Cord Blood-Derived Invariant Natural Killer T Cells as a Platform for Allogeneic Chimeric Antigen Receptor Cell Therapy

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Cord Blood-Derived Invariant Natural Killer T Cells as a Platform for Allogeneic Chimeric Antigen Receptor Cell Therapy

by

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Cord Blood-Derived Invariant Natural Killer T Cells as a Platform for Allogeneic Chimeric Antigen Receptor Cell Therapy

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by

Maison Donald Grefe, B.A.

Houston, Texas

May, 2024
Dedicated to Mom, Dad, Zavier, Grandma Jane, Grandpa Joe, Grandma Cher, and

Grandpa Ron
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Thank you to my advisor and mentor Dr. Jin Im for taking me into her lab and fully supporting my goals as an aspiring scientist. A special thank you to Dr. Jeff Molldrem for his valuable mentorship throughout my project, and Dr. Abel Trujillo-Ocampo who answered my unending questions and was always willing to lend a helping-hand.

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Cord Blood-Derived Invariant Natural Killer T Cells as a Platform for Allogeneic Chimeric Antigen Receptor Cell Therapy

Maison Donald Grefe, B.A.

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Chimeric antigen receptor (CAR) T cells have revolutionized the treatment of hematopoietic malignancies achieving >50% complete response rates in numerous refractory/relapsed B cell malignancies. However, there are challenges that hinder CAR-T efficacy and bar the broader use of this therapy in patients. One approach to address these challenges is to create a safe allogeneic CAR cell product by using cells that do not cause graft versus host disease (GvHD). Invariant natural killer T (iNKT) cells are an ideal candidate as they are restricted to the monomorphic CD1d protein in contrast to HLA restricted αβ-T cells and therefore are safe in the allogeneic setting. Furthermore, iNKT cells possess inherent antitumor mechanisms including the expression of NK activating receptors and the capability to lyse M2 tumor associated macrophages via aberrant glycolipid presentation on CD1d. Previous work from our lab demonstrated iNKT cells derived from cord blood units (CB-iNKT) are enriched in naïve and central memory phenotype compared to iNKT cells derived from adult peripheral blood (AB-iNKT). Patients that receive CAR-T cells with higher proportions of naïve/central memory phenotype cells receive greater therapeutic benefit. Therefore, CB-CAR-iNKT cells may serve as an effective platform for allogeneic adoptive cell therapy. Here we evaluate the preclinical therapeutic potential of CB-CAR-iNKT cells by equipping them with 8F4CAR that is restricted to the acute myeloid leukemia (AML) associated antigen PR1 presented in the context of HLA/A2*01. We show CB-8F4CAR-iNKT cells expand in greater numbers compared to AB-8F4CAR-iNKT cells with equivalent 8F4CAR expression and iNKT cell purity. CB-8F4CAR-iNKT cells exhibit significantly higher CD62L expression
indicating an enriched naïve and central memory phenotype compared to AB-8F4CAR-iNKT cells. While both CB-8F4CAR-iNKT cells and AB-8F4CAR-iNKT cells display potent AML cytotoxicity via 8F4CAR and iNKTCR in vitro, CB-8F4CAR-iNKT cells trend in an increase in proliferation after repeated leukemia challenge. The increased expression of CD62L and the increased proliferative potential of CB-8F4CAR-iNKT cells suggests an improvement in anti-leukemic activity over AB-8F4CAR-iNKT cells in vivo. We go on to show that CB-8F4CAR-iNKT cells are polarized to Th2 cytokine production after 8F4CAR mediated leukemia cytolysis, which is consistent with iNKTCR mediated stimulation of parental CB-iNKT cells. Importantly, CB-8F4CAR-iNKT cells display anti-leukemic activity in vivo. This thesis supports the idea that CB-iNKT cells can be an effective allogeneic CAR therapy and supports the use of CB-iNKT cells as a platform to target hematopoietic malignancies.
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INTRODUCTION:

Acute Myeloid Leukemia

Acute myeloid leukemia (AML) arises from the dysregulation of normal myeloid hematopoiesis and manifests as uncontrolled proliferation of myeloid hematopoietic blasts from leukemic progenitors (1). Aberrant leukemic blast accumulation in the bone marrow compromises the development and function of healthy blood cells resulting in symptoms including anemia, fatigue, neutropenia, and thrombocytopenia (2). AML is the most common form of acute leukemia in adults accounting for ~80% of acute leukemias. Moreover, AML is responsible for ~50% of all deaths from leukemia in the United States (3). The common characteristics between healthy hematopoietic stem cells and leukemic stem cells and blasts, and genetic heterogeneity of AML makes developing targeted therapies challenging. Indeed, AML patients face a <30% 5-year survival rate (4). The emergence and success of cancer immunotherapy in recent years has opened the door to new options to treat AML and improve patient outcomes. This thesis describes the preclinical development of a novel adoptive cell therapy to target AML.

The Use of Chimeric Antigen Receptors to Target Leukemias

The development of immunotherapies to treat leukemias began in 1957 with the first allogeneic stem cell transplant (allo-SCT) performed by Nobel Prize laureate Dr. E. Donall Thomas. Allo-SCT works by replacing the patient’s diseased immune system with the immune system of a healthy donor (5). This therapy was revolutionary as it became a curative option for patients with hematological malignancies and is still part of the standard of care for several leukemias today (6). From these treatments, researchers identified the primary driver of the graft-versus-leukemia (GvL) response as T cells within
the donated immune system (7). This sparked interest in specifically harnessing the power of T cells to eliminate cancer, and ultimately resulted in the development of adoptive cell therapies (ACT) where autologous T cells are isolated from a patient, modified, and reinfused to aid in cancer clearance. Chimeric antigen receptor (CAR) T cells are the most prominent and efficacious type of ACT. CAR-T cells are genetically modified to express a fusion protein on the cell surface that consists of a cancer antigen specific scFv region of an antibody fused with the costimulatory domain of a T cell receptor (TCR) (8). Engagement of the CAR with its cognate antigen results in T cell activation, proliferation, and leukemia cell lysis. The efficacy of CAR-T cells was demonstrated in patients for the first time in 2010 by a group led by Dr. Steven Rosenberg showing a 75% overall response rate in advanced B cell malignancies (9). Since then, numerous clinical trials have been conducted to improve CAR-T cell efficacy against leukemias resulting in six FDA approved CAR-T therapies in the United States (10).

**Challenges of CAR-T Cells and Current Targets**

Despite impressive efficacy, CAR-T cells are not without significant safety concerns. The most common CAR-T related adverse event is cytokine release syndrome (CRS), which occurs in 50-90% of patients (11). CRS is thought to originate upon CAR engagement with cognate antigen inducing rapid CAR-T expansion and the release of a myriad of inflammatory cytokines including IL-6, IFNγ, IL-8, and GM-CSF. CRS is identified clinically and graded by the severity of symptoms including fever, hypotension, and irregular heartbeat (12). Additionally, CAR-T cells can induce neurotoxicity in patients classified as immune effector cell-associated neurotoxicity syndrome (ICANS), and while the pathophysiology is incompletely understood it is generally associated with
The occurrence of ICANS appears dependent on perturbation of the blood-brain barrier where inflammatory cytokines and CAR-T cells gain entry to the central nervous system (13). The spectrum of severity of both CRS and ICANS ranges from mild to severe including the possibility of death due to the CAR-T product itself. Resolving these toxicities will be critical to further improving CAR-T cell efficacy and patient outcomes.

Furthermore, the application of CAR-T cells against AML presents significant challenges. In contrast to the consistent expression of CD19 on B cell malignancies, AML is highly heterogeneous both inter- and intra-patient which makes identifying a suitable target antigen difficult. There have been efforts to develop CAR-T cells against numerous AML associated antigens including CD123, CD33, CD44, CLL-1, CD96, CD47, CD23, CD7 that show promising preclinical activity (14). However, most of the antigens expressed on AML are present in healthy tissue thus causing severe on-target off-tumor activity. Indeed, significant toxicities and deaths have been reported in phase one clinical trials (15). Thus, identifying a homogeneously expressed AML specific antigen will improve CAR-T application to this challenging disease.

8F4CAR and the Leukemia Associated Antigen PR1

The curative potential of allo-SCT is elicited from the cytotoxic T lymphocytes (CTL) from the donor that can lyse the recipient’s leukemic stem cells and blasts. Evidence suggests that the targeting of T cells against leukemia is due to the recognition of a leukemia associated antigen by the TCR (16). Targeting leukemic antigens is becoming central to modern day immunotherapy and thus it is critical to choose an antigen wisely. The Translational Research Working Group of the National Cancer
Institute describes the criteria for antigen selection in the context of AML. The antigen should: be found in leukemia stem cells and blasts with some specificity, demonstrate oncogenicity, elicit an immune response, and be clinically relevant (17). One such AML associated antigen that satisfies these recommendations is PR1. The nonameric peptide (VLQELNVT corresponding to proteasomal degradation of two serine proteases proteinase 3 (P3) and neutrophil elastase (NE) (18). While these proteins are normally contained within the neutrophilic granules of polymorphonuclear leukocytes, P3 and NE are aberrantly expressed in leukemic stem cells and blasts and mis-localized to the cytoplasm rendering them amenable to antigen processing and presentation of PR1 on HLA/A2*01. Indeed, primary patient samples were shown to contain PR1 specific CTL and displayed potent anti-leukemic activity in vitro (18,19).

To target AML in the absence of PR1-specific CTL, a novel TCR-mimetic antibody against the PR1/HLA/A2*01 complex was developed. This antibody, termed 8F4, enabled leukemic blast and leukemic stem cell lysis while sparing healthy hematopoietic stem cells in vitro (20). In an AML mouse model, treatment with the 8F4 antibody significantly decreased leukemia burden and extended mouse survival (21). To further increase potency, 8F4 was converted into a CAR-T therapy (Fig 1) that shows promising anti-leukemic activity in murine models (22). As part of this work we collaborated with Dr. Jeffrey J. Molldrem, the discoverer of PR1-specific CTL and inventor of PR1/HLA/A2*01 targeting therapies, to improve the therapeutic window of 8F4CAR by changing the effector cell it is equipped to.
Figure 1. 8F4CAR Construct. The 8F4CAR construct begins with a signal peptide followed by the variable light chain 2 and variable heavy chain of humanized 8F4 antibody connected by G₄S linker. The antigen recognition domain is hinged to the cell membrane by IgG1 constant heavy chains 2 and 3. The transmembrane domain and stimulatory molecules consists of CD28 paired with CD3ζ.

Potential Advantages of CAR-iNKT Cells Over Conventional CAR-T Cells

Currently, CAR-T cells must be generated from autologous T cells on a case-by-case basis. The use of allogeneic CAR-T cells would risk inducing graft versus host disease (GvHD) due to HLA mismatching between donor and recipient (23). The bespoke manufacturing process required for each patient leads to high production costs, the risk of manufacturing failure, and treatment delays up to 3 weeks to produce the therapy (24). The delay in treatment can be particularly deleterious to patients with aggressive acute leukemias who may see disease progression and a worsened prognosis upon treatment administration. Additionally, autologous CAR-T cells may not be efficacious for some patients due to T cell dysfunction caused by the cancer itself or previous lines of therapy (25). Several groups in the field aim to address these challenges by creating an allogeneic or ‘off-the-shelf’ CAR-T cell product. The potential advantages of allogeneic CAR-T cells include decreased production costs due to large scale manufacturing of cells from a single donor, the ability to cryopreserve treatment cells making them immediately available and amenable to repeated dosing, and the standardization of CAR-T cell fitness quality through careful donor selection (26).
One approach to create an allogeneic CAR-T cell product is to change the effector cell from an HLA restricted αβ T cell. Invariant natural killer T (iNKT) cells are a rare population of T lymphocytes that are uniquely positioned as a bridge between the innate and adaptive immune systems (Fig 2). iNKT cells are often part of the first responders during an immune response, and upon activation iNKT cells upregulate CD40L that can induce the maturation of dendritic cells (DCs) causing IL-12 secretion and stimulation of NK and T cells (27). iNKT cells can also directly modulate the function of other immune cell populations via the secretion of a variety of Th1 and Th2 type cytokines (27). Which Th1/Th2 cytokines iNKT cells produce is determined by their phenotype that is generally defined by CD4 expression. CD4⁺ iNKT cells are polarized to Th2 cytokine production and CD4⁻ iNKT cells are polarized to Th1 cytokine production (28). Importantly, iNKT cells are defined by the expression of the Vα24-Jα18 invariant T cell receptor (iNKTCR) alpha chain paired with a Vβ11 chain and are restricted to glycolipid presentation on the monomorphic CD1d protein (29). Therefore, allogeneic iNKT cells do not cause GvHD as was proven in humans recently (30). Additionally, iNKT cells possess several inherent anti-cancer mechanisms including the expression of natural killer (NK) cell activating receptors such as DNAM-1 and NKG2D, the ability to more effectively infiltrate solid tumors compared to αβ T cells due to high expression of chemokine receptor CCL2, and iNKT cells can specifically eliminate M2 tumor associated macrophages (TAM) through aberrant glycolipid presentation on CD1d (31,32,33).
Figure 2. iNKT cells are a bridge between the innate and adaptive immune systems. iNKT cells can both directly and indirectly modulate the function of other immune cell populations.

Cord Blood Derived iNKT Cells Compared to Adult Peripheral Blood Derived iNKT Cells

While these characteristics make iNKT cells an attractive CAR platform, there are challenges in harnessing iNKT cells from adult peripheral blood. These include low iNKT cell abundance (0.001%-0.1% of T cells in circulation) and an inconsistent CD4 phenotypetype between adult donors (34,35). In previous unpublished work from the Jin S. Im lab, we discovered iNKT cells derived from cord blood units (CB-iNKT) can be effectively expanded \textit{ex vivo} to greater numbers than adult blood derived iNKT (AB-iNKT) cells, display a consistent uniform phenotype, are enriched in naïve and central memory populations, and appear more metabolically flexible compared to AB-iNKT cells based on preliminary RNA-seq data. Based on these findings we decided to investigate the use of CB-iNKT cells as a platform for CAR therapy.
Hypothesis and Specific Aims

We hypothesize that CB-8F4CAR-iNKT cells will exhibit anti-leukemic activity comparable to AB-8F4CAR-iNKT cells in vitro and exert anti-leukemic activity in vivo. To investigate the potential of CB-8F4CAR-iNKT cells, the following aims will be executed:

Aim 1: Evaluate the cytotoxicity and cytokine production profile of 8F4CAR-iNKT cells from both cord and adult donors against AML in vitro.

Aim 2: Evaluate the anti-leukemic activity of CB-8F4CAR-iNKT cells in vivo with a xenogeneic AML model.
RESULTS:

Generation of 8F4CAR-iNKT Cells from Primary Cord and Adult Donors

We generated donor-matched untransduced (UT) and 8F4CAR-iNKT cells from four cord blood units and four adult peripheral blood units following the protocol detailed in methods (page 24) (Figure 3, Figure 4A). We found CB-8F4CAR-iNKT cells expanded ~80 fold more than AB-8F4CAR-iNKT cells when normalized for the number of peripheral blood mononuclear cell (PBMC) source, and there was no statistical difference in the number of UT-iNKT and 8F4CAR-iNKT cells generated per donor source (Figure 3B,C). The iNKT cell purity of all cells alive in culture at the conclusion of the cell generation protocol, measured by CD3 and 6B11 positivity, was >90% for both CB- and AB-iNKT cells (Figure 3D). 8F4CAR expression was first measured by flow cytometry at day 14 and remained consistent until final assessment at day 28 in iNKT cells from both cord and adult donors. Furthermore, CB-8F4CAR-iNKT and AB-8F4CAR-iNKT cells showed similar 8F4CAR expression at the midpoint and the final measurement at ~75% (Figure 3E). After successful generation of 8F4CAR-iNKT cells from both donor types we proceeded to assess phenotypic differences by flow cytometry.
Figure 3. Schematic of 8F4CAR-iNKT cell expansion protocol. iNKT cells were isolated and expanded from cord blood units and adult peripheral blood. Detailed expansion protocol can be found in Methods section.

Figure 4. CB-8F4CAR-iNKT cells expand in greater numbers compared to AB-
8F4CAR-iNKT cells with equivalent iNKT cell purity and 8F4CAR expression.

8F4CAR-iNKT cells were generated from four cord and four adult donors. iNKT cells were enriched and stimulated with aGC-pulsed allogeneic DCs supplemented with IL-2 before 8F4CAR transduction. (A) Representative cord and adult donor flow cytometry analysis of pre- and post-iNKT cell enrichment, the final iNKT cell purity, and the final 8F4CAR expression (blue and red histogram indicates 8F4CAR-iNKT cells and grey is donor-matched UT-iNKT cells). (B) Absolute number of iNKT cells throughout the expansion protocol. (C) Total fold change of iNKT cells following 28-day cell expansion protocol. Bolded numbers indicate the mean number of iNKT cells generated per 500M PBMC. Cells were counted by trypan blue exclusion. Mean +/- SD. ***, P < 0.001; ns, not significant; paired student t-test for intra-donor type comparison; unpaired student t-test for inter-donor type comparison. (D) iNKT cell purity of all live cells in culture determined by both CD3 and 6B11 positivity. Each point is a donor. (E) 8F4CAR expression at days 14 and 28 of the cell generation protocol by gating on IgG+ and 6B11+ iNKT cells. Each point is a donor.

Characterization of 8F4CAR-iNKT Cells

iNKT cells in adult peripheral blood are phenotypically heterogeneous with subsets generally defined by CD4 expression. As expected, there was variation in CD4 expression in AB-iNKT cells (range 23.4%-85.6%) whereas CB-iNKT cells were consistently CD4 positive (range 79%-99%). 8F4CAR transduction trended in an increase in CD4 positive cells with a subsequent decrease in CD4 CD8α double negative cells compared to donor matched UT-iNKT cells for both donor types (Figure 5A,B). We also investigated the memory phenotypes of iNKT cells from both donor groups by measuring CD62L and CD45RA expression. This is particularly important for
CAR-T and CAR-iNKT cell therapy as CAR-cells with a higher proportion of naïve or central memory cells show superior anti-leukemic activity in preclinical models (36). We found CD62L expression to be significantly increased in CB-iNKT cells compared to AB-8F4CAR-iNKT cells, which supports our hypothesis that CB-8F4CAR-iNKT cells may display significant leukemia control in vivo (Figure 5C,D).

**Figure 5.** CB-8F4CAR-iNKT cells maintain a consistent CD4+ phenotype and are significantly enriched in naïve and central memory cells compared to AB-8F4CAR-iNKT cells. Phenotypic analysis of iNKT cells after ex vivo expansion. (A) The percentage of CD4+, CD8α+, and CD4–CD8α+ UT- and 8F4CAR-iNKT cells at expansion.
protocol conclusion. *, P < 0.05; ns, not significant; paired student t-test for intra-donor type comparison. Each point is a donor. (B) A representative flow cytometric analysis of one cord and one adult donor UT- and 8F4CAR-iNKT cells. (C) Percentage of CD62L+ iNKT cells throughout the cell expansion protocol. Mean +/- SD. *, P < 0.05, ** P < 0.01, ****, P < 0.0001; ns, not significant; paired student t-test for intra-donor type comparison; unpaired student t-test for inter-donor type comparison. (D) Representative flow cytometric analysis of the memory phenotype, defined by CD62L and CD45RA, of UT- and 8F4CAR-iNKT cells from one cord and one adult donor throughout the cell expansion protocol.

**Cytotoxicity of 8F4CAR-iNKT Cells Against Leukemia in vitro**

To determine the anti-leukemic activity of 8F4CAR-iNKT mediated by both 8F4CAR and iNKTCR we co-cultured effector cells with HLA/A2 or CD1d expressing leukemia cell lines for 16 hours. Additionally, we performed cytotoxicity assays against primary AML patient samples to identify clinically relevant anti-leukemic activity. We observed no significant killing of WT targets and HLA/A2+ primary AML when incubated with UT- or 8F4CAR-iNKT cells. However, both CB- and AB-8F4CAR-iNKT cells exhibited similar leukemia cytolysis when targets expressed CAR antigen in both the U937 cell line and primary patient samples. Furthermore, both CB- and AB-8F4CAR-iNKT cells retain the ability to lyse CD1d+ cell lines as effectively as UT-iNKT cells (Figure 6A).

To assess the functional fitness of 8F4CAR-iNKT cells we conducted repeat leukemia challenge assays. In this assay we co-culture effector cells with GFP expressing U937 HLA/A2+ cells for 4 days before measuring the number of live leukemia
targets and live effector cells. We then add fresh leukemia cells and this process is repeated for 4-5 cycles (Figure 6B). We observed a trend where CB-8F4CAR-iNKT cells expanded in greater number compared to AB-8F4CAR-iNKT cells, while both donor types controlled leukemia cell growth to a similar extent (Figure 6C,D). As long-term persistence of CD19CAR-T cells is a key determinant in achieving durable responses in patients, these results support the use of CB-CAR-iNKT cells for adoptive cell therapy (38).

**Figure 6.** CB-8F4CAR-iNKT and AB-8F4CAR-iNKT cells display similar leukemia cytotoxicity mediated by 8F4CAR and iNKTCR in vitro. After ex vivo expansion, iNKT cells were frozen and thawed for functional assessment. (A) The indicated leukemia cell lines and primary AML patient samples were co-cultured with indicated effector iNKT

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cells for 16 hours at indicated E:T ratios. Specific killing was calculated by counting the number of live target cells in experimental conditions against the number of live target cells without effector cells. iNKTCR mediated cytolysis was measured by co-culture of αGC pulsed CD1d+ K562 cells. A representative experiment from 3 independent experiments (each with a unique iNKT donor) is shown for each leukemia target. Mean +/- SD, 3 technical replicates. (B) U937 HLA/A2+ GFP+ cells were co-cultured with effector cells at a 1:1 E:T ratio with 50 IU/mL IL-2. Every 3-4 days iNKT cells and target cells were counted and analyzed by flow cytometry and fresh U937 cells were added to maintain an E:T ratio of 1:1. A representative flow cytometric analysis throughout the experiment is shown from two total experiments (each with a unique iNKT cell donor). (C) Total fold change of iNKT cells from a representative experiment (three technical replicates). Mean +/- SD. Student unpaired t test. (D) The specific killing of U937 HLA/A2 targets throughout the serial challenge experiment. Mean +/- SD.

Cytokine Production of 8F4CAR-iNKT Cells

To further evaluate the function of 8F4CAR-iNKT cells we determined their cytokine production profile in response to 8F4CAR mediated leukemia cytolysis. It is well documented that both CD4+ and CD4- iNKT cells can produce Th1 type cytokines, but CD4+ iNKT cells are polarized to Th2 cytokine production. Indeed, previous unpublished work from our lab demonstrated CB-iNKT cells secrete more Th2 cytokines in response to stimulation as expected by their consistent CD4 positivity. Here, we observed that while both AB- and CB-iNKT cells secrete Th1 type cytokines after 8F4CAR mediated cytolysis, CB-iNKT cells are Th2 polarized (Figure 7A,B,C). The bias in Th2 cytokine
production is conserved across both iNKTCR and 8F4CAR mediated activation (Figure 7D).

Figure 7. CB-8F4CAR-iNKT cells have a Th2 polarized cytokine production profile upon 8F4CAR mediated leukemia cytolysis. Th1 and Th2 type cytokine production
profile of CB- and AB-8F4CAR-iNKT cells. Effector cells were co-cultured with leukemia targets for 8 hours and subsequently stained for intracellular cytokines for flow cytometry analysis. Additionally, in a separate experiment, effector cells were co-cultured with leukemia for 24 hours before supernatant was collected and analyzed for cytokine production by ELISA. (A) Representative analysis of IFNγ, TNFα, and IL-4 production after leukemia and effector cell co-culture at a 1:1 E:T ratio. (B) Percent IFNγ+, TNFα+, and IL-4+ CB and AB-8F4CAR-iNKT cells after leukemia co-culture with three technical replicates. Mean +/- SD. **, P < 0.01, ****, P < 0.0001. Student t test. (C) Secretion of IFNγ and IL-4 measured by ELISA after 8F4CAR-iNKT cell and leukemia or dendritic cells (pulsed with glycolipid agonist) co-culture with three technical replicates. Mean +/- SD, *, P < 0.05, student t test, n.d. is not detectable. (D) Ratio of IL-4 to IFNγ production in supernatant. Mean +/- SD, ***, P < 0.001, ***, P < 0.0001. Student t test. Three and four independent experiments (each with a unique iNKT cell donor) were conducted for both intracellular cytokine staining and supernatant cytokine analysis respectively.

**Anti-Leukemic Activity of 8F4CAR-iNKT Cells in vivo**

We then evaluated the ability of CB-8F4CAR-iNKT cells to control leukemia growth in a xenogeneic AML model. NOD scid gamma (NSG) mice were intravenously injected with U937 HLA/A2+ luciferase+ cells followed by intravenous treatment cell injection three days later. We administered increasing doses of CB-8F4CAR-iNKT cells to identify a dose dependent effect, including groups treated with donor matched CB-UT-iNKT cells as a control. We found mice treated with CB-8F4CAR-iNKT cells had significantly decreased leukemia burden compared to mice treated with CB-UT-iNKT cells (Figure 8A,B). Moreover, the lifespan of mice treated with CB-8F4CAR-iNKT cells was significantly extended compared to control groups (Figure 8C,D). These effects
trended in a dose dependent manner. Notably, mice treated with the lowest dose (1.0 x 10^5) of CB-8F4CAR-iNKT cells showed significant survival improvements over controls. There was no visible indication of GvHD-like symptoms throughout the experiment and mouse weight remained stable in all groups until ultimately decreasing due to leukemia burden before death (data not shown).

Figure 8. CB-8F4CAR-iNKT cells reduce leukemia burden and prolong mouse survival. NSG mice were intravenously injected with U937 HLA/A2, luciferase expressing cells followed by indicated treatment three days later. Leukemia progression was monitored by weekly bioluminescence imaging (BLI). (A) BLI images beginning at day 14 post leukemia cell injection. One mouse in the 2.5M CB-UT-iNKT cell group did not show successful leukemia engraftment. (B) BLI signal at the last time point where all
mice were alive. (C,D) Kaplan-Meier curves of mice treated with CB-UT-iNKT and CB-8F4CAR-iNKT cells. *, P < 0.05. Log-rank Mantel-Cox statistical assessment.

DISCUSSION:

CAR-T cells are becoming an increasingly popular therapeutic option for patients with hematologic malignancies due to their impressive efficacy. However, more widespread implementation of CAR-T cell therapy is limited by its time intensive and costly manufacturing protocol. A solution to the complications of CAR-T manufacturing is to create an ‘off-the-shelf’ allogeneic CAR-T product. One approach is to employ an effector cell that can be used safely in an allogeneic setting.

iNKT cells are a promising platform for allogeneic CAR adoptive cell therapy against hematologic malignancies. The therapeutic potential of AB-CAR-iNKT cells have been extensively characterized by Dr. Leonid Metelitsa’s group at Baylor College of Medicine. Preclinical experiments that demonstrate the ability of iNKT cells to modulate the tumor microenvironment (TME) through TAM destruction, infiltrate solid tumors more effectively than T cells, and exert potent anti-tumor effects led to phase 1 clinical trials of autologous GD2CAR-iNKT cells and allogeneic CD19CAR-iNKT cells. These trials confirm that AB-CAR-iNKT cells are safe and well-tolerated in patients (even as an allogeneic therapy) and show anti-tumor efficacy (29,39). In unpublished work from our lab, we identified CB-iNKT cells to possess several promising characteristics that may make them well suited as an allogeneic CAR platform such as: a consistent CD4 phenotype across donors, expansion to greater numbers than AB-iNKT cells, and an enrichment in naïve and central memory populations. In this work we investigate the
preclinical therapeutic potential of CB-8F4CAR-iNKT cells against the particularly hard to treat AML.

In line with previous unpublished findings, we show that CB-8F4CAR-iNKT cells can be effectively expanded to greater number than AB-8F4CAR-iNKT cells. The method of using allogeneic DCs loaded with αGC reliably expands iNKT cells from both donor types yielding highly pure iNKT cells with similar 8F4CAR expression. Notably, 8F4CAR transduction does not drastically alter CD4 phenotype when comparing to donor-matched UT-iNKT cells in either donor type, and CB-8F4CAR-iNKT cells retain a significant increase in CD62L expression compared to AB-8F4CAR-iNKT cells. It is reasonable to postulate that CB-iNKT cells have a greater proliferative potential due to their enrichment in CD62L+ cells (37). Indeed, iNKT cells in adult peripheral blood go through multiple cycles of expansion and contraction throughout a lifetime and are prone to anergy upon repeated activation (40). However, we have also found in unpublished work that CB-iNKT cells have improved metabolic function compared to AB-iNKT cells though this remains to be tested in CB-8F4CAR-iNKT cells.

We go on to show that both CB-8F4CAR-iNKT cells and AB-8F4CAR-iNKT cells exert potent leukemia cytolysis mediated via both 8F4CAR and iNKTCR. Importantly, 8F4CAR-iNKT cells from both donor types show similar primary patient AML cytolysis, which suggests clinical relevance. While the anti-leukemic activity is similar between donor types in vitro, we found CB-8F4CAR-iNKT cells proliferated more than AB-8F4CAR-iNKT cells after repeated leukemia challenge. Because CAR-T cell expansion and persistence are key factors for durable responses in patients, we believe CB-CAR-iNKT cells may perform better than AB-CAR-iNKT cells (38,41). An important experiment to evaluate this hypothesis will be to monitor the differences in CB- and AB-iNKT cell persistence in vivo in tumor free and tumor bearing mice.
iNKT cells secrete a myriad of cytokines upon activation, which is generally determined by their CD4 expression. As expected from CD4+ iNKT cells, we show that CB-8F4CAR-iNKT cells have a Th2 polarized cytokine production profile after 8F4CAR mediated leukemia cytolysis. It has become popular in the field of CAR-T cell engineering to enhance the Th1 cytokine production of effector cells in the hopes of overcoming the immunosuppressive TME (42,43). However, the recent preclinical success of IL-10 expressing CAR-T cells challenges the idea that immunosuppressive cytokine production by CAR-T cells is deleterious. The opposite appears to be true as IL-10 producing CAR-T cells had improved metabolic function in the TME, showed effective proliferation and tumor clearance, and induced memory responses in lymphoid organs that protected mice from tumor rechallenge (44). Additionally, there is evidence that shows GM-CSF (a Th1 type cytokine) secretion by CAR-T cells exacerbates cytokine release syndrome (CRS) in preclinical models (45). Therefore, we view the Th2 biased cytokine production profile of CB-8F4CAR-iNKT cells to be a potentially protective feature.

Finally, we show that CB-8F4CAR-iNKT cells exert anti-leukemic activity in a xenogeneic AML model by reducing leukemia burden and extending mouse survival. We even observe therapeutic activity at the lowest dose of 1.0 x 10^5 CB-8F4CAR-iNKT cells, an effect that is not mirrored by 8F4CAR-T cells tested by the Molldrem lab (unpublished). To confirm this effect, a donor-matched 8F4CAR-iNKT and 8F4CAR-T cell treatment of xenogeneic AML model will need to be tested.

In summary, this work provides supporting evidence for the use of CB-iNKT cells as a platform for allogeneic CAR ACT against leukemia. We demonstrate that 1) CB-8F4CAR-iNKT cells have greater proliferative potential during the cell generation protocol and after repeated leukemia challenge; 2) CB-8F4CAR-iNKT cells have
consistent phenotype enriched in CD62L expressing cells; 3) CB-8F4CAR-iNKT cells exert similar CAR mediated leukemia cytolysis compared to AB-8F4CAR-iNKT cells in vitro; 4) CB-8F4CAR-iNKT cells are polarized to Th2 type cytokine production; and 5) CB-8F4CAR-iNKT cells show anti-leukemic activity in vivo. These findings merit further investigation into the potential of CB-CAR-iNKT cells in treating cancer.
METHODS:

Cell Lines:

Wild-type U937 and K562 cell lines were originally acquired from ATCC and transduced with HLA/A2 or CD1d. U937 HLA/A2 cells were transduced with GFP/Luciferase and provided by the Jeffrey J. Molldrem lab. Primary AML samples were provided by the Tissue Bank from the Hematopoietic Biology and Malignancy Department at MD Anderson Cancer Center. All cells were cultured in complete media (RPMI 1640 [Gibco 11875093], 10% heat-inactivated FBS [Gibco #A5256701], 1mM Glutamine [Gibco #25030081], 50uM Non-essential Amino Acids [Gibco #11140050], 25uM Essential Amino Acids [Gibco #11130051], 10uM HEPES [Gibco #15630080], 10ug/mL gentamicin [Gibco #15710064], 50uM β-mercaptoethanol [Gibco #21985023]) and confirmed to be mycoplasma negative prior to experiments.

Retrovirus Production:

Retrovirus containing 8F4CAR was produced by co-transfecting 293GP cells with RD114 envelope coding plasmid along with 8F4CAR in pSFG vector using the lipofectamine 2000 system (Thermo Fisher #11668019) according to manufacturer’s protocol. In short, 293GP cells were seeded in 10cm Poly-D-Lysine coated plates 16 hours prior to transfection. Media was gently replaced as to not disturb the cells before transfection. Then in 1.5mL OPTI-MEM media (Thermo Fisher #31985070) 9ug of 8F4CAR plasmid was mixed with 4.5ug RD114 plasmid in one conical tube; in another conical tube 60uL of lipofectamine 2000 was mixed in 1.5mL OPTI-MEM. After 5 mins the two solutions were gently mixed and plasmid-lipofectamine complexes were allowed to form for 20 mins prior to dropwise addition to 293GP cells. After 48-72 hrs, retrovirus-
containing supernatant was collected and spun at 2000 g to remove cell debris before aliquoting and storing at -80C.

**Primary iNKT Cell Isolation, Activation, and 8F4CAR Transduction:**

All research was conducted in accordance with the Declaration of Helsinki and The University of Texas MD Anderson Cancer Center Institutional Review Board guidelines. Buffy coats were purchased from MD Anderson Blood Bank, and deidentified cord blood units for general translational research were provided by MD Anderson Cord Blood Bank. Mononuclear cells were isolated by Histopaque (Sigma #10771) density gradient centrifugation, followed by iNKT cell enrichment with anti-iNKT micro-beads according to manufacturer’s protocol (Milyteni Biotec #130-094-842). Enriched iNKT cells were split in half to generate donor-matched UT- and 8F4CAR-iNKT cells and stimulated with irradiated allogeneic dendritic cells (allo-DCs) in complete media that were plated and pulsed with 100nM α-Galactosylceramide (αGalCer) (Avanti #867000) one day prior. After 3 days of stimulation in the presence of 50IU/mL recombinant human IL-2 (Peprotech #200-02), iNKT cells were transduced with RetroNectin (Takara #T100A) according to manufacturer’s protocol. Briefly, one day prior to transduction, RetroNectin was coated on 24 well non-tissue culture treated plates (Thermo #0877251) at 7ug/mL in sterile PBS (Thermo #J61196-AP) for 16hrs at 4C. On the day of iNKT cell transduction, the RetroNectin treated plates were removed from 4C and brought to room temperature. Then the RetroNectin solution was aspirated, and complete media was added for 30 mins in 37C before media was replaced with 1mL retrovirus-containing supernatant. The plate was then wrapped in parafilm and centrifuged at 2000g for 2 hr at 32C. The viral supernatant was then removed and stimulated iNKT cells were added in complete media supplemented with 50IU/mL IL-2. iNKT cells were cultured for 14 days and re-stimulated...
with αGalCer pulsed allogeneic DCs for an additional 14 days before final count and phenotype was assessed by flow cytometry. Cells were then frozen and stored in liquid nitrogen prior to functional analysis.

**Flow Cytometry**

For phenotypic assessment, iNKT cells were collected in 1.5mL Eppendorf tubes and washed with PBS. Cells were then resuspended in fluorochrome conjugated antibody solution (1ug/ml for each antibody) and covered from light for 10 mins at room temperature. Cells were then washed with PBS, labeled with live/dead cell dye (BD #564996), washed again with PBS, and resuspended in PBS for acquisition on the LSRFortessa X-20 Cell Analyzer. For intracellular cytokine staining, 8F4CAR-iNKT cells were stimulated with target leukemia cells at 1:1 E:T ratio for 8 hours, or 30ng/mL PMA (Sigma #P8139) and 1ug/mL Ionomycin (Sigma #I0634) as a positive control, in the presence of protein transport inhibitors Brefeldin A (BD #555029) and Monensin (BD #554724). Cells were then washed with PBS and stained for surface markers as described above before fixation and permeabilization with 1X Fix/Perm buffer (BD #554714). Cells were washed with 1X Perm/Wash buffer (BD #554714) and resuspended in intracellular cytokine antibody solution (in 1X Perm/Wash buffer) for 30 mins at room temperature. Lastly, cells were washed with 1X Perm/Wash buffer and resuspended in PBS for immediate acquisition on the LSRFortessa X-20 (BD). Analysis was performed using FlowJo v10.8.

**Table 1. Antibodies for Flow Cytometry**

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<td>Anti-human iNKT-PE-Cy7 (Clone 6B11)</td>
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**Cytotoxicity Assay:**

Effector cells were thawed and rested in complete media for 16 hrs prior to functional assessment. Target cell lines were cultured for 1 week before use in cytotoxicity assays, and primary leukemia samples were thawed one day prior and rested in complete media. Target cell lines were stained in the dark with 1uM Cell Trace Violet (Thermo #C34557) in PBS at 37C for 20 mins before quenching and washing with 5X volume complete media. Primary leukemia target cells were not labeled with cell trace dye to preserve viability and instead were identified by CD33 and CD34 staining. Target cells were plated at 10K cells/well in 96 well round bottom plates (Corning #3799).
before addition of effector cells at the according E:T ratios. The co-culture was carried out for 16 hrs before cells were washed with PBS, stained with live dead cell dye (and CD33 and CD34 in the case of primary leukemia), counting beads were added (Sigma #C36950), and cells were acquired on the LSRFortessa X-20. Percent specific killing was calculated: \[
\frac{\text{experimental number live target cells} - \text{number live target cells alone in media}}{\text{0} - \text{number live target cells alone in media}} \times 100.
\]

Serial Challenge Assay:

U937 HLA/A2 GFP cells were co-cultured with effector cells at a 1:1 E:T ratio in 96 well round bottom plates in complete media supplemented with 50 IU/mL IL-2. On the same day of experiment set-up, a sample was washed with PBS, stained with Live/Dead cell dye, and counting beads were added to establish the initial absolute number of effector and target cells. Every 3-4 days a sample was taken and counts of target and effector cells were recorded. On the day of acquisition, new U937 HLA/A2 GFP target cells were added to maintain an E:T ratio of 1:1 in fresh complete media supplemented with 50 IU/mL IL-2.

Cytokine Production Analysis by ELISA:

iNKT cells were co-cultured with wild-type U937 or HLA/A2 U937 cells for 24 hours in a 1:1 E:T ratio. Supernatant was collected and analyzed for the presence of IFNγ, IL-4, and IL-10 using BD capture and detection antibody pairs in 96 well high-binding plates (Corning #3690). Streptavidin-HRP was added, and luminescence signal was read on the Cyation 3 (Fisher Scientific).
Table 2. Reagents for Cytokine Analysis by ELISA

<table>
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<th>Catalog Number</th>
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</tr>
<tr>
<td>Streptavidin-HRP</td>
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Xenogeneic Mouse Model:

All animal experiments were performed under the University of Texas Institutional Animal Care and Use Committee (IACUC) approved protocols. Female 6-8 week old NSG (NOD.Cg-Prkdcr<scid>Il2rg<tm1Wjl>/SzJ) mice were obtained from the Jackson Laboratory. NSG mice between the ages of 8 and 12 weeks were irradiated with 200 cGy using the 137Cs irradiator on day -1, and were intravenously (i.v.) injected with 5.0 x 10^3 U937 HLA/A2 GFP/Luc cells on day 0. Three days later treatment cells were administered i.v. at the according doses. Leukemia progression was monitored by BLI
imaging on the IVIS Lumina X5 (PerkinElmer) at the MDACC Small Animal Imaging Facility (Smith Research Building) once weekly.

REFERENCES:


42. Nonaka K, Saio M, Umemura N, Kikuchi A, Takahashi T, Osada S, Yoshida K. Th1 polarization in the tumor microenvironment upregulates the myeloid-derived


Vita:

Maison Donald Grefe completed his Bachelor of Arts in Biology at Concordia College in Moorhead, Minnesota. During his undergraduate training, Maison conducted a biology honors thesis studying the nuclei division autonomy in the model organism *Ashbya gossypii* in Dr. Cori Anderson’s lab. Later he spent a semester abroad at the Scripps Research Institute in Jupiter, Florida working with Dr. Matthew Disney in identifying RNA-binding small molecules. Maison then went on to join Dr. Jin S. Im’s lab at MD Anderson UTHealth Houston Graduate School of Biomedical Sciences as an M.S. student studying the anti-cancer potential of CAR-iNKT cells.