retain cell cycling ability and cytokine production comparable to that of fresh cells when cryopreserved in 5% DMSO and 3% HES.

Although 10% DMSO can retain viability and recovery after cryopreservation, use of a 5% DMSO and 3% HES method may better preserve total cell functional capacity. Cells cryopreserved with 5% DMSO and 3% HES do not behave significantly different than that of fresh cells. Widely used cryopreservation protocols for stem cells include a 10% DMSO solution,[37] however we have shown that cells cryopreserved with this method have a decreased ability in terms of CD3+ cell expansion and IFN-γ production. It has already been shown that cryopreservation of hematopoietic progenitor cells in 5% DMSO is equal or superior to 10% DMSO with regard to viability of CD34+ cells and preservation of mature colony-forming cells.[25,26] However, further study of the function and number of T cells to evaluate the effects of the freeze/thaw process is needed to determine whether all cell types, including T cell subsets, behave similarly.

The functional capacity of T cells can be measured in vitro by subjecting them to stimulation by mitogens, specifically PHA. The response to this plant lectin is thought to be similar to the in vivo T cell response to antigens. PHA provides a two-signal stimulation by binding to lymphocyte surface sugars, which then triggers a second signal which is transmitted into the cell initiating cell growth and proliferation. This signal is transmitted through the mitogen-activated protein kinase (MAPK) pathway which is the main signaling pathway for transcription of genes associated with mitosis, differentiation, cell survival and apoptosis.[6] It has

been postulated that cryopreservation may effect the MAPK signaling pathway because it is known as a stress-induced pathway. However, literature on this matter has only been studied on islet cells, fibroblasts, and murine bone marrow HSCs.[38] This literature indicates a change in the structures of the cell membrane causing a decreased ability to interact and receive signals from its microenvironment.[39] However, studies conducted on T cells indicate that phenotype is least affected by cryopreservation and thaw procedures, which is consistent with our data (Figure 3).[35]

It is clear from our study that cells cryopreserved with 10% DMSO have a decreased functional ability in terms of CD3+ cell expansion and IFN-γ production. In addition, cells cryopreserved in 5% DMSO and 3% HES did not behave significantly different from fresh cells. No decrease in viability was noted in the cells cryopreserved with 10% DMSO (Figure 2) and no significant difference was found in the ability of the cells to cycle. Although the cells were not dead, cryopreservation may have impaired internal signaling ability. Literature suggests that there is little to no effect on the phenotype of lymphocytes after cryopreservation, but there are limited studies on the effects of cytokine receptors.[41] Surface receptor integrity could be altered or possibly the signaling mechanism for these receptors may have been damaged in the cryopreservation process. IL-2 was added to the culture media and is a growth factor for stimulated T cells. It is also secreted by T cells and is responsible for T cell clonal expansion and increased IFN-γ production.[43] Alteration of the surface receptor or signaling through this receptor may have contributed to the behavior of these cells in culture. Upon PHA stimulation, 5% DMSO and 3% HES cryopreserved T cells were not significantly different from fresh cells in the secretion of INF-γ. However, the cells cryopreserved with 10% DMSO have a clear delay and reduced ability to produce IFN-γ (Figure 8). Limited studies have also suggested cryopreservation may enhance the ability of the cells to produce cytokines after long-term storage.[41,42] This could have an implication on DLI therapy because of the major role of

cytokines in the immune system, especially IFN-γ which plays a role in both innate and adaptive immunity.

It is possible that the effect of cryopreservation solutions had an impact on the functional capacity of T cells. DMSO is a colligative cryoprotectant that protects the cells from excessive dehydration as extracellular water is drawn into growing ice crystals. Colligative means it is dependent upon the number of particles (solute), not the composition of the particles. DMSO freely penetrates the hematopoietic cell membrane so the internal and external DMSO concentrations are equal throughout the temperature range. The higher the DMSO concentration, the lower the osmotic stress, but this must be balanced with the toxicity to the cell and the presence of other cryoprotectants. It is essential to reduce the osmotic stress across the cell membrane because the tolerance of cells to freezing depends on their ability to withstand this stress. In order for this to be effective, colligative cryoprotectants (DMSO) must be capable of penetrating the cells to avoid contributing to the molality of the extracellular medium and must be nontoxic to the cells at the concentration required for this effect.[44] It has already been reported that the tolerance of granulocytes is much less than that of lymphocytes, indicating the need for different cryoprotectants for different cell types.[45,46] Macromolecules such as HES have a high molecular weight and do not freely penetrate the cell. This polymeric cryoprotectant protects the cell by forming a viscous, glassy shell that slows the movement of water, preventing dehydration as water is incorporated into extracellular ice crystals. However, this is also temperature dependent and high concentrations are needed to prevent crystallization and its associated mechanical and osmotic stresses, which may be different for stem cells and T cells. These characteristics warrant the need to freeze in more than simple two or three-component solutions.[43]

The majority of literature on this subject deals with the effects on HPCs and more specifically CD34+ cells, an important consideration when creating protocols for preservation of

T cells.[46,47] Membrane permeability is crucial in characterizing the response of a cell to the freezing environment, and stem cells and lymphocytes are unique in their permeability parameters. These differences can be useful in understanding the implications of cryopreservation on different cell types and on mixed cell populations, like in DLI. Great variations in permeability could result in low survival rates if cells are frozen together as illustrated by the lysis of erythrocytes when cryopreserving bone marrow or cord blood using the conventional protocol of 10% DMSO.[45]

There were no significant differences in the ability of the cells to cycle (Figure 6a and 6b), but CD3+ cell expansion and IFN-γ production in cells cryopreserved with 10% DMSO was impaired. This indicates significant lengthening of cell cycle transition periods. CFSE would have been able to measure the number of divisions of cell populations and that information, in addition to the cell cycle data, would have provided valuable proliferation comparison between methods. Unfortunately, without information that would have been obtained from CFSE data, this requires additional study. The cell cycle is a highly regulated process by which signals from both inside and outside the cell intervene inducing or inhibiting cell proliferation. The mechanism by which cells monitor their progression and transition through the cell cycle is poorly understood[6] and it is not the objective of this thesis to study the role of cryopreservation on the molecular mechanisms regulating cell proliferation. It is possible that cryoprotectants may have an effect on the time it takes for this process. Whether this occurs during freezing or thawing is unknown, and the exact mechanisms responsible for damage are not known. A separation will need to be made to distinguish the effects of cell specific or solution dependent damage that may effect the timing of cell cycle progression.

Several groups have studied the responses of T cells post thaw, however, data is conflicting. Upon PHA stimulation, cryopreserved T cells attain a cytokine production level comparable to that of fresh cells for secretion of IL-2, IL-6, TNF-α and IFN-γ. This supports our

data on IFN-γ production when cells are cryopreserved with 5% DMSO and 3% HES (See Figure 9). Also, viability and cell cycle distribution were consistent with our results for cells cryopreserved for up to 50 days.[49] Conversely, another group has seen a decrease in IFN-γ production in T cell products stored for 6 months and an even larger decrease in those cells stored for more than a year.[50] Disis et al[51] found that the use of human serum albumin and 10% DMSO can preserve a T cell proliferative response. Proliferative responses were not consistent with data from our study (Cryo B method, Figure 5), but this may be due to a difference in evaluation methods. Overall proliferative responses were evaluated through radioactive thymadine uptake. In our study, we were able to assess specifically the T cell proliferation and cell cycle distribution by using the BrdU and CFSE methods. These techniques provide a higher level of information based on details of specific cell subsets, in this case T cells. Furthermore, in an attempt to preserve function after cryopreservation, manipulations of T cells have involved the use of additives that might artificially alter native responses such as high concentrations of DMSO and serum additives that are not for clinical use, such as FCS. Our aim was to evaluate T cells cryopreserved with a method that is clinically relevant for immune-based therapies, like DLI. It is clear more studies are needed to examine the potential effects of cryopreservation methods, which can be used clinically, on T cell function and viability post thaw.

The ability to store T cells so that they remain functional and viable is absolutely necessary for immunotherapeutic treatment with DLI. Cryopreservation will also be important for these therapies because it permits the transportation of cells, time for completion of safety and quality control testing and further manipulations, such as the pooling of cells to reach a therapeutic dose. If this can be achieved, national cell processing centers may be developed that can coordinate better patient care regimens. Cryopreservation would allow for less

restrictive donor recruitment and availability, since only a single collection would be necessary. T cells could be separated from the original graft and frozen in dose appropriate aliquots.

This study shows the ability to cryopreserve T cells for therapeutic purposes in a 5%DMSO and 3% HES solution and still retain function and viability compared to that of fresh cells. This media can be used in place of the more common 10% DMSO solution. Based on cell recovery, cell cycling ability and cytokine production in response to PHA, these cryopreserved cells could retain their functional ability for immunotherapeutic purposes.

CONCLUSIONS

Currently, the most accepted cryopreservation solution for donor leukocytes for immunotherapy after allogeneic hematopoietic stem cell transplant contains 10% DMSO as a cryoprotectant. We compared cryopreservation in a 10% DMSO solution to another cryoprotectant solution containing 5% DMSO and 3% HES. These cells were compared to fresh cells (20-hour overnight hold at 4°C) to assess their ability to preserve T cell function, recovery and viability, post thaw. Cells placed in an in vitro cell culture system and stimulated with PHA, were evaluated over the course of four days for cell proliferation, cycle distribution and IFN-γ production.

From these studies, the following conclusions have been noted:

1. The use of 5% DMSO and 3% HES showed a CD3+ cell expansion similar to fresh T cells from the same donor and were highly superior to cryopreserved CD3+ cells in 10% DMSO.

2. Cell cycle entry of cryopreserved T cells did not significantly change, suggesting a normal mitogenic response to PHA. Together with a larger T cell expansion with cells cryopreserved in 5% DMSO and 3% HES, this suggests a significant shortening of the cell cycle transition periods.

3. There was a clear delay in the ability of cells cryopreserved with 10% DMSO to produce IFN-γ, which was statistically significant at the 48 hour time point. In addition the cells frozen with 5% DMSO and 3% HES retained the ability to produce IFN-γ similar to that of the fresh cells.

Additional studies are needed to determine if the 5% DMSO, 3% HES method can provide consistency in the final product and to evaluate long-term storage effects. To further

evaluate cell proliferation in terms of cell cycle, data on the number of cell cycles by each population is needed.

In summary, the cells frozen in 5% DMSO and 3% HES did not behave significantly different than fresh cells in terms of cell expansion and IFN-γ production. A routine method of cryopreservation of cell products used for DLI (10% DMSO) has shown a disadvantage on its ability to support T cell expansion and IFN-γ production in vitro.

REFERENCES

1. Weiden, P.L., Flournoy, N., Thomas, E.D., et al. Antileukemic effect of graft-versus-host disease inhuman recipients of allogeneic-marrow grafts. N Engl J Med 1979; 300:1068-73.
2. Horowitz, M., Gale, R., Sondel, P., et al. Graft-versus-leukemia reactions after bone marrow transplantation. Blood 1990; 75(3):555-62.
3. Kolb, H.J., Schattenberg, A., Goldman, J.M., et al. Graft versus leukemia effect of Donor Lymphocyte transfusion in marrow grafted patients. European Group for Blood and Marrow Transplantation Working Party Chronic Leukemia. Blood 1995; 86:2041-50.
4. Bone Marrow Transplant Written Information [database on the internet] Cleveland (OH) Cleveland Clinic Health Information Center–[cited 2007 Nov 2]. Available from URL www.clevelandclinic.org/transplant/body.cfm?id=84
5. Nash, R.A., Pepe, M.S., Storb, R., et al. Acute graft versus host disease: analysis of risk factors after allogeneic marrow transplantation and prophylaxis with cyclosporine and methotrexate. Blood 1992; 80:1838-45.
6. Abbas, A.K.., Lichtman, A.H., Transplantation Immunology. In: Malley, J., Schmitt B., editors. Cellular and Molecular Immunology. 5th ed. Philadelphia, PA. Elsevier Saunders; 2005. p. 369-390.
7. Lukas, M., Hematopoietic Stem Cells and Cellular Therapy. In: Rudmann, S.V., Wurm, E., editors. Textbook of Blood Banking and Transfusion Medicine. 2nd ed. Philadelphia, PA. Elsevier; 2005. p. 504-521.
8. Craddock, C., Szydlo, R., Klein, J., et al. Estimating leukemia-free survival after allografting for chronic myeloid leukemia: A new method that takes into account patients who relapse and are restored to complete remission. Blood 2000; 96(1):86-90.

9. Kolb, H., Mittermuller, J., Clemm, C., et al. Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients. Blood 1990; 76(12):2462-65.
10. Porter, D.L.., Antin, Joseph. The Graft versus Leukemia effects of allogeneic cell therapy. Annu Rev Med 1999; 50(1):369-86.
11. Jiang, Y.Z., Cullis, J.O., Kanfer, E.J., et al. T cell and NK cell mediated graft-versus-leukaemia reactivity following donor buffy coat transfusion to treat relapse after marrow transplantation for chronic myeloid leukaemia. Bone Marrow Transplant 1993; 11(2):133-38.
12. Collins, R. Jr., Shpilberg, O., Drobyski, W., et al. Donor leukocyte infusions in 140 patients with relapsed malignancy after allogeneic bone marrow transplantation. J Clin Oncol. 1997; 15(2):433-44.
13. Urbano-Ispizua, A., Rozman, C., Pimentel, P., et al. The number of donor CD3+ cells is the most important factor for graft failure after allogeneic transplantation of CD34+ selected cells from peripheral blood from HLA-identical siblings. Blood 2001; 97(2):383-37.
14. McSweeney, P., Niederwieser, D., Shizuru, J., et al. Hematopoietic cell transplantation in older patients with hematologic malignancies: Replacing high-dose cytotoxic therapy with graft-versus-tumor effects. Blood 2001; 97(11):3390-400.
15. Drobyski, W., Hessner, M., Klein, J., et al. T-cell depletion plus salvage immunotherapy with donor leukocyte infusions as a strategy to treat chronic-phase chronic myelogenous leukemia patients undergoing HLA-identical sibling marrow transplantation. Blood 1999; 94(2):434-41.
16. Bunjes, D., Theobald, M., Hertenstein, B., et al. Successful therapy with donor buffy coat transfusions in patients with relapsed chronic myeloid leukemia after bone marrow transplantation is associated with high frequencies of host-reactive interleukin 2-secreting T helper cells. Bone Marrow Transplant 1995;15(5):713-19.

17. Jiang, Y.Z., Kanfer, E.J., Macdonald, D., et al. Graft-versus-leukaemia following allogeneic bone marrow transplantation: Emergence of cytotoxic T lymphocytes reacting to host leukaemia cells. Bone Marrow Transplant 1991; 8(4):253-58.
18. Alyea, E., Soiffer, R., Canning, C., et al. Toxicity and efficacy of defined doses of CD4+ donor lymphocytes for treatment of relapse after allogeneic bone marrow transplant. Blood 1998; 91(10):3671-80.
19. Dodi, I.A., Van Rhee, F., Forde, H.C., et al. CD4(+) bias in T cells cloned from a CML patient with active graft versus leukemia effect. Cytotherapy 2002; 4(4):353-63.
20. Dazzi, F., Szydlo, R.M., Craddock, C., et al. Comparison of single-dose and escalating-dose regimens of donor lymphocyte infusion for relapse after allografting for chronic myeloid leukemia. Blood 2000; 95(1):67-71.
21. Law, P., Graft Processing, Storage and Infusion. In: Ball, E., Lister, J., Law, P., editors. Hematopoietic Stem Cell Therapy. Philadelphia, PA: Churchill Livingstone; 2000. p. 312-321.
22. Eckardt, J.R., Roodman, G.D., Boldt, D.H., et al. Comparison of engraftment and acute GVHD in patients undergoing cryopreserved or fresh allogeneic BMT. Bone Marrow Transplant 1993; 11(2):125-31.
23. Davis J, Rowley S, Braine H, et al. Clinical toxicity of cryopreserved bone marrow graft infusion. Blood 1990; 75(3):781-86.
24. Lane, T.A., Medina, B., Bashey, A., et al. Clinical efficacy of cryopreserved donor lymphocytes for infusion (DLI). Biology of Blood and Marrow Transplantation 2007; 13(2, Supplement 1):127-28.
25. Rowley SD, Feng Z, Chen L, et al. A randomized phase III clinical trial of autologous blood stem cell transplantation comparing cryopreservation using dimethylsulfoxide vs dimethylsulfoxide with hydroxyethylstarch. Bone Marrow Transplantation 2003; 31:1043-51.

26. Abrahamsen, J.F., Rusten, L., Bakken, A.M., et al. Better preservation of early hematopoietic progenitor cells when human peripheral blood progenitor cells are cryopreserved with 5 percent dimethylsulfoxide instead of 10 percent dimethylsulfoxide. Transfusion 2004; 44(5):785-89.
27. Fleming KK, Hubel A. Cryopreservation of hematopoietic and non-hematopoietic stem cells. Transfusion and Apheresis Science 2006; 34(3):309-15.
28. Hubel A. Parameters of cell freezing: Implications for the cryopreservation of stem cells. Transfus Med Rev 1997; 11(3):224-33.
29. Hubel A, Norman J, Darr TB. Cryobiophysical characteristics of genetically modified hematopoietic progenitor cells. Cryobiology 1999; 38(2):140-53.
30. BrdU Labeling and the BrdU Flow Kit Staining Protocol, Labeling of cellls with BrdU. Available at URL www.bdbiosciences.com. Accessed November 2, 2007.
31. Hasbold J, Gett AV, Rush JS, et al. Quantitative analysis of lymphocyte differentiation and proliferation in vitro using carboxyfluorescein diacetate succinimidyl ester. Immunol Cell Biol. 1999 12;77(6):516-22.
32. Liu D, Yu J, Chen H, et al. Statistical determination of threshold for cellular division in the CFSE-labeling assay. J of Immunol Methods 312(1-2):126-36(2006).
33. Lyons AB, Parish CR. Determination of lymphocyte division by flow cytometry. J Immunol Methods 1994; 171(1):131-37.
34. Wang X, Duan X, Liu L, et al. Carboxyfluorescein diacetate succinimidyl ester fluorescent dye for cell labeling. Acta Biochim Biophys Sin (Shanghai) 2005; 37(6):379-85.
35. Costantini A, Mancini S, Giuliodoro S, et al. Effects of cryopreservation on lymphocyte immunophenotype and function. J Immunol Methods 2003; 278(1-2):145-55.
36. Schroder K, Hertzog P, Ravasi T, Hume D. Interferon-{gamma}: An overview of signals, mechanisms and functions. J Leukoc Biol 2004; 75(2):163-89.

37. Berz D, McCormack EM, Winer ES, et al. Cryopreservation of hematopoietic stem cells. Am J Hematol 2007; 82(6):463-72.
38. Kang,L., Tujun, Y., Mansbridge, J. Comparison of the Stress Response to Cryopreservation in Monolayer and Three-Dimensional Human Fibroblast Cultures: Stress Proteins, MAP Kinases, and Growth Factor Gene Expression. Tissue Engineering 2000; 6(5):539-54.
39. Goltsev, A.V., Babenko, N.N., Dubrava, T. G., et al. Modification of the state of bone marrow hematopoietic cells after cryopreservation. International Journal of Refrigeration 2006; 29:358-67.
40. Venkataraman, M., Westerman, M.P. Susceptibility of human T cells, T-cell subsets, and B cells to cryopreservation. Cryobiology 1986; 23(3):199-208.
41. Weinberg, A., Zhang, L., Brown, D., et al. Viability and functional activity of cryopreserved mononuclear cells. Clin Diagn Lab Immunol 2000; 7(4):714-16.
42. Via, C., Finkelman, F. Critical role of interleukin-2 in the development of acute graft-versus-host disease. Int Immunol 1993; 5(6):565-72.
43. Venkataraman M. Effects of cryopreservation of immune responses. XI. heightened secretion of tumor necrosis factor-alpha by frozen human peripheral blood mononuclear cells. Cryobiology 1997; 34(3):276-83.
44. Mazur P. Cryobiology: The freezing of biological systems. Science [Internet]. 168th 1970: 939. Available at URL www.sciencedirect.com. Accessed September 5, 200
45. Frey NV, Lazarus HM, Goldstein SC. Has allogeneic stem cell cryopreservation been given the 'cold shoulder' an analysis of the pros and cons of using frozen versus fresh stem cell products in allogeneic stem cell transplantation. Bone Marrow Transplant 2006; 38(6):399-405.

46. Ojeda-Uribe M, Sovalat H, Bourderont D, et al. Peripheral blood and BM CD34+ CD38- cells show better resistance to cryopreservation than CD34+ CD38+ cells in autologous stem cell transplantation. Cytotherapy 2004; 6(6):571-83.
47. Martin-Henao GA, Torrico C, Azqueta C, et al. Cryopreservation of hematopoietic progenitor cells from apheresis at high cell concentrations does not impair the hematopoietic recovery after transplantation. Transfusion 2005; 45(12):1917-24.
48. Rowley S, Bensinger W, Gooley T, Buckner C. Effect of cell concentration on bone marrow and peripheral blood stem cell cryopreservation. Blood 1994; 83(9):2731-36.
49. Wang, S., Hsu, M., Tzeng, C., et al. The influence of cryopreservation on cytokine production by human T lymphocytes. Cryobiology 1998; 37(1):22-29.
50. Owen RE, Sinclair E, Emu B, et al. Loss of T cell responses following long-term cryopreservation. J Immunol Methods 2007; 326(1):93-115.
51. Disis ML, dela Rosa C, Goodell V, et al. Maximizing the retention of antigen specific lymphocyte function after cryopreservation. J of Immunol Methods 2006; 308(1-2):13-18.