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Optimizing the Manufacturing and Efficacy of γδ T Cell Immunotherapies for the Treatment of Cancer

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Optimizing the Manufacturing and Efficacy of γδ T Cell Immunotherapies for the Treatment of Cancer

By

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B.A., Hamilton College, 2017

Advisor: H. Trent Spencer, Ph.D.

An abstract of a dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Graduate Division of Biological and Biomedical Science,
Molecular and Systems Pharmacology
2021

Abstract

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By Rebecca Elisabeth Burnham

Originally discovered in the 1980's, γδ T cells have become a frontline leader in the development of cellular cancer immunotherapies. Stimulating the *in vivo* expansion of γδ T cells was initially the most common approach for immunotherapies utilizing γδ T cells. However, when it became clear that off-target effects were common, interest in this method diminished. Instead, the field shifted towards investigating the *ex vivo* expansion of both autologous and allogeneic γδ T cell therapies. The ability to use γδ T cells in an allogeneic setting is both a unique and attractive property, as it allows for the development of an off-the-shelf cell therapy. The focus of this dissertation is to optimize the manufacturing process and improve the efficacy of allogeneic γδ T cells as a cancer immunotherapy.

The cellular manufacturing process consists of multiple processes including donor selection, cellular isolation and expansion, and storage. In this work, we have identified exercise as being a critical parameter in donor selection. In comparison to cells from sedentary donors, γδ T cells from exercise donors have greater expansion capabilities. Interestingly, modifying the expansion protocol to include an additional cytokine, IL-21, can rescue the expansion of γδ T cells from sedentary donors. αβ T cell depletions are necessary step in the expansion of γδ T cells to ensure that cells capable of promoting graft versus host disease are removed. Depleting αβ T cells on day 6 of expansion is the most efficient time-point to perform the depletion to minimize cell losses and remain cost effective. Additionally, we report the development of a novel and highly cytotoxic γδ T cell immunotherapy in which γδ T cells from multiple donors are mixed and expanded in culture together. The final step in the cellular manufacturing process, cryopreservation and storage, requires significant consideration in addition to donor selection and cellular expansion, because this step can drastically alter the final cell product. In fact, thawing γδ T cells into their standard culture media results in poor viability and extreme cell losses. Thawing γδ T cells into human serum albumin, offers protection to cell viability. Additionally, chromatin condensation prior to cryopreservation offers additional protection to γδ T cell viability after thawing. However, mitochondria in γδ T cells that have been cryopreserved exhibit high levels of membrane depolarization, suggesting that the mitochondria are critically damaged and further optimization is required.

Improving the tumor homing capabilities of γδ T cells is critical to improving the efficacy of γδ T cells against solid tumors. Currently, efficient tumor homing is a limitation reducing the efficacy of T cell therapies against solid tumors. We describe a novel dual-cell γMSC-γδ T cell immunotherapy which utilizes the pairing between chemokines secreted from γMSCs and chemokine receptors expressed by γδ T cells to increase tumor homing. γδ T cells preferentially migrated to both conditioned media from γMSCs and γMSCs cells *in vitro*. When γMSCs were directly injected into neuroblastoma tumors, γδ T cells preferentially migrated to the γMSCs tumors *in vivo*. Additionally, we provide evidence that direct cell contact between γMSCs and γδ T cells does not impact γδ T cell viability or cytotoxicity. Further development of the dual-cell γMSC-γδ T cell immunotherapy provides promise for increasing T cell tumor homing efficiency.

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Acknowledgements

First and foremost, I would like to thank my advisor, Dr. Trent Spencer, for his mentorship and guidance over the past four years. I truly enjoyed being a part of his laboratory and the ways in which he challenged me to improve my critical thinking and analysis skills. I would also like to thank Dr. Christopher Doering, who served as a second mentor to me in the Spencer-Doering laboratory. Dr. Doering's door was always open and I appreciate the new perspectives and directions he offered in regards to my dissertation work. Dr. Edwin Horwitz served not only as a committee member, but as a third mentor during graduate school. I enjoyed working with Dr. Horwitz on a variety of projects that gave me the chance to gain experience in new research fields and broaden my scientific knowledge. Finally, I would like to thank Dr. Mala Shanmugam for serving on my dissertation committee. I appreciate the encouragement and advice she has offered me throughout graduate school and her helpful suggestions that have improved my dissertation work.

Thank you to my parents and brother for their unwavering support throughout my journey as a scientist. I would not have made it through graduate school without all of your love, advice, and encouragement. Most importantly, I would like to express my deepest appreciation for my husband, Andre. Thank you for taking the time to help with experiments and serve as my in-house editor. I would not have made it through my dissertation without your love and support.

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Chapter 1: Introduction

1.1 Cancer Immunotherapies

A. Early cancer immunotherapies

Classical treatments for cancer include surgery, chemotherapy, and radiation; however, the field of immunotherapy has rapidly progressed over the last century to become a frontline treatment for cancer. The earliest reports of using the immune system to fight cancer date back to the ancient Egyptians. The physician Imhotep (c 2600 BC) induced infections in patients as a way to generate spontaneous tumor regression (Ebers Papyrus, 1937). Although there have been reports of tumors miraculously disappearing for thousands of years, it wasn't until the late 18th century that scientists were able to have definitive evidence linking the immune system and cancer progression. Two German physicians, Wilhelm Busch and Friedrich Fehleisen, reported tumor regression in patients that were infected by the bacteria erysipelas (Stanley J Oiseth, 2017). Busch pursued this observation in 1868 by intentionally infecting a patient with erysipelas and was successful in reducing the size of the patient's tumor, an experiment Fehleisen was able to repeat four years later. The next breakthrough in the field of cancer immunotherapy came in 1891 and can be attributed to William Coley, an American physician. In addition to Busch and Fehleisen, Coley also observed tumors spontaneously regress when he intentionally infected his patients with erysipelas. However, Coley recognized that administering erysipelas to patients was not the best option for cancer treatment; in some patients, erysipelas treatment produced no response, while in others, the infection proved to be fatal. Instead, Coley developed the first cancer vaccine, termed "Coley's toxins", which was created from a mixture of heat inactivated bacteria. Coley's toxins were used to cure or regress cancer in over 1,000 patients due to the treatment's ability to activate the immune system. Over time, as more standardized treatments such as radiotherapy and chemotherapy were developed, the use of Coley's Toxins slowly

diminished (Kienle, 2012). Today, there are many approaches to developing immune-activating therapies including immune checkpoint inhibitors, cancer vaccines, antibody therapies, cytokine therapies, and adoptive cell transfer therapies.

B. Immune Checkpoint Therapy

Lymphocytes have a complex system of activation and inhibitory pathways that play a critical role in tumor immunosurveillance. As our understanding of immune system regulators has progressed, three targets for immune checkpoint therapy have emerged: T-lymphocyte-associated protein 4 (CTLA-4), programed cell death protein 1 (PD-1), and PD-1 ligand 1 (PD-L1).

CTLA-4. CTLA-4 was the first receptor to be identified as a negative regulator of T cell activation. CTLA-4 recognizes both CD80 and CD86, two molecules that play a critical role in T cell activation, at a higher affinity than the T cell co-stimulatory receptor, CD28 (Rudd et al., 2009). One mechanism through which CTLA-4 expression on T cells can impair T cell function is by degrading CD80 and CD86 via trans-endocytosis, directly limiting CD28 co-stimulation (Qureshi et al., 2011). Additionally, when CTLA-4 is crosslinked to the TCR and CD28, T cell proliferation and Il-2 secretion is inhibited (Krummel and Allison, 1995).CTLA-4 expression on CD4+, CD8+ and CD4+FOXP3+ regulatory T cells (Tregs) acts as an immune checkpoint through different mechanisms. For example, while CTLA-4 regulates the responses of naïve CD4+ T cells, (Krummel and Allison, 1996) it regulates the responses of memory CD8+ T cells (Chambers et al., 1998). In Tregs, CTLA-4 deficiency results in lymphocyte proliferation and T cell-mediated autoimmune disease, suggesting that CTLA-4 plays a critical role in the ability of Tregs to suppress the immune system (Wing et al., 2008). Due to CTLA-4's role in the negative regulation of T cell activation, it has become a therapeutic target as a way to increase effector T

cell activity. *In vivo* studies with anti-CTLA-4 antibodies have had promising results, leading to FDA approval for the first immune checkpoint inhibitor, ipilimumab. A clinical trial (NCT00094653) using Ipilimumab effectively improved survival in a study of 676 patients with unresectable stage III or IV melanoma, who had previously received treatment for metastatic melanoma (Hodi et al., 2010). Based on the results of this trial, in 2011, the FDA approved the use of ipilimumab for patients with unresectable or metastatic melanoma. Since its initial approval in 2011, ipilimumab has been approved for use in other cancers, such as advanced renal cell carcinoma, and in combination with the anti-PDL1 antibody nivolumab (Vaddepally et al., 2020).

PD-1. PD-1 was first discovered in 1992, as a cell surface protein thought to play a role in programmed cell death (Ishida et al., 1992). However, when it was discovered that mice bearing PD-$1^{-/-}$ T cells developed autoimmune disease, it became clear that PD-1 has a regulatory role in T cell function (Nishimura et al., 1999). There are two known ligands that bind to PD-1: PD-L1 and PD-L2. Binding of PD-L1 to PD-1 can inhibit TCR-mediated T cell proliferation, decrease cytokine secretion, and reduce co-stimulation through CD28 (Freeman et al., 2000). Similarly, engagement of PD-L2 with PD-1 can inhibit CD28 co-stimulation at low antigen concentrations and reduce cytokine secretion at high antigen concentrations (Latchman et al., 2001). Although PD-1 can inhibit T cell proliferation and reduce cytokine secretion from T cells, its primary role in immune suppression is promoting the loss of effector function, also known as T cell exhaustion. In fact, PD-1 expression is upregulated on exhausted T cells (Barber et al., 2006) and T cells isolated from both solid tumors such as hepatocellular carcinoma, melanoma, and renal cell carcinoma, and non-solid tumors such as Hodgkin's lymphoma and chronic myeloid leukemia (Gehring et al., 2009; Mumprecht et al., 2009; Patil et al., 2015; Yamamoto et

al., 2008). Furthermore, PD-1 expression on immune cells in patients has been correlated with a poor prognosis. In patients with renal cell carcinoma, PD-1 expression on tumor-infiltrating immune cells was associated with advanced metastasis and tumor necrosis, while PD-1+ immune cells were indicative of larger and higher-grade tumors (Thompson et al., 2007).

The first monoclonal antibody targeting PD-1, nivolumab, was approved for clinical use by the FDA in 2014. Approval for this therapy was given following a clinical trial in previously treated patients with unresectable or metastatic melanoma, who had improved response rates and reduced toxicity when treated with nivolumab, compared to chemotherapy (Weber et al., 2015). Nivolumab has since been approved for use in a range of cancers in which it has had demonstrated improved clinical outcomes for patients including: advanced squamous-cell lung cancer, non-small cell lung cancer (NSCLC), advanced renal cell cancer, recurrent squamous-cell carcinoma of the head and neck, metastatic urothelial carcinoma, colorectal cancer, and Hodgkin's lymphoma (Ansell et al., 2015; Brahmer et al., 2015; El-Khoueiry et al., 2017; Ferris et al., 2016; Motzer et al., 2015; Overman et al., 2017; Sharma et al., 2017; Younes et al., 2016). A second anti-PD-1 monoclonal antibody, pembrolizumab, has also received FDA approval as a frontline immune checkpoint immunotherapy for use in patients with unresectable or metastatic melanoma, NSCLC, Hodgkin's lymphoma, head and neck squamous cell carcinoma, urothelial carcinoma, gastric/gastroesophageal junction adenocarcinoma, and colorectal cancer (Bellmunt et al., 2017; Chen et al., 2017; Chow et al., 2016; Fuchs et al., 2018; Herzka and Nil, 1989; Ribas et al., 2015; Robert et al., 2015; Shimizu et al., 1989). In 2018, a third anti-PD-1 antibody, cemiplimab, received approval for the treatment of patients with metastatic cutaneous squamous cell carcinoma or locally advanced cutaneous squamous cell carcinoma and is currently under investigation in a number of solid tumor clinical trials, testing the ability of this antibody against

cancers such as ovarian cancer, cervical cancer, melanoma, multiple myeloma, and glioblastoma (Dubikaitis Iu and Fedotova, 1985; Markham and Duggan, 2018).

PD-L1. Inhibiting the PD-1/PD-L1 interaction through the blockade of PD-L1 has also proven to be a viable therapeutic strategy. Atezolizumab was the first anti-PD-L1 antibody approved for use in patients with locally advanced or metastatic urothelial carcinoma or NSCLC. Two additional PD-L1 immune checkpoint inhibitors have been approved for clinical use as well: avelumab and durvalumab. Avelumab had significant response rates in phase I and phase II clinical trials in patients with metastatic urothelial carcinoma and metastatic Merkel cell carcinoma (Kaufman et al., 2016; Patel et al., 2018). Of note, avelumab is the first FDA approved treatment for Merkel cell carcinoma, an aggressive skin cancer that rarely has durable responses to chemotherapy. Similarly, durvalumab was also approved for the treatment of metastatic urothelial carcinoma, in addition to NSCLC (Antonia et al., 2017).

C. Cancer vaccine therapy

Along with a greater understanding of the role of cancer in immune suppression in patients, comes a renewed interest in exploring cancer vaccines as an option for therapeutically activating the immune system against cancer. Overall, cancer vaccines have lacked clinical efficacy; however, the development of successful vaccines for hepatitis B virus (HBV) to prevent liver cancer and human papillomavirus (HPV) to prevent cervical cancer, offer hope to the field (Fernandez-Rodriguez and Gutierrez-Garcia, 2014; Roden and Stern, 2018). There are currently two broad classes of cancer vaccines that have been the focus for development: cancer vaccines targeting antigens and vaccine vectors. The most challenging component of developing a cancer vaccine is choosing a target that is preferentially expressed on all cancer cells, one that is critical for the cancer cell's survival, and one that is able to produce a strong immune response. The

majority of cancer vaccines have focused on targeting tumor associated antigens such as: cancer/germline antigens (MAGE-A1, MAGE-A3, NY-ESO-1), cell lineage differentiation antigens (tyrosinase, gp100, MART-1, PSA, PAP), and antigens overexpressed by cancer cells (hTERT, HER2, mesothelin, MUC-1). Cancer vaccines have also been developed to target oncogenic viral antigens, such as the vaccine developed for HBV. In regions where HBV infection is common, it has been associated with hepatocellular carcinoma in up to 80% of diagnosed patients (Lee et al., 1999). Cancer vaccines targeting neoantigens offer promise due to the specificity of neoantigens to cancer cells and because the immune system recognizes them as foreign, reducing immune tolerance. Although clinical trials using neoantigen targeted vaccines have produced clinical responses in up to two-thirds of patients (Ott et al., 2017; Sahin et al., 2017), this type of therapy is a precision medicine that must be developed individually for each patient, making it difficult to scale clinically.

Another area of focus in cancer vaccine therapies is the development of vaccine vectors used to stimulate the activation and proliferation of lymphocytes. Different strategies have been employed to develop vaccine vectors, including the use of cellular vaccines, virus vector vaccines, peptide vaccines, DNA vaccines, and RNA vaccines. The first cancer vaccine approved by the FDA, sipuleucel-T, is a dendritic cell (DC) vaccine in which DCs are isolated from patients, activated with granulocyte-macrophage colony-stimulating factor (GM-CSF) fused to an antigen, and infused back into the patient (Santos and Butterfield, 2018). Sipuleucel-T has shown modest effects in patients and a low toxicity profile, which is significant because it gives strong evidence that DC vaccines can be effective (Kantoff et al., 2010). Preclinical studies using a vaccine composed of tumor cells modified to secrete GM-CSF to activate dendritic cells (DCs), both induced an immune response and tumor regression in mouse models (Dranoff et al., 1993;

Dunussi-Joannopoulos et al., 1998). In contrast, clinical trials using this vaccine were able to produce an immune response in patients, but not achieve objective responses (Laheru et al., 2008; Lipson et al., 2015; Small et al., 2007). Studies using peptide vaccines have advanced from using short peptides, which weakly activate CD4+ T cell responses, to using synthetic long peptides, which are processed by DCs and stimulate strong CD4+ and CD8+ T cell responses (Cho et al., 2013; Hailemichael et al., 2013; Toes et al., 1996a; Toes et al., 1996b; van Duikeren et al., 2012; Zhu et al., 2007). Additionally, there has been interest in developing viral vectors with viruses engineered to express antigens and immunostimulatory molecules, however phase II and III clinical trials with these vaccines have failed to show clinical efficacy, suggesting this strategy needs further preclinical development (Amato et al., 2010; Oudard et al., 2011; Sato-Dahlman et al., 2020; Tosch et al., 2017). Unlike viral vector vaccines, which can be neutralized by the antiviral immune response, DNA and RNA vaccines can be given effectively in multiple doses because the body is less likely to develop immunity against them. VGX-3100, a DNA vaccine expressing HPV E6 and E7 antigens, is currently being tested in a phase III clinical trial in cervical cancer after a phase IIb trial showed 48.2% of patients had tumor regression after treatment (Trimble et al., 2015). RNA vaccines have also shown promise in clinical trials, with a recent phase I trial in patients with melanoma showing not only stimulation of T cells, but regression in metastatic lesions or stabilization of disease (Kranz et al., 2016). Advancement in vaccine technology has led to improvements in cancer vaccines in recent years. With the speed at which vaccines have been developed to target COVID-19, it is possible that there will be renewed interest and faster therapeutic developments in the field of cancer vaccine therapies. Additionally, discoveries regarding the immunosuppressive tumor microenvironment have guided research on how to modify the TME to make vaccines more effective against cancer

(Hollingsworth and Jansen, 2019). Further research on how cancer vaccines can be co-administered with other therapies, such as checkpoint inhibitors, will clarify the relevance of vaccine therapy against cancer.

D. Antibody therapies

Therapeutic monoclonal antibodies (mAbs) offer a targeted approach to treating cancer, in comparison to other therapies, such as chemotherapy. In addition to the immune checkpoint inhibitors previously described, the use of mAbs has become widespread in the treatment of both hematological and solid tumors. Antibodies have been developed to target multiple pathways, including disrupting growth factor signaling, regulating immune cell responses, and the tumor microenvironment. The epidermal growth factor receptor (EGFR) family has produced multiple targets for mAB therapeutics including EGFR, HER2, HER3 and HER4, through different mechanisms of action. Cetuximab and panitumumab, both EGFR-specific antibodies approved for use in patients with colorectal cancer, disrupt EGFR signaling by blocking ligand binding (Kim, 2009; Li et al., 2005; Sunada et al., 1986). In contrast, Trastuzumab, an antibody targeting HER2 in metastatic breast cancer, inhibits receptor dimerization, induces endocytic destruction of the receptor, and activates the immune system (Hudis, 2007). Vascular endothelial growth factor (VEGF), often expressed by solid tumors, is a primary target for mAbs targeting the tumor microenvironment (TME). Bevacizumab, a mAB targeting VEGF that is approved for use as a combination therapy in breast, colorectal, and non-small cell lung cancer, disrupts the binding of VEGF to its receptor. More recently, mAbs targeting VEGF receptors, specifically VEGFR1 and VEGFR2, have been developed. Ramucirumab, targeting VEGFR2, had promising results in preclinical studies and is approved for use in advanced gastric cancer, non-small cell lung cancer, metastatic colorectal cancer, and hepatocellular cancer (Krupitskaya and Wakelee, 2009; Saeed

et al., 2021). Phase I studies with the VEGFR1 mAB, icrucumab, have shown the antibody to be safe and potential for antitumor responses based on the achievement of stable disease in 23% of the patients tested (LoRusso et al., 2014). Additional studies targeting the TME have focused on the immunosuppressive cytokine, transforming growth factor β (TGFβ), which is involved in tumor immune evasion, for patients with malignant melanoma and renal cell carcinoma (Grutter et al., 2008; Ny et al., 1988).

In addition to the mechanisms described above, therapeutic mAbs promote anti-cancer effects through antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). ADCC occurs when the constant fragment (Fc) of the antibody binds to effector cells (T cells, NK cells, macrophages, neutrophils) via the FC γ-receptor (FcγR) and the fragment of antigen binding (Fab) binds to antigen on the tumor cell. Once the effector cell and cancer cell are linked via the antibody, the effector cell can exert cytotoxic mechanisms to lyse the cancer cell. ADCC is an important mechanism in a number of mABs including trastuzumab, rituximab, cetuzimab, and dinutuximab (Bibeau et al., 2009; Clynes et al., 2000; Keyel and Reynolds, 2019; Weng and Levy, 2003). Additionally, mAbs can activate the complement system to engage in CDC. CDC occurs when the antibody binds to an antigen on a cancer cell, which activates the complement pathway and forms a membrane attack complex (MAC) to lyse the target cell. Rituximab, alemtuzumab, and ofatumumab are all at least in part dependent on CDC for clinical efficacy, as preclinical work has shown depletion of complement reduces therapeutic results (Cragg and Glennie, 2004; Di Gaetano et al., 2003; Racila et al., 2008).

E. Cytokine therapies

Cytokines are small proteins integral in cell signaling processes and the regulation of immune cells. The role of cytokines in tumor progression has been investigated in a number of

studies and results have shown that while cytokines can have protective effects against tumor progression, in some contexts they also have pro-tumor effects. For example, mice with impaired interferon-γ (IFNγ) were more susceptible to tumor formation when exposed to carcinogens, as compared to mice with functional IFNγ (Kaplan et al., 1998; Qin et al., 2002; Street et al., 2001). Similar results were obtained when mice with impaired IL-12 and IL-23, two cytokines important in IFNγ production, were exposed to carcinogens (Smyth et al., 2000). In contrast, aberrant cytokine production has been associated with tumor formation. A number of cytokines including IL-15, tumor-necrosis factor-α (TNFα), IL-6, macrophage colony-stimulating factor (M-csf), and IL-1 have been associated with increased tumor growth and metastasis when overexpressed (Fehniger et al., 2001; Hilbert et al., 1995; Lattanzio et al., 1997; Lin et al., 2001; Moore et al., 1999; Voronov et al., 2003).

Cytokines have been administered to patients in a number of studies in an attempt to manipulate the tumor environment favorably. Clinical trials of systemic administration of IFNα have shown a reduced risk of recurrence in patients with malignant melanoma and improved survival in patients with chronic myeloid leukemia (CML) (Kirkwood et al., 2001; Kirkwood et al., 1996). Systemic administration of high doses of IL-2 had modest patient responses for renal-cell carcinoma and melanoma, while low doses of IL-2 increased the number of circulating NK cells (Fyfe et al., 1995; Rosenberg et al., 1993; Soiffer et al., 1996). Similarly, systemic administration of GM-CSF has shown benefit in patients with melanoma, prostate cancer, and pulmonary metastases (Anderson et al., 1999; Rini et al., 2003; Spitler et al., 2000). However, the systemic administration of cytokines is not a targeted approach and off-target side effects have been observed, such as the expansion of Tregs in IL-2 therapies and severe toxicities in TNFα and IL-12 therapies (Atkins et al., 1997; Furtado et al., 2002; Lienard et al., 1992).

Because of the limited clinical benefit and toxicities associated with systemic cytokine administration, other approaches have been investigated. Injecting cytokines directly into the tumor has shown promise in preclinical studies through improved activation of host immune cells, although this approach could be limited in clinical application (Forni et al., 1988; Zhang et al., 2018). Cytokine-based vaccines have also shown promise and are under development. Approaches to cytokine vaccine development includes engineering autologous and allogeneic tumor cells to express molecules such as GM-CSF and IL-12 (Dranoff et al., 1997). The final approach to improve cytokine therapy is through the use of engineered cytokines. Cytokines such as IL-2, IL-4, IL-15, and IFN have been engineered to reduce off-target effects and in other studies, fused to antibodies to increase tumor-specificity (Conlon et al., 2019). As cytokines play an important role in tumor progression, future research should investigate the role that improved cytokine therapies can have in combination with other cancer immunotherapies.

F. Adoptive cell therapy

Unlike other immunotherapies that rely on a therapeutic to activate the patient's immune system, adoptive cell therapy (ACT) overcomes challenges with activating immune cells *in vivo* by directly administering large quantities of lymphocytes to the patient. ACT was originally described in 1988, however the development of a lymphodepleting regimen given in preparation for ACT has been responsible for the remarkable results achieved in recent years (Rosenberg et al., 1988). In a clinical trial of patients with refractory metastatic melanoma, 51% of patients experienced clinical responses to treatment with a lymphodepleting agent followed by ACT (Dudley et al., 2005). The ability to use both unmodified and genetically engineered autologous or allogeneic lymphocytes has extended the clinical applications of ACT in cancer patients. Current advances in ACT will be covered in depth in the following section regarding cellular

immunotherapies. Overall, the field of cancer immunotherapy has progressed rapidly over the past century. There are a number of strategies currently under investigation to utilize the strengths of the immune system to target cancer and it is possible that a combination of these strategies will result in the most effective cancer immunotherapy treatment.

1.2 Cellular Immunotherapies

A. Tumor infiltrating lymphocyte therapy

Tumor infiltrating lymphocyte (TIL) ACT originated in the 1980s, when the combination of cyclophosphamide and TIL was used to cure mice with colon adenocarcinoma of advanced hepatic metastasis (Rosenberg et al., 1986). Current protocols for TIL therapy involve surgically resecting a patient's tumor, isolating and expanding lymphocytes from the tumor, then treating the patient with a preconditioning lymphodepleting regimen before infusing the *ex vivo* expanded lymphocytes back into the patient. To date, advances in TIL therapy have come from knowledge gained from clinical trials treating patients with metastatic melanoma. Early trials using autologous TIL and high-dose IL-2 resulted in an overall response rate of 34% in patients (Rosenberg et al., 1994). Follow-up studies revealed that clinical responses could be correlated to the *in vitro* cytotoxic activity of TIL, the tumor trafficking of TIL, the length and doubling time of cells in culture prior to infusion, and the persistence of the cells once infused into patients (Aebersold et al., 1991; Rosenberg et al., 1990; Schwartzentruber et al., 1994). Optimization of the *ex vivo* expansion protocol for TIL led to the development of a cell product that had specific tumor recognition against melanoma in 81% of patients tested (Dudley et al., 2003). Furthermore, the development of an improved lymphodepletion regimen of cyclophosphamide/fludarabine combined with 2 Gy or 12 Gy TBI, in addition to TIL therapy,

further increased response rates in melanoma patients from 49% to 52% and 75%, respectively (Dudley et al., 2008). Although the response rates in this trial were promising, they were accompanied by higher rates of toxicity, suggesting that further optimization of the lymphodepleting regimen is necessary. More recently, TILs have been successfully expanded from a number of cancer types including renal cell carcinoma, breast cancer, cervical cancer, ovarian cancer, non-small cell lung cancer, gastrointestinal cancer, head and neck cancer, pancreatic cancer, and cholangiocarcinoma (Andersen et al., 2018; Ben-Avi et al., 2018; Fujita et al., 1995; Junker et al., 2011; Lee et al., 2017; Stevanovic et al., 2015; Tran et al., 2014; Turcotte et al., 2013). Preliminary studies have offered promise that TIL therapy can be applied broadly across different types of cancer, however further research is necessary to confirm this. One company in particular, PACT Pharma, is at the forefront of this research. PACT Pharma has developed a precision medicine therapy where they identify and isolate neoepitope sequences, which recognize tumor-specific mutations, within the TCR from a patient's CD8+ T cells. After this, they collect and isolate CD8+ and CD4+ T cells to reengineer their TCRs with the neoepitope-specific TCRs designed. Finally, the engineered CD8+ and CD4+ T cells are expanded and reinfused into the patient. The development of patient and tumor-specific therapies such as the one developed by PACT Pharma hold promise as a potent cancer therapeutic.

B. Engineered TCR (TCR-T) therapy

Genetically engineering T cells offers the ability to increase tumor specificity of T cell therapies. First described in the 1980s, TCR therapy involves engineering T cells to express either native or novel αβ- T cell receptors (TCR) to increase their recognition of tumor antigens (Bluthmann et al., 1988). TCRs have different affinities for peptides presented by the major

histocompatibility complex (MHC), which determines if TCR-peptide engagement and T cell activation will occur. As with other immunotherapies, proper selection of the target antigen is necessary to promote specificity and efficacy of the therapy. TCR-T cells have been tested in a variety of cancers. In one of the most successful TCR-T trials, 80% (16/20) of advanced multiple myeloma patients achieved remission after being treated with New York esophageal squamous cell carcinoma (NY-ESO-1)- specific T cells (Rapoport et al., 2015). MART-1 and Gp100-specific TCR-T cells were tested in patients with metastatic melanoma and produced objective tumor responses in 30% and 19% of patients, respectively (Johnson et al., 2009). Patients treated with TCR-T cells specific for MAGE-A3 developed myocardial damage that resulted in death, giving evidence that although TCR-T cells were designed to have superior on-target specificity, off-target toxicity is still possible (Linette et al., 2013). Interestingly, TCR-T cells specific to MAGE-A4 persisted in patients for up to 5 months after injection, however tumor progression was observed in 70% of patients only 2 months after the treatment was administered (Kageyama et al., 2015). To date, success with TCR-T therapy is limited and several strategies have been employed to improve the functionality of this therapy. Currently under investigation are studies testing the optimization of TCR pairing through the use of cysteine-modified TCRs, a TCR heterodimer, or CD3-independent TCRs, all strategies that have either improved antigen binding or TCR-T cell functionality (Govers et al., 2010).

C. Chimeric antigen receptor (CAR)-T cell therapy

CAR-T cell therapy is unique from engineered TCR therapy in that it allows T cells to recognize target cells in a MHC-independent fashion. Since the development of first-generation CAR-T cells in 1993, three additional generations have been developed, each with increasing

costimulatory activity. First generation CARs consisted of a single-chain variable fragment (scFv) and an intracellular CD3ζ domain, which could successfully stimulate IL-2 production upon antigen recognition and target cell lysis, but could not sufficiently prime resting T cells for expansion and activation (Brocker and Karjalainen, 1995; Eshhar et al., 1993). Second- and third-generation CARs contain a second costimulatory molecule, usually CD28 or OX40/4-1BB, to further stimulate T cell activity (Finney et al., 1998; Hombach et al., 2012; Kofler et al., 2011; Maher et al., 2002; Pule et al., 2005; Savoldo et al., 2011). Finally, fourth-generation CAR constructs, also known as T cell redirected for universal cytokine-mediated killing (TRUCKs), are comprised of a second-generation dually expressing the CAR and IL-12.

Clinical trials using CAR-T cells have been successful against a range of hematological cancers, while challenges remain in the development CAR-T cells to treat solid tumors. CARs targeting CD19, a B cell marker, have been especially successful in patients with leukemias and lymphomas. Tisagenleucel was the first CAR-T cell product approved by the FDA for use in patients with acute lymphoblastic leukemia (ALL). Tisagenleucel has had striking results in clinical trials, with one study showing that just one infusion led to an 81% 3-month survival rate and a 76% 12-month survival rate (Maude et al., 2018). Not only did this study show benefits to patient survival, but in addition, tisagenleucel could be detected in the blood for up to 20 months after infusion, showing evidence of long-term persistence. Positive patient responses to tisagenleucel treatment have also been observed in patients with relapsed/refractory (R/R) chronic lymphocytic leukemia, follicular lymphoma, and multiple myeloma (Schuster et al., 2017; Vitale and Strati, 2020).

Although anti-CD19 CAR-T cell therapy induces positive responses in many patients, side effects such as cytokine release syndrome, neurotoxicity, cytopenia and normal B cell aplasia are observed (Porter et al., 2011; Ramos et al., 2016). The effects of these toxicities can be reduced through administering therapeutics, such as IL-1 and IL-6, to block molecular pathways involved or through additional engineering of the T cells transduced with the CAR (Giavridis et al., 2018; Norelli et al., 2018; Sachdeva et al., 2019; Sterner et al., 2019). Another challenge with anti-CD19 CAR therapies is that some patients experience relapse due to mechanisms such as the loss of CD19 expression, CD19 antigen masking, and trogocytosis (Hamieh et al., 2019; Ruella et al., 2018). Interestingly, the infusion of a second CAR-T cell targeting CD22, another molecule expressed on leukemias and lymphomas, has been an effective way to treat patients that relapse after initial anti-CD19 CAR-T cell therapy (Fry et al., 2018).

The success of CAR-T cell therapies in hematological tumors has broadened their application into the field of solid tumor therapy as well. CAR-T cell therapy in solid tumors has been slower to develop because unlike hematological tumors, which widely express CD19, there lacks a universal antigen widely expressed by multiple solid tumors that would be safe to target. A range of CAR-T cells targeting tumor associated antigens are currently being developed for a variety of cancers including: neuroblastoma, glioblastoma, non-small cell lung cancer, breast cancer, gastric cancer, liver cancer, pancreatic cancer, colon cancer, renal cell cancer, prostate cancer, ovarian cancer, osteosarcoma, and melanoma (Li et al., 2018). Unable to produce clinical results, initial trials with CAR-T cells targeting the alpha-folate receptor (FR-α) in ovarian cancer and CAIX in metastatic renal cell carcinoma were disappointing (Kershaw et al., 2006; Lamers et al., 2016; Sorensen and Erlandsen, 1990). In a phase I clinical trial of biliary tract and pancreatic cancers, 5 of 6 patients treated with HER2-specific CAR-T cells achieved stable

disease (Feng et al., 2018). Similarly, treatment with HER2 CMV CAR-T cells produced stable disease in 7 of 17 patients with HER2-positive glioblastoma that were CMV positive (Nabil Ahmed, 2015). More promising results were obtained when GD2-specific CAR-T cells were administered to patients with neuroblastoma. Of the 11 patients with active disease enrolled in the trial, 3 achieved complete remission and CAR-T cells were detected for up to 192 weeks post-infusion, signaling durable long-term persistence (Louis et al., 2011). Although some clinical successes are beginning to emerge from solid tumor immunotherapy with CAR-T cells, challenges remain. Because there is no universal antigen expressed by solid tumors, patients treated with CAR-T cells often exhibit off-tumor toxicities. This issue could potentially be addressed by the further development of bispecific CARs that contain at least two extracellular antigen recognition domains. Additionally, optimization of the cell manufacturing procedure could lead to increased CAR-T cell persistence *in vivo* and produce cells with the correct effector phenotype (Li et al., 2018).

D. **Natural Killer (NK) Cell Therapy**

NK cells are innate lymphocytes that have the ability to kill cancer cells through multiple mechanisms. One unique advantage of NK cells is that they don't induce graft versus host disease (GVHD), making it possible to develop an off-the-shelf allogeneic NK cell therapy (Miller et al., 2005; Rubnitz et al., 2010; Ruggeri et al., 2002). Therapies using NK cells have been investigated in a number of contexts including as autologous cell therapies, allogeneic cell therapies, and CAR-NK cell therapies. Clinical studies using autologous NK cell products have not been effective at producing positive patient responses, which could be due to the poor functionality of patient-derived NK cells (Geller et al., 2011; Parkhurst et al., 2011; Sakamoto et al., 2015). Additionally, hematopoietic stem cell transplantation (HSCT) studies using

haploidentical donors gives evidence of a greater graft-versus-leukemia (GvL) effect by NK cells than when HLA-compatible donors are used, suggesting that NK cells have enhanced alloreactivity when donor and recipient killer-cell immunoglobulin-like receptors (KIRs) are mismatched (Ruggeri et al., 1999; Ruggeri et al., 2002; Ruggeri et al., 2007). This finding led to several clinical trials using haplo-identical NK cells, which have had promising results. A clinical trial using haplo-identical NK cells in patients with AML showed that after treatment with cyclophosphamide and fludarabine, donor NK cells expanded *in vivo* and 5 of 19 patients achieved complete remission (Miller et al., 2005). Another clinical trial in a pediatric population of 18 patients with AML achieved a 100% 2-year event-free survival using a similar treatment regimen of cyclophosphamide, fludarabine, and haplo-identical NK cells (Rubnitz et al., 2010). Other strategies to increase the efficacy of NK cell therapies include engineering NK cells to express CARs, administration of bispecific killer cell engagers (BiKEs) and tri-specific killer cell engagers (TriKEs) to activate NK cells *in vivo*, and the administration of immune checkpoint inhibitors to restore NK function *in vivo* (Benson et al., 2010; Bryceson et al., 2006; Gleason et al., 2012; Hsu et al., 2018; Tang et al., 2018).

E. γδ T Cell Therapy

γδ T cells are a small class of T cells, distinct from αβ T cells with a TCR composed of γ and δ chains, instead of α and β chains. γδ T cells typically comprise between 1-10% of the peripheral blood and are enriched in the gut and epithelial tissues. Unique from αβ T cells, γδ T cells can recognize antigens in a human leukocyte antigen (HLA)-unrestricted fashion. Additionally, they have properties of both the innate and adaptive immune system, making them versatile T cells that play a protective role in wound healing, tissue homeostasis, infection, and

cancer surveillance (Girardi et al., 2001; Nielsen et al., 2017). In fact, a study in 2015 reported that of all immune cells, the infiltration of γδ T cells into tumors was the strongest predictor of a favorable outcome for cancer patients (Gentles et al., 2015). Not only do γδ T cells possess a range of direct cytotoxic mechanisms against cancer cells, but they can also indirectly exert cytotoxic mechanisms such as stimulating antigen presenting cell maturation, αβ T cell maturation, cytokine secretion, and macrophage recruitment (Ismaili et al., 2002; Meuter et al., 2010; Wang et al., 2006). Although the field of γδ T cell cancer immunotherapy holds tremendous promise, challenges remain in the development of effective therapies, which will be discussed in the section on 'γδ T Cell Immunotherapies'.

1.3 Pharmacology of Cell Therapies

A. Development of a cellular therapy

Compared to traditional "drugs", cellular therapies are more difficult to define. During the drug development process, a new potential therapy must meet strict guidelines on safety, purity, and potency, developed by the Food and Drug Administration (FDA). Typically, drugs are comprised of a molecular structure that can be chemically defined and whose function can be predicted and tested. In contrast, cellular therapies are often comprised of multiple cell types, which can further be divided into cells with different phenotypes and functions. For example, a T cell product may be purified to contain >99% of αβ T cells, however those cells may be distributed across four phenotypes: naïve, central memory, effector memory, and effector memory CD45RA+. Furthermore, due to variation that occurs between donors, one manufacturing protocol may result in two entirely different cell products from two donors. Because of the challenges that are specific to the development of cellular therapies, the FDA's

guidance on determining the safety, purity, and potency of a therapy will vary based on the type of therapy being proposed.

B. Potency

Potency is a critical parameter when characterizing cell product composition and developing the release criteria of a product. However, potency, defined as the quantity of drug required to achieve a defined response, is difficult to determine for cellular therapies. During the drug development process, potency of a cellular therapy is typically determined through assays that test cell functionality or expression of markers that correlate with functionality. For example, potency of the CD19 CAR T cell therapy, tisagenlecleucel, is tested by measuring by the amount of IFNγ produced when exposed to tumor cells *in vitro*. In contrast, potency of the product Xcellerated T cells, an activated T cell product used to treat chronic lymphocytic leukemia, is tested using flow cytometry to measure expression of CD154, a molecule highly correlated with the cell product's activity (Bravery et al., 2013). Currently, the FDA does not require *in vivo* assays to measure the potency of a cellular therapy, but it is difficult to correlate the activity of cells *in vitro* with how they will perform *in vivo* due to a number of factors. Challenges that must be addressed when developing potency assays for cell therapies include variability in cell phenotype and functionality between donors, the stability of the product after expansion or cryopreservation, and the effects of all cell types included in the final product. In addition to these challenges, potency measures are further complicated by the fact that cellular therapies can continue to expand once administered *in vivo* (Ghorashian et al., 2019).

C. Biodistribution.

Understanding the biodistribution of a cell product is important in determining the potential off-target effects that could occur. Cells administered intravenously typically follow a similar distribution pattern, with a large proportion of cells trafficking to organs such as the lungs, spleen, liver and kidneys. Biodistribution studies have shown that T cells rapidly clear from the peripheral blood once injected. One study measuring lymphocyte distribution after a peripheral blood stem cell graft showed that 30 minutes after injection, only 15% of administered cells could be found in peripheral blood, which decreased to less than 3% of infused lymphocytes at one-hour post-infusion (Storek et al., 2002). Overall, biodistribution has been more extensively investigated for CAR-T cells compared to γδ T cells, however CAR-T cells typically persist *in vivo* longer than γδ T cells, suggesting that they have different distribution kinetics. Clinical trials using CAR T cells have shown that the concentration of T cells in the peripheral blood declines after infusion as the cells distribute throughout the body, but then increases to reach a maximum concentration (Cmax) anywhere between 7-14 days post-infusion (Gardner et al., 2017; Hu et al., 2017; Kochenderfer et al., 2015; Kochenderfer et al., 2017; Lee et al., 2015; Locke et al., 2017; Wang et al., 2016b). CAR-T cells usually persist *in vivo* for 2-3 months, however in some cases they can persist for up to 5 years (Davila et al., 2014; Porter et al., 2015). Unlike CAR-T cells, γδ T cells persist *in vivo* for up to one week, suggesting that their Cmax occurs earlier. To date, studies quantifying γδ T cell trafficking have shown that only 1-3% of injected cells can be found at the site of the tumor (Beck et al., 2010; Zoine et al., 2019). Individual tissue analysis shows that the majority of administered γδ T cells can be found in the lungs, spleen, liver, and kidneys (Beck et al., 2010; Wang et al., 2020).

D. Toxicity

Engineered T cell therapies have been associated with two major toxicities: cytokine release syndrome (CRS) and neurotoxicity. CRS is the most common adverse event associated with CAR-T cell therapies and side-effects include fever, chills, and myalgia. However, up to 43% of patients treated with CD19 CAR-T cells exhibit severe CRS, which can lead to death (Brentjens et al., 2011; Davila et al., 2014; Fitzgerald et al., 2017; Maude et al., 2014). Although less frequent than CRS, neurotoxicity is another common adverse advent observed in patients treated with engineered T cell therapies. Clinical trials with both TCR-T and CAR-T cell products have reported cases of mild to moderate encephalopathy (Gust et al., 2017; Morgan et al., 2013). Neurotoxicity in patients treated with engineered T cells is incompletely understood and requires further investigation, although the majority of cases are reversible. In contrast to treatment with engineered T cell therapies with αβ T cells, γδ T therapies have been associated with a minimal risk for adverse events. Not only do γδ T cells have a reduced risk of GVHD, but their short-term persistence *in vivo* is beneficial as well. While CAR-T cells are able to persist *in vivo* for years after they are administered, γδ T cells only persist for up to one week after injection, reducing any potential off-target effects that they could have.

1.4 γδ T cell immunotherapies

A. γδ T cell subsets

γδ T cells can be categorized into subsets based on their expression of the γ-chain and δ-chain of the TCR. In humans, Vδ1 and Vδ2 are the most common subsets and will be the focus of this discussion, however other subsets such as Vδ3 and Vδ5 have also been reported (Fichtner et al., 2020; Pang et al., 2012; Sullivan et al., 2019). Although both subsets belong to the γδ T

cell family, Vδ1 and Vδ2 cells have distinct differences in TCR arrangement, distribution throughout the body, and function. While Vδ1 γδ T cells can pair with the multiple Vγ chains including Vγ2, Vγ3, Vγ4, Vγ5, Vγ8, and Vγ10, Vδ2 cells pair almost exclusively with Vγ9 (Sant et al., 2019). Vδ1 cells are widely distributed throughout the body and can be found in tissues such as the skin, intestine, lymph nodes, liver and spleen (Mikulak et al., 2019). In contrast, Vδ2 cells are primarily found in the peripheral blood, where they usually comprise greater than 90% of the γδ T cell compartment. Additionally, a study in patients with triple-negative breast cancer revealed that infiltration of Vδ1 cells into tumors was positively correlated with remission and survival, but not the infiltration of Vδ2 cells or overall γδ T cell infiltration (Wu et al., 2019). These results suggest that Vδ1 and Vδ2 cells may not be equally effective at treating the same types of cancer, a distinction that could help guide future studies.

Vδ1 cells have shown promise in a number of preclinical studies, however challenges remain in developing an expansion protocol that could be used in a clinical setting. *In vitro* and *in vivo* studies using Vδ1 cells have had success against different types of cancers including leukemia, non-Hodgkin lymphoma, colon cancer, breast cancer, lung cancer, and neuroblastoma (Catellani et al., 2007; Dolstra et al., 2001; Ferrarini et al., 1996; Schilbach et al., 2008; Wu et al., 2015). Unlike Vδ2 cells, Vδ1 cells do not respond to aminobisphosphonates (N-BP), making it more difficult to manufacture a clinical-grade Vδ1 cell product (Tanaka et al., 1995b; Tanaka et al., 1994). Common reagents used in the *ex vivo* expansion of Vδ1 cells include plant mitogens, cytokines, and irradiated feeder cells, however these protocols lack clinical applicability (Siegers et al., 2011; Wu et al., 2015). More recently, a clinical-grade expansion protocol using a combination of an antibody (OKT-3) and cytokines (IL-4, IFNγ, IL-21, IL-1β)

was developed, although it has yet to be tested in patients (Almeida et al., 2016). Another area of concern in the development of a Vδ1 therapy is the evidence that Vδ1 cells can have a pro-tumor role. IL-17 producing Vδ1 cells have been implicated in promoting tumor progression in a number of studies, further complicating the development of Vδ1 therapies (Wu et al., 2014). To progress Vδ1 immunotherapies into clinical trials, it is necessary to develop a clinical grade expansion protocol that exclusively produces $V\delta1^{+}$ IL-17^{-} cells.

Vδ2 immunotherapies have rapidly progressed from initial proof of concept studies into clinical trials. Vδ2 cell expansion is stimulated by phosphoantigens (pAgs), such as bromohydrin pyrophosphate (BrHPP) or (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), and the aminobisphosphonate (n-BP) zoledronic acid (ZOL). BrHPP and HMBPP are synthetic and natural pyrophosphates, respectively, that increase the concentration of isopentenyl pyrophosphate (IPP), allowing for increased recognition of target cells by γδ T cells (Yazdanifar et al., 2020). Similary, n-BPs increase the intracellular concentration of IPP and geranyl pyrophosphate (GPP) by inhibiting farnesyl pyrophosphate synthase (FPPS) in the mevalonate pathway. It is thought that IPP and GPP can induce a change in butyrophilin-3 family molecules on antigen presenting cells (APC) or target cells, which leads to the recognition and activation of Vδ2 cells through interactions with the γδ TCR (Yazdanifar et al., 2020). To date, two approaches have been used in the development of Vδ2 cancer immunotherapies: administration of activating molecules, such as pAgs and n-BPs, to facilitate the expansion of γδ T cells directly *in vivo*, or through the isolation and expansion of γδ T cells *ex vivo* for autologous or allogeneic cell transplant.

B. In vivo expansion

Strong preliminary data showing that γδ T cells are cytotoxic against a variety of cancers facilitated research aimed at expanding the cells directly *in vivo*. In these studies, n-BPs or pAgs were administered directly to patients, often with IL-2, to activate and expand γδ T cells *in vivo* (Bennouna et al., 2010; Dieli et al., 2003; Dieli et al., 2007; Kunzmann et al., 2012; Lang et al., 2011; Meraviglia et al., 2010; Pressey et al., 2016; Wilhelm et al., 2003). Although some studies were successful in increasing the number of circulating γδ T cells, the overall patient response rates were disappointing. Several limitations with the *in vivo* expansion strategy have emerged. First, the most commonly used n-BP, zoledronate, is rapidly cleared from the peripheral blood and sequestered in the bone, limiting the amount of drug available to effectively increase expansion (Chen et al., 2002). Additionally, the administration of activating agents directly into the patient is a non-specific approach to γδ T cell expansion and off target effects, such as the expansion of Tregs, have been observed in a number of studies (Pressey et al., 2016). Due to these limitations, current studies have shifted focus to the development of *ex vivo* expansion strategies.

C. Ex vivo expansion

One of the greatest advantages that γδ T cell therapies have over other cellular therapies is their potential use as an off-the-shelf allogeneic product that can be stored and ready for immediate use. In comparison, αβ T cell therapies must be developed as autologous cell products or the donor and patient must undergo HLA-matching to reduce the risk of GVHD. A number of expansion strategies have been developed, however the most common is the use of ZOL (1-5 μM) in combination with IL-2, as these reagents are effective at stimulating γδ T cell expansion and are approved for use in humans. Clinical trials conducted in patients with renal cell cancer, multiple myeloma, non-small cell lung cancer, breast cancer, cervical cancer, ovarian

cancer, colorectal cancer, and gastrointestinal cancer have shown that autologous *ex vivo* expanded γδ T cell therapies are safe and well-tolerated (Abe et al., 2009; Bennouna et al., 2008; Izumi et al., 2013; Kobayashi et al., 2011; Kobayashi et al., 2007; Nakajima et al., 2010; Nicol et al., 2011; Sakamoto et al., 2011). More recently, the field has progressed to testing allogeneic γδ T cell products in clinical trials. A number of clinical trials have been initiated within recent years and are currently recruiting patients. Although early clinical data suggests that allogeneic γδ T cell products are safe (Xu et al., 2020), little is known about their efficacy in comparison to autologous γδ T cell products. If current clinical trials with allogeneic γδ T cells can't improve patient responses, other strategies such as genetic engineering could be employed.

D. Challenges that remain

In comparison to the exciting breakthroughs made in preclinical studies with γδ T cells, clinical trials have had disappointing results, suggesting not only that there is room for improvement, but also that the use of γδ T cells could be maximized in combination with other therapeutic strategies. For example, one group has developed a bispecific antibody that recognizes Vγ9 on γδ T cells and Her2 on tumor cells that successfully increases γδ T cell cytotoxicity against tumor cells expressing Her2 (Oberg et al., 2015; Oberg et al., 2014). Combining the use of γδ T cells with checkpoint inhibitors is also of interest, as *ex vivo* expanded cells express exhaustion markers. More specifically, blocking PD-1 and TIM-3 on γδ T cells can increase their cytotoxicity (Li et al., 2018; Rossi et al., 2019). Another therapeutic strategy of interest is engineering γδ T cells to express a CAR. γδ T cells expressing CARs directed against CD19 and GD2 have been developed, although further studies are necessary to develop them therapeutically.

Chapter 2: Characterization of donor variability for γδ T cell ex vivo expansion and development of an allogeneic γδ T cell immunotherapy.

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The work contained in this chapter was published in Frontiers in Medicine: Gene and Cell Therapy in 2020.

Citation:

Burnham RE, Zoine JT, Story JY, Garimalla SN, Gibson G, Rae A, Williams E, Bixby L, Archer D, Doering CB, Spencer HT. Characterization of Donor Variability for γδ T Cell *ex vivo* Expansion and Development of an Allogeneic γδ T Cell Immunotherapy. Front Med (Lausanne). 2020 Nov 13;7:588453. doi: 10.3389/fmed.2020.588453. PMID: 33282892; PMCID: PMC7691424.

2.1 Abstract

Gamma delta (γδ) T cells recently emerged as an attractive candidate for cancer immunotherapy treatments due to their inherent cytotoxicity against both hematological and solid tumors. Moreover, γδ T cells provide a platform for the development of allogeneic cell therapies, as they can recognize antigens independent of MHC recognition and without the requirement for a chimeric antigen receptor. However, γδ T cell adoptive cell therapy depends on *ex vivo* expansion to manufacture sufficient cell product numbers, which remains challenging and limited by inter-donor variability. In the current study, we characterize the differences in expansion of γδ T cells from various donors that expand (EX) and donors that fail to expand, i.e. non-expanders (NE). Further, we demonstrate that IL-21 can be used to increase the expansion potential of NE. In order to reduce the risk of graft versus host disease (GVHD) induced by an allogeneic T cell product, αβ T cell depletions must be considered due to the potential for HLA mismatch. Typically, αβ T cell depletions are performed at the end of expansion, prior to infusion. We show that γδ T cell cultures can be successfully αβ depleted on day 6 of expansion, providing a better environment for the γδ T cells to expand, and that the αβ T cell population remains below clinically acceptable standards for T cell-depleted allogeneic stem cell products. Finally, we assess the potential for a mixed donor γδ T cell therapy and characterize the effects of cryopreservation on γδ T cells. Collectively, these studies support the development of an improved allogeneic γδ T cell product and suggest the possibility of using mixed donor γδ T cell immunotherapies.

2.2 Introduction

Gamma delta (γδ) T cells are a unique and promising candidate for the development of cancer immunotherapy treatments. γδ T cells are key players in the innate immune system, inducing cytotoxicity directly through the expression of cell surface receptors such as the γδ T-cell receptor and natural killer group 2D (NKG2D) (Nedellec et al., 2010; Wrobel et al., 2007). Upon activation, γδ T cells release cytotoxic cytokines and chemokines that directly contribute to the cytolysis of tumor cells (Tikhonov et al., 2006; Todaro et al., 2009). Additionally, γδ T cells serve as modulators in the adaptive immune system and can target tumor cells indirectly by priming αβ T cells, recruiting B cells to assist with antibody production, and activating dendritic cells (Caccamo et al., 2006; Ismaili et al., 2002; Wang et al., 2001). A variety of cytotoxic mechanisms have been shown to contribute to γδ T cell anti-tumor activity in preclinical studies (Beck et al., 2010; Kang et al., 2009; Lozupone et al., 2004; Otto et al., 2005; Santolaria et al., 2013), and moderate patient responses to γδ T-cell immunotherapies have been observed in early phase clinical trials (Bennouna et al., 2008; Dieli et al., 2007; Kobayashi et al., 2007; Kunzmann et al., 2012; Lang et al., 2011; Meraviglia et al., 2010; Nakajima et al., 2010; Sakamoto et al., 2011).

There are two main subsets of γδ T cells currently under investigation: Vδ1 and Vδ2. Vδ1 T cells, while enriched in tissues such as the intestine, colon, and dermis, comprise only a small percentage of circulating peripheral blood γδ T cells. While studies have shown that Vδ1 T cells may have increased cytotoxic potential against certain hematopoietic malignancies and solid tumors (Almeida et al., 2016; Mikulak et al., 2019; Wu et al., 2019), challenges remain in developing a good manufacturing practice (GMP) compliant expansion protocol. Unlike Vδ2 T cells, Vδ1 T cells neither respond to N-BP nor phosphoantigen (pAG)-mediated stimulation (Bukowski et al., 1995; Tanaka et al., 1995b; Tanaka et al., 1994). Protocols have been developed

to successfully expand Vδ1 T cells using plant mitogens, cytokines and irradiated feeder cells (Knight et al., 2012; Siegers et al., 2011; Wu et al., 2015), and some are advancing to clinical trials. In addition, recent evidence has implicated IL-17 producing Vδ1 T cells in promoting tumor progression (Silva-Santos et al., 2015; Wu et al., 2014), revealing the importance of characterizing IL-17 production and ensuring that γδ T-cell therapies do not promote tumor growth. Given these challenges and the fact that clinical-grade N-BP and pAG are available and approved for use in humans, Vδ2 T cells have been a primary focus for clinical development.

Clinical trials using γδ T-cell based immunotherapies have tried to expand Vδ2 T cells *in vivo* through direct administration of stimulating agents (Dieli et al., 2007; Kunzmann et al., 2012; Lang et al., 2011; Meraviglia et al., 2010) or *ex vivo* through the collection and stimulation of γδ T cells in a pool of peripheral blood mononuclear cells (PBMCs) (Kobayashi et al., 2007; Nakajima et al., 2010; Sakamoto et al., 2011). *In vivo* expansions of γδ T cells using a combination of the amino-bisphosphonate (N-BP), zoledronate, and the cytokine, IL-2, have proved difficult due to the off-target expansion of regulatory T cells (Koreth et al., 2011; Pressey, 2016) and dose-limiting toxicities associated with cytokine therapies (Kammula et al., 1998). Due to these challenges, γδ T cells have been investigated in the context of adoptive cell transfer, in which autologous cells are expanded *ex vivo* and reinfused into the patient (Abe et al., 2009; Bennouna et al., 2008; Nakajima et al., 2010; Nicol et al., 2011; Sakamoto et al., 2011). This approach allows for the selective expansion of γδ T cells and the complete characterization of effector cells. However, the feasibility of adoptive cell transfer can be reduced due to challenges faced in expanding γδ T cells from patient derived PBMCs. Due to their ability to recognize target cells independently of human leukocyte antigen (HLA) mediated antigen presentation, γδ T cells are a viable candidate for allogeneic cell therapies in which third party donor cells are expanded *ex vivo*

and either immediately infused into a patient or cryopreserved until needed for treatment.

Expanding a clinically relevant number of γδ T cells from PBMCs remains a significant challenge in the development of an allogenic γδ T cell immunotherapy. Our lab has developed a GMP compliant protocol for the serum-free expansion of Vδ2 T cells from PBMCs using zoledronate and IL-2 (Sutton et al., 2016; Zoine JT and Sutton KS, 2019). However, we and others have reported significant donor to donor variability in the *ex vivo* expansion of γδ T cells (Khan et al., 2014; Salot et al., 2007). Recent studies have shown up to an 80-fold difference between donor expansions, and while γδ T cells from some donors' PBMCs expand to comprise greater than 90% of the total PBMC culture at the end of expansion, other donors never achieve more than 30% of the total culture (Khan et al., 2014). Understanding the differences in inter-donor variability in order to select donors whose PBMCs will successfully expand is essential for clinical use of γδ T cells in an allogeneic cell therapy setting.

The goals of these studies were to characterize the variability in the expansion of healthy donor γδ T cells and to optimize the expansion process for the development of a mixed donor γδ T cell immunotherapy. We report herein (i) a characterization of the variability in expansion of healthy donor γδ T cells; (ii) evidence that γδ T cell expansion from NE can be "rescued" with the addition of IL-21; (iii) optimization of the protocol for the depletion of αβ T cells during the expansion of γδ T cells, reducing the amount of reagents necessary for this procedure; (iv) the development of a novel allogeneic mixed γδ T cell immunotherapy; and (v) a characterization of the effects of cryopreservation on γδ T cells.

2.3 Methods

Expansion of γδ T cells and classification of donors

Peripheral blood (40 mL) from 16 healthy donors was collected through the Emory Children's Clinical and Translational Discovery Core (IRB00101797). Donors were pre-selected based on self-reported levels of exercise and age. The 8 donors in the exercise category self-reported intense physical exercise between 4-7 days a week, while the 8 sedentary donors reported exercising 0-1 day a week. To reduce the impact of age on γδ T cell expansion, all donors that participated in this study were under the age of 35. PBMCs were isolated from whole blood via density gradient centrifugation with Ficoll-Paque Plus (GE Healthcare Life Sciences). After isolation, PBMCs were cultured in OpTmizer media (Life Technologies), supplemented with OpTmizer T-cell expansion supplement, 1% penicillin/streptomycin and 2 mM L-glutamine, and stimulated with zoledronate (Sigma-Aldrich) and IL-2 (Peprotech). Cell counts were performed using a Cellometer (Nexelcom) and cells were resuspended in fresh media at $1.5x10^6$ cells/mL every 3 days. Zoledronate (5 μM) and IL-2 (500 IU/mL) were added on day 0 and 3 of culture. IL-2 (1000 IU/mL) was added on days 6, 9, and 12 of expansion. Flow cytometry was used to determine the percentage of γδ T cells in culture on days 0, 6, and 12 or 14 of expansion. Cell growth experiments were performed by plating 5×10^6 cells for each donor and taking cell counts of the expansion every 3 days.

While testing the supplementation of other cytokines, all expansions received zoledronate (5 μM) and IL-2 (500 IU/mL) on day 0 and 3 of culture. The "normal" expansion conditions received 1000 IU/mL of IL-2 on day 6, 9, and 12 of expansion. The other conditions received 10 ng/mL of IL-15 (Peprotech), IL-21 (Peprotech), or IL-15+IL-21 from day 0 through day 12 of expansion and 500 IU/mL of IL-2 on day 6, 9, and 12 of expansion. As stated above, cell growth experiments were performed by plating 5×10^6 cells for each donor and taking cell counts of the expansion every 3 days.

RNA-sequencing

RNA-sequencing was performed on *ex vivo* expanded and cell sorted γδ T cells from 3 donors. Reads were aligned with Kallisto and transcripts per gene were collapsed to a gene. Kallisto reported 26,898 genes present and these genes were sorted to include genes present in 3 samples with an average of at least 0.2 transcripts per million reads (TPM), generating a list of 13,693 genes. A value of 1 was added to each collapsed TPM and the values were converted to $\log_2$ to create a relative expression range of 0 to 14.0.

Flow Cytometry

Cells were washed with phosphate buffer saline (PBS) and spun at 300 x g in flow cytometry tubes. The supernatant was decanted and replaced with eBioscience Fixable Viability Dye eFluor780 (ThermoFisher) for 30 minutes. Cells were washed in 10 x PBS and resuspended with the appropriate antibodies. Antibodies from BD Biosciences include: BV421 Mouse Anti-Human CD3 (Clone UCHT1), PE Mouse Anti-Human γδ TCR (Clone 11F2), BUV395 Mouse Anti-Human CD56 (Clone NCAM16.2), BUV395 Mouse Anti-Human CD56, BV711 Mouse Anti-Human CD178 (Clone NOK-1), BV786 Mouse Anti-Human CD107a (Clone H4A3), BV480 Mouse Anti-Human CD3 (Clone UCHT1), APC-R700 Mouse Anti-Human CD56 (Clone NCAM16.2), BV711 Mouse Anti-Human CD27 (Clone M-T271), BUV496 Mouse Anti-Human CD16 (Clone 3G8), BV421 Mouse Anti-Human CD57 (Clone NK-1), BV786 Mouse Anti-Human PD1 (Clone EH12.1), PE-CF594 Mouse Anti-Human PDL1 (Clone MIH1), BUV737 Mouse Anti-Human FAS (Clone DX2), PE Mouse Anti-Human FASL (Clone NOK-1), and BUV395 Mouse Anti-Human CD107a (Clone H4A3). Cells were analyzed using a LSRII (BD Biosciences), an Aurora (CYTEK), and a BD FACSymphony (BD Biosciences).

αβ T Cell Depletions

αβ T cell depletions were performed according to the manufacturer's protocol (Miltenyi Biotec). Briefly, cells were washed in autoMACS Rinsing Solution containing 0.5% BSA (Miltenyi Biotec) and spun at 300 x g for 5 min. Cells were incubated with Anti-TCRα/β-Biotin (Miltenyi Biotec) for 10 minutes at 4°C, then washed in autoMACS Rinsing Solution and filtered through a 0.4 μM filter. Cells were then incubated with Anti-Biotin Microbeads (Miltenyi Biotec) for 15 minutes at 4°C, washed in autoMACS Rinsing Solution, and passed through an LD Column (Miltenyi Biotec). After depletion, cells were counted and resuspended in OpTmizer Media with 1000 IU/mL of IL-2. Flow cytometry was used to assess the efficiency of depletion as described above.

Cytotoxicity Assays

Flow cytometry-based cytotoxicity assays were performed to test the *in vitro* cytotoxic potential of *ex vivo* expanded γδ T cells against a malignant cell line (26, 27). The target cell line used in this study was the chronic myelogenous leukemia cell line K562 (ATCC). Target cells were labelled with Violet Proliferation Dye 450 (BD Biosciences) and incubated with γδ T cells at effector to target cell ratios of 1:1 and 5:1 for 4 hours at 37°C. Flow cytometry was used to measure target cell death, using the dead stain eBioscience Fixable Viability Dye eFluor 780 (ThermoFisher) and the early apoptosis stain Annexin V (BioLegend). In studies using the mixed γδ T cell product after thawing, additional target cell lines were tested including: Nomo-1, MOLT-4, SEM, Nalm-6, and Jurkats. The Nomo-1 and MOLT-4 cell lines were a gift from the laboratory of Dr. Douglas Graham (Emory University). The SEM and Nalm-6 cells lines were a gift from the laboratory of Dr. Curtis Henry (Emory University). The Jurkats cells were obtained from ATCC. γδ T cell cytotoxicity was calculated by subtracting the background cell death of each target cell line from each experimental sample.

Cell Mixing

γδ T cells from individual donors were grown in culture through day 6 of expansion. After performing an αβ depletion on day 6, $2x10^6$ γδ T cells from 3 donors were mixed together at a ratio of 1:1:1 and expanded under normal conditions. Flow cytometry was used to assess the percentage of γδ T cells in culture to determine if γδ T cells from different donors could grow in culture together.

Cryopreservation of γδ T cells

To freeze, γδ T cells were washed with PBS and spun at 300 x g for 5 min. Cells were resuspended at a concentration of 1 x 10^7 cells/mL in Human Albumin U.S.P. Albutein 5% (Grifols Therapeutics Inc.) and 10% DMSO. Cells were frozen at a rate of -1°C per minute and moved to liquid nitrogen storage when they reached a temperature of -80°C. To thaw, γδ T cells were removed from liquid nitrogen and incubated in a 37°C water bath. When the cells were nearly thawed, they were removed from the water bath and diluted in media. Cells were spun at 300 x g for 5 min and then resuspended in media containing IL-2 (1,000 IU/mL).

Statistical Analysis

All figures and statistics were generated in GraphPad Prism Software, Version 8.2.1. Data were analyzed using a Student's *t* test or a two-way analysis of variance (ANOVA) with Sidak's multiple comparisons post hoc tests. Corresponding tests and p-values are stated in the figure legends.

2.4 Results

Expansion of γδ T cells from healthy donors

Variability in the expansion of γδ T cells from healthy donor PBMCs has been reported by multiple groups and poses a challenge in the development of immunotherapies utilizing γδ T cells.

To characterize donor variability, donors were either classified as Non-Expanders (NE) or Expanders (EX) according to the percentage of γδ T cells in culture on the final day of expansion. Donors that had 60% or more γδ T cells on the final day of expansion were classified as EX, while donors that had less than 60% of γδ T cells were classified as NE (Fig. 1A).

In this study, we examined 16 donors and demonstrated that 62.5% expanded (10 of the 16 donors). Interestingly, 100% of self-reported exercisers were classified as EX, while only 25% of sedentary donors were EX (Table 1). The relationship between these variables was significant ($p= 0.007$) such that γδ T cells from donors who exercise were more likely to expand. There was no difference in the starting percentage of γδ T cells in culture between NE (2.91% ± 0.75%) or EX (2.48% ± 0.52%) on day 0 of expansion. However, EX had a higher percentage (76.16% ± 2.05%) of γδ T cells in culture when compared to NE (28.47% ± 6.62%) on day 14 of expansion (Fig. 1B). In contrast, when comparing αβ T cell and NK cell growth on day 14, NE had an average of 33.5% ± 10.54% αβ T cells and 37.83% ± 8.98% NK cells as compared to the 12.1% ± 0.93% αβ T cells and 11.7% ± 1.98% NK cells in EX cultures (Fig. 1C).

Total cell growth and fold expansion was greater for EX ($6.75 \times 10^6 \pm 1.59 \times 10^6$, 2.41-fold ± 0.63) compared to NE ($3.28 \times 10^6 \pm 3.67 \times 10^5$, 0.74-fold ± 0.40) (Fig. S1). There was no difference in the starting number (NE= $1.30 \times 10^5 \pm 3.38 \times 10^4$, EX= $1.16 \times 10^5 \pm 2.18 \times 10^4$) of γδ T cells in culture on day 0 of expansion. However, EX had an average number of $1.57 \times 10^6 \pm 3.50 \times 10^5$ γδ T cells in culture by day 6 of expansion compared to $4.69 \times 10^5 \pm 1.30 \times 10^5$ γδ T cells for NE. Additionally, EX had an average number of $8.63 \times 10^6 \pm 2.56 \times 10^6$ γδ T cells in culture on day 14, while NE had an average of $1.27 \times 10^6 \pm 8.44 \times 10^5$ γδ T cells (Fig. 1D). Although NE had a higher percentage of αβ T cells and NK cells on day 14, we found no significant differences in the numbers of αβ T cells and NK cells between NE ($5.8 \times 10^5 \pm 1.35 \times 10^5$ and

$1.53x10^6 \pm 7.85\ x10^5$) and EX ($1.23x10^6 \pm 2.74x10^5$ and $9.92x10^5 \pm 1.87x10^5$) (Fig. 1E). This difference in growth contributed to a γδ T cell fold expansion of 104.2-fold ± 32.84 for EX compared to 14.97-fold ± 11.93 for NE (Fig 1F).

To test the reproducibility of donor expansion across time, γδ T cells from two EXs were expanded from PBMCs isolated from two different blood draws. We found no significant differences in the percentage of γδ T cells or the number of γδ T cells in culture for either donor across the two time points (Fig. S2).

Table 1. A chi-square analysis comparing the relationship between exercise and *ex vivo* γδ T cell expansion (p= 0.007). Values indicate the number of donors in each category.

	Expander	**Non-Expander**
Exerciser	8	0
Sedentary	2	6

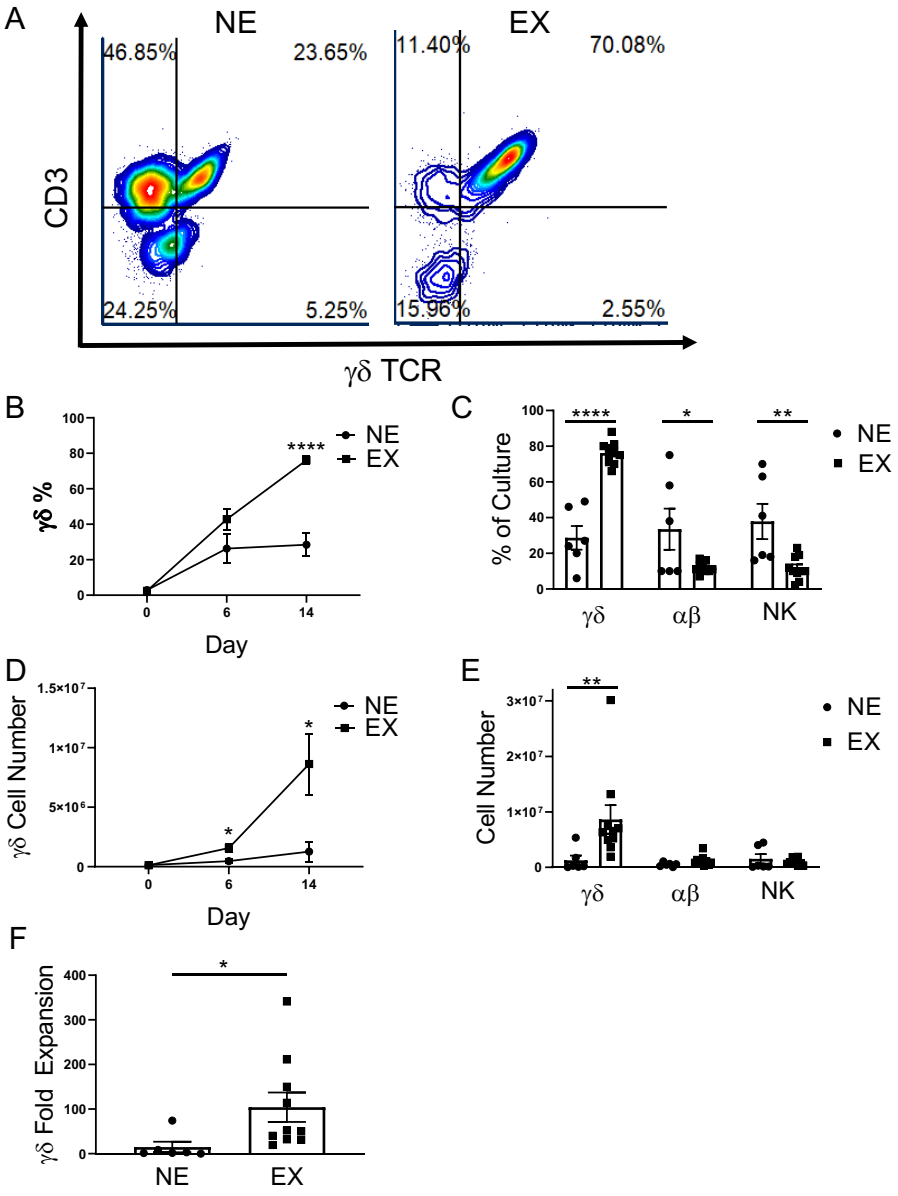


Figure 2.1. **Expansion of healthy donor γδ T cells.** γδ T cells were expanded from healthy donors, labeled either Non-Expanders (NE, n=6) or Expanders (EX, n=10) in two independent experiments. A) Representative flow cytometry plots showing the percentage of γδ T cells (CD3+ γδTCR+), αβ T cells (CD3+ γδTCR-) and NK cells (CD3- γδTCR-CD56+) in culture on day 14 of expansion. B) The percentage of γδ T cells was significantly higher for EX by day 14 of expansion (Student's *t* test, $p < 0.000001$). C) On day 14, EX had a greater percentage of γδ T cells in culture

($p < 0.0001$), while NE had a greater percentage of αβ T cells ($p = 0.022$) and NK cells ($p = 0.003$) (two-way ANOVA). D) Compared to NEs, EXs had a larger number of γδ T cells in culture on day 6 (Student's *t* test, $p = 0.037$) and 14 (Student's *t* test, $p = 0.048$) of expansion. E) EX had a greater number of γδ T cells compared to NE ($p = 0.002$), however there were no differences between EX and NE for the number of αβ T cells or NK cells in culture (two-way ANOVA). F) Due to greater cell growth, EX also had a greater fold expansion of γδ T cells (Student's *t* test, $p = 0.031$).

Characterization of NE and EX at the end of expansion

Flow cytometry was used to characterize Vδ receptor subset type and the expression of chemokine receptors, which are influential in the ability of T cell trafficking towards tumors. In evaluating γδ T cells for Vδ receptor subset and the expression of chemokine receptors, γδ T cells expanded from both NE and EX were primarily composed of the Vδ2 subtype, rather than Vδ1 (Fig. 2A). Based on RNA-seq data from 3 EX donors (Fig. 2B), 5 CC and 4 CXC chemokine receptors were chosen for further evaluation by flow cytometry. There was no difference in the baseline expression of chemokine receptors on day 0 of expansion between NE and EX (Fig. 2C-D). Of the chemokine receptors we analyzed on day 12 of expansion, CCR2 had the highest expression on γδ T cells from both NE (89.28% ± 2.19%) and EX (92.92% ± 0.83%). CCR4, CCR6, CCR7, CXCR1, CXCR3, and CXCR4 were all moderately expressed at similar levels on NE and EX γδ T cells (Fig. 2C-D). CCR3 was expressed similarly on γδ T cells from NE (3.89% ± 2.19%) and EX (2.14% ± 0.29%), although it had the lowest expression of the receptors tested (Fig. 2D).

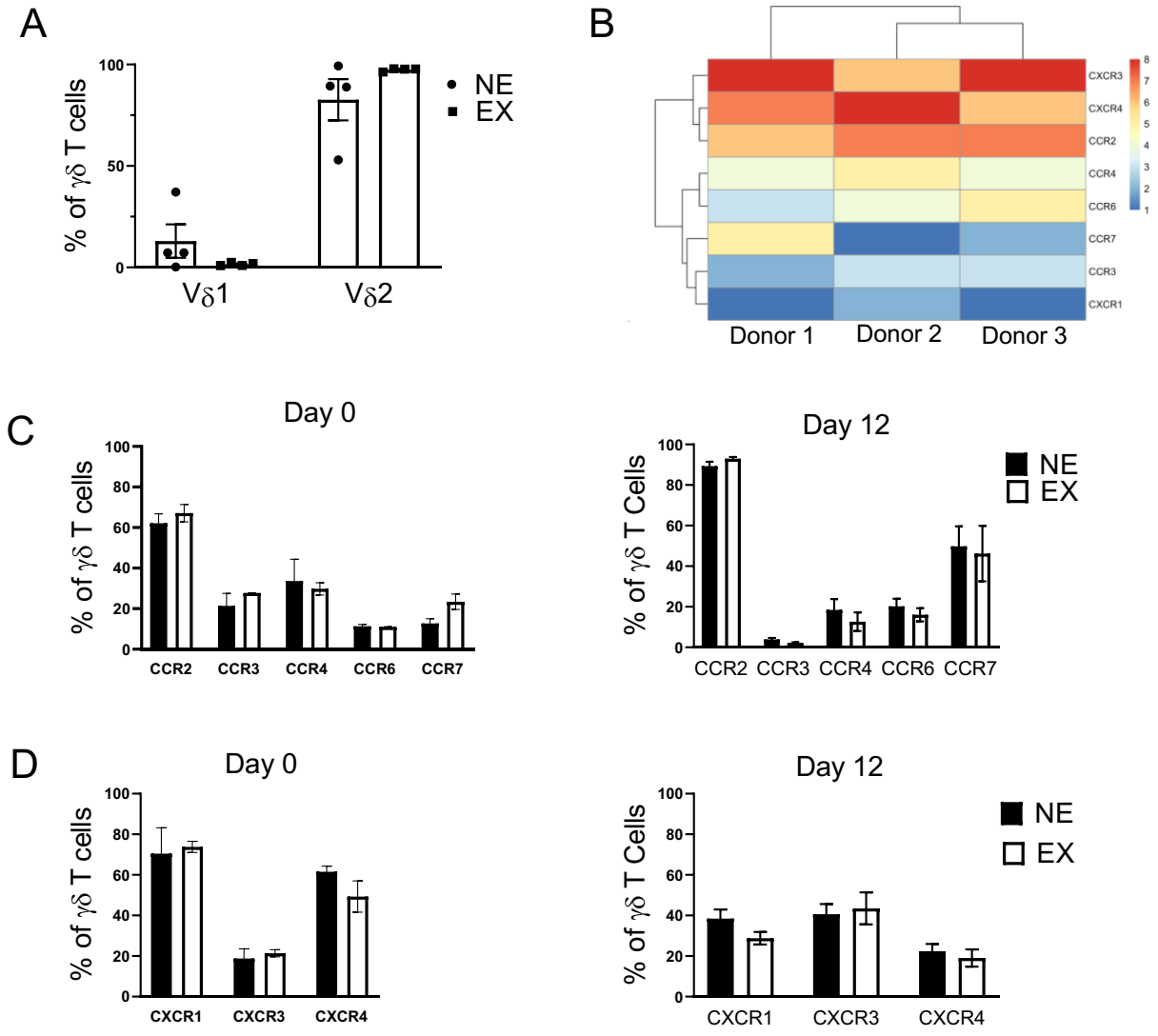


Figure 2.2 γδ T cell receptor subtype and chemokine receptor expression analyzed via flow cytometry and RNA-sequencing. A) The majority of γδ T cells expanded from both NE (n=4) and EX (n=4) were of the Vδ2 subset. B) RNA-sequencing revealed that CCR2, CXCR3, and CXCR4 had the highest expression of the chemokine receptors expressed on γδ T cells at the end of expansion. C) There was no difference in CC receptor expression on γδ T cells from NE or EX on day 0 or day 12 of expansion. Of the CC chemokine receptors, CCR2 was highly expressed, while CCR4, CCR6, and CCR7 were only moderately expressed. CCR3 had the lowest expression

of any chemokine receptors evaluated. D) CXCR1, CXCR2, and CXCR4 were expressed at moderate levels on γδ T cells from both NE and EX on both day 0 and day 12 of expansion.

IL-21 increases the percentage and number of γδ T cells from NE

Common gamma chain cytokines, including IL-2, IL-7, IL-15, and IL-21, have been implicated in the expansion of T cells. Therefore, we investigated the use of these cytokines in combination with IL-2 to determine if they could provide benefit in the expansion of γδ T cells. The effects of IL-7, IL-15, IL-21, and IL-15 + IL-21 on the expansion of γδ T cells was determined for NE and EX. IL-7 decreased the expansion of γδ T cells for both EX and NE and was not investigated further (data not shown). IL-15 had no effect on the percentage or number of γδ T cells in culture for NE. The addition of IL-21 into the culture increased the percentage and number of γδ T cells in culture for NE by day 12 of expansion (Fig. 3A-B, Fig. S3). Similarly, the combination of IL-15 + IL-21 also increased the percentage of γδ T cells in culture for NE by day 12 and had a trend towards increased γδ T cell number. The addition of IL-15, IL-21, and IL-15 + IL-21 had no effect on the γδ T cell percentage for EX. However, as shown in Fig. 3C, EX had significantly more γδ T cells on day 12 of expansion under normal conditions as compared to NE. Although there was a trend toward fewer cell numbers overall for NE as compared to EX, the addition of IL-15, IL-21, and IL-15 + IL-21 increased NE γδ T cell numbers to levels similar to those of EX.

The use of IL-21 in the expansion of γδ T cells has been associated with a reduction in cytotoxic capacity (Barjon et al., 2017). Therefore, we characterized the γδ T cells expanded with IL-21 to ensure that it had no negative impact on functionality or phenotype. The addition of IL-21 into our expansion conditions had no impact on the activation of γδ T cells for NE or EX (S4A), as assessed by the expression of CD69. Similarly, there was no effect of IL-21 on the cytotoxicity or degranulation of γδ T cells when incubated with K562 cells (S4B and C). Additionally, there was no difference in expression of exhaustion (PD-1, TIM3, CD244, and CTLA-4) or senescence

(KLRG1 and CD85j) markers on day 12 for cultures expanded under normal conditions or with the addition of IL-21 (S4D).

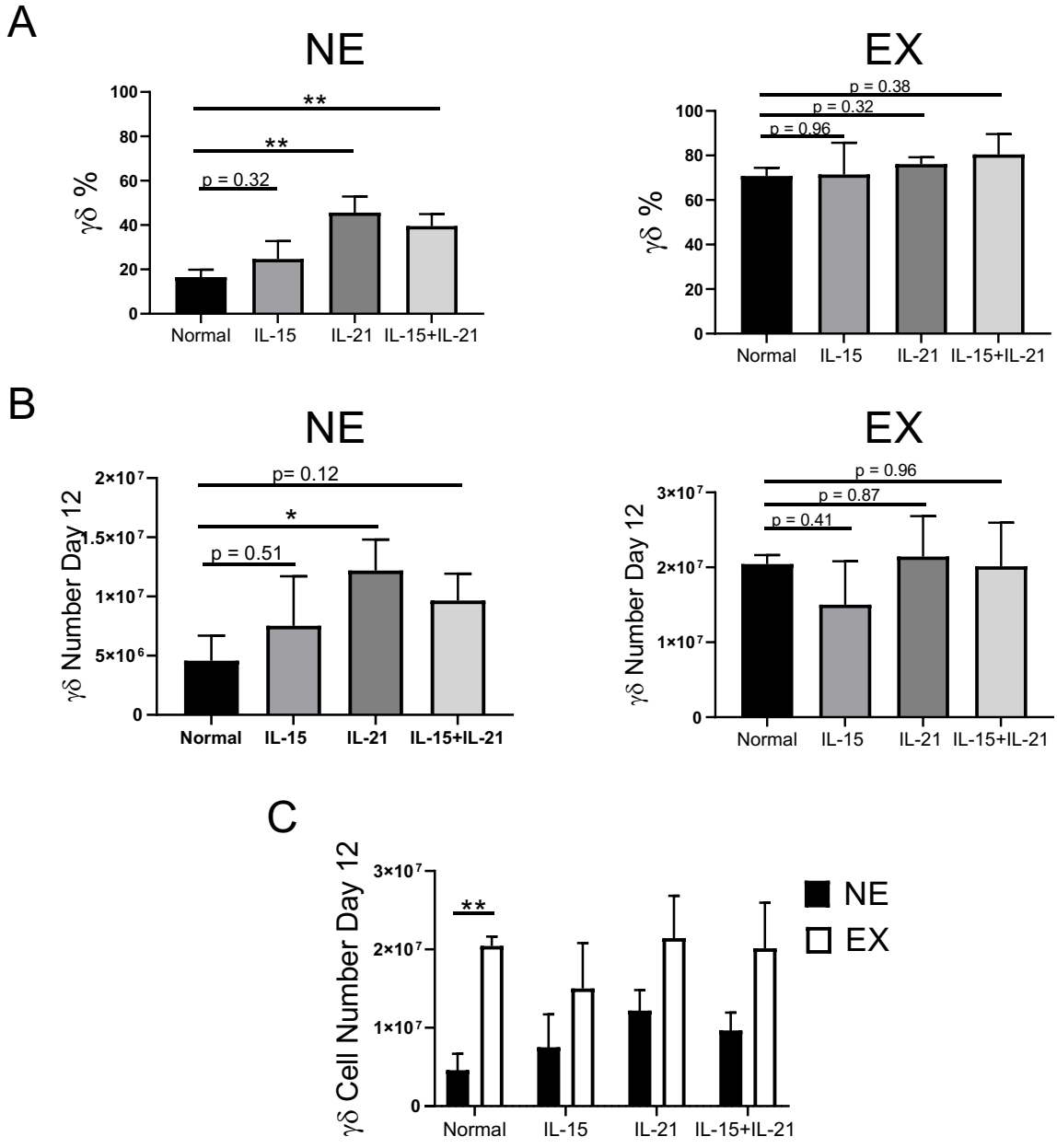


Figure 2.3. Effects of IL-15, IL-21, and IL-15+IL-21 on expansion across 3 independent experiments. A) The addition of IL-21 (Student's *t* test, p= 0.0027) and IL-15+IL-21 (Student's *t* test, p= 0.0035) increased γδ T cell percentage by day 12 of NE (n=7) in comparison to normal expansion conditions. Adding IL-15, IL-21, and IL-15+IL-21 had no effect on the percentage of γδ T cells in culture on day 12 for EX (n=3) (Student's *t* test, p > 0.05). B) The number of γδ T

cells in culture on day 12 for NE was increased with the addition of IL-21 (Student's *t* test, p= 0.0427), but not for IL-15 or IL-15+IL-21 (multiple Student's *t* tests, $p > 0.05$). There was no difference in the number of γδ T cells in culture on day 12 for EX under normal expansion conditions or with the addition of IL-15, IL-21, or IL-15+IL-21 (multiple Student's *t* tests, $p > 0.05$). C) EX had a greater number of γδ T cells on day 12 under normal expansion conditions as compared to NE (Student's *t* test, p= 0.002), however there was no difference in the number of γδ T cells in culture on day 12 between NE and EX with the addition of IL-15, IL-21, and IL-15+IL-21 (multiple Student's *t* tests, $p > 0.05$).

αβ depletion during expansion

To determine if αβ depletions could be performed early in the expansion process, γδ T cells were αβ-depleted on day 0, 3, 6, and 9 of expansion. As observed in Fig. S1A, cell growth peaks at day 12 of expansion and because of this, day 12 was used as the endpoint analysis for all further studies. Depleting αβ T cells on day 0 or 3 resulted in a significant loss of γδ T cells, and due to low initial numbers of γδ T cells was not evaluated further (data not shown)**.** Cultures depleted of αβ T cells on day 6 had a lower percentage of γδ T cells post-depletion (75.34% ± 5.88%) compared to the day 9 depleted cultures (91.69% ± 1.53%) due to a larger starting population of NK cells. Similar to reports characterizing the efficiency of αβ T cell depletion, we report a recovery of γδ T cells between 47-77% (Fig. S5A). Of the γδ T cells lost during the αβ depletion procedure, a percentage was recovered in the αβ fraction (Fig. S5B), and these cells could also be expanded, although not to cell numbers relevant for therapeutic use (Fig. S5C).

For cultures depleted on day 6, the percentage of γδ T cells increased through day 12, and by the end of expansion, we found no difference in the percentage of γδ T cells for day 6 (87.93% ± 2.63%) and day 9 (90.59% ± 1.54%) depleted cultures (Fig. 4A). Following αβ T cell depletion, γδ T cells continued to expand, regardless of the day of depletion (Fig. 4B). Although the depletions were performed on day 6 or day 9 of expansion, the cultures remained depleted through day 12, with less than 1% αβ T cells in culture (Fig. 4C). Additionally, the depletion process did not induce an up-regulation of senescent markers on the γδ T cells, as compared to non-depleted cultures (Fig. 4D).

Although less than 1% of our expanded γδ T cell cultures are typically comprised of αβ T cells, the phenotype of the remaining αβ T cells has important implications for graft versus host disease (GVHD) risk. Therefore, the remaining αβ T cell phenotypes were characterized in culture

(Fig. 4E). Of the αβ T cells in culture, 84.44% ± 1.81% were effector memory (EM) cells, 2.49% ± 0.76% were effector memory CD45RA+ (EMRA) cells, 11.15% ± 1.79% were central memory cells (CM), and 1.93% ± 0.36% were naïve cells.

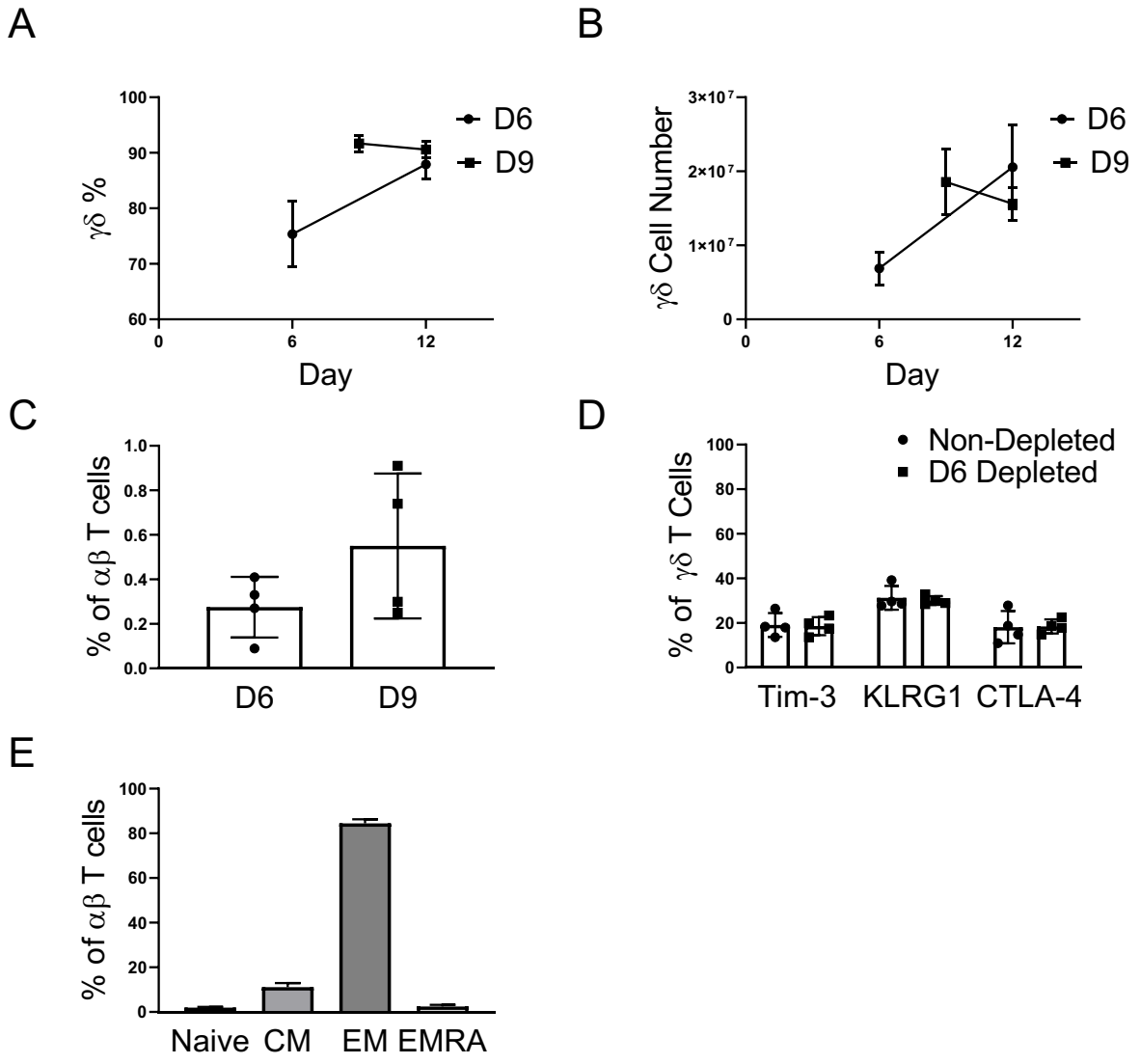


Figure 2.4. αβ depletions performed on day 6 (D6) and 9 (D9) of expansion (2 independent experiments). A) The percentage of γδ T cells post-depletion was lower when the depletion was performed on day 6 (n=6) of expansion versus day 9 (n=6) of expansion, however by day 12 there was no difference in the percent of γδ T cells in culture. B) The number of γδ T cells in culture post-depletion to the end of expansion followed the same trend as the percentage of γδ T cells in culture. C) Following depletion, αβ T cells made up less than 1% of the culture. D) Performing the

αβ depletion did not induce senescence in the γδ T cells by the end of expansion, as compared to a non-depleted culture. E) Flow cytometry was performed determine the proportion of naïve (CD27+CD45RA+), central memory (CD27+CD45RA-), effector memory (CD27-CD45RA-), and effector memory CD45RA+ (CD27-CD45RA+) αβ T cells in culture. Of the αβ T cells in culture on day 12, the majority of cells were of the EM phenotype.

Mixed donor γδ T cell immunotherapy.

γδ T cells provide an opportunity for a mixed donor T cell immunotherapy because of their low risk for GVHD and cross-sample cytotoxicity due to the lack of HLA-γδ TCR engagement. The aim of these experiments was to determine if γδ T cells from different donors could be mixed and expanded in culture together to create a uniform cellular product. After αβ T cell depletion on day 6, the γδ T cells from three individual donors were mixed. The percentage of γδ T cells in the mixed donor γδ T cell product remained constant throughout expansion (Fig. 5A). In contrast, when the αβ fractions from three separate donors were mixed post-depletion (on day 6), three distinct products were observed in the expansion (Fig. S6A). The γδ T cell mixed product continued to grow throughout day 12 (Fig. 5B), with an average fold expansion of 2.81-fold ± 0.16 after mixing (Fig. 5C), while the αβ mixed samples had only a small increase in fold expansion (1.10-fold ± 0.21; Fig. S6B-C).

Similar to γδ T cells expanded from individual donors, the mixed product was comprised of 92.78% ± 1.57% γδ T cells, 0.27% ± 0.06% αβ T cells, and 6.96% ± 1.52% NK cells on day 12 of expansion (Fig. 5D). Moreover, the individual donors used for the mixed cell product expanded to a final composition made up of 92.07% ± 1.24% γδ T cells, 0.23% ± 0.02% αβ T cells, and 7.69% ± 1.25% NK cells. Comparing the mixed γδ T cell product to individual donor γδ T cells, we observed increased cytotoxicity towards K562 myeloid leukemia cells at a ratio of 1:1 and 5:1 (Fig. 5E).

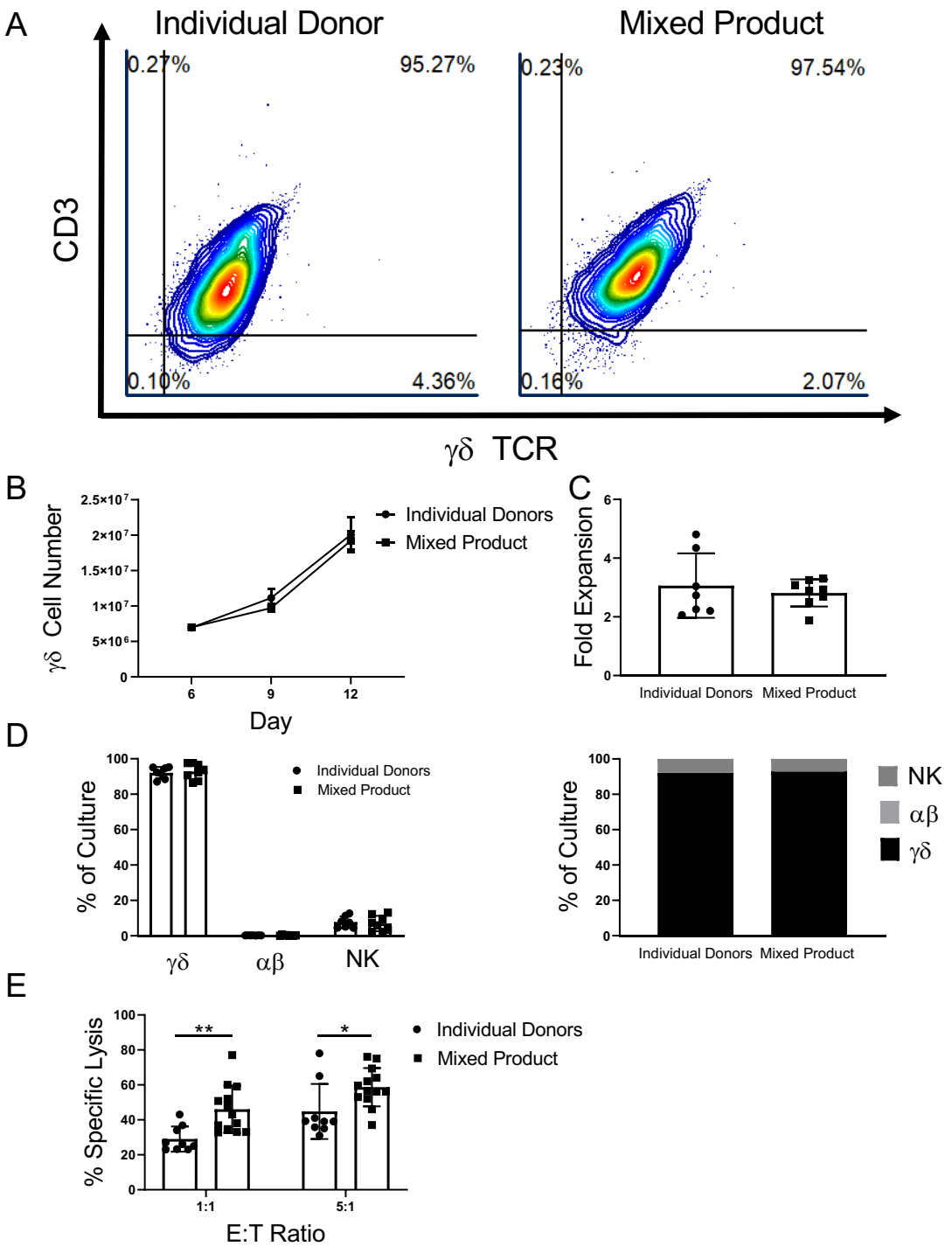


Figure 2.5. Characterization of a mixed γδ T cell product. A) Representative flow cytometry plots showing that γδ T cells from individual donors and the mixed product expand to a uniform

cellular product by day 12 of expansion (2 independent experiments). B) There was no difference in γδ T cell growth between γδ T cells from the individual donors (n=7) and the mixed product (n=8) between day 6 and day 12 of expansion (two-way ANOVA). C) No difference was seen in the fold expansion of individual donor γδ T cells or the mixed product between day 6 and day 12 of expansion (Student's *t* test, $p > 0.05$). D) On day 12 of expansion, there was no difference in the percentage of γδ T cells, αβ T cells, or NK cells in the cultures from individual donors and the mixed product (two-way ANOVA, $p > 0.05$). E) Cytotoxicity of the mixed cell product against K562 cells was greater at the 1:1 ($p= 0.006$) and 5:1 ($p= 0.03$) ratios when compared to the cytotoxicity of individual donor γδ T cells (two-way ANOVA).

Cyropreservation of the γδ T cell product.

After cryopreservation, there was a significant decrease in the percentage of Vδ2 γδ T cells in culture (Fig. 6A). Vδ1 γδ T cells made up less than 4% of all γδ T cells in culture, and cryopreservation had no effect on this percentage. The percentage of NK cells significantly increased post-thaw, likely due to the decrease in total viable Vδ2 γδ T cells.

Phenotypic populations of γδ T cells are difficult to differentiate using the common markers, CD27 and CD45RA. Instead, a recent report has shown that γδ T cells can be differentiated into four main phenotypes using CD27, CD28, and CD16: $\gamma\delta^{28+}$ (CD28+CD27+CD16-), $\gamma\delta^{28-}$ (CD28-CD27+CD16-), $\gamma\delta^{16+}$ (CD28-CD27-CD16+), and $\gamma\delta^{16-}$ (CD28-CD27-CD16-), with $\gamma\delta^{28+}$ being the most common phenotype. In assessing the surface marker (CD27, CD28, and CD16) phenotypes of our mixed donor cell product before and after cryopreservation, we demonstrated an increase in the percentage of $\gamma\delta^{28+}$ and $\gamma\delta^{28-}$ Vδ2 cells, a decrease in $\gamma\delta^{16+}$ Vδ2 cells, and no change in the percentage of $\gamma\delta^{16-}$ cells (Fig. 6B). Of note, these classifications made up no more than 7% of our mixed product, and as a result, we characterized the remaining bulk population of γδ T cells into $\gamma\delta^{28+16+}$ (CD28+CD27+CD16+) and $\gamma\delta^{28-16+}$ (CD28-CD27+CD16+) to account for the majority of the cell population. The $\gamma\delta^{28+16+}$ phenotype comprised 77.2 ± 1.5% of the Vδ2 population, which decreased to 65.7 ± 2.1% post-thaw. The $\gamma\delta^{28-16+}$ population increased from 16.2 ± 1.1% pre-freeze to 24.7 ± 2.3% post-thaw (Fig. 6C). Cryopreservation had no effect on the PD1 or FAS expression on Vδ2 γδ T cells in the mixed product. However, expression of their ligands, PDL1 and FASL, was decreased post-thaw (Fig. 6D), and CD62L and CD107a expression decreased post-thaw, while CD57 remained unchanged.

To ensure the mixed γδ T cell product retained functionality after undergoing a freeze/thaw cycle, the mixed product was tested in a cytotoxicity assay against a range of leukemia cell lines. After thawing, the cells were retained cytotoxicity at the 1:1 and 5:1 effector to target ratio against K562 (24.3% ± 3.8% and 25.3% ± 0.3%), Nomo-1 (10.8% ± 2.4% and 17% ± 2.2%), MOLT-4 (30.0% ± 4.3% and 55.8% ± 9.2%), SEM (38.4% ± 2.9% and 49.4% ± 2.0%), Nalm-6 (43.2% ± 2.2% and 64.8% ± 1.9%), and Jurkats (65.3% ± 5.7% and 70.5% ± 3.1%) cell lines (Fig 6E and S7).

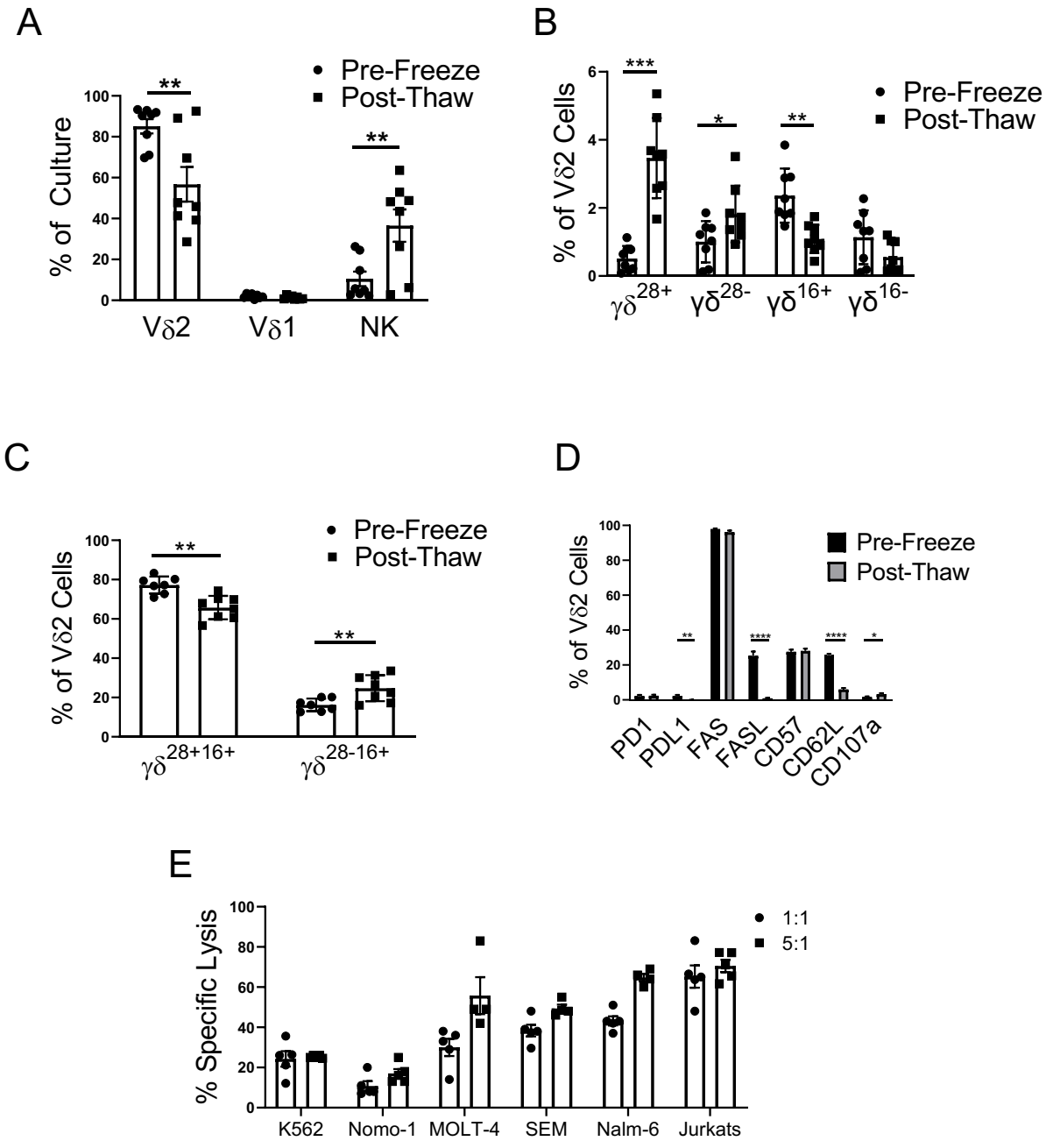


Figure 2.6. Cryopreservation of the mixed cell product (n=8, 2 independent experiments). A) The mixed cell product experienced a decrease in the percentage of Vδ2 γδ T cells post-thaw (Student's *t* test, p= 0.008), while there was no change in the percentage of Vδ1 γδ T cells (Student's *t* test, p > 0.05), and there was an increase in the percentage of NK cells (Student's *t*

test, p= 0.01). B) γδ T cells were categorized into four phenotypes using CD28, CD27, and CD16: $\gamma\delta^{28+}$ (CD28+CD27+CD16-), $\gamma\delta^{28-}$ (CD28-CD27+CD16-), $\gamma\delta^{16+}$ (CD28-CD27-CD16+), and $\gamma\delta^{16-}$ (CD28-CD27-CD16-). There was no difference in the percentage of $\gamma\delta^{16-}$ Vδ2 γδ T cells post-thaw. The percentage of $\gamma\delta^{28+}$ cells (Student's *t* test, p= 0.0005) and $\gamma\delta^{28-}$ (Student's *t* test, p= 0.04) increased post-thaw, while the percentage of $\gamma\delta^{16+}$ decreased (Student's *t* test, p= 0.009). C) To account for the bulk population of γδ T cells, the Vδ2 cells were also categorized into $\gamma\delta^{28+16+}$ (CD28+CD27+CD16+) and $\gamma\delta^{28-16+}$ (CD28-CD27+CD16+). There was a decrease in the percentage of $\gamma\delta^{28+16+}$ cells post-thaw (Student's *t* test, p= 0.000009), while the percentage of $\gamma\delta^{28-16+}$ cells increased (Student's *t* test, p= 0.0013). D) The percent of Vδ2 cells that expressed PD1, FAS, and CD57 did not change after thawing. The percent of PDL1+ (Student's *t* test, p= 0.001), FASL+ (Student's *t* test, p< 0.000001), and CD62L+ (Student's *t* test, p< 0.000001) Vδ2 cells increased post-thaw, while the percent of CD107a+ cells increased (Student's *t* test, p= 0.03). E) Cytotoxicity of the mixed γδ T cell product 48 hours post-thaw against a range of leukemia cell lines: K562, Nomo-1, Molt-4, SEM, Nalm-6, and Jurkats.

2.6 Discussion

Variation in the expansion of γδ T cells from different donors has been reported across the literature. Donors that expand are ideal candidates for the development of an allogeneic γδ T cell therapy, as they have a higher percentage and fold expansion of γδ T cells and therefore also provide a greater number of cells to use for product manufacturing. Additionally, we have shown that individual donor expansion is reproducible. In the context of an allogeneic therapy, having donors fail to expand can delay treatment and increase the cost of production for the therapy. To better define the starting cell population, we analyzed the starting percentage of γδ T cells for NE and EX and found no difference, suggesting that the percentage of circulating γδ T cells in the peripheral blood cannot be used as a predictor for the success of a donor's expansion.

Treating PBMCs with zoledronate and IL-2 results in the selective expansion of γδ T cells. αβ T cells and NK cells also grow in a donor-dependent manner. Our data show that γδ T cells from EX grow preferentially over αβ T cells and NK cells in our specified culture conditions. In contrast, we find no preferential expansion of γδ T cells in a culture of PBMCs from donors that are NE. In fact, of the six NE in this study, half of the cultures were primarily comprised of αβ T cells at the end of expansion, while the other half were primarily comprised of NK cells. It is possible that the donor variability observed in this study, as well as others, could be accounted for by variability in the donors' lifestyles. Exercise immediately prior to PBMC isolation has been shown to increase the *ex vivo* expansion of γδ T cells (Baker et al., 2019). In this study, a donor's level of exercise was predictive of their γδ T cell expansion potential. While 100% of donors who reported high levels of exercise were expanders, only 25% of sedentary donors were classified as expanders. Taken together, these studies suggest that a donor's level of physical activity can be

indicative of whether or not their γδ T cells will expand *ex vivo*. To better understand the difference in expansion potential of NE and EX, further studies characterizing the starting cellular populations are necessary. RNA-sequencing performed immediately after isolation from the blood, paired with expansion data, could give insight into a marker that could predict whether γδ T cells from a particular donor will expand.

There is substantial variation in cell culture methods used to expand γδ T cells. Variation can be found in the media (RPMI, IMDEM, OpTmizer), the addition or absence of serum (FBS, human AB serum), and the type of stimulatory molecules used (i.e., phosphoantigens, aminobisphosphonates, and cytokines). We chose to use the serum-free protocol developed by our laboratory, which uses a combination of zoledronate and IL-2. It is well known that IL-2 is beneficial for the selective expansion of γδ T cells *ex vivo.* Additional cytokines have also been implicated in the expansion of γδ T cells. In this study, we investigated common gamma chain cytokines that have a role in the expansion of T cells: IL-7, IL-15, and IL-21 (Drake et al., 2016; Van Acker et al., 2016; Zheng et al., 2001). IL-7 decreased the expansion of γδ T cells (data not shown) and was not investigated further. Van Acker et al. reported successful γδ T cell expansion with the addition of IL-15 (Van Acker et al., 2016), however, we found that it had no benefit in our culture conditions for either NE or EX. These conflicting results could be due to differences in the expansion protocol used. Our studies are unique in that we assess the benefits of common gamma chain cytokines under serum-free conditions, which is more clinically relevant compared to serum-containing protocols.

The addition of IL-21 increased the expansion of γδ T cells from NE, but had no effect on the expansion of γδ T cells from EX. IL-21 has been shown to induce the proliferation of natural

killer (NK) cells and increase proliferation of activated T cells (Parrish-Novak et al., 2000). Vermijlen et al. showed that IL-21 increased γδ T cell expansion, although not to levels greater than expansion induced with IL-2 (Vermijlen, 2007). Additionally, it is known that IL-21 can enhance the effects of IL-2 and IL-15 on T cell proliferation, which might explain the increase in γδ T cell percentage observed in the IL-15 plus IL-21 condition for NE. These results are significant in the context of adoptive cell therapies in which a patient must receive γδ T cells from HLA-matched donors. Being able to increase the *ex vivo* expansion of NE γδ T cells by the addition of a cytokine would suggest that a successful γδ T cell therapy could be produced from any donor, not just an EX.

αβ T cell depletions are a necessary step in the development of an allogeneic γδ T cell immunotherapy due to the risk for graft versus host disease (GVHD), which is initiated by naïve αβ T cells (Abdelhakim et al., 2017; Korngold and Sprent, 1987). Typically, αβ T cell depletions are performed at or near the end of γδ T cell expansions. However, our aim was to successfully deplete αβ T cells in an EX culture at an earlier time point. Here, we have demonstrated that αβ T cells can be depleted on day 6 or day 9 of expansion and that the culture remains depleted of αβ T cells through day 12. Most importantly, αβ T cells accounted for less than 1% of the total cell population on day 12 and of those that remain, less than 2% were naïve cells. This shows the αβ T cells remaining in culture present an extremely low risk for GVHD (Bleakley et al., 2015). Depleting the αβ T cells in the middle of expansion, compared to the end, requires the use of fewer reagents, which is practical in the context of creating a viable and cost effective cell therapy. Additionally, for the donors tested in this study, depleting the αβ T cells earlier in the expansion resulted in a reduction in the NK cell population by day 12, as compared to the non-depleted cultures.

Creating a mixed donor γδ T cell product is a novel approach towards improving γδ T cell therapies. Due to variability in the expansion and cytotoxicity of γδ T cells from different donors, a mixed donor cell product provides an opportunity to increase the therapeutic efficacy of γδ T cell cancer immunotherapies. Unlike αβ T cells, the risk of developing GVHD is extremely low for patients treated with γδ T cells, making a cell product combined from different donors feasible. We report that γδ T cells from multiple donors can be mixed after αβ T cell depletion and successfully expanded to create a uniform cellular product comprised of 93% γδ T cells and 7% NK cells, on average. When compared to γδ T cells from individual donors, the mixed product had greater cytotoxicity towards leukemia cells *in vitro*. These results suggest that γδ T cells mixed from different donors may have a synergistic effect on each other, resulting in a cell product with greater overall cytotoxicity. Although NK cells comprised 7% of the mixed product, depletion was not considered because NK cells also present low risk for GVHD, are cytotoxic against a range of cancers, and may contribute to the cytotoxicity seen in the γδ cell product.

Cryopreservation is a necessary step in the translation of an off-the-shelf cell product and often requires optimization. Limited information is known about the cryopreservation of γδ T cells and the effects of a freeze/thaw cycle on their health. We characterized the composition of the cell product before and after freezing to determine if there were any phenotypic changes associated with cryopreservation. In the present work, the composition of the mixed product changed dramatically after thawing, with a significant reduction in the percent of Vδ2 γδ T cells and an increase in the percent of NK cells. A recent study extensively characterized the phenotype of γδ T cells based on CD28, CD27, and CD16 expression (Ryan et al., 2016). This group found that there were four main phenotypes that could be used to classify γδ T cells: $\gamma\delta^{28+}$, $\gamma\delta^{28-}$, $\gamma\delta^{16+}$, and $\gamma\delta^{16-}$. However, these four phenotypes could only be used to classify up to 7% of our mixed cell

product and we further characterized our cell product into two additional phenotypes: $\gamma\delta^{28+16+}$ and $\gamma\delta^{28-}$. Ryan et al. found that CD27 expression on γδ T cells was an indicator of expansion potential, while CD16 expression was an indicator of higher levels of cytotoxicity (41). Over 93% of γδ T cells in the mixed product were CD27+CD16+, which could account for their high levels of expansion and cytotoxicity. Additionally, high expression of CD27 on these γδ T cells is important in determining the role that they could have in promoting tumor progression. Studies have shown that a subset of γδ T cells, specifically those that produce IL-17, play a role in tumor progression. IL-17 producing γδ T cells either do not express CD27 or express low levels of CD27 (Caccamo et al., 2011), suggesting that γδ T cells expanded using this protocol will not promote tumor progression *in vivo.*

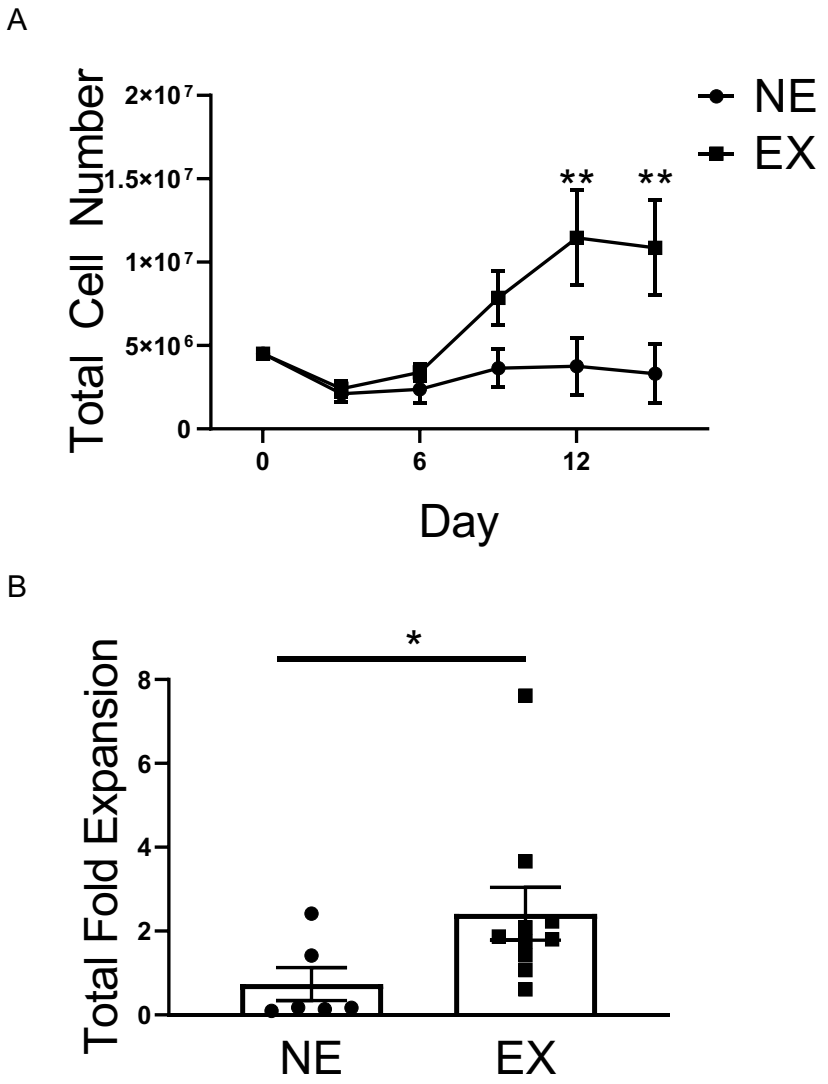
After thawing, the final mixed product was comprised of an average of 66% $\gamma\delta^{28+16+}$ and 25% $\gamma\delta^{28-16+}$ γδ T cells, which is similar to levels our laboratory has previously published on γδ T cells expanded from neuroblastoma patient-derived apheresis products (27). Cryopreservation significantly decreased the population of $\gamma\delta^{28+16+}$ Vδ2 γδ T cells and increased the population of $\gamma\delta^{28-16+}$ cells. As the loss of CD28 on T cells can be an indicator of senescence (Effros, 1997), the increase in $\gamma\delta^{28-16+}$ cells suggests that cryopreservation increases levels of senescence in γδ T cells after thawing. However, the decreased expression of PDL1 and FASL suggests that the mixed product may be less susceptible to tumor-induced apoptosis (Liu et al., 2016) and activation-induced cell death (Tanaka et al., 1995a) after a freeze/thaw cycle. Although we show that cryopreserved γδ T cells were cytotoxic against a range of leukemia cell lines, the overall cytotoxicity of the mixed product against K562 cells before and after freezing was reduced. These results indicate that cryopreservation impacts the composition, phenotype, and functionality of

γδ T cells and further optimization is necessary to reduce the harmful effects of the cryopreservation process.

In addition to optimizing the negative impacts of cryopreservation, an important consideration to optimize the health of the mixed γδ T-cell product is the timing with which the cells will be administered to patients during a regimen of chemotherapy. Chemotherapy negatively impacts the cytotoxicity of resident γδ T cells (Bruni et al., 2019), suggesting that it could also negatively impact cells being infused into a patient if the two treatments are administered closely together. Further studies will be necessary to determine the impacts of chemotherapy on the mixed γδ T cell product and to optimize the course of treatment.

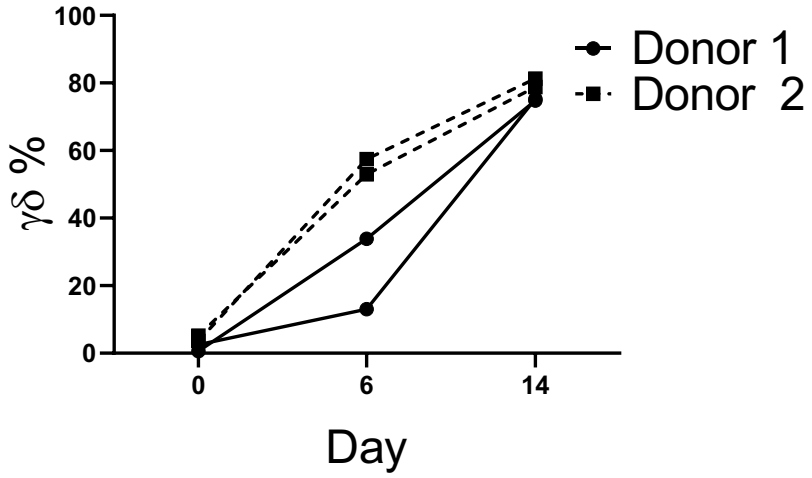
The characterization of the expansion of healthy donor γδ T cells from NE and EX is informative because it shows that the success of an expansion can not be predicted based upon initial γδ T cell percentages. Instead, further research is necessary to determine if there are differences between γδ T cells from NE and EX after isolation from a donor. In the autologous cell transplant setting, where cells from cancer patients usually do not expand well, the use of IL-21 can "rescue" the expansion so that enough cells can be manufactured for treatment. Depleting αβ T cells during the expansion of γδ T cells greatly reduces the amount of reagents necessary for this procedure, allowing for a more cost-effective therapy that can be easily scaled up to clinical levels. Additionally, αβ T cell depletion during expansion allows for the development of a novel allogeneic mixed donor γδ T cell immunotherapy. We show here that a mixed donor γδ T cell immunotherapy has increased cytotoxicity in comparison to an individual donor γδ T cell immunotherapy. As clinical trials with γδ T cell immunotherapies have had limited efficacy, the mixed donor cell product should be considered for development as a more effective treatment.

2.6 Supplemental Figures

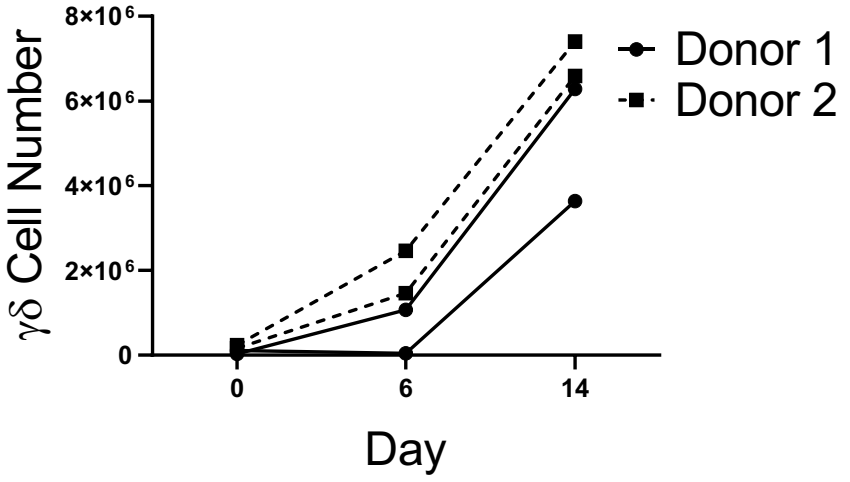


Supplemental Figure 2.1. Overall cell growth was evaluated for NE (n=6) and EX (n=10) in 2 independent experiments. A) The total cell growth was greater for EX on day 12 (p= 0.0028) and 14 (p= 0.0034) of expansion, when compared to NE (two-way ANOVA). B) EX also had a greater fold expansion as compared to NE (Student's *t* test, p= 0.039).

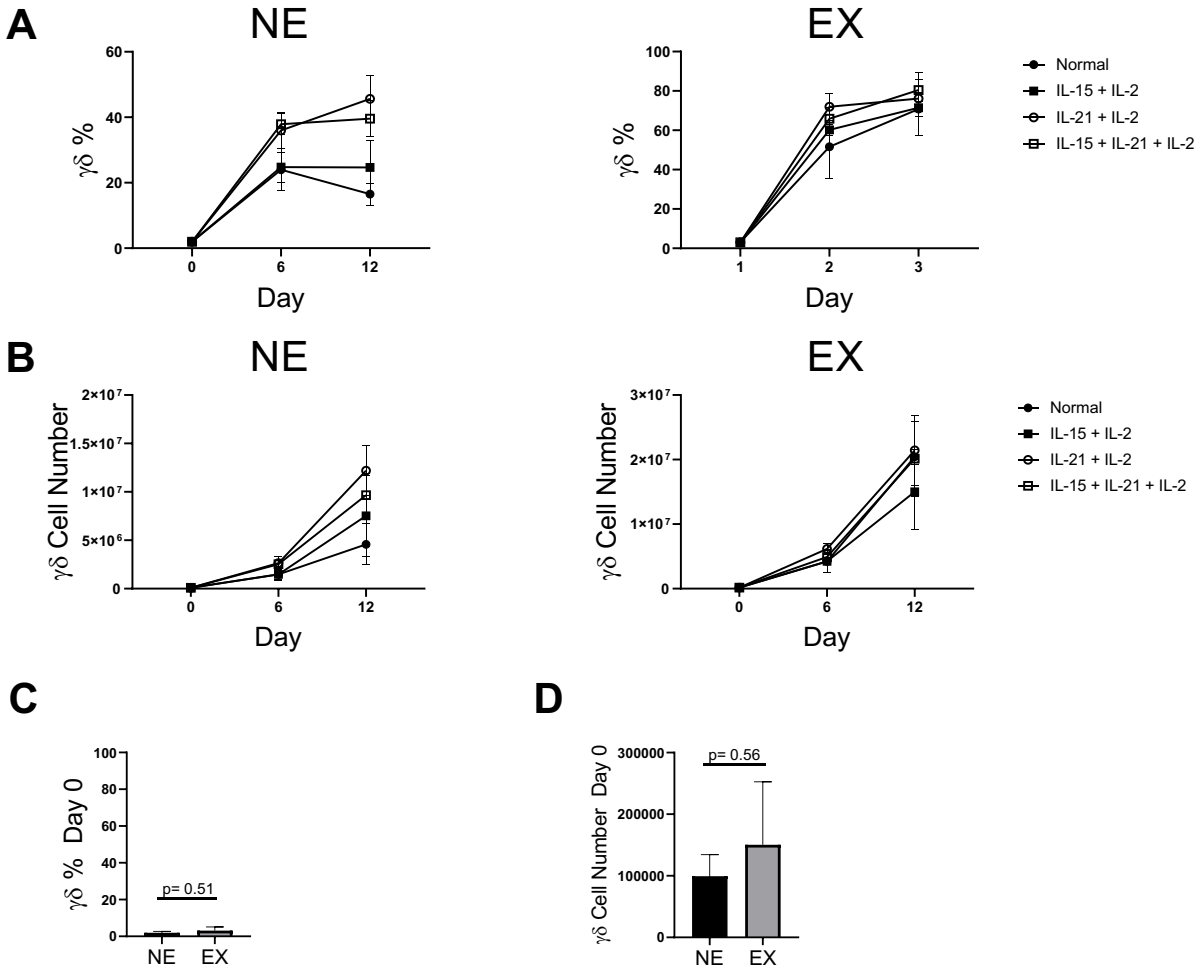
A



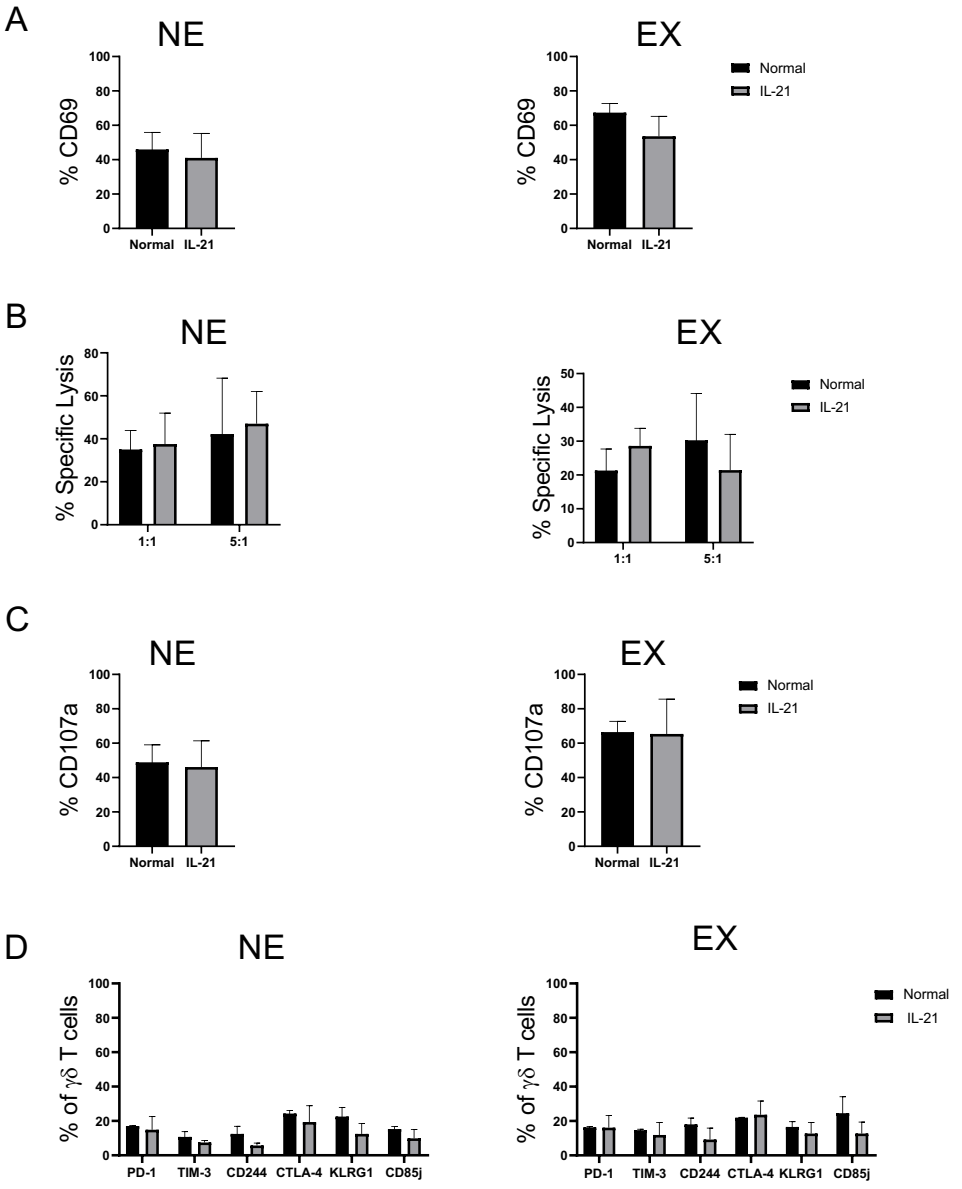
B



Supplemental Figure 2.2. To test the reproducibility of donor expansion across time, two EX were expanded at two different time points (2 independent experiments). A) There was no difference between the two expansions in the percentage of γδ T cells for either donor (paired Student's *t* test, $p > 0.05$). B) Similarly, there was no difference in the number of γδ T cells expanded from either donor at the two time points (paired Student's *t* test, $p > 0.05$).

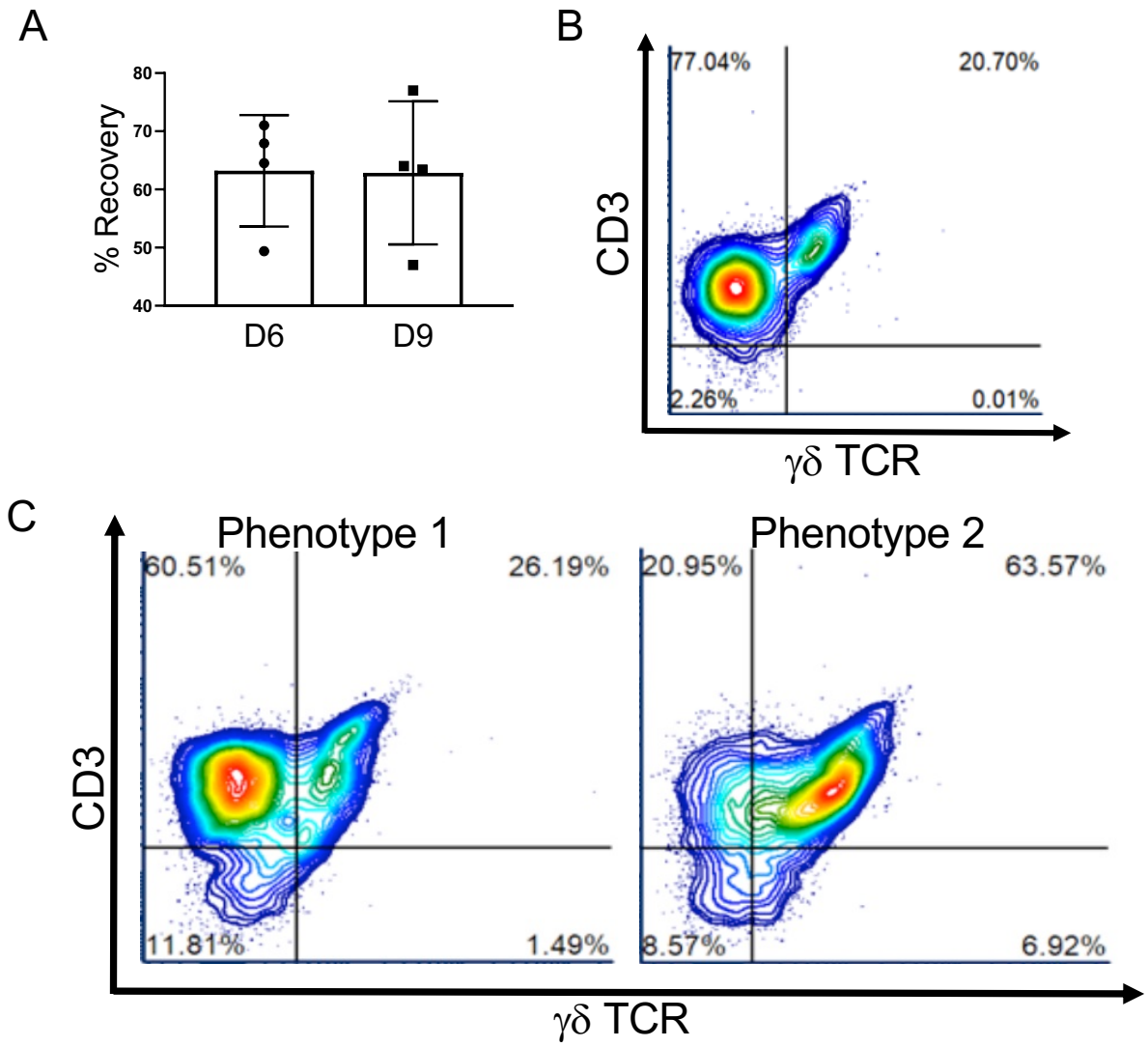


Supplemental Figure 2.3. γδ T cell growth and percentage over time with the supplementation of IL-15, IL-21, or IL-15 and IL-21 in the culture conditions across 3 independent experiments. A) The percentage of γδ T cells in culture for NE and EX on day 0, 6, and 12 of expansion. B) γδ T cell growth for NE and EX. C) There was no difference in the starting percentage of γδ T cells between NE or EX (p= 0.56). D) The number of γδ T cells in culture on day 0 was not statistically significant between NE and EX (p= 0.51).

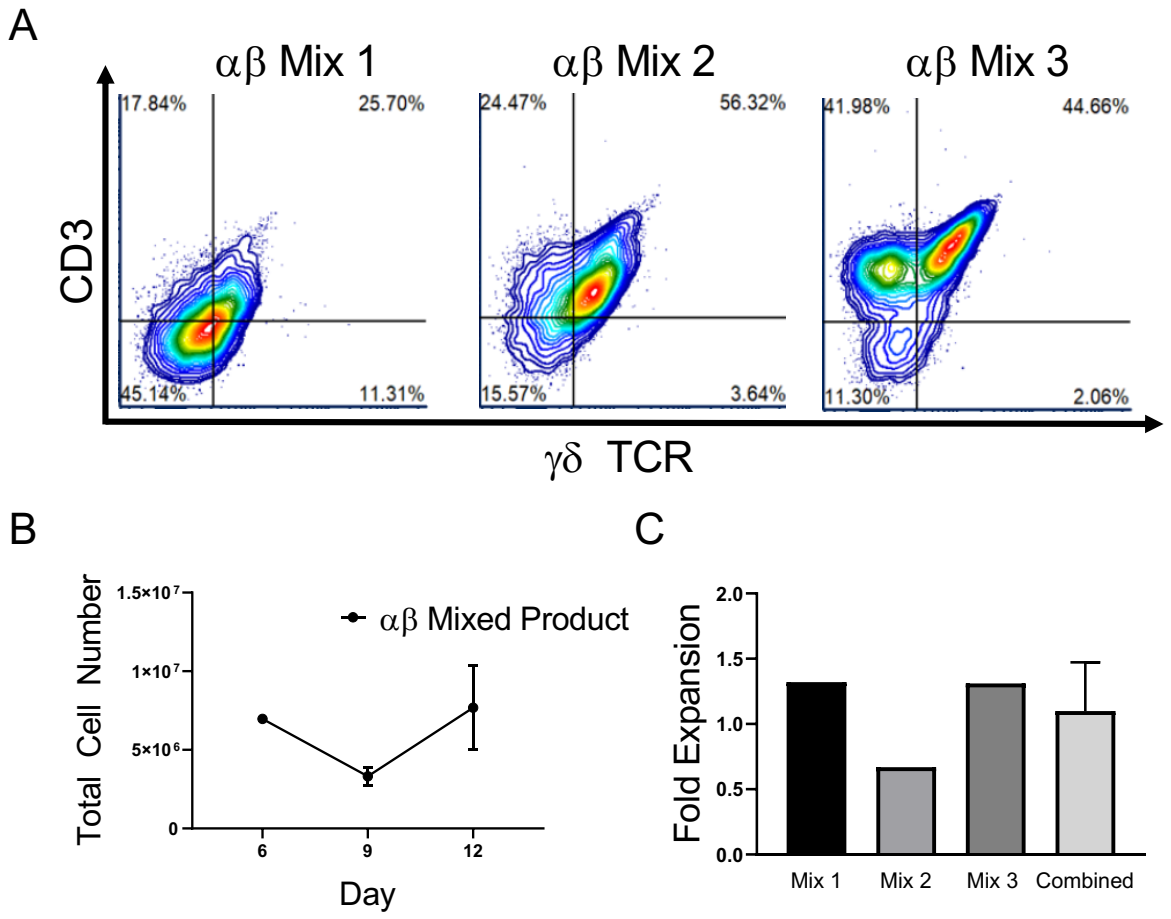


Supplemental Figure 2.4. Characterization of the activation, cytotoxicity, degranulation, exhaustion and senescence for γδ T cells expanded with IL-21 supplementation for NE (n=2) and EX (n=2) (1 independent experiment). Activation, cytotoxicity, and degranulation were assessed on day 12 of expansion after γδ T cells were incubated with K562 cells at a 1:1 ratio (activation, cytotoxicity, degranulation) or a 5:1 ratio (cytotoxicity) for 4 hours. A) There was no difference in CD69 expression for cultures expanded with and without IL-21 for either NE or EX.

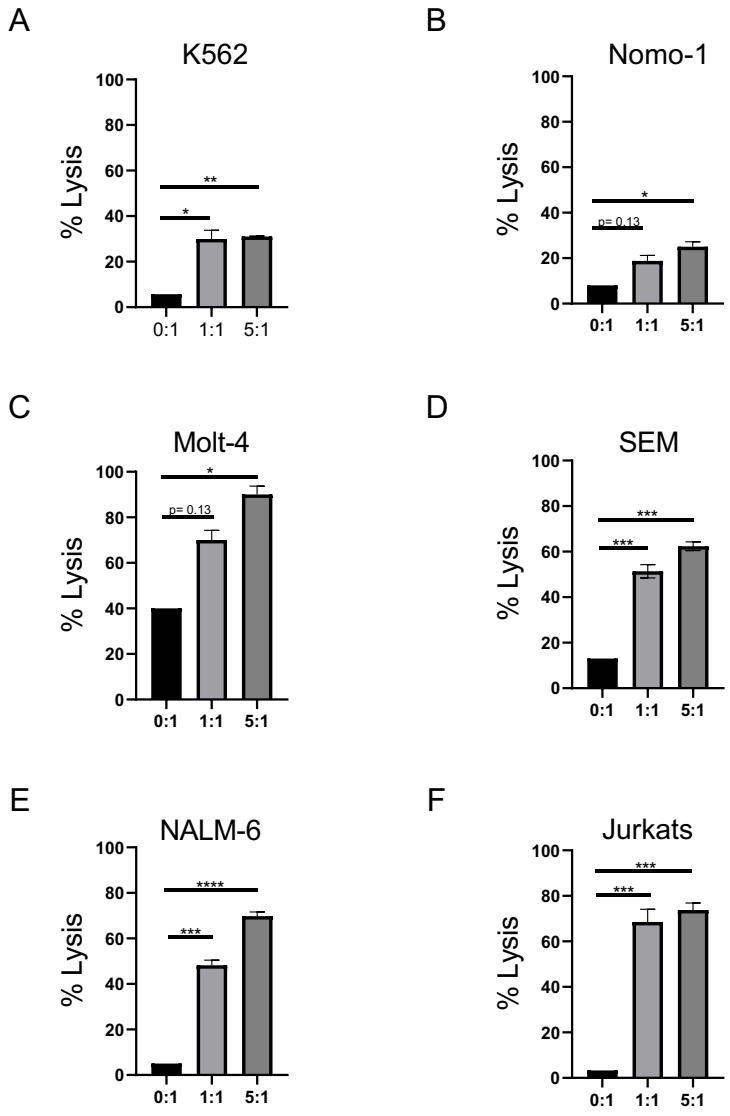
B) Supplementation of IL-21 in the expansion conditions had no impact on cytotoxicity against K562 cells for either NE or EX. C) Similarly, IL-21 supplementation did not affect degranulation when γδ T cells were incubated with K562 cells for 4 hours. D) γδ T cells expanded with Il-21 did not have increased expression of exhaustion (PD-1, TIM3, CD244, CTLA-4) or senescence (KLRG1, CD85j) markers on day 12 as compared to cultures expanded under normal conditions.



Supplemental Figure 2.5. Analysis of the αβ depletion process. A) After the αβ depletion was performed, between 47-77% of the starting number of γδ T cells was recovered. B) A representative flow cytometry plot showing that some γδ T cells were lost to the αβ fraction after depletion. C) Representative flow cytometry plots showing that when the αβ fractions are cultured post-depletion, two phenotypes emerge. Phenotype 1 is primarily comprised of αβ T cells, while Phenotype 2 is primarily comprised of γδ T cells.



Supplemental Figure 2.6. Mixture of the αβ fraction post-depletion does not create a uniform cell product (n= 3, 1 independent experiment). A) Mixture of the αβ fraction after depletion from three donors resulted in three distinct cell products by day 12 of expansion. B) The total cell growth of the αβ mixed products decreased directly after mixing, but recovered to the initial starting number by day 12. C) Two of the three αβ mixed products had a small increase in the fold expansion between day 6 and day 12 of expansion, while the third had a decrease in fold expansion.



Supplemental Figure 2.7. Raw cytotoxicity data showing the background cell death of each negative control (0:1) for the K562, Nomo-1, Molt-4, SEM, Nalm-6, and Jurkats cell lines. Data graphed at the 1:1 and 5:1 effector:target ratios represents the raw cytotoxicity data before the background cell death was subtracted out. One-way ANOVAs were performed for each cell

line to determine if target cell death was statistically significant at the 1:1 and 5:1 ratios as compared to the negative controls.

Chapter 3: Human serum albumin and chromatin condensation rescue *ex vivo* expanded γδ T cells from the effects of cryopreservation

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The work contained in this chapter was published in Cryobiology in 2021.

Citation:

Burnham RE, Tope D, Branella G, Williams E, Doering CB, Spencer HT. Human serum albumin and chromatin condensation rescue ex vivo expanded γδ T cells from the effects of cryopreservation. Cryobiology. 2021 Jan 21:S0011-2240(21)00011-0. doi: 10.1016/j.cryobiol.2021.01.011. Epub ahead of print. PMID: 33485898.

3.1 Abstract

Clinical applications of gamma delta (γδ) T cells have advanced from initial interest in expanding γδ T cells *in vivo* to the development of a manufacturing process for the *ex vivo* expansion. To develop an "off-the-shelf" allogeneic γδ T cell product, the cell manufacturing process must be optimized to include cryopreservation. It is known that cryopreservation can dramatically reduce viability of primary cells and other cell types after thawing, although the exact effects of cryopreservation on γδ T cell health and functionality have not yet been characterized. Our aim was to characterize the effects of a freeze/thaw cycle on γδ T cells and to develop an optimized protocol for cryopreservation. γδ T cells were expanded under serum-free conditions, using a good manufacturing practice (GMP) compliant protocol developed by our lab. We observed that cryopreservation reduced cell survival and increased the percentage of apoptotic cells, two measures that could not be improved through the use of 5 GMP compliant freezing media. The choice of thawing medium, specifically human albumin (HSA), improved γδ T cell viability and in addition, chromatin condensation prior to freezing increased cell viability after thawing, which could not be further improved with the use of a general caspase inhibitor. Finally, we found that cryopreserved cells had depolarized mitochondrial membranes and reduced cytotoxicity when tested against a range of leukemia cell lines. These studies provide a detailed analysis of the effects of cryopreservation on γδ T cells and provide methods for improving viability in the post-thaw period.

3.2 Introduction

Gamma delta (γδ) T cells are a unique subset of T cells that provide a promising avenue for the development of an allogeneic cell immunotherapy for use against both hematological and solid tumors. Unlike αβ T cells, γδ T cells have the ability to recognize malignant cells independently

of Human Leukocyte Antigen (HLA) molecules on antigen-presenting cells (Grusby et al., 1993; Kalyan and Kabelitz, 2013; Morita et al., 1995; Wang et al., 2010), and thus, present a low risk of graft versus host disease (GVHD) in an allogeneic setting (Drobyski et al., 1999). The use of γδ T cells has been investigated in a number of clinical trials, yet significant challenges remain in regards (Abdelhakim et al., 2017) to the manufacturing protocol used for the expansion of these cells. Additionally, there is a lack of information detailing the effects of cryopreservation on γδ T cell health and functionality (Li et al., 2019). Cryopreservation is a critical process in the development of a commercial cell therapy and further investigation is necessary to ensure that the use of cryopreserved γδ T cells are a viable option for therapy.

The use of γδ T cells has clinical applications in a variety of cancers (Beck et al., 2010; Lamb et al., 2013; Lozupone et al., 2004; Santolaria et al., 2013; Zoine et al., 2019), yet only two studies have explored the impact of cryopreservation on γδ T cells (Berglund et al., 2018; Zoine et al., 2019). Although both reports found that γδ T cells expanded from peripheral blood mononuclear cells (PBMCs) remained cytotoxic after cryopreservation, neither provided an in depth characterization of the effects of cryopreservation. Additionally, neither used an αβ T cell-depleted γδ T cell product, a necessary step for the use of an allogeneic therapy, suggesting that these results might not translate to a clinically relevant allo-γδ T cell product. Patients enrolled in clinical trials using γδ T cells receive on average between three to ten doses of cells throughout the course of their treatment (Bennouna et al., 2008; Kobayashi et al., 2007; Nakajima et al., 2010; Sakamoto et al., 2011), a strategy that poses many disadvantages when using fresh γδ T cells. Using a cryopreserved cell product could overcome disadvantages including the potential for delayed treatment if manufacturing is unsuccessful or time limitations on safety and quality

control testing. The use of cryopreserved γδ T cells would eliminate these disadvantages, providing an "off-the-shelf" immunotherapy product.

In this study, we assessed the effects of cryopreservation on γδ T cell health and functionality, hypothesizing that the negative effects of cryopreservation could be reduced once a better understanding was achieved. We used a protocol, readily adaptable to good manufacturing practice (GMP) guidelines, developed by our lab to expand γδ T cells (Sutton et al., 2016). Initially, we characterized the viability of cryopreserved cells post-thaw, then aimed to optimize the cryopreservation protocol using a variety of GMP-compliant freezing and thawing media. To further reduce the effects of cryopreservation, we tested the effectiveness of chromatin condensation and caspase inhibitors on γδ T cell viability post-thaw. Using an optimized cryopreservation protocol, we identified a potential mechanism for the initiation of apoptosis after thawing and tested the functionality of cryopreserved γδ T cells against a range of leukemia cell lines.

3.3 Methods

Expansion of γδ T cells

Whole blood was collected from healthy donors through the Emory Children's Clinical and Translational Discovery Core (IRB0010797) and peripheral blood mononuclear cells (PBMCs) were isolated via Ficoll-Paque Plus (GE Healthcare Life Sciences) density centrifugation. PBMCs were cultured in OpTmizer media supplemented with OpTmizer T-cell expansion supplement (Life Technologies), 1% penicillin/streptomycin, and 2 mM L-glutamine. Cell counts were performed using a Cellometer (Nexelcom) and cells were resuspended in fresh media at $1.5x10^6$ cells/mL every 3 days. To selectively expand γδ T cells from PBMCs, 5 μM of Zoledronate and 500 IU/mL of IL-2 were added on day 0 and 3 of expansion. αβ T cells were

depleted from the culture on day 6 of expansion using a GMP-compliant protocol (Miltenyi Biotec). Briefly, cells were washed in autoMACS Rinsing Solution containing 0.5% BSA (Miltenyi Biotec), incubated with Anti-TCRα/β-Biotin for 10 minutes at 4°C, washed in autoMACS Rinsing Solution and then filtered through a 0.4μM filter. Cells were then incubated with Anti-Biotin Microbeads (Miltenyi Biotec) for 15 minutes at 4°C, washed in autoMACS Rinsing Solution and passed through an LD Column (Miltenyi Biotec). Post-depletion, 1000 IU/mL of IL-2 was added on day 6 and 9 of expansion.

Cryopreservation of γδ T cells

On day 12 of expansion, γδ T cells were washed and prepared for cryopreservation. For studies comparing different GMP-grade freezing media, γδ T cells were washed and resuspended at 1 x 10^7 cells/mL in one of the 5 media tested: Synth-a-Freeze Cryopreservation Medium (ThermoFisher Scientific), GIBCO Recovery Cell Culture Freezing Medium (ThermoFisher Scientific), STEM-CELLBANKER (amsbio), Nutrifreez D10 Cryopreservation Medium (Biological Industries), or 5% human albumin (2.5g human albumin in 50 mL aqueous diluent, Grifols Therapeutics) with 10% Me2SO. For all subsequent experiments, γδ T cells were cryopreserved in 5% human albumin (2.5g human albumin in 50 mL aqueous diluent, Grifols Therapeutics) with 10% Me2SO. Cells were frozen at a rate of -1°C per minute. When the cells reached -80°C, they were promptly moved to liquid nitrogen.

Thawing of γδ T cells

γδ T cells were removed from liquid nitrogen storage and thawed in a water bath (37°C). When the cells were nearly thawed, they were removed from the water bath and washed with 10X OpTmizer media pre-warmed to 37°C. Cells were spun at 300 x g and resuspended in thawing medium with 1,000 IU/mL of IL-2. As noted, OpTmizer media was used initially as the thawing

medium to characterize the effects of cryopreservation on γδ T cells. Additional thawing media tested include human platelet lysate (HPL, Emory University Hospital), 5% human albumin (HSA) (2.5g human albumin in 50 mL aqueous diluent, Grifols Therapeutics), and human serum collected from the blood and pooled after the isolation of γδ T cells from whole blood. For studies comparing the effects of thawing into 1% HSA and 2.5% HSA, HSA was added to OpTmizer media at the appropriate concentrations. For all subsequent studies, γδ T cells were thawed into 5% HSA with IL-2 (1,000 IU/mL).

Flow cytometry

To prepare cells for flow cytometry, cells were washed with 10 X phosphate buffer saline (PBS) and spun at 300 x g. Cells were stained with eBioscience Fixable Viabililty dye eFluor780 (ThermoFisher) for 30 minutes. After incubation with the viability dye, cells were washed in 10 X PBS and resuspended with the appropriate antibodies. Antibodies from BD Biosciences include: BV421 Mouse Anti-Human CD3 (Clone UCHT1, 5 µL/100 µL sample), PE Mouse Anti-Human γδ TCR (Clone 11F2, 5 µL/100 µL sample for a final concentration of 1.25 µg/mL), and APC-R700 Mouse Anti-Human CD56 (Clone NCAM16.2, 5 µL/100 µL sample). APC Annexin V was acquired from Biolegend. Cells were analyzed using an Aurora (CYTEK) flow cytometer.

Chromatin Condensation

Chromatin condensation was induced by incubating γδ T cells in hyperosmotic media (Falk et al., 2018) OpTmizer media has an osmolarity of 290 mOsm. To raise the osmolarity of OpTmizer to 570 mOsm, the osmolarity required to induce chromatin condensation according to the literature, 1 mL of 20 X PBS (2.8 mM NaCl, 54 mM KCl, 30 mM KH_2PO_4, 120 mM

Na_2PO_4, adjusted to pH 7.4) was added to 19 mL of OpTmizer. γδ T cells were incubated in the hyperosmotic media for 12 min, then spun down at 300 x g and prepared for cryopreservation

Caspase Inhibitor Treatment

The pan-caspase inhibitor Z-VAD-FMK (TOCRIS) was used for all caspase inhibitor studies. For studies in which cells were treated with Z-VAD-FMK prior to cryopreservation, cells were spun down for 5 min at 300 x g and resuspended in OpTmizer containing 50 μM Z-VAD-FMK for 30 min at 37°C. After this incubation, cells were spun down and immediately prepared for cryopreservation. For studies in which cells were treated with Z-VAD-FMK after cryopreservation, cells were thawed as described above and resuspended in 5% HSA containing 50 μM of Z-VAD-FMK and 1,000 IU/mL of IL-2.

Cytotoxicity assays

The *in vitro* cytotoxicity of fresh and cryopreserved γδ T cells was evaluated using a flow cytometry-based cytotoxicity assay. The target cell lines used in these studies include: K562, Jurkats, Kasumi-1, Nomo-1, Molt-4, MV411, and 697. The K562 and Jurkats cell lines were obtained from American Type Culture Collection (ATCC). The Nomo-1, Kasumi-1, 697, and MOLT-4 cell lines were kindly provided by the laboratory of Dr. Douglas Graham (Emory University). The MV411 cell line was kindly provided by the laboratory of Dr. Kevin Bunting (Emory University). Before the cytotoxicity assay, target cells were labelled with Violet Proliferation Dye 450 (VPD450). γδ T cells were mixed and incubated with target cells at a ratio of 1:1 or 5:1 and the cells were incubated together for 4 hours at 37°C. Target cell death was assessed via flow cytometry using eBioscience Fixable Viability Dye eFlour780 (Thermofisher) and the early apoptosis stain Annexin V (Biolegend). γδ T cell cytotoxicity was calculated by subtracting the background cell death of each target cell line from each experimental sample.

Mitochondrial Membrane Potential

The $\Delta\psi_m$ was measured by flow cytometry, using the Mitochondrial Membrane Potential Kit (Millipore Sigma) according to the manufacturer's instructions. This kit uses JC-10, a cationic lipophilic dye that changes emission from 590 nm to 525 nm when mitochondria shift from a polarized to depolarized state. Briefly, 5 x 10^5 cells were spun down and resuspended in 500 µL of the JC-10 dye loading solution and incubated at 37 °C for 30 min in the dark. Cells were then spun down and resuspended in PBS containing antibodies for BV421 Mouse Anti-Human CD3 (Clone UCHT1) and PE Mouse Anti-Human γδ TCR (Clone 11F2) (BD Biosciences). Treatment with 10 µM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Millipore Sigma) for 30 min prior to incubation with the JC-10 dye loading solution was used as a positive control for mitochondrial membrane depolarization. Cells were analyzed using an Aurora (CYTEK) flow cytometer.

Statistical analysis

Figures and statistics were generated using GraphPad Prism Software, Version 8.2.1. Corresponding statistical tests and p-values are stated in the results. Student's t tests were used when comparing two groups. One-way ANOVAs were used to compare three or more groups and two-way ANOVAs were used when comparing three or more groups across two variables. Post-hoc analysis was performed when the one- or two-way ANOVA produced significant results ($p< 0.05$). All data are reported as mean ± standard error of the mean (SEM).

3.4 Results

Characterization of γδ T cells after cryopreservation

To characterize the effects of cryopreservation on γδ T cells, we assessed changes in cell number and viability after thawing (post-thaw). Substantial cell loss was observed when γδ T cells were

thawed into their standard culture medium, OpTmizer T Cell expansion medium. Two hours after thawing, 88.0% ± 5.3% of cells survived, which decreased to 68.5% ± 3.9% after four hours post-thaw. Twenty-four hours after thawing, half of the initial population of cells remained (51.6% ± 4.4%) and this percentage was stable throughout 48 hours post-thaw (47.8% ± 6.0%) (Fig. 1A). Viability, as assessed by Trypan Blue staining, was 68.9% ± 5.5% immediately post-thaw and 70.1% ± 5.7% at two hours post-thaw. However, viability decreased to 55.2% ± 3.5% at four hours post-thaw and 39.0% ± 3.0% at 24 hours post-thaw. γδ T cell viability remained stable between 24 and 48 hours (38.0% ± 2.3%) (Fig. 1B).

γδ T cell viability was further assessed by flow cytometry (Fig. 1C). Immediately after thawing, 50.5% ± 3.4% of γδ T cells stained positively for Annexin V. Two hours later, 63.4% ± 7.0% of γδ T cells stained positively for Annexin V, which increased slightly at four hours post-thaw (68.5% ± 6.4%) (Fig. 1D). Twenty-four hours post-thaw, 79.5% ± 5.8% of the culture was comprised of apoptotic cells (Fig. 1D). Based on these data, the aim of our following experiments was to determine if the viability of γδ T cells could be increased in the post-thaw period.

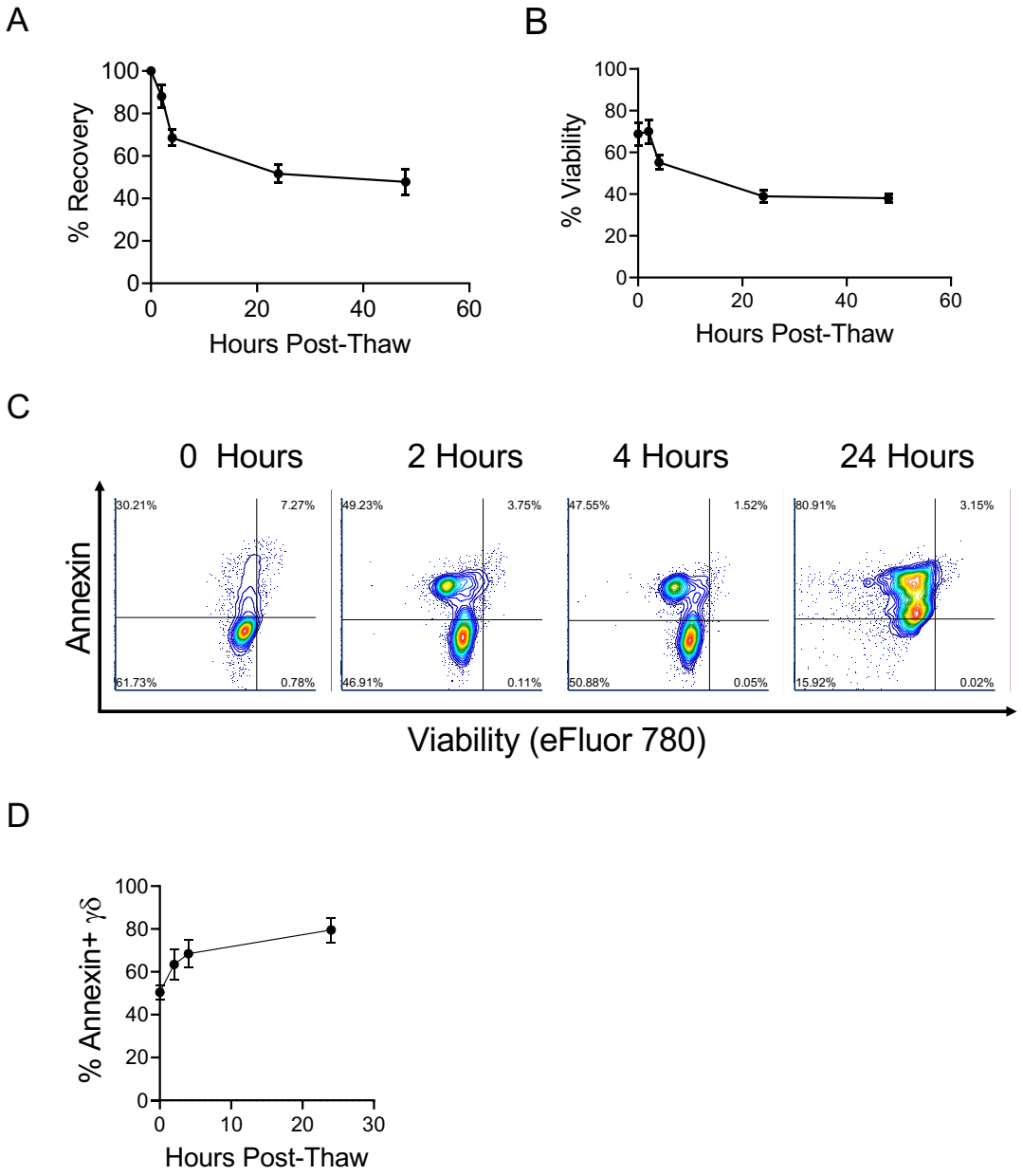

Figure 3.1 Survival and viability of γδ T cells thawed into OpTmizer media. A) Cell counts were performed at 0, 2, 4, 24, and 48 hours post-thaw and the percent recovery was calculated by normalizing counts to the number of cells recovered at 0 hours post-thaw. B) Viability of γδ T cells was measured via Trypan Blue Staining at 0, 2, 4, 24, and 48 hours post-thaw. C) Representative flow cytometry plots visualizing the shift in cell populations up to 24 hours post-

thaw by Annexin V and eFluor780 (Live/Dead viability stain) staining. D) Quantification of the percentage of γδ T cells in culture staining positively for Annexin V.

Comparison of good manufacturing practice (GMP) grade freezing media

In this study, five different good manufacturing practice (GMP)-grade freezing media were tested to determine if any provided benefit to cell viability after thawing compared to the standard freezing medium of 5% HSA 10% Me2SO. None of the GMP-grade freezing media tested, including Synth-a-Freeze, Recovery Cell Culture, Stem-Cell Banker, Nutrifreeze, or Cryostor CS10 increased the percentage of cells recovered immediately post-thaw (one-way ANOVA, $p= 0.79$) (Fig. 2A). Additionally, none of the GMP-grade freezing media provided benefit in terms of decreasing the percentage of Annexin V+ γδ T cells at 0, 2, or 4 hours post-thaw when compared to 5% HSA 10% Me2SO (mixed-effects analysis, $p= 0.86$) (Fig. 2B).

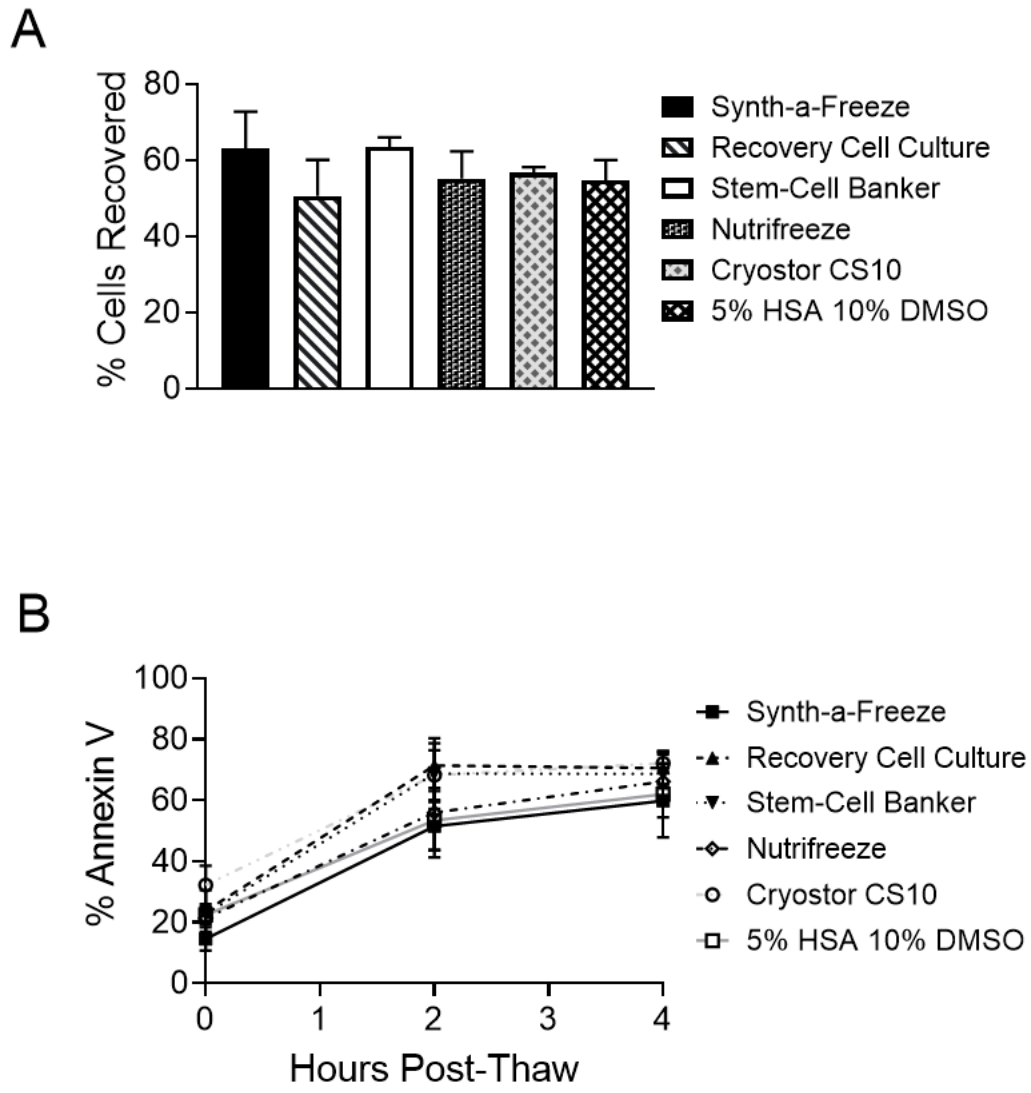

Figure 3.2. Cyropreservation of γδ T cells in different GMP grade freezing media. In this experiment, γδ T cells were frozen in either Synth-a-Freeze, Recovery Cell Culture, Stem-Cell Banker, Nutrifreeze, Cryostor CS10, or 5% HSA and 10% Me2SO and thawed into OpTmizer media. A) The percentage of cells recovered post-thaw after γδ T cells were cryopreserved into different freezing mediums. B) The percentage of γδ T cells staining positively for Annexin V at 0, 2, and 4 hours post-thaw.

Thawing medium impacts γδ T cell viability post-thaw

To determine if the type of thawing media used could influence cell viability, γδ T cells were thawed into their culture medium, OpTmizer T cell medium, and three human-derived products: 100% human platelet lysate (HPL), 5% human albumin (HSA), and 100% human serum (Serum). Thawing γδ T cells into 5% HSA, but not HPL or Serum, significantly decreased the percentage of Annexin V+ γδ T cells up to four hours after thawing (Fig. 3A). There was a significant increase in the percentage of apoptotic γδ T cells at 2 and 4 hours post-thaw, as compared to 0 hours post-thaw, when cells were thawed into OpTmizer (one-way ANOVA and Tukey's multiple comparisons test, p= 0.03and p= 0.03), HPL (one-way ANOVA and Tukey's multiple comparisons test, p= 0.003 and p= 0.005), and 5% HSA (one-way ANOVA and Tukey's multiple comparisons test, p=0.02 and p= 0.002) (Fig. 3B).

Immediately post-thaw, there was no significant difference in the percentage of apoptotic γδ T cells for the various thawing media tested. However, there were significantly fewer apoptotic γδ T cells at 2 and 4 hours (Fig. 3C) post-thaw when cells were thawed into 5% HSA as compared to thawing into OpTmizer, HPL, and Serum (one-way ANOVA, p= 0.0003, p=0.003 and p= 0.0002).

In addition to the difference in the percentage of apoptotic cells in culture post-thaw, the choice of thawing medium also impacted the percentage of γδ T cells in culture after thawing. For cells thawed into OpTmizer, there was a significant decrease in the percentage of γδ T cells between 0 and 4 hours post-thaw and 2 and 4 hours post-thaw (one-way ANOVA and Tukey's multiple comparisons test, p = 0.001 and p= 0.001). Cells thawed into HPL showed similar decreases in the percentage of γδ T cells in culture, with significant decreases between 0 and 4 hours post and 2 and 4 hours after thawing (one-way ANOVA and Tukey's multiple

comparisons test, $p<0.0001$ and $p = 0.0002$). There was no change in the percentage of γδ T cells in culture at 2 or 4 hours post-thaw for cells thawed into 5% HSA (one-way ANOVA, $p= 0.09$) or serum (one-way ANOVA, $p=0.10$) (Fig. 3D).

To determine if HSA could be used as a supplement in OpTmizer after thawing, we compared the effects of 1% HSA in OpTmizer (1% HSA) and 2.5% HSA in OpTmizer (2.5% HSA) to 5% HSA between 0 and 4 hours post-thaw. There were significantly fewer apoptotic γδ T cells for cells thawed into 5% HSA as compared to 1% HSA at 0, 2, and 4 hours post-thaw (Tukey's post-hoc multiple comparisons test, $p= 0.002$, $p< 0.0001$, and $p< 0.0001$, respectively). Cells thawed into 2.5% HSA had significantly lower levels of apoptosis at 2 and 4 hours post-thaw compared to cells thawed into 1% HSA (two-way ANOVA and Tukey's post-hoc multiple comparisons test, $p= 0.02$ and $p= 0.03$). Additionally, cells thawed into 2.5% HSA had significantly higher levels of apoptosis when compared to cells in 5% HSA at 2 and 4 hours post-thaw (Tukey's post-hoc multiple comparisons test, $p= 0.001$ and $p= 0.0008$) (Fig. 3E).

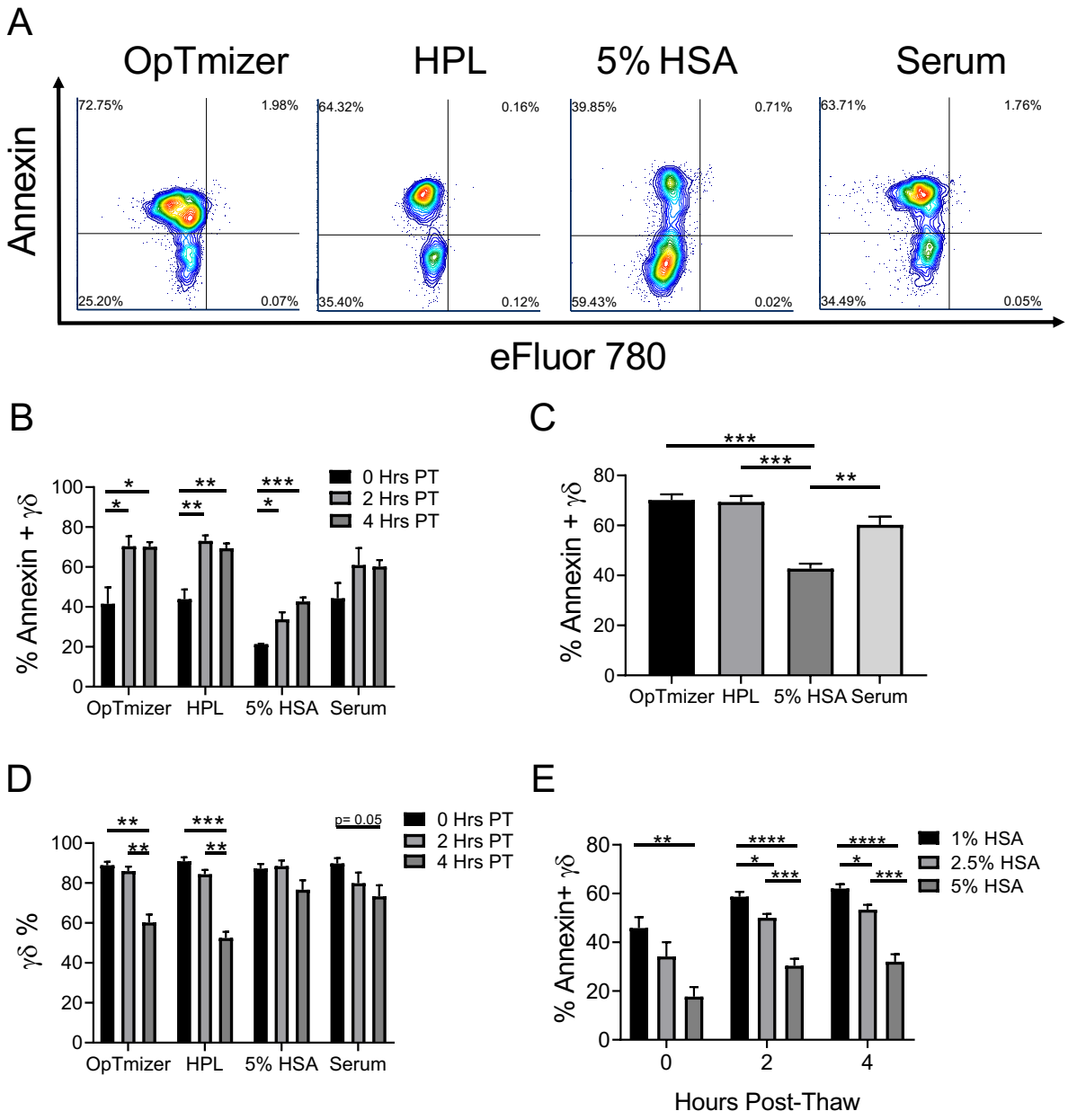

Figure 3.3. Characterization of γδ T cells frozen in 5% HSA and 10% Me2SO and thawed into different solutions. A) Representative flow cytometry plots visualizing the percentage of live and apoptotic γδ T cells 4 hours post-thaw when thawed into OpTmizer media, human platelet lysate (HPL), 5% human albumin (HSA), and human serum (Serum). B) Quantification of the percentage of Annexin V+ γδ T cells thawed into OpTmizer, HPL, 5% HSA, and S at 0, 2,

and 4 hours post-thaw. C) Comparison of the percentage of Annexin V+ γδ T cells in culture at 4 hours post-thaw when thawed into different solutions. D) The percentage of γδ T cells in culture, assessed by flow cytometry, over the course of 4 hours post-thaw. E) Comparison of the percentage of Annexin V+ γδ T cells at 0, 2, and 4 hours post-thaw when thawed into OpTmizer + 1% HSA (1% HSA), OpTmizer + 2.5% HSA (2.5% HSA), or 5% HSA.

Chromatin condensation improves γδ T cell viability post-thaw

Previous studies using normal human foreskin fibroblasts (NHDF) cells showed benefits to cell survival if chromatin condensation, induced by incubating cells in a hypertonic solution, was performed prior to cryopreservation (Falk et al.). Therefore, chromatin condensation was performed prior to cryopreservation to determine if it could provide protection to γδ T cell viability post-thaw. Representative flow cytometry images show that chromatin condensation decreased levels of apoptotic γδ T cells immediately post-thaw (Fig. 4A). Chromatin condensation decreased the percentage of apoptotic γδ T cells by an average of 10% immediately post-thaw into 5% HSA (Tukey's post-hoc multiple comparisons test, $p= 0.0004$); however there was no advantage at 2 or 4 hours post-thaw (Tukey's post-hoc multiple comparisons test, $p= 0.52$ and $p= 0.99$). Of the chromatin condensed γδ T cells that were thawed into 5% HSA, only 11.5% ± 0.62% were Annexin V+, as compared to 44.4 ± 3.2% Annexin V+ γδ T cells when neither chromatin condensed nor thawed into OpTmizer (Fig. 1D).

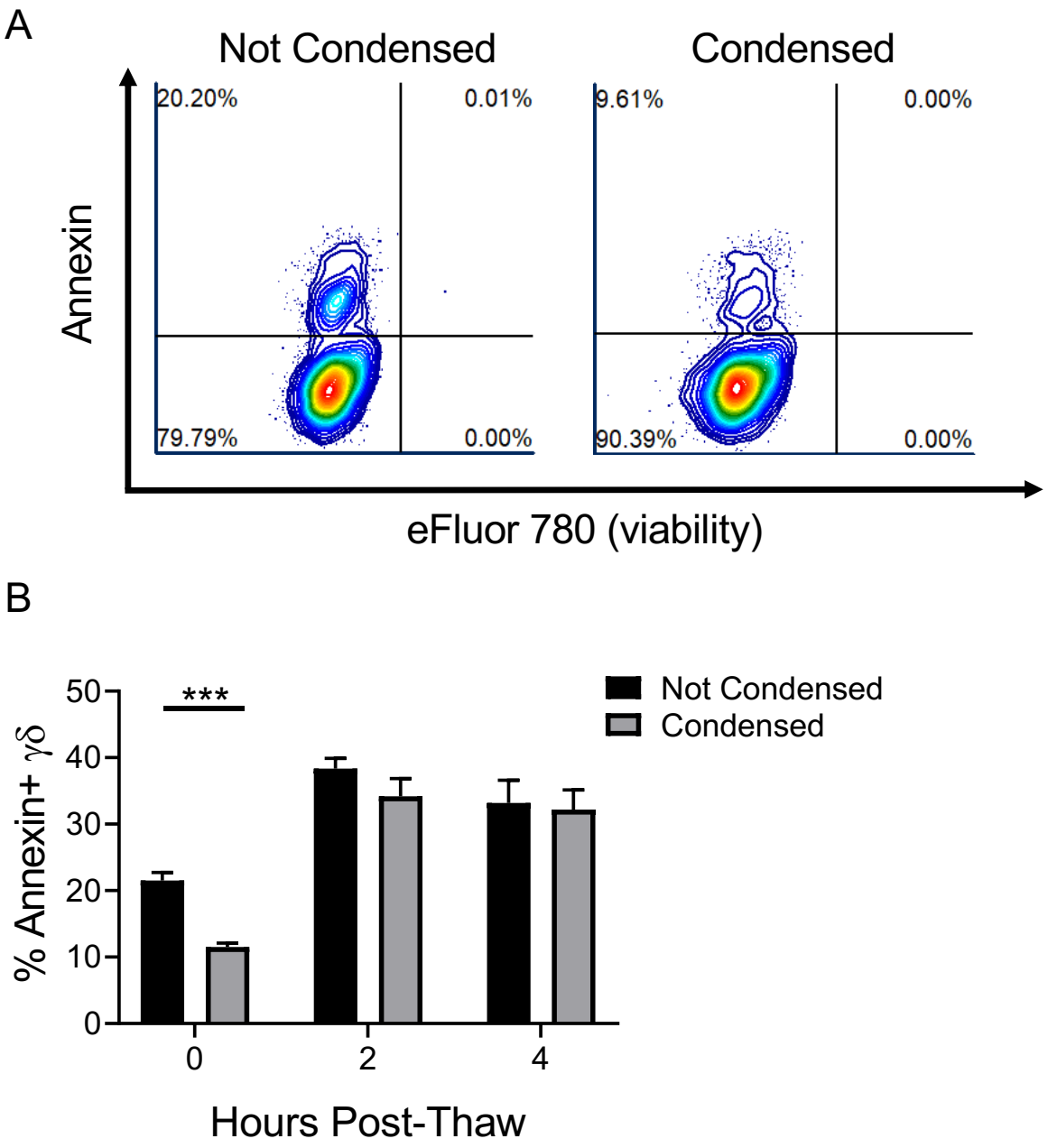

Figure 3.4. The effect of chromatin condensation on γδ T cell viability post-thaw when thawed into 5% HSA. A) Representative flow cytometry plots visualizing the difference in the percentage of Annexin V+ γδ T cells at 0 hours post-thaw. B) Quantification of the percentage of Annexin V+ γδ T cells at 0, 2, and 4 hours post-thaw when cryopreserved under normal conditions or after chromatin condensation.

Caspase inhibitor treatment provides no additional benefit post-thaw

γδ T cells were treated with a general caspase inhibitor (CI) either prior to freezing or post-thaw to determine if inhibiting the caspase pathway could reduce levels of apoptosis. As previously described, chromatin condensation prior to freezing decreased the percentage of apoptotic γδ T cells immediately post-thaw (one-way ANOVA and Sidak's post-hoc multiple comparisons test, $p= 0.003$). γδ T cells treated with the CI prior to freezing had decreased levels of apoptosis immediately post-thaw when compared to cells frozen under non condensed conditions (one-way ANOVA and Sidak's post-hoc multiple comparisons test, $p= 0.01$). Cells that underwent chromatin condensation and CI treatment prior to freezing also had a decrease in levels of apoptosis immediately post-thaw (one-way ANOVA and Sidak's post-hoc multiple comparisons test, $p= 0.003$). However, there was no additional benefit of CI treatment prior to cryopreservation when compared to the chromatin condensation treatment (one-way ANOVA and Sidak's post-hoc multiple comparisons test, $p= 0.88$) (Fig. 5A). Additionally, neither chromatin condensation nor CI treatment provided benefit as compared to non-condensed cryopreservation conditions at 2 or 4 hours post-thaw (Fig. 5B).

Because the CI treatment prior to freezing provided no benefit in the post-thaw period, we treated γδ T cells with the CI immediately post-thaw as well. In this study, γδ T cells underwent chromatin condensation prior to freezing and immediately post-thaw were resuspended in 5% HSA containing the CI. There were no statistical differences in the percentages of apoptotic γδ T cells at 0, 2, or 4 hours post-thaw when the cells were treated with the CI as compared to the cells that were not treated with the CI (two-way ANOVA, $p= 0.51$).

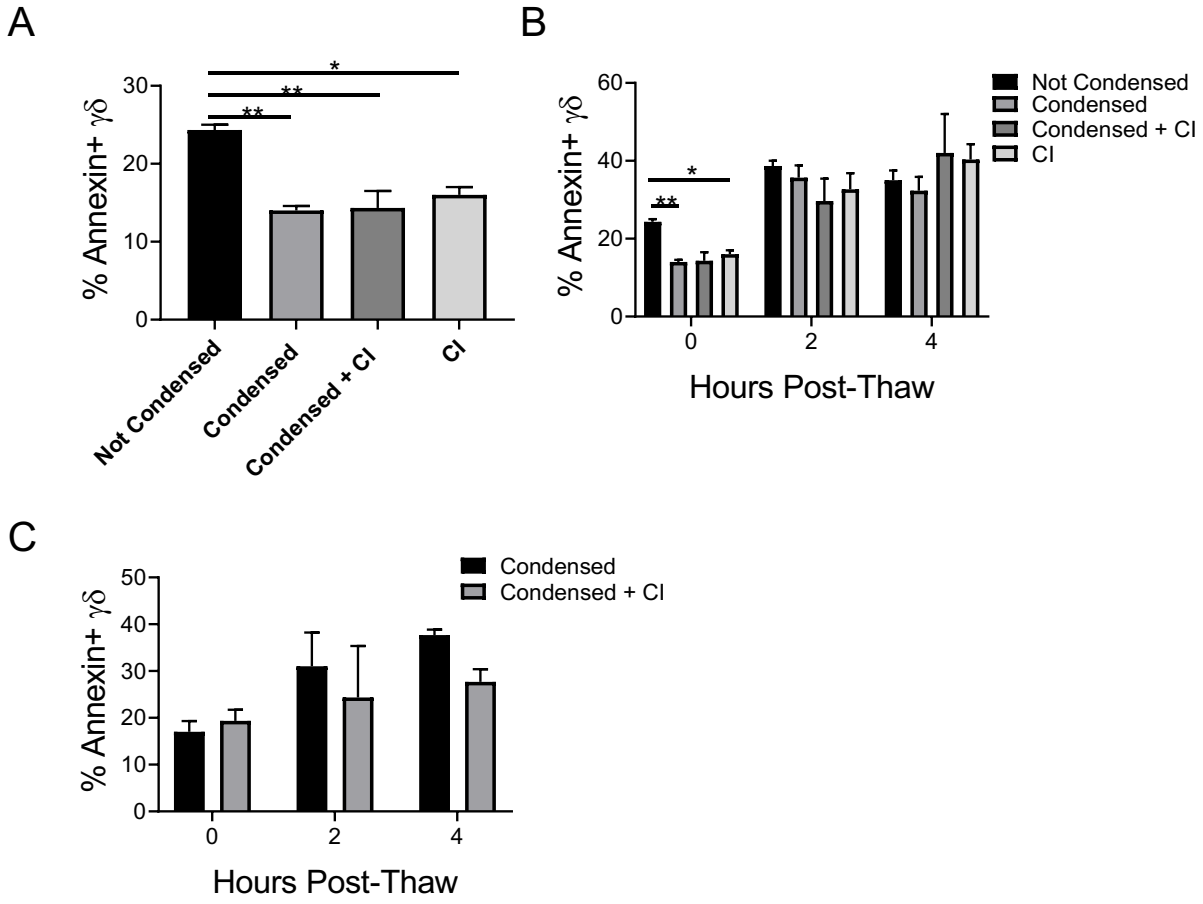

Figure 3.5. The effects of a general caspase inhibitor (CI) on γδ T cell viability when treated with the CI prior to and after cryopreservation. A) Quantification of the percentage of Annexin V+ γδ T cells at 0 hours post-thaw when cryopreserved under normal conditions, chromatin condensed conditions, chromatin condensed + CI treatment, or CI treatment alone. B) Comparison of the effects of chromatin condensation and CI treatment at 0, 2, and 4 hours post-thaw. C) The effects of CI treatment post-thaw on γδ T cells cryopreserved after chromatin condensation.

Mitochondrial membrane depolarization post-thaw

The percentage of cells with depolarized mitochondrial membranes was investigated because of the role that mitochondria play in initiating apoptosis through the intrinsic apoptotic pathway. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used as a positive control to determine maximum mitochondrial depolarization for γδ T cells. Prior to freezing, 21.1% of γδ T cells had mitochondria with depolarized membranes. Immediately after thawing, this increased to 56.9% of cells with depolarized mitochondrial membranes (two-way ANOVA and Dunnett's post-hoc multiple comparisons test, $p= 0.002$). At 2 and 4 hours after thawing, 74.6% (two-way ANOVA and Dunnett's post-hoc multiple comparisons test, $p< 0.0001$) and 72.8% (two-way ANOVA and Dunnett's post-hoc multiple comparisons test, $p= 0.0001$) of γδ T cells had mitochondria with depolarized membranes, respectively (Fig. 6A). There was no difference in the percentage of γδ T cells with depolarized mitochondrial membranes at 0, 2, or 4 hours after thawing for cells that underwent chromatin condensation prior to freezing (Condensed) and cells that did not (Not Condensed) (Two-way ANOVA, $p= 0.24$) (Fig. 6B).

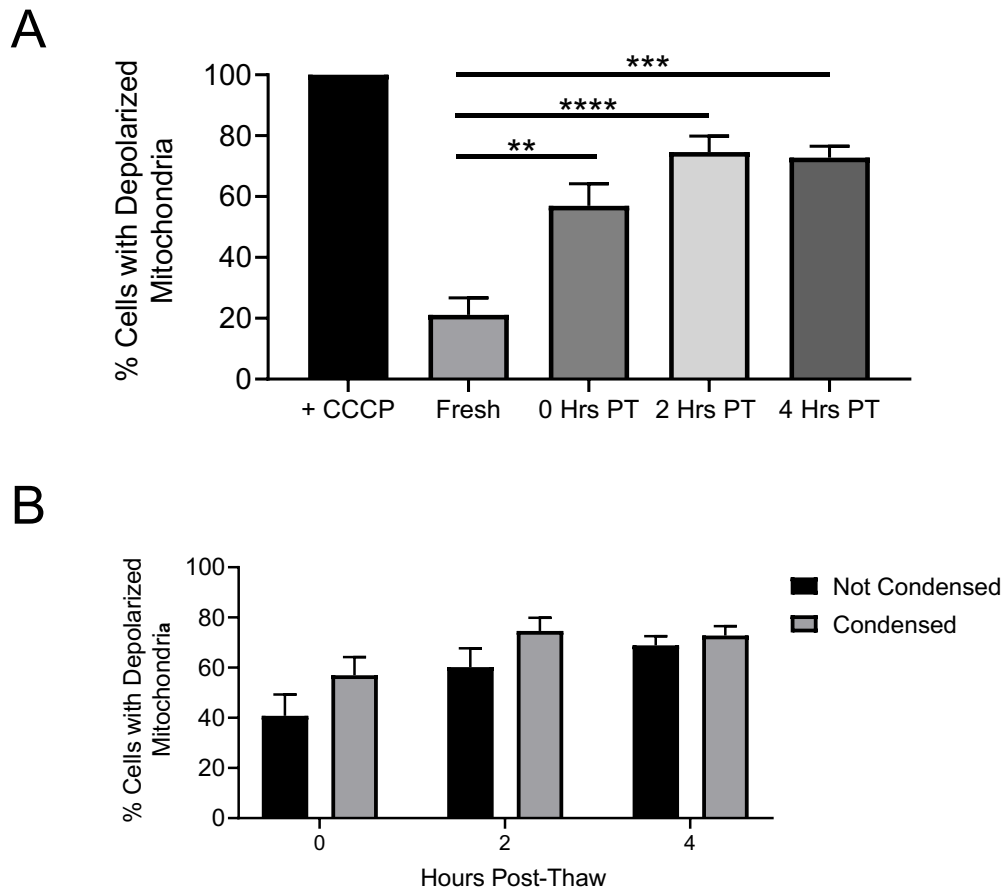

Figure 3.6. Mitochondrial membrane depolarization post-thaw. A). The percentage of chromatin condensed cells staining positively for depolarized mitochondria at 0, 2, and 4 hours after thawing, as compared to their pre-freeze (fresh) values. B) Comparison of the percentage of cells with depolarized mitochondria between cells that underwent chromatin condensation prior to freezing (Condensed) and those that did not (Not Condensed).

Cytotoxicity of γδ T cells post-thaw

γδ T cell functionality after thawing was assessed using *in vitro* cytotoxicity assays in which the effector: target ratios were determined based on live cell numbers via Trypan Blue staining. When γδ T cells were tested in a cytotoxicity assay immediately after thawing against K562 cells, there was a trend towards decreased cytotoxicity at a 1:1 effector:target ratio (two- way ANOVA and Sidak's post-hoc multiple comparisons test, p= 0.08) and a significant decrease in cytotoxicity at a 5:1 ratio (two-way ANOVA and Sidak's post-hoc multiple comparisons test, p = 0.005) when compared to γδ T cells that recovered for 1-hour post-thaw (Fig. 7A). There was no difference in γδ T cell cytotoxicity against K562 cells when they were incubated in OpTmizer or 5% HSA for the 1-hour recovery period prior to being tested in the assay (two-way ANOVA, p= 0.37) (Fig. 7B), indicating that the choice of incubation medium does not impact the cytotoxicity of viable cells that survive the thawing process.

γδ T cells were tested against a panel of leukemia cell lines at a 1:1 effector:target ratio, allowing for measurement of changes in cytotoxicity prior to freezing and after thawing. γδ T cells that did not undergo chromatin condensation prior to freezing exhibited decreased cytotoxicity post-thaw against K562 (Student's t-test, p= 0.003), Jurkats (Student's t-test, p= 0.04), Kasumi-1 (Student's t-test, p=0.049), Molt-4 (Student's t-test, p= 0.006), and 697 cells (Student's t-test, p= 0.001). There was no difference in cytotoxicity at the 1:1 ratio when γδ T cells were tested against Nomo-1 (Student's t-test, p= 0.83) or MV411 cells (Student's t-test, p= 0.37). Similar trends were seen in chromatin condensed γδ T cells with decreased cytotoxicity post-thaw against K562 (Student's t-test, p= 0.003), Jurkats (Student's t-test, p= 0.004), Kasumi-1 (Student's t-test, p=0.004), Molt-4 (Student's t-test, p= 0.004), and 697 cells (Student's t-test, p= 0.0001), with no difference in cytotoxicity when tested against Nomo-1 (Student's t-test, p=

0.32) or MV411 cells (Student's t-test, p= 0.28). Additionally, there was no difference in cytotoxicity between chromatin condensed γδ T cells and cells that did not undergo chromatin condensation for any of the cell lines tested (Fig. 7C).

As a measure of γδ T cell health, we assessed the percentage of live γδ T cells (Annexin-eFluor780-) in the cytotoxicity assay against K562 cells when the assay was complete. When tested prior to cryopreservation, 82.2% ± 0.7% of γδ T cells were alive at the 1:1 ratio and 84.0% ± 2.0% were alive at the 5:1 ratio. When assessed after cryopreservation, γδ T cells that were resuspended in 5% HSA for a 1-hour recovery period prior to the assay had an average of 55.7% ± 2.2% live γδ T cells at the 1:1 ratio and an average of 47.6% ± 4.9% live γδ T cells at the 5:1 ratio, which were significantly decreased as compared to pre-freeze values (Sidak's post-hoc multiple comparisons test, p= 0.0004 and p< 0.0001) (Fig. 7E).

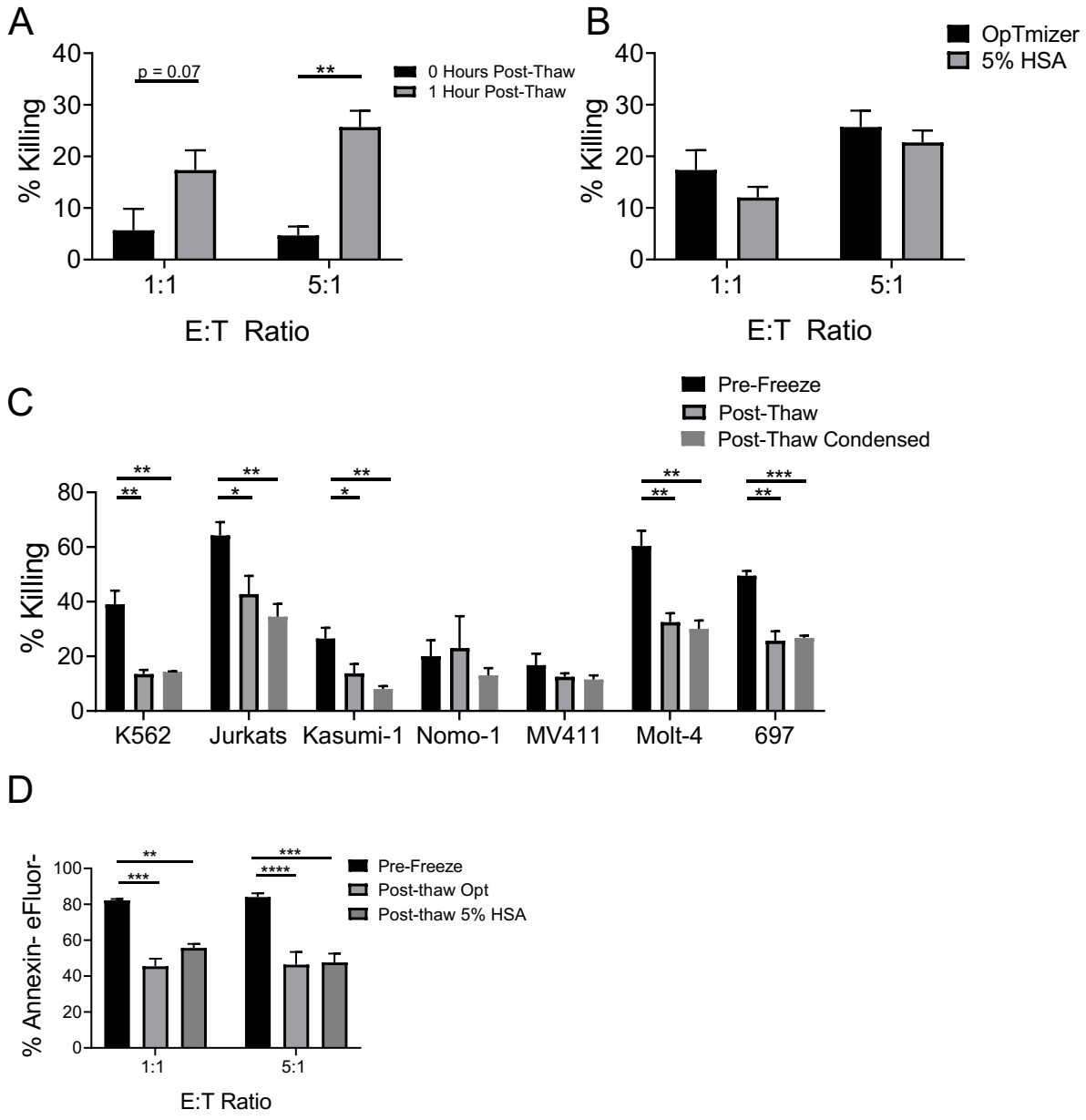

Figure 3.7. γδ T cell cytotoxicity post-thaw. A) Cytotoxicity of γδ T cells against K562 cells when the cytotoxicity assay was initiated immediately post-thaw (0 Hrs PT) or after allowing the cells to recover for 1 hour (1 Hr PT). B) Cytotoxicity of γδ T cells against K562 cells when the γδ T cells were recovered for 1 hour in OpTmizer media or 5% HSA. C) γδ T cell cytotoxicity against a range of leukemia cell lines at a target:effector ratio of 1:1. D) Analysis of the

percentage of live γδ T cells (Annexin-eFluor-) after a cytotoxicity assay against K562 cells when recovered in 5% HSA for 1 hour post-thaw.

3.5 Discussion

Cryopreservation is a critical process in the development and manufacturing of off-the-shelf cell therapies. The process of freezing and thawing cells can lead to cell damage, resulting in decreased cell viability and functionality. γδ T cells have the potential to be applied as an allogeneic cell therapy, making the cryopreservation process essential to the manufacturing protocol. This study is the first report of the effects of cryopreservation on γδ T cell health and functionality and provides an optimized protocol for good manufacturing (GMP) compliant cryopreservation of γδ T cells.

The impact of cryopreservation on T cells is currently not well understood. Post-thaw viabilities of CD3+ T cells, primarily comprised of αβ T cells and a small proportion of γδ T cells, can range from 27%-93% after cryopreservation (Liseth et al., 2009; Stroncek et al., 2011). Additionally, a 2.9-30-fold increase in cell death can be seen in CD3+ T cells after thawing (Steininger et al., 2013). Our studies show that cryopreservation has a dramatic effect on γδ T cell health during the post-thaw period. We observed that the majority of cryopreserved γδ T cells undergo apoptosis within four hours post-thaw. By 24 hours post-thaw, the cells are primarily apoptotic, with only 51% of cryopreserved γδ T cells surviving up to the 24-hour time point. Cryopreservation leads to significant loss of the total cell product when γδ T cells are cultured *ex vivo* post-thaw. However, it is unknown if the cell losses seen in an overnight culture are representative of what would occur *in vivo* if the cell product was injected immediately upon thawing, which requires further, ongoing, studies.

The choice of freezing medium and cryoprotective agent (CPA) used for cryopreservation is an important consideration, as the correct combination can limit osmotic stress during the freeze/thaw cycle. Cryopreserving cells without cryoprotectant results in up to

90% cell death, which can be avoided through the use of reagents such as Me2SO, Trehalose, or the antifreeze fusion protein TrxA-ApAFP752 (AFP). Me2SO is the most effective cryoprotectant in reducing cell death after cryopreservation as it penetrates the nucleus, condenses chromatin, and reduces the nuclear envelope size. AFP and Trehalose are also used, however they are less effective at condensing chromatin and result in reduced cell viability after thawing (Kratochvilova et al., 2019). In this study, we compared five GMP quality freezing media to the standard γδ freezing medium of 5% human albumin (HSA) and 10% dimethyl sulfoxide (Me2SO). Although each of the five reagents tested are effective at preserving other cell lines throughout the cryopreservation process (Ivics et al., 2011; Miki et al., 2016; Nicoud et al., 2012; Nishishita et al., 2015; Parker et al., 2018; Saliem et al., 2012), none of the cryopreservation specific media improved post-thaw recovery or viability of γδ T cells in comparison to 5% HSA 10% Me2SO. The freezing medium used to cryopreserve γδ T cells requires further optimization that could be achieved with investigation into a formula that properly mimics the intracellular composition of γδ T cells to limit osmotic stress and cell damage.

While the majority of clinical trials using T cell therapies inject cryopreserved cells immediately upon thawing (Cruz et al., 2013; Desreumaux et al., 2012; Garfall et al., 2015; Kalos et al., 2011; Porter et al., 2011; Till et al., 2008), this protocol does not allow for any post-thaw manipulations, such as genetic engineering. In an effort to increase post-thaw viability, we compared the effects of thawing cryopreserved γδ T cells into three human derived products: human platelet lysate (HPL), 5% human albumin (HSA), and human serum. Thawing γδ T cells into 5% HSA increased cell viability over the first four hours in the post-thaw period compared to the other thawing media tested. HSA is known to have anti-oxidant effects and can both limit

the production of free radicals and act as a free radical scavenger (Fanali et al., 2012). Antioxidant treatments have successfully decreased the effects of oxidative stress induced by cryopreservation in other cell lines and 5% HSA may be offering protection through this pathway (Aliakbari et al., 2016; Bai et al., 2020; Valadbeygi et al., 2016). Supplementing OpTmizer with HSA was not sufficient to rescue the viability of γδ T cells in the post-thaw period, suggesting that any manipulations made to the cells after thawing would need to be performed in 5% HSA for optimal viability. Based on these studies, clinical trials using unmodified γδ T cells should consider washing and resuspending the cells in 5% HSA before injection.

Cryopreservation can induce extensive physical damage to cells through the formation of intracellular ice crystals (Mandumpal et al., 2011; Mazur et al., 1972). The use of CPAs, such as Me2SO, protects cells during the cryopreservation process by decreasing the formation of intracellular ice crystals (Fahy et al., 1984; Rall and Fahy, 1985). Me2SO influences the ability of ice crystals to form due to its molecular composition, which includes an oxygen anion and a sulphur cation. Additionally, water molecules do not form hydrogen bonds with Me2SO, which directly impacts interactions between water molecules and the formation of ice in the solution. Compared to other CPAs, solutions cryopreserved via Me2SO form smaller ice crystals, which contributes to increased viability after thawing (Kratochvilova et al., 2017). Me2SO also offers protection during cryopreservation by penetrating the cell nucleus and effectively condensing chromatin (Falk et al., 2018; Kratochvilova et al., 2019). A recent report found that artificial chromatin condensation prior to freezing improves the viability of cells after thawing, offering chromatin additional protection from the effects of ice formation and cellular dehydration during the freezing process (Falk et al., 2018). In our study, we found that chromatin condensation prior

to cryopreservation did indeed increase γδ T cell viability immediately after thawing, bringing the percentage of healthy γδ T cells up to 90%. The combination of chromatin condensation prior to cryopreservation and thawing into HSA increased viability dramatically compared to our initial preoptimized conditions. Although chromatin condensation prior to cryopreservation increased cell viability after thawing, there was no benefit of this method at later timepoints, such as 24 hours post-thaw, suggesting that further optimization is necessary. As Me2SO can condense chromatin on its own, it is possible that thawing into a combination of Me2SO and HSA could offer better protection to cell viability overall.

The use of caspase inhibitors to prevent apoptosis in cryopreserved cells has been widely investigated (Bissoyi and Pramanik, 2014; Pero et al., 2018; Yagi et al., 2001). Treatment with the general caspase inhibitor, Z-FAD-FMK, both prior to and after cryopreservation decreased apoptosis in γδ T cells immediately post-thaw, however it provided no additional benefit over chromatin condensation in reducing apoptosis. Caspase release can be initiated through either the extrinsic apoptotic pathway, in which there is ligation of a death receptor that initiates the caspase cascade, or through the intrinsic apoptosis pathway, where release of cytochrome C from the mitochondria activates the caspase cascade (Hengartner, 2000). We show here that cryopreservation induces mitochondrial dysfunction, as seen by high levels of mitochondria with depolarized membranes. Taken together, these data suggest that apoptosis is occurring through the extrinsic apoptotic pathway. Cryopreservation is likely damaging the mitochondria to such an extent that it is not possible to rescue the cells from apoptosis with the use of a caspase inhibitor. Surprisingly, the combination of chromatin condensation prior to freezing, which protects the chromatin from the effects of a freeze/thaw cycle, and caspase inhibitor treatment, which protects the cells from the effects of initiation of the caspase cascade, provided no additional

benefit to γδ T cells. Because the viability of γδ T cells that undergo chromatin condensation prior to cryopreservation can be as high as 90% when thawed, it is possible that viability cannot be significantly increased. Additionally, the mitochondria may be fatally damaged during the cryopreservation process, which would not allow for either treatment to be effective hours after thawing.

Although γδ T cells displayed decreased cytotoxicity post-thaw, they remained functional against a range of leukemia cell lines. Immediately post-thaw, γδ T cell cytotoxicity was greatly reduced, but increased when the cells were allowed to rest for one hour after thawing. These results are similar to a study characterizing the expansion of Natural Killer (NK) cells, which showed that cryopreserved NK cells had increased cytotoxicity when allowed to recover before the cytotoxicity assay, although they were not able to regain full cytotoxic potential as compared to fresh NK cells (Lapteva et al., 2014). Because viability quickly decreases in γδ T cells after thawing, this biological response to freezing can be overcome by increasing the administered cell dose, as the remaining cells are viable and functional.

Significant progress has been made regarding the improvement of *ex vivo* expansion and use of γδ T cells. Moreover, clinical trials have shown that the γδ T cell therapies are safe, supporting their use in a number of therapeutic settings. This study suggests that cryopreservation significantly impacts γδ T cell health, and thus, further optimization, including comparing the safety and efficacy of cryopreserved γδ T cells to fresh γδ T cells, is necessary. A convenient solution would be to increase the cell dose for patients treated with cryopreserved cells. However, further research is needed to determine if this is a feasible route. Herein, we report a GMP compliant method for the expansion and cryopreservation of γδ T cells in which cells from healthy donors are expanded in OpTmizer with Zoledronate and IL-2, depleted of αβ

T cells on day 6 of expansion, and cryopreserved on day 12 in 5% HSA and 10% Me2SO after chromatin condensation. Additionally, we show that γδ T cells should be given a recovery period for at least one hour after thawing into 5% HSA to achieve optimal cytotoxicity.

Chapter 4: Increasing the efficiency of γδ T cell tumor homing using a novel dual-cell γMSC-γδ T cell cancer immunotherapy.

Author contributions: Rebecca E. Burnham designed and performed experiments, analyzed data and wrote the manuscript; Hunter C. Jonas, Andre J. Burnham, and Kristopher Knight performed experiments; Christopher B. Doering, Edwin M. Horwitz, and H. Trent Spencer designed experiments and edited that manuscript

This work is unpublished.

4.1 Abstract

A current limitation in the use of T cell products for solid tumor immunotherapies is the low efficiency of tumor homing. Only a small percentage of therapeutic cells infused into patients reach the tumor, suggesting that there is room for optimization. One way to increase tumor homing is through manipulation of chemokine secretion from the tumor microenvironment. Mesenchymal stromal cells primed with interferon-gamma (γMSCs) secrete a range of chemokines that correspond to the expression of chemokine receptors on γδ T cells. In this study, we show that γδ T cells preferentially migrate to conditioned media from γMSCs and to γMSC cells *in vitro*. Additionally, we provide evidence that direct cell contact between γMSCs and γδ T cells does not influence γδ T cell viability or cytotoxicity *in vitro*. *In vivo* studies showed that when γMSCs were directly injected into IMR5 neuroblastoma tumors, γδ T cells migrated preferentially to γMSC-tumors as compared to tumors injected with MSCs or tumors alone. In this work, we suggest a novel dual-cell γMSC-γδ T cell therapy that can be utilized to increase γδ T cell homing to the tumor.

4.2 Introduction

Efficient tumor trafficking is critical to the success of a cancer cellular therapy and is currently a major obstacle in the development of anti-cancer T cell therapies. T-cell trafficking is a complex process comprised of four steps including rolling, adhesion, extravasation, and chemotaxis. Although cellular therapies have had great success in treating patients with hematological tumors, treatments against solid tumors have generated mixed results. A key limitation in the use of T-cell therapies, including both chimeric antigen receptor (CAR)-T cells and γδ T cells, against solid tumors is the inability of the cells to efficiently home to the site of the tumor. Both

preclinical animal models and clinical studies have shown that of the relatively large number of *ex vivo* expanded T cells injected *in vivo*, only a small proportion traffic to the tumor following IV infusion (Bernhard et al., 2008; Hong et al., 2011; John et al., 2013; Pockaj et al., 1994). Inefficient tumor homing may be attributed to a downregulation in adhesion molecules, aberrant tumor vasculature, or a discrepancy between the chemoattractive molecules secreted by the tumor and the corresponding receptors expressed by the T cells, notably chemokines and chemokine receptors (Donnadieu et al., 2020; Slaney et al., 2014).

Chemokines are potent modulators of T-cell trafficking and have been investigated as therapeutic targets to increase tumor homing. The correlation of T-cell infiltration into tumors secreting chemokines that correspond to chemokine receptors expressed on the T cells is well documented for a variety of chemokines including, CXCL9, CXCL10, CXCL11, CXCL16, CCL2, CCL3, CCL4, and CCL5 (Guirnalda et al., 2013; Harlin et al., 2009; Hong et al., 2011; Messina et al., 2012; Tannenbaum et al., 1998). CCL2 has been of particular interest in recent studies and has emerged as a powerful T-cell chemoattractant. The addition of CCR2, the classic chemokine receptor for CCL2, into two independent CAR-T cell constructs effectively increased the ability of CAR-T cells to traffic towards tumors secreting CCL2 (Craddock et al., 2010; Moon et al., 2011). Additionally, loss of function studies in CCR2 deficient mice have suggested that the recruitment of γδ T cells to the site of the tumor via the CCR2-CCL2 chemotactic axis plays a protective role in tumor development (Lanca et al., 2013).

In addition to CAR-T cell therapies, γδ T cell therapies have recently emerged as an attractive candidate for cancer immunotherapies. However, unlike CAR-T cell therapies comprised of αβ T cells, γδ T cell therapies have the unique advantage of human leukocyte

antigen (HLA)-independent target cell recognition and can be developed as an allogeneic cell product with limited donor-host alloreactivity. We have recently developed a good manufacturing practice (GMP) compliant protocol to expand γδ T cells for use as an allogeneic cell therapy. This expansion protocol produces γδ T cells with a high expression of CCR2 and moderate expression of CCR4, CCR6, CCR7, CXCR1, CXCR3, and CXCR4 (Burnham et al., 2020), making our γδ T cell product a prime candidate for investigating the chemotactic axis of γδ T-cell homing to the tumor microenvironment (TME). Modifying the construction of the TME and thus the composition of secreted factors may increase homing of IV infused γδ T cell therapies to the site of the tumor. Introducing autologous or donor-derived Mesenchymal Stromal Cells (MSCs), for example, may present a viable strategy for such an approach.

MSCs are spindle-shaped, multipotent cells that ubiquitously present through the body in a variety of tissues, (e.g., bone marrow, blood, and adipose tissue) (Horwitz et al., 2005). MSCs may be applied as a vehicle for manipulating the TME for a number of reasons. For example, clinical studies have shown that MSCs are safe to use in patients, which is a critical factor in the consideration of a dual-cell immunotherapy (Kallekleiv et al., 2016). When primed with interferon-gamma (IFNγ), MSCs secrete a range of chemokines corresponding to the receptors expressed on our γδ T cell product, including CCL2, CCL5, CCL7, CCL8, CXCL9, CXCL10, and CXCL11 (de Witte et al., 2015; Noronha et al., 2019; Wang et al., 2016a). Moreover, IFNγ-primed MSCs (γMSCs) are under investigation as a cancer therapeutic, and once in the TME, may polarize tumor-associated macrophages to the more favorable, anti-tumor M1 phenotype (Relation et al., 2018). We describe a novel cell therapy in which γMSCs are injected into the TME of a neuroblastoma animal model in order to increase the secretion of chemoattractant

molecules from the TME, and thus increase the tumor homing capabilities of a γδ T cell immunotherapy.

4.3 Methods

Expansion of γδ T cells.

Whole blood was collected from healthy donors through the Emory Children's Clinical and Translational Discovery Core (IRB0010797) and peripheral blood mononuclear cells (PBMCs) were isolated via FicollPaque Plus (GE Healthcare Life Sciences) density centrifugation. PBMCs were cultured in OpTmizer media supplemented with OpTmizer T-cell expansion supplement (Life Technologies), 1% penicillin/streptomycin, and 2 mM L-glutamine. Cell counts were performed using a Cellometer (Nexelcom) and cells were resuspended in fresh media at 1.5 × 106 cells/mL every 3 days. To selectively expand γδ T cells from PBMCs, 5 μM of Zoledronate and 500 IU/mL of IL-2 were added on day 0 and 3 of expansion. αβ T cells were depleted from the culture on day 6 of expansion using a GMP-compliant protocol (Miltenyi Biotec). Briefly, cells were washed in autoMACS Rinsing Solution containing 0.5% BSA (Miltenyi Biotec), incubated with Anti-TCR α/β-Biotin for 10 min at 4 ◦C, washed in autoMACS Rinsing Solution and then filtered through a 0.4 μM filter. Cells were then incubated with Anti-Biotin Microbeads (Miltenyi Biotec) for 15 min at 4 ◦C, washed in autoMACS Rinsing Solution and passed through an LD Column (Miltenyi Biotec). Post-depletion, 1000 IU/mL of IL-2 was added on day 6 and 9 of expansion.

MSC cell culture and conditioned media.

Human MSCs were isolated from healthy donor bone marrow provided by the Emory University GMP lab. MSCs were cultured in DMEM with 10% FBS, 1% penicillin/streptomycin, and 2 mM L-glutamine. MSCs were primed with 1 ng/mL of human INFγ (Peprotech) for 48 hours in complete media to create γMSC conditioned media. MSC conditioned media was created by incubating cells in complete media for 48 hours.

Transwell migration.

Transwell assays were performed using Polycarbonate 6.5mm Transwell plates with a 3.0 uM pore (Corning Life Sciences). For assays using conditioned media, 600 μL of conditioned media was placed in the lower chamber and 500,000 γδ T cells were placed in the top chamber. After 4 hours, the number of γδ T cells that had migrated across the barrier to the bottom chamber was assessed using a Cellometer (Nexelcom). Migration assays in which γδ T cells migrated to MSC or γMSC cells were performed by plating MSCs in the bottom chamber where they received fresh media or media supplemented with IFNγ for 48 hours. After 48 hours, the media was replaced and γδ T cells were placed in the top chamber for 4 hours, after which a cell count was performed to determine how many cells migrated to the bottom chamber. Fresh media was used as a control for background, unspecific migration. Specific migration was calculated with the following equation:

$$Specific\ Migration = \frac{Number\ of\ cells\ migrated\ to\ experimental\ media - number\ of\ cells\ migrated\ to\ control\ media}{Total\ number\ of\ cells\ added\ to\ top\ chamber}$$

Flow cytometry.

To prepare cells for flow cytometry, cells were washed with 10 X phosphate buffer saline (PBS) and spun at 300×g. Cells were washed in 10 X PBS and resuspended with the appropriate antibodies. Antibodies from BD Biosciences include: BV421 Mouse Anti-Human CD3 (Clone UCHT1, 5 μL/100 μL sample), PE Mouse Anti-Human γδ TCR (Clone 11F2, 5 μL/100 μL sample for a final concentration of 1.25 μg/mL), and APC-R700 Mouse Anti-Human CD56 (Clone NCAM16.2, 5 μL/100 μL sample). APC Annexin V and 7-AAD were acquired from Biolegend. Cells were analyzed using an Aurora (CYTEK) flow cytometer.

Cytotoxicity assays.

The in vitro cytotoxicity of γδ T cells was evaluated using a flow cytometry-based cytotoxicity assay. The target cell line used in this assay was the IMR5 neuroblastoma cell line. Before the cytotoxicity assay, target cells were labelled with Violet Proliferation Dye 450 (VPD450). γδ T cells were mixed and incubated with target cells at a ratio of 5:1 and the cells were incubated together for 4 h at 37 ◦C. Target cell death was assessed via flow cytometry using 7-AAD (Biolegend) and the early apoptosis stain Annexin V (Biolegend). γδ T cell cytotoxicity was calculated by subtracting the background cell death of each target cell line from each experimental sample.

CD107a degranulation assays.

CD107a degranulation assays were performed by incubating γδ T cells at a 1:1 ratio with IMR5 cells for 4 hours. Additional conditions included testing the effects of γδ T cells in combination with IMR5 cells and MSC or γMSC cells at a 1:1:1 ratio for 4 hours. After 4 hours, the cells

were washed and prepared for flow cytometry. The antibody used to measure CD107a was obtained from BD Biosciences.

In vivo biodistribution and tracking.

Animal experiments were performed in a pathogen-free environment using NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSG) mice purchased from the Jackson Laboratory. Experiments and animal care were conducted on protocols approved by the Institutional Animal Care and Use Committee (IACUC). Male and female five-week-old mice were inoculated with IMR5-luciferase cells subcutaneously. When tumors were established, either 5 x 10^5 MSCs or γMSCs were injected directly into the tumor. Twelve hours later, 2.5 x 10^6 DiR labelled γδ T cells were infused via the tail vein and migration throughout the animals was determined using the IVIS Spectrum imaging system (PerkinElmer). Isoflurane anesthesia was used during the course of imaging. Living Image software was used to acquire and analyze fluorescence and bioluminescence data, which were uniformly scaled for analysis. Whole body images were captured to determine distribution of fluorescence throughout the body. To quantify the fluorescence at the site of the tumor, the lungs, head, and tail were covered and images of the tumor were captured. Bioluminescence images of the tumors were used to develop tumor-specific gates that could be used to quantify fluorescence.

4.4 Results

γδ T cell migration. In this study, we tested the ability of γδ T cells to migrate to both conditioned media (CM) (Fig. 1A) and cells (Fig. 1D) to determine if γδ T cells preferentially migrate to γMSCs as compared to MSCs or media. Our results show that a larger number of γδ T

cells migrated to MSC CM as compared to media alone ($p = 0.0001$). Additionally, a larger number of γδ T cells migrated to γMSC CM as compared to MSC CM ($p = 0.001$) or media alone ($p = 0.0001$) (Fig. 1B). This resulted in γδ T cells specifically migrating to γMSC CM at a higher rate than MSC CM ($p = 0.01$) (Fig. 1C). Similarly, a larger number of γδ T cells migrated to γMSC cells than either MSC cells ($p = 0.01$) or media alone ($p = 0.001$) (Fig. 1E), which resulted in a greater specific migration towards γMSC cells than MSC cells ($p = 0.01$) (Fig. 1F).

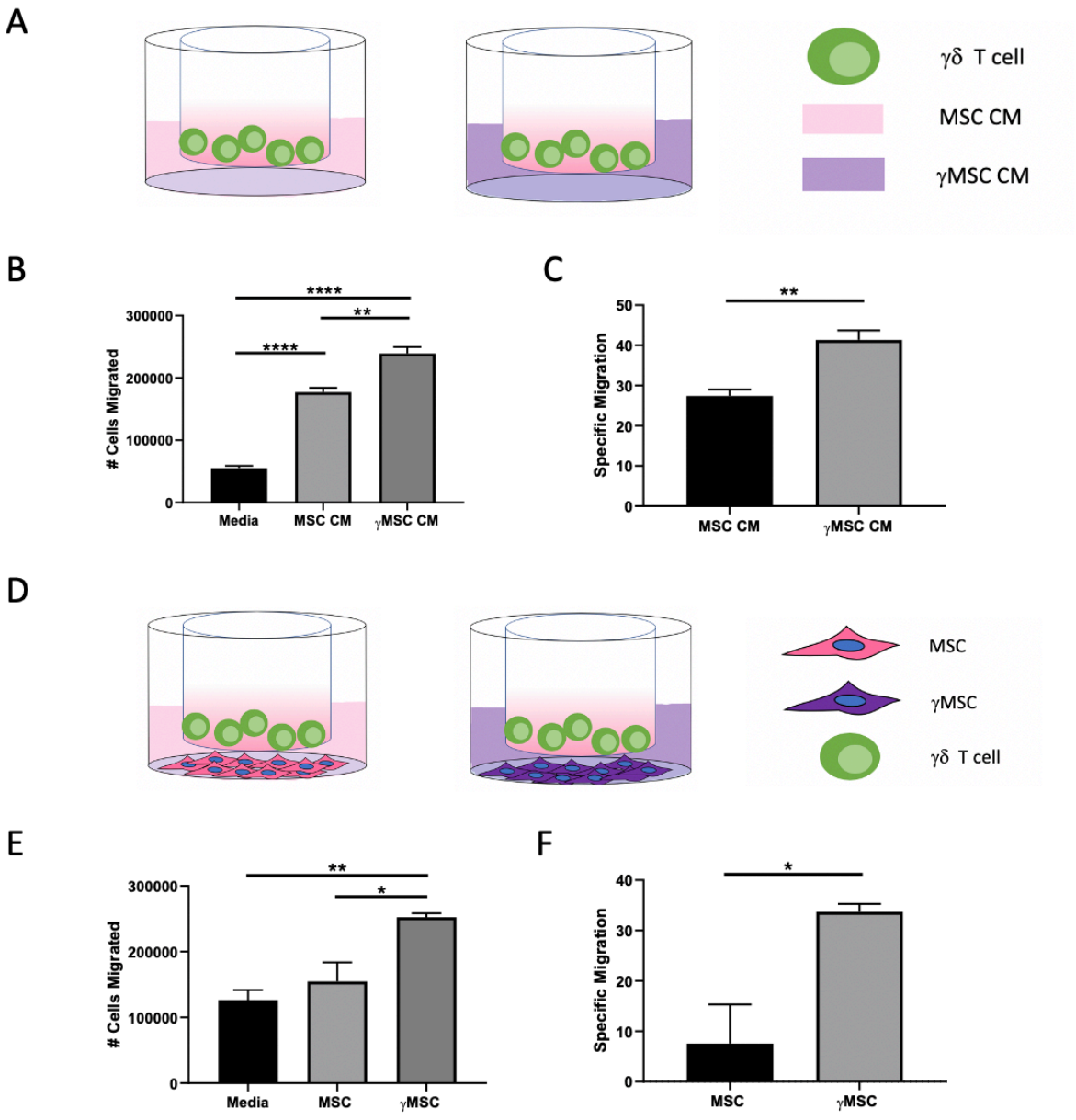

Figure 4.1. Transwell migration of γδ T cells. A) Schematic representing the transwell migration to conditioned media. B)Transwell migration of γδ T cells to media, MSC conditioned media (CM), or γMSC CM. C) Specific migration of γδ T cells to MSC CM or γMSC CM. D) Schematic representing the transwell migration to MSC or γMSC cells. D) Transwell migration of γδ T cells to media alone, MSC cells, or γMSC cells. E) Specific migration of γδ T cells to MSC cells and γMSC cells.

γδ T cell health and cytotoxicity in the presence of MSCs or γMSCs. In this study, we aimed to determine if MSCs influence the health and functionality of our γδ T cell product in a cell-contact dependent manner. Incubating γδ T cells with MSCs or γMSCs at a 1:1 ratio for 4 hours had no effect on viability (92.9% and 90.9% viable, respectively), as assessed by flow cytometry staining for Annexin V (apoptosis marker) and 7-AAD (viability marker) (Fig. 2A). In a cytotoxicity assay, γδ T cells had significantly higher cytotoxicity against IMR5 cells at a 5:1 ratio as compared to either MSCs ($p = 0.01$), or γMSCs ($p = 0.01$) (Fig. 2B). As expected, both MSCs and γMSCs also exhibited cytotoxicity towards IMR5 cells, although not at the levels displayed by the γδ T cells. Additionally, there was no difference in the cytotoxicity of γδ T cells against IMR5 cells when the experiment was performed in the presence of MSCs or γMSCs (Fig. 2C). Similarly, there was no change in degranulation, assessed by flow cytometry staining for CD107a, when γδ T cells were incubated alone, with IMR5 cells (1:1 ratio), or with MSCs or γMSCs (1:1:1 ratio) (Fig. 2D).

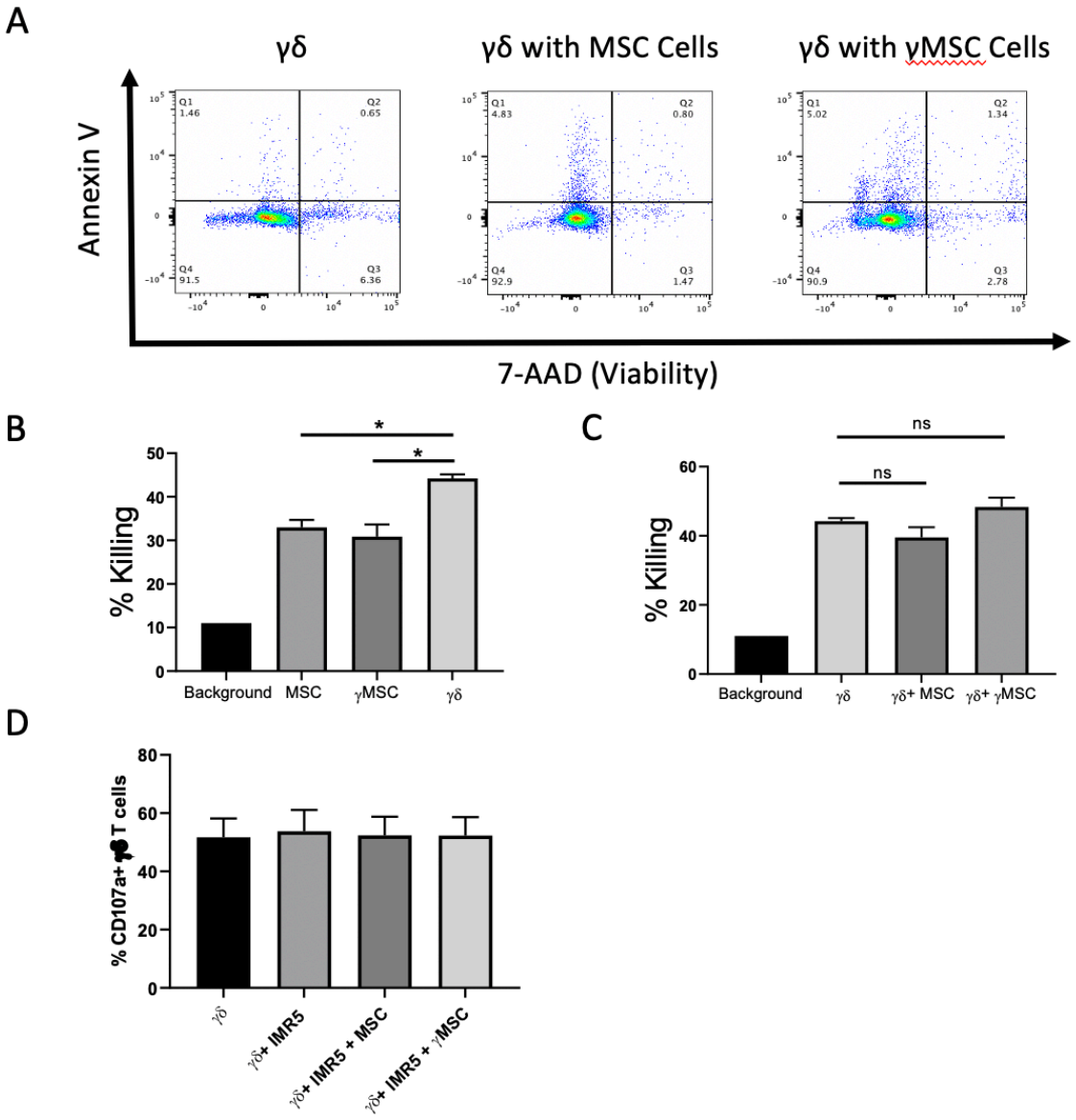

Figure 4.2. Effects of MSC and γMSC cells on γδ T cell health and cytotoxicity. A) γδ T cells were incubated with MSC cells or γMSC cells at a 1:1 ratio for four hours and γδ T cell health was assessed via flow cytometry staining for Annexin V (apoptosis) and 7-AAD (viability). B) γδ T cells were more cytotoxic against IMR5 cells than either MSCs or γMSCs when tested at a 5:1 ratio. C) There was no difference in the cytotoxicity of γδ T cells against IMR5 cells at a 5:1

ratio when tested alone, or in combination with either MSCs or γMSCs. D) There was no change in basal CD107a expression on γδ T cells when incubated with IMR5 cells or a combination of IMR5 cells and MSCs or γMSCs.

In vivo biodistribution and tracking. To determine if we could increase γδ T cell tumor homing via direct tumor injections of MSCs or γMSCs, γδ T cells labelled with DiR and the IVIS imaging system was used to track migration throughout the body over time. The number of MSCs and γMSCs was optimized based on previous studies characterizing the anti-tumor effects of γMSCs on neuroblastoma (Relation et al., 2018). By 24 hours after IV-infusion, γδ T cells had spread throughout the body, including to regions consistent with head, neck, lungs, spleen, liver, and tumor (Fig. 3A). The fluorescent signal in the tumor increased steadily from 4 hours post-infusion up to 24 hours post-infusion for mice treated with γδ T cells alone, γδ T cell and MSCs, or γδ T cells and γMSCs (Fig 3B), suggesting that an increasing number of T cells were migrating to the tumor over time. Although there was a trend towards increased signal at 24 hours post-infusion for mice treated with γδ T cells and γMSCs as compared to mice treated with γδ T cells or γδ T cells and MSCs, this difference was not statistically significant ($p = 0.057$; Fig. 3C). However, when tumors were dissociated and flow cytometry was used to quantify the percentage of the tumor comprised of γδ T cells, there was a significantly greater percentage of γδ T cells in the tumors from mice treated with γδ T cells and γMSCs, as compared to mice treated with γδ T cells alone ($p < 0.05$). There was no difference in the percentage of γδ T cells in tumors from mice treated with γδ T cells and MSCs or γδ T cells alone ($p > 0.05$; Fig. 3D).

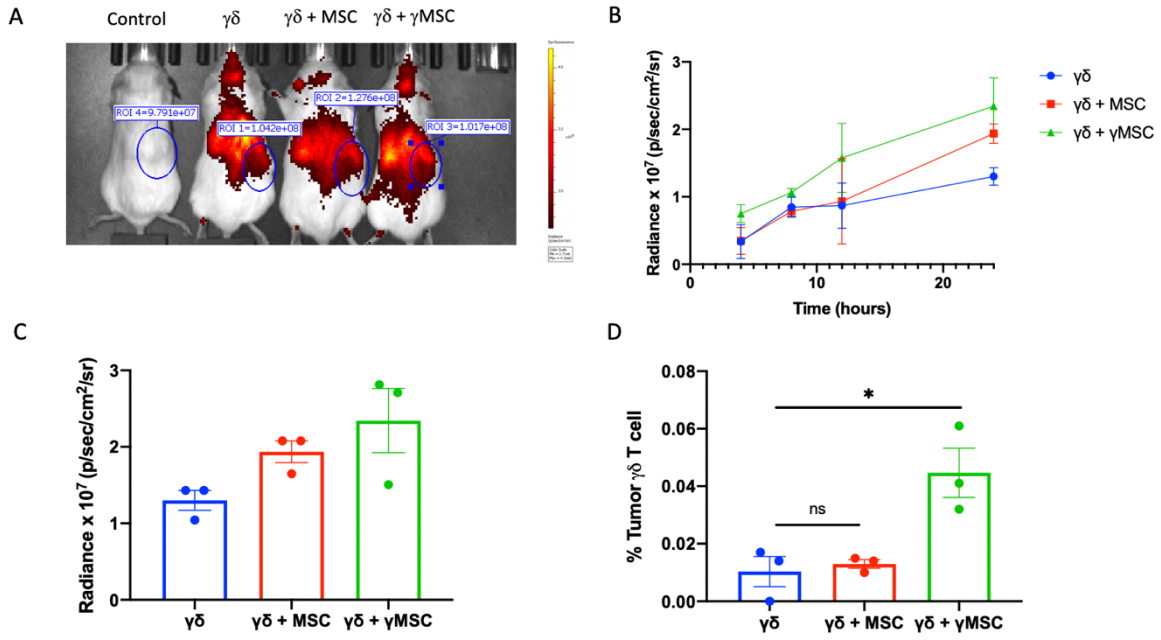

Figure 3. *In vivo* biodistribution and tumor trafficking of IV-infused γδ T cells in a murine IMR5 neuroblastoma model in the presence of MSCs or γMSCs. A) Representative IVIS image demonstrating the *in vivo* biodistribution of DiR labelled γδ T cells 24 hours post infusion. B) Quantitative analysis showing the increase in γδ T cell trafficking per tumor through time (4 to 24 hours post infusion). C) Mean fluorescent signal (radiance) of DiR labelled γδ T cells at a tumor-specific region of interest 24 hours post infusion. D) Flow cytometry analysis of the percentage of γδ T cells per tumor 24 hours post infusion.

4.4 Discussion

Early successes with T-cell therapies in hematological malignancies suggest the future is promising for solid tumors as well, however, the limitation of inefficient tumor trafficking needs to be addressed to improve current therapies. Evidence from clinical studies suggests that there is a positive correlation between the number of infused cells that reach the tumor and favorable outcomes in patients (Pockaj et al., 1994). As only a small percentage of infused T cells ever reach the tumor, developing strategies to increase T-cell homing could drastically improve the effectiveness of these therapies. Moreover, developing strategies to retain T cells at the site of the tumor is also an important consideration, as it has been reported that infused cells will traffic to and from the site of the tumor once injected (Torcellan et al., 2017). Our novel dual-cell immunotherapy reported here was developed to address both the issue of increasing γδ T cell homing to the tumor and retention at the site of the tumor.

To efficiently increase T cell homing to tumors via chemotaxis, there is a need for chemokine ligands secreted from the TME to bind to corresponding chemokine receptors expressed on the surface of the migrating T cells. Interestingly, after being activated with IFNγ, MSCs secrete a range of chemokines that are associated with T cell homing, and many of the corresponding receptors are expressed by our γδ T cell product. In these studies, we report that γδ T cells specifically migrate to γMSCs. The γδ T cells produced by our expansion protocol express high levels of CCR2 (>90%), in addition to CCR4, CCR6, CCR7, CXCR1, CXCR3, and CXCR4 (Burnham et al., 2020). γMSCs are known to secrete high levels of CCL2, CCL5, CCL7, CCL8, CXCL9, CXCL10, and CXCL11, making our dual-cell immunotherapy comprised of γMSCs and γδ T cells a promising pairing (Guirnalda et al., 2013; Harlin et al., 2009; Hong et

al., 2011; Messina et al., 2012; Tannenbaum et al., 1998). Preliminary blocking experiments from our lab suggest that approximately 50% of γδ T cell migration to γMSCs can be attributed to CCL2. These data suggest that additional chemokines secreted from γMSCs, most likely CXCL9, CXCL10, and CXCL11, may also play a role in γδ T cell homing to γMSCs. Identifying the exact chemotactic mechanism responsible for γδ T cell migration to γMSCs could be useful to further increase migration through modification of either MSC chemokine secretion or γδ T cell chemokine receptor expression. From our data, however, we conclude that the molecules responsible for γδ T cell migration are likely secreted factors from γMSCs, as γδ T cells preferentially migrated to both γMSC conditioned media and γMSC cells. Importantly, our data give evidence that γMSCs may increase γδ T-cell homing both *in vitro* and in an *in vivo* tumor model.

In our *in vivo* imaging studies, we demonstrated that homing of γδ T cells to the tumor increases through time. This was true of animals that received γδ T cells alone, γδ T cells and MSCs, and γδ T cells and γMSCs. The imaging data only showed a trend towards increased γδ T cell homing to tumors injected with γMSCs and it is possible that cells may have been migrating to other tissues within the volume of our designated region of interest. However, when whole tumors were dissected, dissociated and analyzed by flow cytometry, this trend became statistically significant; γδ T cells preferentially homed to tumors with γMSCs as compared to tumors alone or tumors injected with MSCs. Of note, a large proportion of γδ T cells migrated throughout the body to additional tissues, likely liver, spleen, and lungs based on signal observed relative to surface anatomy. Additional research is necessary to determine more precisely where the γδ T cells are migrating throughout the body. Although outside the scope of this study,

additional research is also needed to understand if the increase in tumor homing that we achieved is sufficient to therapeutically reduce tumor burden and increase survival.

There are conflicting reports describing the effects of MSCs on γδ T cell health and functionality. Various groups have reported that MSCs can inhibit γδ T cell expansion and cytokine production, acting in an immunosuppressive fashion (Fechter et al., 2017; Prigione et al., 2009). It is important to note that these studies were investigating γδ T cells as part of a population of peripheral blood mononuclear cells, indicating that these results are not directly translatable to the γδ T cells used in this report. In this study, direct cell contact with MSCs and γMSCs did not increase the level of apoptosis or cell death in the γδ T cells tested. It has also been reported that MSCs inhibit the cytotoxicity and degranulation of γδ T cells *in vitro* (Liu et al., 2015; Martinet et al., 2009). In contrast, our studies suggest that neither the cytotoxicity nor the degranulation capacity of γδ T cells is impacted by the presence of MSCs or γMSCs. Overall, these results suggest that the γMSCs injected as part of our proposed dual-cell therapy will not inhibit the ability of γδ T cells to exert cytotoxic effects if the two cell types come into contact at the site of the tumor. This is notable because of the literature describing the immunosuppressive effects of MSCs on lymphocytes.

The novel dual-cell immunotherapy proposed in this report has potential to be further developed as a therapy that can be applied to a variety of cancer types. We used a neuroblastoma tumor model in this study as a proof of concept, however future research is necessary to determine how our proposed model may be applied to additional malignancies. Classical strategies for developing cancer immunotherapies have suggested focusing on the pairing of tumor types with T cell therapies based on the chemokine secretion profile of the individual

tumor (Rafiq et al., 2020). The therapy proposed in this report, where γMSCs are injected directly into the tumor to manipulate the tumor microenvironment, eliminates the need for matching the tumor type to the proposed therapy. Instead, the combination γMSC-γδ T cell therapy can be applied to many cancer types, as γMSCs appear to secrete sufficient chemotactic factors that increase γδ T cell homing to the tumor. Once at the site of the tumor, continuous chemokine secretion from γMSCs could function to increase the retention of γδ T cells at the site of the tumor. Additionally, γMSCs have been associated with producing anti-cancer effects and could act in unison with the γδ T cells to eradicate the tumor. Overall, our studies provide evidence for the development of a dual-cell immunotherapy that utilizes the pairing of chemokines secreted from γMSCs with the chemokine receptors expressed on γδ T cells to increase tumor homing and the effectiveness of infused γδ T cell products.

Chapter Five: General Discussion

5.1 Summary of Results

Since the discovery of γδ T cells in the mid-1980's, the use and development of γδ T cell cancer immunotherapies has progressed rapidly. Although investigators were originally interested in stimulating the expansion of γδ T cells *in vivo*, the number of studies using this approach quickly declined as it became apparent that off-target effects were likely and that this approach offered limited clinical benefit (Pauza et al., 2018; Pressey et al., 2016). As the use of *in vivo* expansion methods declined, the field of γδ T cell immunotherapies shifted towards the development of autologous therapies, using patient-derived γδ T cells. However, the successes seen in pre-clinical studies were not translated into favorable patient outcomes in clinical trials. Although these studies confirmed that autologous γδ T cell therapies were safe, their limited clinical efficacy has been attributed to challenges common to many solid tumor T cell immunotherapies. As γδ T cells can be used in an allogeneic setting, current efforts in the field have shifted to focus on the development of third-party donor γδ T cell immunotherapies. Within the past few years, clinical trials have been initiated to test the safety and efficacy of allogeneic γδ T cell therapies. Further efforts are aimed at the development of modified γδ T cell therapies, which could become the next main focus in the field of γδ T cell cancer immunotherapies. Based on recent trends in the field, this dissertation focused on the development of an allogeneic γδ T cell therapy and modification of the tumor microenvironment to address the issue of inefficient tumor trafficking, a common limitation found in solid tumor cancer cell immunotherapies.

As the interest in allogeneic γδ T cell therapies increased, it became apparent that the field lacked a proven expansion method to consistently expand γδ T cells. Chapter Two of this dissertation focused on the development of a good manufacturing process (GMP)-compliant protocol to consistently and efficiently expand γδ T cells. One of the main findings was that

donor variation seen in γδ T cell expansion could be attributed to exercise. We found that γδ T cells from donors who exercise consistently expand, in striking contrast to the poor expansion rates of γδ T cells from sedentary donors. However, the addition of IL-21 in the expansion protocol has the potential to rescue the expansion of γδ T cells from sedentary donors. Additionally, this chapter analyzed the timing and efficiency of αβ depletion within the expansion protocol. Depleting the αβ T cells within the culture was most efficient halfway through the expansion protocol and importantly, the γδ T cell product remained depleted of αβ T cells through the end of expansion. Furthermore, the percentage of αβ T cells that remained in the culture was within the threshold necessary to use this cell product in an allogeneic setting. Finally, Chapter Two described the development of a novel mixed donor γδ T cell immunotherapy and the impacts of cryopreservation. The mixed donor γδ T cell immunotherapy, to our knowledge, is the first proposed therapy combining γδ T cells from different donors. The mixed donor therapy had increased cytotoxicity compared to the individual donor γδ T cells, suggesting that when combined, γδ T cells from different donors act synergistically together. Cryopreservation had a negative impact on the mixed donor γδ T cell therapy, stimulating further investigation into improving the freeze/thaw process of γδ T cells.

In an effort to optimize the expansion and manufacturing protocol further, the focus of Chapter Three was on characterizing and negating the effects of cryopreservation on γδ T cell health and functionality. The standard cryopreservation protocol used by our laboratory for γδ T cells resulted in an average cell loss of 50% and a cell product with sub-optimal viability. The use of commercially available GMP-grade cryopreservation media was not able to improve γδ T cell health after a freeze/thaw cycle. Interestingly, the media that γδ T cells were thawed into

made a significant impact on improving overall cell health. The use of Human Serum Albumin (HSA) as a thawing media significantly improved γδ T cell viability immediately in the post-thaw period. Chromatin condensation prior to cryopreservation further improved γδ T cell health post-thaw and when combined with the use of HSA as a thawing media, viability was as high as 90%. Although these manipulations improved γδ T cell health immediately in the post-thaw period, high levels of mitochondrial membrane depolarization suggest that cryopreservation may be fatally damaging a high proportion of cells. The cryopreservation process also reduced the cytotoxic capacity of γδ T cells, suggesting there is room for further improvement.

After optimizing the expansion and cryopreservation process of γδ T cells, Chapter Four focused on addressing an important limitation for solid tumor cell immunotherapies: inefficient tumor trafficking. In this chapter, we propose utilizing the chemotactic axis to increase tumor homing using a dual-cell immunotherapy composed of a combination of γMSCs and γδ T cells. γMSCs secrete a range of chemokines to which γδ T cells express the corresponding receptors on their cell surface. We found that γδ T cells migrate preferentially to both conditioned media from γMSCs and to the γMSC cells themselves *in vitro*. When we injected γMSCs directly into tumors *in vivo*, we found that γδ T cells migrated to these tumors at higher rates than to tumors alone or tumors injected with MSCs. Live animal imaging revealed that once infused, γδ T cells migrate throughout the body to other organs as well, likely including the lungs, spleen, and liver based on the signal observed. Additionally, in contrast to reports in the literature that MSCs have an immunosuppressive effect on γδ T cells, we found no evidence that γMSCs negatively impacted γδ T cell health or cytotoxicity. These studies concluded that the proposed dual-cell γMSC-γδ T cell immunotherapy has potential to increase tumor homing, although future research is needed to determine the therapeutic capacity of this therapy.

5.2 Implications of Findings

The focus of this dissertation centered on developing a GMP-compliant protocol for an off-the-shelf allogeneic γδ T cell cancer immunotherapy and a strategy to improve the efficiency of γδ T cell tumor homing. Current γδ T cell immunotherapies are limited in clinical applicability because they lack manufacturing protocols that consistently and efficiently produce the number of γδ T cells necessary for a cellular therapy. Furthermore, the majority of γδ T cell therapies described in the literature fail to address the issue of inefficient tumor homing, a step that is critical in being able to therapeutically reduce tumor burden. The studies described in this dissertation not only provide a GMP-compliant protocol capable of consistently producing a clinically relevant number of γδ T cells, but also offer insight into a potential dual-cell immunotherapy that could be used in increase the tumor homing capabilities of γδ T cells.

Understanding the cause behind donor variability in γδ T cell expansion is an important preliminary step in the development of a successful expansion protocol. Clinical-scale expansions of γδ T cells are costly and time-consuming. Being able to select cell donors based on objective characteristics that would suggest a high probability of expansion is a cost-effective approach that reduces manufacturing time and makes the development of an off-the-shelf cell product feasible. Chapter Two of this dissertation provides evidence that donors who exercise are more likely to have successful γδ T cell expansions than sedentary donors. These studies provide criteria that can be used in the donor selection process for clinical trials and companies manufacturing allogeneic γδ T cell therapies. A second group has described the positive effects of exercise on γδ T cell expansion, suggesting that level of exercise is an important consideration when selecting donors for clinical-scale expansions (Baker et al., 2019).

Although the FDA does not currently allow cell products comprised of cells from different donors, the mixed donor γδ T cell immunotherapy described in Chapter Two has important implications for the field. As with the donor variability seen in the expansion capacity of γδ T cells from different donors, there is also variability seen in the cytotoxic capacity. Even among donors who exercise, we give evidence for inter-donor variation in cytotoxic abilities. Interestingly, the mixed donor γδ T cell immunotherapy we developed had an increased cytotoxic capacity in comparison to the cytotoxicity of individual donor γδ T cell products. These results suggest that somehow the γδ T cells are synergizing with each other, making a superior cell product that can kill cancer cells more effectively. Understanding the mechanism behind the synergy seen when γδ T cells from different donors are mixed together could give insight into how to develop a γδ T cell therapy with a more standardized level of cytotoxicity. This mechanism could be important in determining how to increase the cytotoxic capacity of γδ T cells that do not effectively kill cancer cells.

The method of cryopreservation and cell recovery after thawing is an important aspect when considering the entire manufacturing process of a γδ T cell therapy. Although Chapter Three describes mechanisms to increase cell health after thawing, we also gave evidence that mitochondria are fatally damaged during the cryopreservation process. This suggests that manipulating γδ T cells prior to cryopreservation could provide the most effective way of ensuring optimal cell health and functionality post-thaw. Chromatin condensation was effective at increasing cell viability, however it did not impact cytotoxicity, suggesting that further optimization of the γδ T cell cryopreservation process is necessary.

Finally, the results in Chapter Four describe a potential dual-cell immunotherapy capable of increasing γδ T cell tumor homing. The tumor homing capacity of a cellular immunotherapy is

critical to its ability to treat and cure cancer. Multiple groups have published studies showing that the number of infused T cells that arrive at the tumor is a small fraction of the number of cells originally infused into the patient (Bernhard et al., 2008; Hong et al., 2011; John et al., 2013; Pockaj et al., 1994). Additionally, clinical studies have given evidence for a positive correlation between the proportion of infused cells that home to the tumor and favorable outcomes for patients (Pockaj et al., 1994). Our dual-cell immunotherapy utilizes a unique pairing of γMSCs and γδ T cells. γMSCs are known to secrete a range of chemokines that correspond to receptors expressed on the γδ T cells produced by our manufacturing protocol. Using γMSCs to increase γδ T cell tumor homing accomplishes two tasks: manipulating the TME to secrete chemokines that will call in γδ T cells and utilizing the anti-cancer properties of γMSCs, such as their ability to polarize tumor associated macrophages (Relation et al., 2018). The results presented in Chapter Four give evidence that γMSCs can effectively increase γδ T cell tumor homing *in vivo* and further investigation is needed to determine if the proposed dual-cell immunotherapy can decrease tumor burden.

5.3 Limitations and Future Directions

Although we successfully developed a GMP-compliant manufacturing protocol that can be scaled to accommodate clinical-sized expansions, a limitation in this work includes the small scale at which the studies were conducted at. Our study characterizing donor variability in expansion included 16 donors and self-reported levels of exercise. Although another group has confirmed that exercise prior to blood collection can increase γδ T cell expansion, further research could identify the type and amount of exercise likely to produce a γδ T cell expander across a broader range of donors (Baker et al., 2019).

To reduce the risk of GVHD, the αβ-depletion process is one of the most important steps in the manufacturing protocol. The IRB protocol for our study only allowed for the collection of a small proportion of the amount of blood that is typically collected during a clinical cell expansion protocol. Because of this, we concluded that the best time to perform the αβ-depletion step was midway through expansion. It is possible that the αβ-depletion could be completed successfully earlier in the expansion process. However, if the αβ-depletion step is performed earlier in the expansion process, a complete characterization of the final γδ T cell product would be necessary to ensure the phenotype is similar to γδ T cells produced by the current manufacturing protocol.

The mixed donor γδ T cell immunotherapy provides an interesting avenue of development for future therapies. To our knowledge, Chapter Two of this dissertation described the first attempt at developing a γδ T cell therapy comprised of cells from different donors. Our proof-of-concept studies give evidence that γδ T cells from different donors can successfully expand in culture together and that when combined, they result in a cell product with enhanced cytotoxicity. Future research is necessary not only to determine the mechanism behind the synergy observed in these studies, but to determine the safety and efficacy of a mixed donor γδ T cell therapy. Although γδ T cells can be used in an allogeneic setting, it is possible the mixed donor therapy presents a higher risk for an adverse reaction than a γδ T cell therapy comprised of cells from an individual donor. Testing the mixed γδ T cell therapy *in vivo* would give insight into the potential risk of adverse reactions and the feasibility of pursuing this therapy further.

Chapter Three presents several strategies that successfully improved γδ T cell health immediately post-thaw; however these strategies failed to protect the mitochondria during cryopreservation and maintain the cytotoxic capacity of the cells. Future research should focus

on developing strategies to maintain the integrity of the mitochondrial membrane during the cryopreservation process. Our results also suggest that cryopreserved γδ T cell lose some of the cytotoxic capacity after a freeze-thaw cycle. Additional research should address the issue of how effective a cryopreserved γδ T cell therapy is in comparison to a fresh γδ T cell therapy. Studies using CAR-T cells suggest that cryopreserved products function as efficiently as fresh products *in vivo*, although it is possible that αβ T cells are less susceptible to the cryopreservation process in comparison to γδ T cells (Panch et al., 2019).

Finally, the dual-cell γMSC-γδ T cell immunotherapy described in Chapter Four presents an opportunity for future investigation. In these studies, γδ T cells preferentially homed to γMSCs both *in vitro* and *in vivo*. Inefficient tumor homing is a limitation that is diminishing the success of T cell immunotherapies in solid tumor applications. Studies with both γMSCs and γδ T cells show that these cells are safe to use in patients, increasing the likelihood that the FDA could approve a dual-cell immunotherapy such as the one we describe. Additional research should confirm that the γMSC-γδ T cell immunotherapy is effective at increasing overall survival and reducing tumor burden. Further optimization of the strategy is necessary to determine how many doses of γMSCs and γδ T cells are necessary to produce an effective treatment and favorable patient outcomes.

5.4 Conclusions

This dissertation describes a GMP-compliant γδ T cell manufacturing protocol and the development of two novel γδ T cell immunotherapies. The γδ T cell manufacturing protocol described in this work includes criteria for donor selection and an optimized protocol to consistently expand γδ T cells. Although currently ahead of its time, we explored the feasibility of a mixed donor γδ T cell immunotherapy that had enhanced cytotoxicity in comparison to

individual donor γδ T cell therapies. We have also developed strategies to enhance γδ T cell health and functionality after cryopreservation, although we believe these strategies could be optimized further. Finally, we describe a novel dual-cell γMSC-γδ T cell immunotherapy that can effectively increase the tumor homing capabilities of γδ T cells.

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