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Original Article

Effect of Cryopreservation on Autologous Chimeric Antigen Receptor T Cell Characteristics

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As clinical applications for chimeric antigen receptor T cell (CART) therapy extend beyond early phase trials, commercial manufacture incorporating cryopreservation steps becomes a logistical necessity. The effect of cryopreservation on CART characteristics is unclear. We retrospectively evaluated the effect of cryopreservation on product release criteria and *in vivo* characteristics in 158 autologous CART products from 6 single-center clinical trials. Further, from 3 healthy donor manufacturing runs, we prospectively identified differentially expressed cell surface markers and gene signatures among fresh versus cryopreserved CARTs. Within 2 days of culture initiation, cell viability of the starting fraction (peripheral blood mononuclear cells [PBMNCs]) decreased significantly in the cryo-thawed arm compared to the fresh arm. Despite this, PBMNC cryopreservation did not affect final CART fold expansion, transduction efficiency, CD3%, or CD4:CD8 ratios. *In vivo* CART persistence and clinical responses did not differ among fresh and cryopreserved final products. In healthy donors, compared to fresh CARTs, early apoptotic cell-surface markers were significantly elevated in cryo-thawed CARTs. Cryo-thawed CARTs also demonstrated significantly elevated expression of mitochondrial dysfunction, apoptosis signaling, and cell cycle damage pathways. Cryopreservation during CART manufacture is a viable strategy, based on standard product release parameters. The clinical impact of cryopreservation-related subtle micro-cellular damage needs further study.

INTRODUCTION

In a historic action in 2017, the US Food and Drug Administration (FDA) ushered in 2 new gene therapies for the treatment of hematologic malignancies in the United States.[1,2] As living drugs, these reprogrammed autologous chimeric antigen receptor T cells (CARTs) represent a new age of innovative cancer treatment. Concurrently, they present unique manufacturing[3] and commercialization[4] challenges that differ from those of traditional pharmaceuticals. Most CARTs are manufactured from autologous peripheral blood mononuclear cells (PBMNCs) collected by leukapheresis, followed by T cell selection, activation, gene modification, and expansion.[5] The expanded CARTs are then formulated for infusion into the patient. Each semi-automated processing step above may introduce inter-product variability.

Commercial large-scale manufacturing of autologous CARTs further requires cell transportation to and from centralized processing facilities, and cell cryopreservation becomes a logistical necessity. As a result of the time required for safety and potency testing that includes assessing transduction efficiency (TE), sterility testing, and testing for the absence of replication-competent virus, most commercial manufacturers have elected to cryopreserve the final CART product. The autologous PBMNC starting material is also often cryopreserved to allow for flexibility in scheduling manufacturing.[6]

While hematopoietic stem cells survive cryopreservation and thawing well, and cryopreserved hematopoietic stem cell transplantation has been performed for many years,[7,8] the effects of the cryopreservation on cultured T cells is less certain. The data available suggest that the overall recovery of cryo-thawed T cells is marginally worse than that of hematopoietic stem cells.[9,10] Recently, one institution reported that CARTs cultured over prolonged periods (9–14 days), particularly in fully automated systems, may have worse post-thaw recovery[11,12] than other cultured T cells (Zhu et al., 2019, Transplantation & Cellular Therapy, annual meeting).

As a center manufacturing CARTs for early phase clinical trials since 2012, we often manufacture CARTs from cryopreserved PBMNCs, and we cryopreserve the final CART products. However, given the advanced nature of the hematologic malignancies of many patients and the need for urgent treatment, fresh, non-cryopreserved

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Table 1. Demographics

Protocol	12-C-0112	14-C-0059	14-C-0168	15-C-0029	16-C-0054	17-C-0048	Total
CART type	CD19, CD28	GD2, CD28, O × 40	BCMA, CD28	CD22, 41BB	CD19, CD28	CD30, CD28	–
Age group	pediatric + adult	pediatric + adult	adult	pediatric + adult	adult	adult	–
Diagnosis	ALL, DLBCL	osteosarcoma, neuroblastoma	multiple myeloma	ALL, DLBCL	ALL, DLBCL, MCL, FL, BL	Hodgkin's lymphoma	–
Infusion, n (%)	56 (38.1)	12 (8.2)	20 (13.6)	37 (25.2)	20 (13.6)	2 (1.4)	147 (100)
Age (years) (mean ± SD)	14.3 (±6.7)	17.8 (±5.9)	56.7 (±4.9)	18.3 (±7.0)	56.4 (±11.5)	35.4(±8.9)	27.7 (±19.9)
Male:female	45:11	10:2	10:10	25:12	14:6	2:0	106:41
Weight (kg) (mean ± SD)	46.5 (±20.9)	60.5 (±21.4)	89 (±25.7)	52.6 (±17.8)	80.3 (±16.4)	115 (±27.6)	60.7 (±26.3)
CAR-T cell dose (million/kg) (mean ± SD)	1.2 (±0.6)	2.9 (±3.9)	5.4 (±3.8)	1.1 (±0.7)	2.5 (±2.1)	0.3	2.0 (±2.5)
Delivery vector	gamma-retrovirus	gamma-retrovirus	gamma-retrovirus	lentivirus	lentivirus	lentivirus	–
Pre-culture lymphocyte enrichment (n)	elutriation (5), bead enriched (46), both(5)	elutriation (5), bead enriched (7), both (0)	Ficoll (20)	elutriation (7), bead enriched (15), both (13), none (2)	Ficoll (20)	Ficoll (2)	–

ALL, acute lymphoblastic leukemia; DLBCL, diffuse large B cell lymphoma; MCL, mantle cell lymphoma; FL, follicular lymphoma; BL, Burkitt's lymphoma.

PBMNCs have been used to manufacture the CARTs, and the final CART products have been infused fresh immediately after the completion of manufacturing.

The primary objective of this study was to determine the impact of cryopreservation on CARTs. We evaluated factors used in current clinical practice for product safety, purity, potency, and consistency ascertainment. These included post-thaw CART recovery and post-thaw changes in TE, CD3%, and CD4:CD8 ratios. In a subset of patients who received fresh or cryopreserved final products at a standard dose, we compared *in vivo* CART levels, persistence over time, and clinical response. We also investigated the effects of cryopreserved autologous PBMNCs on the manufacturing process. Specifically, we studied post-thaw PBMNC recovery, fold expansion (FE), TE, and CD4:CD8 ratios during the manufacturing process. In addition, in 3 healthy volunteer donor CART manufacturing runs, we prospectively examined more subtle markers of cryopreservation-related cell damage.

RESULTS

Patient and Product Characteristics

Data were obtained on 145 patients with a total of 158 consecutive autologous CART cultures; 12 patients received more than one infusion in the same protocol. The total numbers of infusions in 6 clinical trials of different CART types were as follows: CD19 CART pediatric (P) trial, n = 58; GD2 CARTs, n = 14; BCMA CARTs, n = 21; CD22 CARTs, n = 42; CD19 adult (A) CART trial, n = 21; and CD30 CARTs, n = 2. After discarding 11 products due to manufacturing failure during culture, 147 CARTs were prepared for infusion into pediatric or adult patients with hematologic malignancies (135, 91.8%) or solid tumors (12, 8.2%). The mean age was 27.7 years (±19.9) and weight was 60.7 kg (±26.3), with a median CART dose of 1×10^6 CAR-transduced viable CD3 ± cells/kg (range: 1×10^5–1×10^7). Gamma-retroviral or lentiviral vectors were used for CAR delivery in 88 (59.9%) and 59 (40.1%) infusates, respectively. See Table 1 for demographics.

Of the 147 infusates, the starting fraction (PBMNCs) was cryopreserved for 70 infusions. For 79 infusions, the final harvested CART product was cryopreserved and thawed prior to infusion. For 50 CART products, both the PBMNCs and final CART product were cryopreserved. The median duration of cryopreservation was 6 days (range: 3–868) and 9 days (range: 1–408) for the starting PBMNCs and the final harvested CARTs, respectively. See Figure 1 and Table S1 for overall manufacturing schema and studies described in the Materials and Methods, and see Table S2 for product cryopreservation details by protocol.

Manufacturing CARTs Using Cryopreserved PBMNCs

At 2 days after PBMNC culture initiation (stimulation period), viable total nucleated cell (TNC, %) was significantly lower for cultures initiated from cryopreserved PBMNCs compared to fresh cells (Figure 2A). A separate analysis of the processing of CD19 CARTs (P) confirmed this finding (Figure 2B). The CD3% was unavailable for all products on day 2 of T cell culture. Despite the decrease in cell counts on day 2, all products contained the required cell quantity for starting the transduction and expansion process.

Compared with cultures that were initiated with fresh PBMNCs, cryopreservation-thawed PBMNC cultures did not demonstrate a significant difference in FE, TE, CD3%, or CD4:CD8 ratios at the time of final CART harvest. Data stratified by protocol and all data combined are summarized in Table S4. Figures S1A–S1D demonstrate the lack of a significant correlation between duration of PBMNC cryopreservation and FE, TE, CD3%, and CD4:CD8 ratios. These correlations remained non-significant, even when data were stratified by CART type. The day of final CART harvest varied

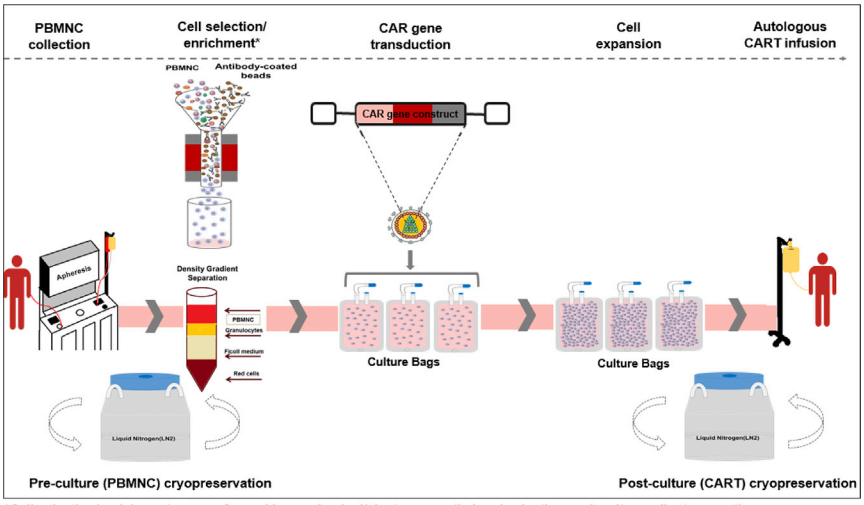

Figure 1. CART Manufacturing Schema

A simplified diagram of the CART manufacturing process across the various manufacturing protocols at the NIH Center for Cellular Engineering. Starting with collection of PBMNCs by apheresis, the fresh or cryo-thawed cells (PBMNCs) are processed by mechanical or electromagnetic bead selection or density gradient centrifugation. Following the cell selection and enrichment process, CAR gene transduction, cell expansion, and harvest are performed over 7–9 days. After testing for sterility, the final CART product is either released fresh for infusion or cryopreserved again for future thaw and infusion. At the time of release, viability (post-thaw) and counts are repeated on cryo-thawed CARTs for cell dose calculations.

by type, ranging from 7 to 9 days. We evaluated the same correlations on day 7 for all CART types (data not shown) and identified no difference.

Recovery, Expansion, and Survival of Cryopreserved CARTs

In Vitro *Post-thaw Recovery of Cryopreserved CARTs*

For the 79 cryopreserved CART products, the mean TNC recovery of the thawed fraction was 97% ± 17.4%. Before cryopreservation and after thaw, CART CD3%, TE(%), and CD4:CD8 ratios were 98% ± 2.1% and 98% ± 2.4%, 56% ± 21% and 55% ± 23%, and 2.2 ± 3.9 and 2.3 ± 4.0, respectively. No significant changes were observed in these parameters across the different types of CARTs. CD19(A), CD19(P), and CD22 CARTs with adequate sample sizes were analyzed separately, and no significant changes were identified post-thaw. Mean values and SDs of all the measured parameters varied by protocol, as expected, due to differences in patient population, vector type, and culture conditions (Figure 3).

As a control, we compared the post-thaw recovery of 78 CART products with the 69 PBMNC products that had been cryopreserved prior to the start of manufacturing. For these 69 PBMNCs, the viable post-thaw TNC recovery was significantly lower compared to CARTs (77% ± 12% versus 97% ± 17%, $p < 0.0001$) (Figure 4A). Viable post-thaw CD3% recovery trended lower for PBMNCs compared to CARTs, but the difference was not statistically significant (87% ± 17% versus 97% ± 17%, $p = 0.07$) (Figure 4C). Overall, CD3% recovery for thawed PBMNCs was better compared to TNC recovery, suggesting that non-CD3+ T cells accounted for most of the cell loss post-thaw.

We also evaluated these differences in the post-thaw recovery of CARTs and PBMNCs using 50 cases where both the PBMNCs and CART products were cryopreserved ($n = 50$). Of these products cryopreserved at both ends, most (47, 94%) were manufactured for the CD19(A) and CD22 CART clinical trials. These CARTs also demonstrated higher TNC recovery at thaw (96% ± 19%) compared to the starting PBMNCs at thaw (76% ± 11%, $p < 0.001$) (Figure 4B). However, these differences were not as evident in CD3% recovery: 96% ± 19% for CARTs versus 92% ± 24% for PBMNCs, $p = 0.2$ (Figure 4D).

Comparison of Expansion, Survival, and Clinical Response to CARTs Infused Fresh and/or Post-cryopreservation

In vivo comparisons between fresh and cryo-thawed CART infusions were feasible for 2 protocols with adequate numbers in each group, at a pre-specified dose level. At a dose level of 1×10^6 transduced viable CD19 CART(P) cells/kg, maximum CART levels, CART persistence *in vivo*, clinical responses, and occurrence of cytokine release syndrome were not significantly different between patients receiving fresh CARTs ($n = 17$) and cryo-thawed CARTs ($n = 30$) (Figures 5A, 5C, and 5E). For CD22 CARTs, the mean maximum CART levels and mean persistence (in days) *in vivo* were greater than those seen with the CD19 CART(P), which was likely due to differences in the vector and co-stimulatory domains, manufacturing methods, and patient-related differences.

Maximum CD22 CART levels *in vivo* were higher in patients receiving fresh CARTs compared to cryo-thawed CARTs ($p = 0.03$) (Figure 5B); however, no significant differences were identified in the persistence of CARTs between fresh and cryo-thawed CD22 CARTs (Figure 5D). Upon further evaluation, the number of patients who received CD22 CARTs with high disease burden was greater in the cryo-thawed CART group (5/6, 83%) compared to the fresh group (6/9, 67%). This difference was not statistically significant ($p = 0.5$). Different cell selection methods used to manufacture these products are also illustrated in Figures 5A and 5B. In this clinical trial as well, clinical responses and occurrence of cytokine release syndrome were no different between patients receiving fresh and cryo-thawed CARTs (Figures 5E and 5F).

For the CD19 CART(P), the proportion of patients that did not show any CARTs in the peripheral blood post-infusion (i.e., had CARTs below the limit of detection [<0.01% of T cells] for the protocol by

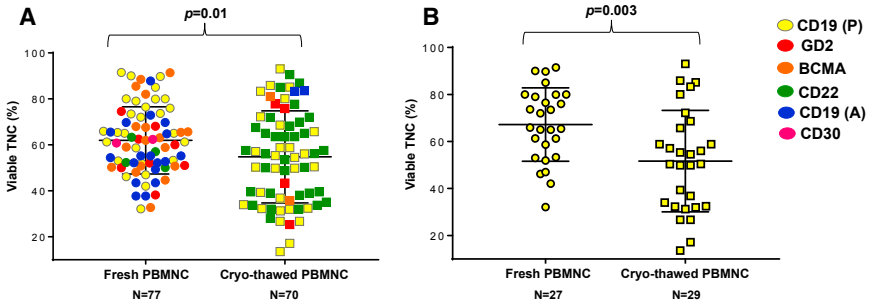

Figure 2. Comparison on Culture Day 2 of Viable TNC Quantities (%) between Fresh and Cryopreserved PBMNCs

The results for PBMNCs from all protocols are shown in (A) [CD19 (P) CARTs, yellow; GD2 CARTs, red; BCMA CARTs, orange; CD22 CARTs, green; CD19 (A) CARTs, blue; and CD30 CARTs, magenta], and the results for PBMNCs from the CD19 CART (P) protocol are shown in (B) (yellow). The scatter dot plots with bars demonstrate mean + SD. p values were calculated for unpaired t tests.

fluorescence-activated cell sorting [FACS] analysis at all times after infusion) was higher among cryopreserved CD19 CARTs (85.7%, 6 of 7) compared to the fresh infused products (14.3%, 1 of 7; p = 0.01). None of the CD22 CART products demonstrated a complete absence of detection *in vivo* post-infusion.

Role of Cryopreservation on Manufacturing Failures

Of the original 158 products, 11 (6.9%) failed manufacture and were not infused. These were non-uniformly distributed across CART types. The causes for manufacturing failure were variable. Of the 11 that failed, 5 cultures were initiated from a cryopreserved starting parent product and 6 from fresh PBMNCs. Overall, 5 of 75 (6.7%) cryopreserved PBMNCs and 6 of 83 (7.2%; p = 0.9) fresh PBMNCs failed manufacturing. Following failure, remanufacture from a cryopreserved PBMNC fraction was attempted for 6 cases, and all 6 resulted in the successful production and infusion of a CART product. These 6 remanufacturing cases included 3 that were originally manufactured from cryopreserved PBMNCs (Table S5).

Phenotypic Evaluation of Fresh versus Cryopreserved CARTs in Healthy Donor Samples

To study T cell phenotypic changes prospectively, 3 healthy donor PBMNCs were used to manufacture CART using 4 different manufacturing schemes, as described in the Materials and Methods. As with the clinical products analyzed previously, mean CART FE, TE, CD4%, and CD8% were no different across the 4 schemes in healthy donor samples (Figures S2A–S2E). Viable (7-amino actinomycin D [7AAD]^{neg}, 7AAD^{dim}) CD3+ T cell percentage was similar in the 4 manufacturing arms (Figures 6A, 6C, 6E, 6G, and 6I). This population also corresponded to the viable cells identified by trypan blue staining (data not shown). However, after eliminating early apoptotic cells (Annexin-V positive and/or Helix NP positive) destined for eventual cell death, viability was significantly lower in the arms where the final CART product was cryopreserved (Figures 6F, 6H, and 6J) compared to the arms where the final CARTs were tested fresh (Figures 6B, 6D, and 6J). This result was independent of the state (cryo-thaw or fresh) of the starting PBMNCs. Analysis of the CD4+ and CD8+ cells within the CART products revealed that the proportion of naive, central memory, and effector memory T cells did not differ significantly across the 4 manufacturing arms (Figure S3).

Global Gene Expression Analysis of Fresh versus Cryopreserved CARTs in Healthy Donor Samples

Global gene expression changes were also studied prospectively in the manufacturing arms above (categories described in the Materials and Methods). A total of 30 samples was analyzed. Of these, 6 samples were uncultured PBMNCs (fresh or cryo-thawed). 12 others were CARTs from the 3 healthy donors, with each donor sample subjected to one of the 4 manufacturing schemes detailed above. As controls, 12 more samples were cultured simultaneously using all the same conditions, except they were not transduced. Principal-component analysis (PCA) of 28 samples was available for further evaluation. Two samples were eliminated from analysis due to sample-processing discrepancies. All the samples clustered into 3 groups. One group contained all of the PBMNCs that were not cultured, the second group contained fresh final CARTs and cultured T cells that were analyzed fresh, and the third contained all cryo-thawed final CARTs and cryo-thawed cultured T cells (Figure 7A). Unsupervised hierarchical clustering analysis also grouped the samples into the same 3 categories (Figures 7B and 7C). As expected, the effect of cryopreservation eclipsed the effects of vector transduction and inter-donor variability.

Eliminating the untransduced control samples from the analysis decreased sample size significantly. PCA of the smaller sample set demonstrated inter-donor variability alone as a significant factor (data not shown).

Further, a total of 2,124 genes were differentially expressed between the fresh CARTs and cultured T cells that were analyzed fresh (group 1) and the cryo-thawed CARTs and cryo-thawed cultured T cells (group 2). The 1,139 genes overexpressed in cryo-thawed CARTs were more likely to belong to apoptotic and cell cycle damage pathways (unfolded protein response, endoplasmic reticulum [ER] stress pathways, mitochondrial dysfunction, apoptosis signaling, protein ubiquitination, and cell cycle damage pathways) (Figure 7D). The 985 genes overexpressed in fresh CARTs and culture-expanded T cells were more likely to belong to signaling pathways specific to T cell cytolysis (Toll-like receptor signaling, B cell-activating factors, and leukemia-signaling pathways) (Figure 7E).

DISCUSSION

More than 70% of therapeutic agents that demonstrate success in early phase clinical trials for refractory solid organ and hematologic

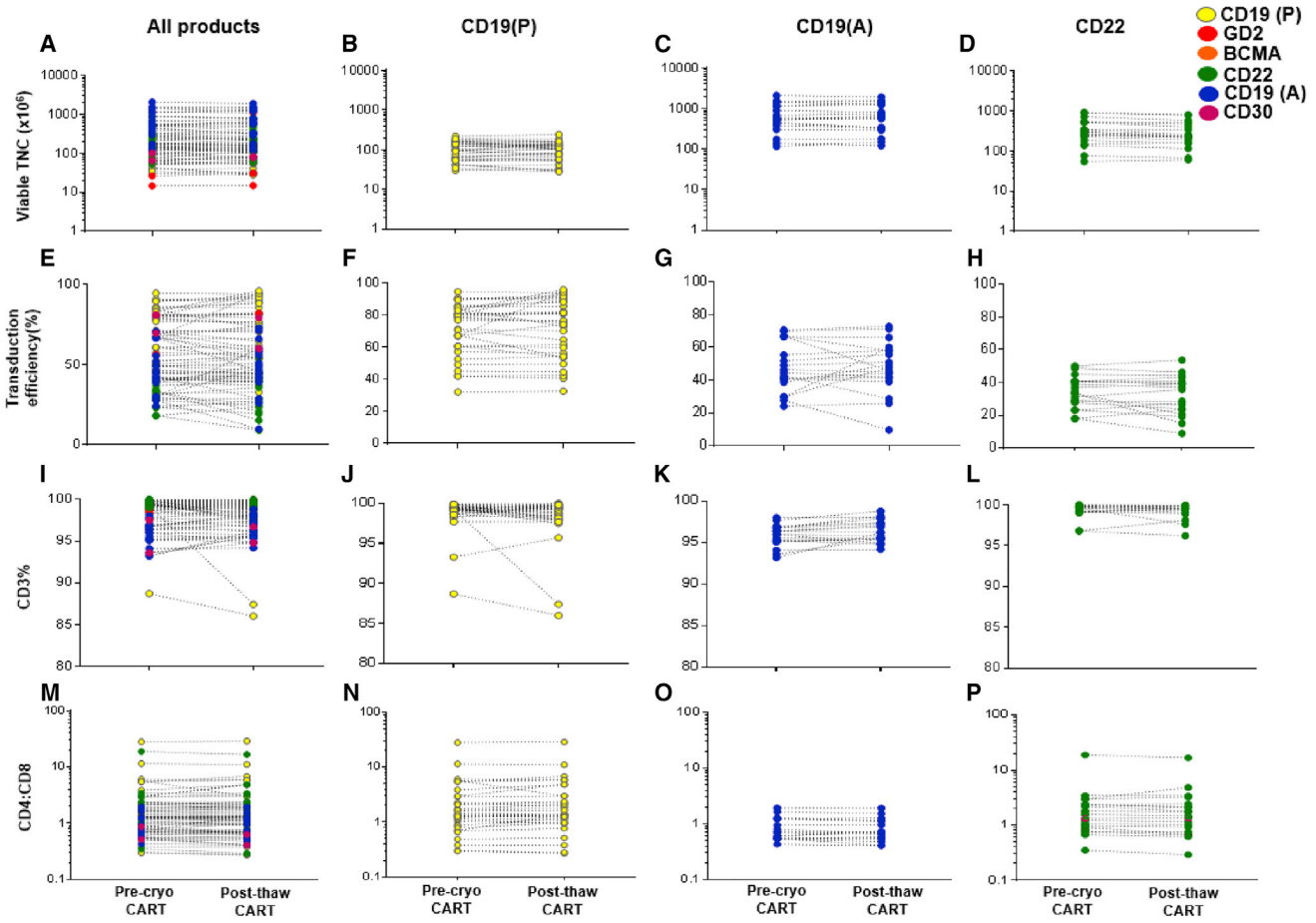

Figure 3. Comparisons of CART Characteristics at Cryopreservation and Post-thaw

Viable TNC (row 1), TE (row 2), CD3% (row 3), and CD4:CD8 ratio (row 4) are compared for the final CART product at cryopreservation and following thaw. Column 1 (A, E, I, and M) shows all CART products. Column 2 (B, F, J, and N) shows CD19-CART (P) products, column 3 (C, G, K, and O) shows CD19 CART(A) products, and column 4 (D, H, L, and P) shows CD22-CART products. The p values were calculated for paired t tests.

malignancies fail efficacy endpoints in larger trials.[13,14] Further, with cell therapies, the conversion rate from a phase III study to regulatory approval is estimated to be at 14.3%, which is considerably lower than the conversion rate (48.7%) of mature pharmaceutical drug classes that demonstrate new drug application success with the FDA.[15] This is due in part to the fact that small changes in manufacturing practices during the scale-out of cell therapies can greatly impact final product safety, purity, and potency.[16,17] While smaller single institution studies often use fresh PBMNCs for CART manufacturing and/or fresh final CART products for autologous infusion, the short stability and shelf life of liquid-stored T cells makes cryopreservation essential for advancement to multicenter trials and licensure, with centralized manufacturing. Hence, it is essential to verify that cryopreserved products are equivalent in safety and efficacy to fresh CARTs, prior to commercialization.

Despite the optimized use of intracellular cryoprotective cocktails,[18] slow cooling in controlled rate freezers,[19] and quick-thaw processes prior to cell use, some decrease in post-thaw cell viability is inevitable.[20] This limitation may be overcome by increasing cell numbers used for cryopreservation. Other issues, including phenotypic and functional drifts in cell subtypes, occur following the freeze-thaw process.[21] Consequently, the biophysical and physiologic properties of thawed mature cells cultured *ex vivo* may vary from those for freshly isolated cells that encounter the same stressors.[22,23] To better understand the clinical impact of these phenomena, we studied relevant phenotypic and functional cellular alterations during and after the manufacture of CARTs cryo-thawed at either or both ends of cell manufacturing.

The results of this study suggest that the CARTs that are cryopreserved at the end of manufacturing and thawed immediately before infusion are as clinically effective as CARTs given fresh. Furthermore, cryopreserved CD19 CARTs have a similar *in vivo* survival and peak *in vivo* levels as CD19 CARTs that are infused fresh.

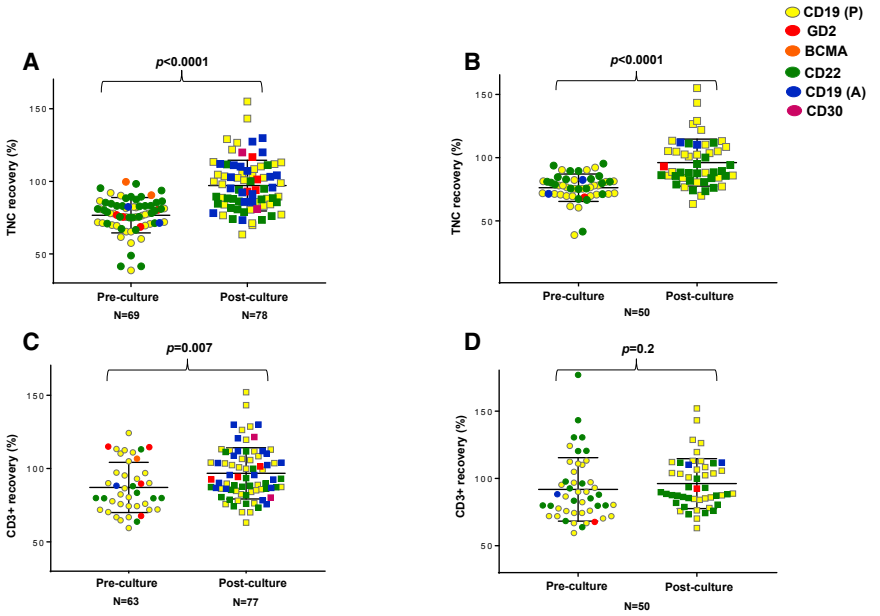

Figure 4. Comparison of Post-thaw Characteristics of PBMNCs and CARTs

(A) The post-thaw TNC recovery of all PBMNCs (pre-culture) and all CARTs (post-culture). (B) The post-thaw TNC recovery only for pairs of PBMNCs and CARTs that underwent double-end cryopreservation, i.e., the PBMNCs were cryopreserved prior to culture (pre-culture) and the CARTs were again analyzed post-cryopreservation-thaw (post-culture). (C) The post-thaw CD3+ cell recovery of all PBMNCs (pre-culture) and all CARTs (post-culture). (D) The post-thaw CD3+ cell recovery for pairs of PBMNCs that were cryopreserved prior to culture (pre-culture) and CARTs that were analyzed post-cryopreservation (post-culture). To calculate cell recovery, viability was measured by the standard trypan blue assay as part of clinical release testing. The scatter dot plots with bars demonstrate mean + SD. p values were calculated for unpaired (A and C) and paired t tests (B and D).

It is not certain if the dose of CARTs administered should be increased if cryopreserved cells are given. We administered CARTs as transduced viable (based on post-thaw trypan blue viability) T cells per kilogram of recipient weight, and we used the same dose for fresh and cryopreserved CARTs. While the *in vivo* recovery and survival of fresh and cryopreserved CD19 CARTs was similar, in a small number of CD22 CARTs the survival of cryopreserved cells was less than that of fresh cells. Further, based on *in vitro* data from healthy volunteer donor CART products, despite similar viability (based on traditional viability assays) between fresh and cryo-thawed CARTs, using markers for early apoptosis and including a novel nucleic acid-binding dye[24] identified a significantly larger number of cells among the viable cells post-thaw, which were destined for eventual demise. Gene expression profiling also identified an increased expression of pathways involved in cell cycle damage and apoptosis in the cryo-thawed CART products.

While we routinely measure the post-thaw viability of CARTs and calculate cells based on viable transduced CD3+ cells, this measure is of limited accuracy. Since cells were infused immediately post-thaw, delayed onset cell death (DOCD) could not be assessed. Furthermore, the *in vivo* expansion of CARTs is also dependent on tumor burden in addition to cell dose. Patients enrolled in 2 clinical protocols received fresh or cryo-thawed final products at the same dose level (1×10^6 transduced viable CARTs/kg). In both protocols, CART persistence did not differ by cryopreservation status. This aligned with data from another recent retrospective analysis.[25] In 1 of the 2 protocols, maximum CART numbers were higher for the fresh infusions compared to cryo-thawed infusions. However, differences in patient disease burden at baseline and variability in cell selection methods during manufacture, which are known to impact *in vivo* CART levels,[26–28] precluded definitive conclusions on this protocol. Of the infusions that failed to demonstrate any CART appearance *in vivo*, 86% and 0% were cryo-thawed in the CD19 and CD22 CART protocols, respectively. Testing, validation, and routine use of commercially available current good manufacturing practice (cGMP) grade cryoprotectant solutions may possibly minimize inter-product variability seen with home-brewed counterparts.[29,30]

We also found that CARTs can be manufactured from cryopreserved PBMNCs. We did observe a reduction of T cells over the first 2 days of culture for cryopreserved, but not fresh, PBMNCs. This DOCD is known to occur between 6 and 48 h post-thaw, and it may occur due to a transcriptional upregulation of key apoptotic markers, including the proteolytic activation of caspase-3 post-thaw. Immediately post-thaw these early apoptotic cells destined for secondary necrosis are not identified by the standard dye-exclusion methods (such as the trypan blue assay used for product release), which only identify losses in structural integrity. Despite this difference in the quality of cells early in culture, we had a sufficient quantity of cells to transduce, and we had similar numbers of CARTs at the end of manufacturing for both fresh and cryopreserved PBMNCs. As with the PBMNCs post-thaw and within the initial culture period, DOCD likely occurs hours to days *in vivo* after CART administration, followed by a partially compensatory expansion.[20] The *in vivo* levels of cryopreserved CARTs may have been higher had a greater dose of cells been administered.

The cryo-thawed starting fraction, PBMNCs, demonstrated significantly lower TNC recovery than the final cryo-thawed CART product, TNCs. This may be attributable to a higher number of non-lymphocytic mononuclear cells, including granulocytes and monocytes, in the starting fraction. These cells are known to have lower post-thaw viability (~7% and 36%, respectively) compared to

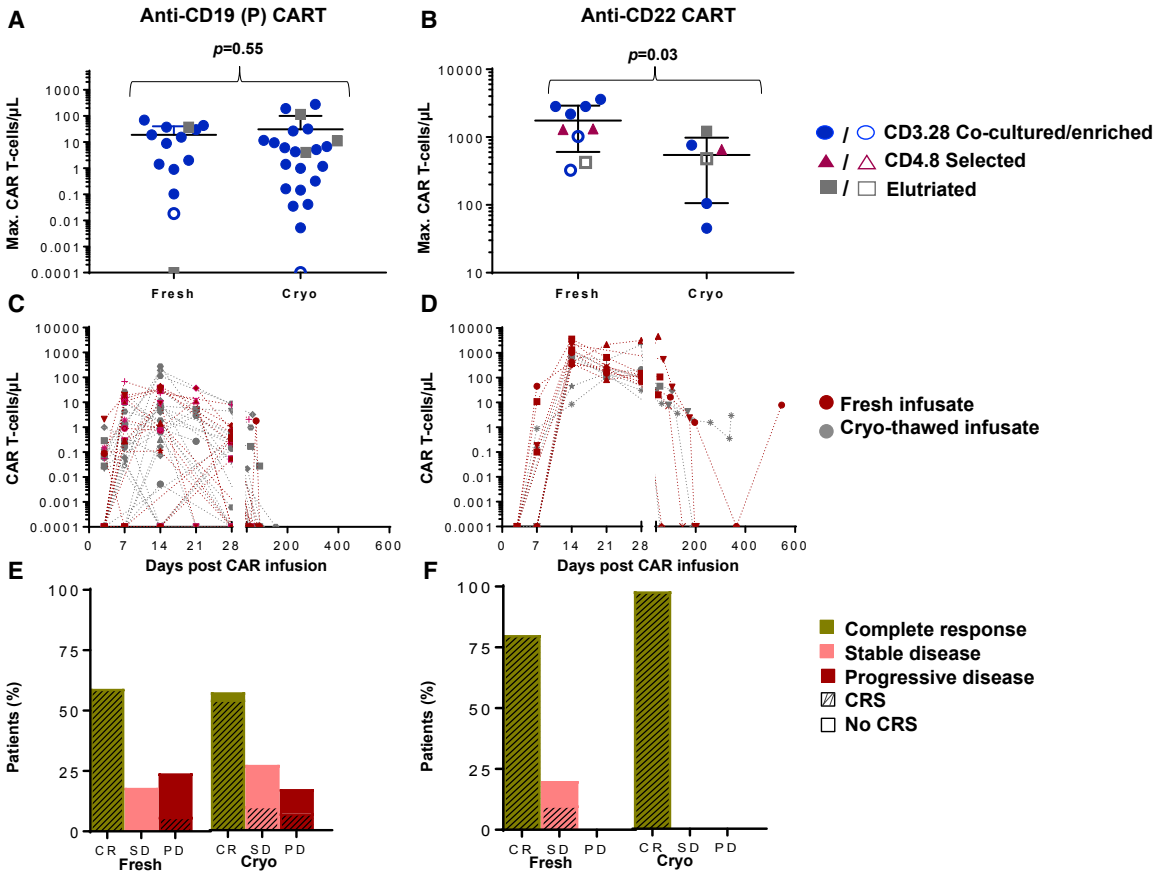

Figure 5. Comparison of *In Vivo* Levels and Persistence of CARTs Infused Fresh or after Cryopreservation

(A and B) The clinical risk stratification (solid shape, high disease burden; outline shape, low disease burden) and maximum levels of peripheral blood T cells expressing CD19 CARTs (A, n = 47) and CD22 CARTs (B, n = 15) in patients who received a first infusion of fresh or cryo-thawed product at a dose of 1×10^6 cells/kg. (C and D) The post-infusion persistence of CARTs in the peripheral blood of patients receiving CD19 CARTs (C) and CD22 CARTs (D). All patients received a dose of 1×10^6 CART cells/kg. Patients receiving fresh CARTs are indicated by pink shapes (CD19 CARTs, n = 17 and CD22 CARTs, n = 9). Patients receiving cryopreserved CARTs are indicated by gray shapes (CD19 CARTs, n = 30 and CD22 CARTs, n = 6). The limit of CART detection by FACS in the peripheral blood was determined for each protocol. For CD19 CARTs and CD22 CARTs, the limits of detection were defined as CART cell fractions of 0.01% and 0.001% of total T cells in the peripheral blood, respectively. In patients receiving fresh or cryopreserved CD19 CARTs (E) and CD22 CARTs (F), clinical response at day 28 and proportion of patients experiencing any grade CRS are shown. All patients received a dose of 1×10^6 CARTs/kg. A total of 17 patients received fresh and 30 received cryopreserved CD19 CARTs and 9 patients received fresh and 7 cryopreserved CD22 CARTs. CR, complete response; SD, stable disease; PD, progressive disease; CRS, cytokine release syndrome. p values were calculated for non-parametric tests, where applicable.

lymphocytes alone (74%).[31] This finding is consistent with what was previously reported for TNC recovery in stem cell products, wherein global TNC recovery was recognized to be lower than that of the more resilient CD34+ cell subsets (post-thaw viability ~80%).[32,33] Upon further analysis of CD3+ T cell subsets, viable post-thaw recovery of T cells in the starting fraction was not significantly different from that in the final manufactured CARTs, which were harvested and cryo-thawed prior to infusion. This was in contrast to a previous report suggesting lower viability of transduced T cells cultured over a period of 7 days in patients with immune deficiencies.[23]

Cryopreservation of PBMNCs and/or of the final manufactured CARTs did not impact TE, CD3%, or CD4:CD8 ratios at harvest or before infusion, respectively. Neither were the proportion of T cell subsets, including naive, effector, and central memory T cells, impacted significantly in our prospective donor cell analysis. Prior studies of other cell types identified phenotypic changes in T cell subsets with cryopreservation[34] and functional impairments due to transient warming events, which occurred during prolonged hypothermic storage.[35] While we were unable to evaluate the frequency and duration of transient warming events in our study, we noticed no significant effect of duration of cryopreservation on T cell phenotype and functional characteristics, during or after manufacture. Further, of the products that failed manufacture, none was specifically related to cryopreservation.

Efforts are underway to add inhibitors of apoptosis and secondary necrosis (zVAD-fmk, p38 mitogen-activated protein kinase [MAPK],

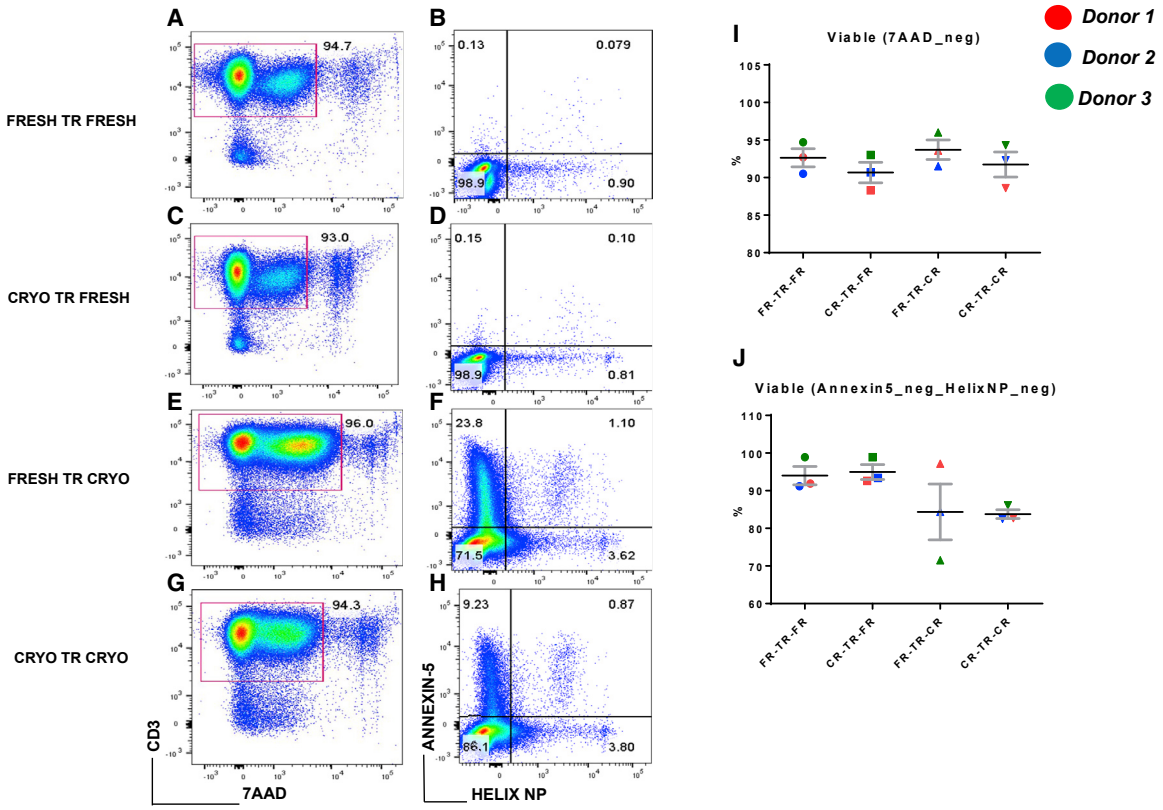

Figure 6. Viability and Apoptosis Analysis of Fresh and Cryopreserved CARTs

CARTs were prepared from fresh or cryopreserved PBMNCs and were analyzed fresh or following cryopreservation. CARTs were prepared from 3 healthy subjects and were transduced (TR) with murine stem cell virus (MSCV)-CAR1922-woodchuck hepatitis virus (WHP) posttranscriptional regulatory element (WPRE) vector. Representative dot plots of fresh CARTs from donor 1 prepared from fresh PBMNCs (Fresh TR Fresh) for 7AAD expression is shown in (A) and for Annexin-V, Helix NP in (B). Dot plots of fresh CARTs from donor 1 prepared from cryopreserved PBMNCs (Cryo TR Fresh) for 7AAD expression is shown in (C) and for Annexin-V, Helix NP in (D). Dot plots of cryopreserved CARTs from donor 1 prepared from fresh PBMNCs (Fresh TR Cryo) for 7AAD expression is shown in (E) and for Annexin-V, Helix NP in (F). Dot plots of cryopreserved CARTs from donor 1 prepared from cryopreserved PBMNCs (Cryo TR Cryo) for 7AAD expression is shown in (G) and for Annexin-V, Helix NP in (H). The $7AAD^{dim}$ population in (A), (C), (E), and (G) was also the cells that were Annexin-V bright in the corresponding adjacent figures, likely representing early apoptotic cells. However, in column 1, the combined viable cell fraction, which included $7AAD^{bright}$ and $7AAD^{dim}$ populations, corresponded with the trypan blue viability used to test clinical products. (I and J) The results for combined analysis CARTs from all 3 donors tested and prepared under the 4 conditions are summarized. The results of the expressions of (I) 7AAD and (J) Annexin-V, Helix NP are shown. Bars in (I) and (J) represent means + SD.

and ROCK inhibitors) to cryopreservation cocktails and culture media.[20,36] Further, cryobiologists are evaluating the role of epigenetic changes and phenotypic and functional drifts in cryo-thawed cells, either due to cryopreservation itself or from a proliferative stress imposed on the surviving cells post-thaw.[37] The need to study DOCD and obtain accurate viable cell counts in the immediate post-thaw period is critical after manufacture, as dose calculations for cell infusion rely on viable transduced T cell counts. To our knowledge, our study presents the most detailed assessment to date of phenotypic, functional, and gene expression alterations in manufactured CARTs.

In summary, cryopreservation at either end of CART manufacture is a viable strategy. Specifically, the cryo-thaw process does not significantly affect clinically relevant CART phenotypic and/or functional parameters, including FE, TE, CD3%, and CD4:CD8 ratios. Further, manufacturing failures, which accounted for approximately 7% of all products in our study, were unrelated to cryopreservation. *In vivo* CART levels and persistence may not be affected by cryopreservation. However, larger controlled studies are necessary to further understand functional and phenotypic alterations with cryopreservation at a molecular level and to assess the occurrence of and mechanisms underlying DOCD *in vitro* and *in vivo*. These studies will inform the need for standardized dose adjustments that may be required to account for the structurally viable albeit early apoptotic cells in cryo-thawed CARTs prepared for infusion into patients. With an increasing demand for large-scale manufacture of these autologous CART therapies of high logistical and regulatory complexity, standardizing and optimizing cryopreservation steps are pivotal to maintaining downstream cell quantity and quality and to enhancing patient outcomes.

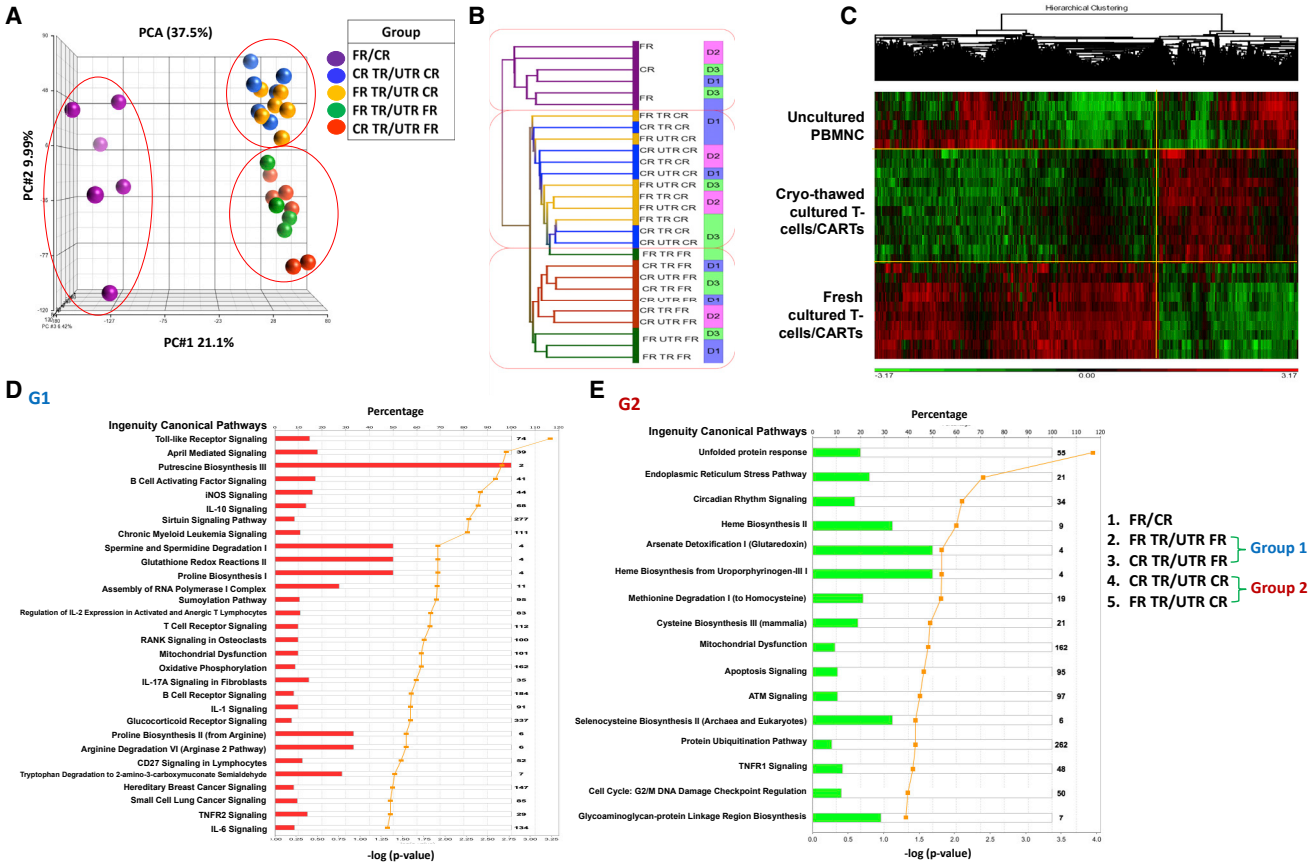

Figure 7. Gene Expression Analysis of Fresh and Cryopreserved CARTs

CARTs were manufactured from 3 healthy subjects, and fresh (C-FR) and cryopreserved (C-CR) CARTs were analyzed by global gene expression analysis. The CARTs were produced from both fresh PBMNCs (P-FR) and cryopreserved PBMNCs (P-FR), and they were transduced (TR) with MSCV-CAR1922-WPRE vector. Untransduced (UTR) fresh and cryopreserved PBMNCs and cultured PBMNCs were also analyzed as a control. The 28 samples were available for global gene expression profiling, and the results were analyzed by principal-component analysis (A) and unsupervised hierarchical clustering analysis (B, cluster dendrogram; C, hierarchical clustering heatmap). (D and E) Ingenuity pathway analysis of genes expressed by cryopreserved and fresh CARTs and cultured T cells are shown. A comparison of the transcriptome of cryopreserved CARTs (n = 6) and cultured T cells (n = 6) with that of fresh CARTs (n = 6) and cultured T cells revealed 2,124 differentially expressed genes. The results of ingenuity pathway analysis of 1,139 genes whose expression was greater in cryopreserved cells is shown in (D) and ingenuity pathway analysis of 985 genes whose expression was greater in fresh cells is shown in (E).

MATERIALS AND METHODS

Study Design

From January 2012 to July 2017, a retrospective data analysis was performed on all CARTs manufactured at the NIH, Center for Cellular Engineering. Clinical protocols on which data were obtained are listed in Table S1. All subjects were enrolled in protocols approved by the National Cancer Institute (NCI) institutional review board (IRB). Protocols comprised phase I dose escalation studies using the various CART constructs against antigens overexpressed in hematologic malignancies or solid tumors. Data on products that failed manufacture were collected separately.

CART manufacturing on the clinical protocols followed 1 of 2 methods. In the protocols treating adult patients (B cell maturation antigen [BCMA], CD19[A], and CD30 CARTs), fresh or cryo-thawed PBMNCs collected by apheresis underwent density gradient separation. T cell stimulation was performed with interleukin-2 (IL-2) and soluble anti-CD3 monoclonal antibody (OKT3) in culture bags, followed by gamma retroviral or lentiviral transduction and expansion, as shown in Figure 1 and Table S3. Between days 7 and 9, cells were harvested, concentrated, and infused fresh or cryopreserved for thaw and infusion at a later time. In the pediatric patient trials (CD19[P], GD2, and CD22 CARTs), fresh or cryo-thawed PBMNC concentrates underwent cell enrichment steps (CD4/8 selection, monocyte depletion by flask [plastic] adherence, elutriation, and/or CD3/CD28 paramagnetic bead enrichment and stimulation). This was followed by gamma-retroviral transduction and expansion with culture harvest between days 7 and 11 for fresh or cryo-thawed infusion (Figure 1; Table S4). See the Supplemental Materials and Methods I for additional CART manufacturing details.[38–42]

Further, to study T cell phenotypic changes and gene expression prospectively, 3 healthy donor PBMNCs were used to manufacture CARTs using 4 different manufacturing schemes: (1) starting manufacturing with fresh PBMNCs and analysis of fresh CARTs, (2) starting with fresh PBMNCs and analysis of cryopreserved CARTs, (3) starting with cryopreserved PBMNCs and analysis of fresh CARTs, and (4) starting with cryopreserved PBMNCs and analysis of cryopreserved CARTs.

Cryopreservation and Thaw

Products that were cryopreserved used a controlled rate freezer (Kryosave, Integra, Planer, Sunbury-on-Thames, UK) and 5% DMSO and 6% pentastarch with 4% human serum albumin as a cryoprotectant. The cryopreserved polyfluoroethylene bags (KryoSure, American Fluroseal, Gaithersburg, MD) or vials (Nunc Cryotube Vials, Roskilde, Denmark) were stored in both the vapor and liquid phases of liquid nitrogen. Thaw was performed in a water bath at 37°C. Immediately after thawing, the products were diluted to a final required volume with Plasma-Lyte A (Baxter Healthcare) containing 10 U/mL preservative-free heparin.

Laboratory Assays

Flow cytometry staining included CD3, CD45, 7-AAD (BD Biosciences), cell surface CAR expression (biotin-labeled protein L, GenScript), Annexin-V (BD Biosciences), and Helix NP (BioLegend). T cell subset analysis used CCR7 and CD45RA (BD Biosciences). RNA purification and microarray data analysis were done as described previously.[43]

Statistical Analysis

Non-parametric Wilcoxon signed-rank tests were used to estimate strength of effects. Multiple linear regression analysis was performed to examine the effect of cryopreservation on the CART production, adjusting for other factors such as different protocols, cell manipulation methods, and infusion dose levels. Statistical analyses were performed using SAS version (v.)9.4 software (Cary, NC). See the Supplemental Materials and Methods II.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.ymthe.2019.05.015.

AUTHOR CONTRIBUTIONS

S.R.P., S.K.S., N.E., N.N.S., and D.F.S. designed the study and wrote the manuscript. A.M., S.L., P.J., S.L.H., and P.D. contributed to data collection and laboratory experiments and data analysis. J.N.K., T.J.F., C.L.M., and D.L. recruited patients in the clinical trials from which data were obtained. They also contributed to study design and manuscript preparation.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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REFERENCES

1. O'Leary, M.C., Lu, X., Huang, Y., Lin, X., Mahmood, I., Przepiorka, D., Gavin, D., Lee, S., Liu, K., George, B., et al. (2019). FDA Approval Summary: Tisagenlecleucel for Treatment of Patients with Relapsed or Refractory B-Cell Precursor Acute Lymphoblastic Leukemia. Clin. Cancer Res. *25*, 1142–1146.
2. Mullard, A. (2017). Second anticancer CAR T therapy receives FDA approval. Nat. Rev. Drug Discov. *16*, 818.
3. Ruella, M., Xu, J., Barrett, D.M., Fraietta, J.A., Reich, T.J., Ambrose, D.E., Klichinsky, M., Shestova, O., Patel, P.R., Kulikovskaya, I., et al. (2018). Induction of resistance to chimeric antigen receptor T cell therapy by transduction of a single leukemic B cell. Nat. Med. *24*, 1499–1503.
4. Iyer, R.K., Bowles, P.A., Kim, H., and Dulgar-Tulloch, A. (2018). Industrializing Autologous Adoptive Immunotherapies: Manufacturing Advances and Challenges. Front. Med. (Lausanne) *5*, 150.
5. Vormittag, P., Gunn, R., Ghorashian, S., and Veraitch, F.S. (2018). A guide to manufacturing CAR T cell therapies. Curr. Opin. Biotechnol. *53*, 164–181.
6. Wang, X., and Rivière, I. (2016). Clinical manufacturing of CAR T cells: foundation of a promising therapy. Mol. Ther. Oncolytics *3*, 16015.
7. Aziz, J., Morris, G., Rizk, M., Shorr, R., Mercer, D., Young, K., and Allan, D. (2017). Cryopreservation of adult unrelated donor products in hematopoietic cell transplantation: the OneMatch experience and systematic review of the literature. Transfusion *57*, 2782–2789.
8. Pavlů, J., Auner, H.W., Szydlo, R.M., Sevillano, B., Palani, R., O'Boyle, F., Chaidos, A., Jakob, C., Kanfer, E., MacDonald, D., et al. (2017). Analysis of hematopoietic recovery after autologous transplantation as method of quality control for long-term progenitor cell cryopreservation. Bone Marrow Transplant. *52*, 1599–1601.
9. Berens, C., Heine, A., Müller, J., Held, S.A., Mayer, K., Brossart, P., Oldenburg, J., Pötzsch, B., Wolf, D., and Rühl, H. (2016). Variable resistance to freezing and thawing of CD34-positive stem cells and lymphocyte subpopulations in leukapheresis products. Cytotherapy *18*, 1325–1331.
10. Fisher, V., Khuu, H., David-Ocampo, V., Byrne, K., Pavletic, S., Bishop, M., Fowler, D.H., Barrett, A.J., and Stroncek, D.F. (2014). Analysis of the recovery of cryopreserved and thawed CD34+ and CD3+ cells collected for hematopoietic transplantation. Transfusion *54*, 1088–1092.
11. Zhu, F., Shah, N.N., Schneider, D., Xu, H., Chaney, K., Luib, L., Keever-Taylor, C., Dropulic, B., Orentas, R., Hari, P., and Johnson, B.D. (2019). Automated Manufacturing of CD20.19 Bi-Specific Chimeric Antigen Receptor T (CAR-T) Cells at an Academic Center for a Phase I Clinical Trial in Relapsed, Refractory NHL. Biol. Blood Marrow Transplant. *25*, S62, (*Suppl*).
12. Zhu, F., Shah, N.N., Schneider, D., Xu, H., Chaney, K., Luib, L., Keever-Taylor, C.A., Dropulic, B., Orentas, R., Hari, P., and Johnson, B. (2018). Point-of-Care Manufacturing of CD20.19 Bi-Specific Chimeric Antigen Receptor T (CAR-T) Cells in a Standard Academic Cell Processing Facility for a Phase I Clinical Trial in Relapsed, Refractory NHL. Blood *132* (*Suppl 1*), 4553.
13. Hay, M., Thomas, D.W., Craighead, J.L., Economides, C., and Rosenthal, J. (2014). Clinical development success rates for investigational drugs. Nat. Biotechnol. *32*, 40–51.
14. Khozin, S., Liu, K., Jarow, J.P., and Pazdur, R. (2015). Regulatory watch: Why do oncology drugs fail to gain US regulatory approval? Nat. Rev. Drug Discov. *14*, 450–451.
15. Aijaz, A., Li, M., Smith, D., Khong, D., LeBlon, C., Fenton, O.S., Olabisi, R.M., Libutti, S., Tischfield, J., Maus, M.V., et al. (2018). Biomanufacturing for clinically advanced cell therapies. Nat. Biomed. Eng. *2*, 362–376.

16. Lipsitz, Y.Y., Timmins, N.E., and Zandstra, P.W. (2016). Quality cell therapy manufacturing by design. Nat. Biotechnol. *34*, 393–400.

17. Lipsitz, Y.Y., Bedford, P., Davies, A.H., Timmins, N.E., and Zandstra, P.W. (2017). Achieving Efficient Manufacturing and Quality Assurance through Synthetic Cell Therapy Design. Cell Stem Cell *20*, 13–17.

18. Nicoud, I.B., Clarke, D.M., Taber, G., Stolowski, K.M., Roberge, S.E., Song, M.K., Mathew, A.J., and Reems, J.A. (2012). Cryopreservation of umbilical cord blood with a novel freezing solution that mimics intracellular ionic composition. Transfusion *52*, 2055–2062.

19. Dijkstra-Tiekstra, M.J., Setroikromo, A.C., Kraan, M., Gkoumassi, E., and de Wildt-Eggen, J. (2014). Optimization of the freezing process for hematopoietic progenitor cells: effect of precooling, initial dimethyl sulfoxide concentration, freezing program, and storage in vapor-phase or liquid nitrogen on in vitro white blood cell quality. Transfusion *54*, 3155–3163.

20. Baust, J.G., Snyder, K.K., Van Buskirk, R., and Baust, J.M. (2017). Integrating Molecular Control to Improve Cryopreservation Outcome. Biopreserv. Biobank. *15*, 134–141.

21. Golab, K., Grose, R., Placencia, V., Wickrema, A., Solomina, J., Tibudan, M., Konsur, E., Ciepły, K., Marek-Trzonkowska, N., Trzonkowski, P., et al. (2018). Cell banking for regulatory T cell-based therapy: strategies to overcome the impact of cryopreservation on the Treg viability and phenotype. Oncotarget *9*, 9728–9740.

22. Hubel, A., Norman, J., and Darr, T.B. (1999). Cryobiophysical characteristics of genetically modified hematopoietic progenitor cells. Cryobiology *38*, 140–153.

23. Hubel, A., Darr, T.B., and Norman, J.A. (1999). Freezing characteristics of genetically modified lymphocytes for the treatment of MPS II. Cell Transplant. *8*, 521–530.

24. Jiang, L., Tixeira, R., Caruso, S., Atkin-Smith, G.K., Baxter, A.A., Paone, S., Hulett, M.D., and Poon, I.K. (2016). Monitoring the progression of cell death and the disassembly of dying cells by flow cytometry. Nat. Protoc. *11*, 655–663.

25. Chong, E.A., Levine, B.L., Grupp, S.A., Davis, M., Siegel, D.L., Maude, S.L., Gladney, W.L., Frey, N.V., Porter, D.L., June, C.H., and Schuster, S.J. (2018). CD19-Directed CAR T-Cell (CTL019) Product Viability and Clinical Outcomes in Non-Hodgkin Lymphomas and B-Cell Acute Lymphoblastic Leukemia. Blood *132* (*Suppl 1*), 197.

26. June, C.H., and Sadelain, M. (2018). Chimeric Antigen Receptor Therapy. N. Engl. J. Med. *379*, 64–73.

27. Ramos, C.A., Rouce, R., Robertson, C.S., Reyna, A., Narala, N., Vyas, G., Mehta, B., Zhang, H., Dakhova, O., Carrum, G., et al. (2018). In Vivo Fate and Activity of Second- versus Third-Generation CD19-Specific CAR-T Cells in B Cell Non-Hodgkin's Lymphomas. Mol. Ther. *26*, 2727–2737.

28. Turtle, C.J., Hanafi, L.A., Berger, C., Gooley, T.A., Cherian, S., Hudecek, M., Sommermeyer, D., Melville, K., Pender, B., Budiarto, T.M., et al. (2016). CD19 CAR-T cells of defined CD4+:CD8+ composition in adult B cell ALL patients. J. Clin. Invest. *126*, 2123–2138.

29. Woods, E.J., Thirumala, S., Badhe-Buchanan, S.S., Clarke, D., and Mathew, A.J. (2016). Off the shelf cellular therapeutics: Factors to consider during cryopreservation and storage of human cells for clinical use. Cytotherapy *18*, 697–711.

30. Worsham, D.N., Reems, J.A., Szczepiorkowski, Z.M., McKenna, D.H., Leemhuis, T., Mathew, A.J., and Cancelas, J.A.; Biomedical Excellence for Safer Transfusion (BEST) Collaborative Group (2017). Clinical methods of cryopreservation for donor lymphocyte infusions vary in their ability to preserve functional T-cell subpopulations. Transfusion *57*, 1555–1565.

31. Creer, M.H., Lemas, M.V., and Mathew, A.J. (2015). Practical Handbook of Cell Therapy Cryopreservation (American Association of Blood Banks).

32. Laroche, V., McKenna, D.H., Moroff, G., Schierman, T., Kadidlo, D., and McCullough, J. (2005). Cell loss and recovery in umbilical cord blood processing: a comparison of postthaw and postwash samples. Transfusion *45*, 1909–1916.

33. Regan, D.M., Wofford, J.D., and Wall, D.A. (2010). Comparison of cord blood thawing methods on cell recovery, potency, and infusion. Transfusion *50*, 2670–2675.

34. Xu, H., Cao, W., Huang, L., Xiao, M., Cao, Y., Zhao, L., Wang, N., and Zhou, J. (2018). Effects of cryopreservation on chimeric antigen receptor T cell functions. Cryobiology *83*, 40–47.

35. Chabot, D., Tremblay, T., Paré, I., Bazin, R., and Loubaki, L. (2017). Transient warming events occurring after freezing impairs umbilical cord-derived mesenchymal stromal cells functionality. Cytotherapy *19*, 978–989.

36. Bissoyi, A., Nayak, B., Pramanik, K., and Sarangi, S.K. (2014). Targeting cryopreservation-induced cell death: a review. Biopreserv. Biobank. *12*, 23–34.

37. Chatterjee, A., Saha, D., Niemann, H., Gryshkov, O., Glasmacher, B., and Hofmann, N. (2017). Effects of cryopreservation on the epigenetic profile of cells. Cryobiology *74*, 1–7.

38. Stroncek, D.F., Ren, J., Lee, D.W., Tran, M., Frodigh, S.E., Sabatino, M., Khuu, H., Merchant, M.S., and Mackall, C.L. (2016). Myeloid cells in peripheral blood mononuclear cell concentrates inhibit the expansion of chimeric antigen receptor T cells. Cytotherapy *18*, 893–901.

39. Fry, T.J., Shah, N.N., Orentas, R.J., Stetler-Stevenson, M., Yuan, C.M., Ramakrishna, S., Wolters, P., Martin, S., Delbrook, C., Yates, B., et al. (2018). CD22-targeted CAR T cells induce remission in B-ALL that is naive or resistant to CD19-targeted CAR immunotherapy. Nat. Med. *24*, 20–28.

40. Lee, D.W., Kochenderfer, J.N., Stetler-Stevenson, M., Cui, Y.K., Delbrook, C., Feldman, S.A., Fry, T.J., Orentas, R., Sabatino, M., Shah, N.N., et al. (2015). T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. Lancet *385*, 517–528.

41. Ali, S.A., Shi, V., Maric, I., Wang, M., Stroncek, D.F., Rose, J.J., Brudno, J.N., Stetler-Stevenson, M., Feldman, S.A., Hansen, B.G., et al. (2016). T cells expressing an anti-B-cell maturation antigen chimeric antigen receptor cause remissions of multiple myeloma. Blood *128*, 1688–1700.

42. Brudno, J.N., Somerville, R.P.T., Shi, V., Rose, J.J., Halverson, D.C., Fowler, D.H., Gea-Banacloche, J.C., Pavletic, S.Z., Hickstein, D.D., Lu, T.L., et al. (2016). Allogeneic T Cells That Express an Anti-CD19 Chimeric Antigen Receptor Induce Remissions of B-Cell Malignancies That Progress After Allogeneic Hematopoietic Stem-Cell Transplantation Without Causing Graft-Versus-Host Disease. J. Clin. Oncol. *34*, 1112–1121.

43. Liu, S., de Castro, L.F., Jin, P., Civini, S., Ren, J., Reems, J.A., Cancelas, J., Nayak, R., Shaw, G., O'Brien, T., et al. (2017). Manufacturing Differences Affect Human Bone Marrow Stromal Cell Characteristics and Function: Comparison of Production Methods and Products from Multiple Centers. Sci. Rep. *7*, 46731.

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Supplemental Information

Effect of Cryopreservation on Autologous Chimeric Antigen Receptor T Cell Characteristics

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Supplementary Tables

2012	2013	2014	2015	2016	2017
12-C-0112 **(CD19 CAR T-CELLS)**	12-C-0112 (CD19 CAR T-CELLS)	12-C-0112 (CD19 CAR T-CELLS)	12-C-0112 (CD19 CAR T-CELLS)	12-C-0112 (CD19 CAR T-CELLS)	
		14-C-0059 **(GD2 CAR T-CELLS**	14-C-0059 (GD2 CAR T-CELLS)	14-C-0059 (GD2 CAR T-CELLS)	
		14-C-0168 **(BCMA CAR T-CELLS)**	14-C-0168 (BCMA CAR T-CELLS)	14-C-0168 (BCMA CAR T-CELLS)	14-C-0168 (BCMA CAR T-CELLS)
			15-C-0029 **(CD22 CAR T-CELLS)**	15-C-0029 (CD22 CAR T-CELLS)	15-C-0029 (CD22 CAR T-CELLS)
				16-C-0054 **(CD19 CAR T-CELLS)**	16-C-0054 (CD19 CAR T-CELLS)
					17-C-0048 **(CD30 CAR T-CELLS)**
1	1	3	4	5	6

Supplementary Table S1. Retrospective review of all CART infusates manufactured at the NIH/CC/DTM/CPS on clinical protocols from 2012-2017

Protocol **CAR-T Cell Type**	12-C-0112 **CD19 CART**	14-C-0059 **GD2 CART**	14-C-0168 **BCMA CART**	15-C-0029 **CD22 CART**	16-C-0054 **CD19 CART**	17-C-0048 **CD30 CART**	TOTAL ---
Pre-culture: **fresh** Post-culture: **fresh**	20	5	18	4	1	0	**48**
Pre-culture: **fresh** Post-culture: **cryo**	7	3	0	0	17	2	**29**
Pre-culture: **cryo** Post-culture: **fresh**	2	3	2	13	0	0	**20**
Pre-culture: **cryo** Post-culture: **cryo**	27	1	0	20	2	0	**50**
Total Products Infused	**56**	**12**	**20**	**37**	**20**	**2**	**147**
Days of pre-culture cryo median (range)	5 (3-220)	4 (3-13)	437.5 (7-868)	12 (3-418)	6.5 (6-7)	--	**6 (3-868)**
Days of post-culture cryo median (range)	6.5 (1-312)	13.5 (1-63)	---	9.5 (2-345)	14 (2-408)	184 (2-367)	**9 (1-408)**

Supplementary Table S2. Number of fresh and cryopreserved CART products manufactured across all protocols from January 2012 to July 2017 with duration of storage.

Protocol	12-C-0112 (P)	14-C-0059 (P)	14-C-0168 (A)	15-C-0029 (P)	16-C-0054 (A)	17-C-0048 (A)
CAR-T cell type	CD19(P), CD28	GD2, CD28, O×40	BCMA,CD28	CD22,41BB	CD19(A),CD28	CD30,CD28
Pre-culture cell Manipulation (N)	Mono-depletion (5) Bead enrichment/select-ion (46) Both (5)	Mono-depletion(5) Bead enrichment/select-ion (7) Both (0)	Ficoll (20)	Mono-depletion (7) Bead enrichment/selection (15) Both (13); None (2)	Ficoll (20)	Ficoll (2)
T-cell stimulation	CD3/CD28 enrichment	CD3/CD28 enrichment	Anti-CD3 monoclonal Ab: OKT3	CD3/CD28 enrichment; CD4/CD8 selection	Anti-CD3 monoclonal Ab: OKT3	Anti-CD3 monoclonal Ab: OKT3
Cytokine for T-cell culture	IL2-40IU(initiation) 300IU(expansion)	IL2-40IU(initiation) 300IU(expansion)	IL2-40IU(initiation) 300IU(expansion)	IL2-40IU(initiation) 100IU(expansion)	IL-2 300IU(expansion)	IL-2 300IU(expansion)
Delivery vector	Gamma-retrovirus	Gamma-retrovirus	Gamma-retrovirus	Lentivirus	Lentivirus	Lentivirus
Transduction assay	Anti-idiotype antibody by FACS	GD2 scFv	BCMA+ T cells	CD22 Fc	CD3 and Protein L	CD3 and Protein L
Conditioning regimen	Cytoxan(900mg/m^2) (-2) + Fludarabine (25mg/m^2) on days -4,-3, -2) or Arm2 with FLAG, Ifos/etoposide, or high-dose Cytoxan/fludarabine	Cytoxan (1800mg/m^2) (-3, -2)	Cytoxan(300mg/m^2)+ Fludarabine (30mg/m^2) on days -5,-4,-3)	Cytoxan(900mg/m^2) (-2) + Fludarabine (25mg/m^2) on days -4,-3, -2)	Cytoxan(300mg/m^2)+ Fludarabine (30mg/m^2) on days -5,-4,-3)	Cytoxan(300mg/m^2)+ Fludarabine (30mg/m^2) on days -5,-4,-3)

Supplementary Table S3. Manufacturing methods, assays, conditioning regimens and other testing across CART protocols included in the study. Pediatric and Adult trials are marked by the suffixes, (P) and (A), respectively.

A. FOLD EXPANSION (FE)			
Protocol ID	**Fresh PBMNC**	**Cryo-thawed PBMNC**	**p-value**
CD19 (P)	17.7 (±17.4) N=27	9.0 (±5.6) N=29	0.01
GD2	4.2 (±3.6) N=8	9.8 (±5.3) N=4	0.05
BCMA	6.9 (±3.8) N=18	10.5 (±12.0) N=2	0.31
CD22	41.2 (±27.8) N=4	19.6 (±9.0) N=33	0.001
CD19 (A)	5.1 (±3.3) N=18	5.6 (±0.8) N=2	0.85
CD30	4.0 (±0.5) N=2	_	_
Total	11.7 (±14.9) N=77	14.0 (±9.1) N=70	0.27

B. TRANSDUCTION EFFICIENCY (TE)			
Protocol ID	**Fresh PBMNC**	**Cryo-thawed PBMNC**	**p-value**
CD19 (P)	67.8 (±21.3) N=27	69.4 (±18.8) N=29	0.76
GD2	67.8 (±19.7) N=8	45.7 (±13.1) N=4	0.07
BCMA	54.0 (±19.9) N=18	40.8 (±0.2) N=2	0.37
CD22	39.5 (±8.1) N=4	34.4 (±9.5) N=33	0.32
CD19 (A)	44.1(±15.5) N=18	59.9 (±15.2) N=2	0.19
CD30	75.3 (±7.8) N=2	_	_
Total	57.7 (±21.4) N=77	50.5 (±21.9) N=70	0.04

C. CD3 %			
Protocol ID	**Fresh PBMNC**	**Cryo-thawed PBMNC**	**p-value**
CD19 (P)	99.3 (±0.6) N=27	97.8 (±4.9) N=29	0.11
GD2	96.6 (±4.3) N=8	98.1 (±0.5) N=4	0.52
BCMA	94.4 (±5.6) N=18	82.9 (±23.5) N=2	0.06
CD22	99.1 (±0.8) N=4	99.5 (±0.7) N=33	0.30
CD19 (A)	95.9 (±1.3) N=18	96.8 (±1.7) N=2	0.36
CD30	96.0 (±2.2) N=2	_	_
Total	97.0 (±3.7) N=77	98.2 (±5.1) N=70	0.10

B. CD4/CD8 ratio			
Protocol ID	**Fresh PBMNC**	**Cryo-thawed PBMNC**	**p-value**
CD19 (P)	1.8 (±1.4) N=27	3.2 (±5.4) N=29	0.19
GD2	1.1 (±0.9) N=8	1.0 (±0.5) N=4	0.84
BCMA	1.5 (±1.1) N=18	0.6 (±0.1) N=2	0.27
CD22	0.5 (±0.3) N=4	2.2 (±3.1) N=33	0.30
CD19 (A)	0.9 (±0.4) N=18	1.3 (±1.0) N=2	0.32
CD30	0.7 (±0.3) N=2	_	_
Total	1.3 (±1.1) N=77	2.5 (±4.1) N=70	0.02

Supplementary Table S4. Stratified and cumulative data comparing cultures that were initiated with fresh PBMNC or cryopreserve-thawed PBMNC. Difference in FE (A), TE (B), CD3% (C), or CD4:CD8 ratios (D) at the time of final CART harvest are summarized.

SI number	CART Protocol	Patient ID	Starting fraction	Product infused (Yes/No)	Reason	Subsequent successful manufacture from cryo-preserved parent product
1	BCMA	19	Cryopreserved	No	Failed sterility testing	No
2	CD22	28	Cryopreserved	No	Low TE	Yes
3	CD22	33	Fresh	No	Clumping (>90% Blasts)	No
4	CD22	36	Cryopreserved	No	Low post-thaw viability	Yes
5	CD22	47	Cryopreserved	No	Low TE	Yes
6	CD22	50	Cryopreserved	No	Low cell count	No
7	CD19(A)	67	Fresh	No	Low cell count	No
8	CD19(P)	112	Fresh	No	Low TE	Yes
9	CD19(P)	116	Fresh	No	Low TE & viability	Yes
10	GD2	86	Fresh	No	Low TE	No
11	GD2	84	Fresh	No	Low TE	Yes

Supplementary Table S5. Products that failed manufacture in each protocol, with results of a subsequent culture using a different aliquot from the original apheresis sample.

Supplementary Methods I

Manufacturing CART

Peripheral blood mononuclear cell (PBMNC) concentrates were collected using a blood cell separator (COBE Spectra or Spectra Optia, Terumo BCT, CO), and 10 to 15 liters of blood were processed. On Day 0, a fresh or cryopreserved PBMNC concentrates containing CD3+ cells underwent manufacturing steps over 7-9 days as specified in each CART manufacturing protocol below.

Manufacturing CART: Protocol specific data

CD19 CART(P)

For the manufacture of CD19 CART(P): 600×10^6 CD3+ cells were co-incubated for 2 hours at room temperature with CD3/CD28 antibodies bound to paramagnetic beads (Dynabeads ClinExVivo CD3/CD28, Invitrogen) at a ratio of 3:1 (beads: cells), followed by a Dynal ClinExVIVO MPC magnetic enrichment (Invitrogen). A total of 100×10^6 cells in the CD3+ fraction were resuspended at a concentration of 1×10^6 cells/mL in PermaLife bags (OriGen Biomedical) at 37°C in 5% CO2 in AIM V medium (Gibco), supplemented with 5% heat-inactivated human AB Serum (Valley Biomedical), 1% Glutamax (Gibco), 40 IU/mL interleukin-2 (Novartis Vaccines and Diagnostics). The cells were transduced twice with clinical grade MSGV-FMC63-28Z recombinant retroviral vector supernatant, once on day 2 and once on day 3, in retronectin-coated bags. The cells were maintained in culture for 7 to 11 days. The cell concentration was maintained at 0.4×10^6 cells/mL by adding fresh medium every other day. On the day of harvest, the CD3/CD28 paramagnetic beads were removed using the Dynal ClinExVIVO MPC magnet (Invitrogen), washed and concentrated, and quality control assessment was performed.

Anti-GD2 CART

A similar process was used to manufacture GD2- CART. Viral transduction was performed with an anti-GD2.28.z.OX40.ICD9 retroviral vector supernatant over 1 or 2 days. For some CD19 and GD2 CART manufacturing procedures a step to deplete monocytes by plastic adherence was used by incubating CD3/CD28 magnetic beads with PBMNC in T flasks rather than in bags. After 2 h the non-adherent cells were collected, and the cells were processed as described.

Anti CD22 CART

CD22 CART were manufactured starting with a population of enriched T cells using the methods described for CD19 CART (P) (N=25) or were T cell selected using CD4/CD8 double positive selection on the CliniMACS Plus instrument (Miltenyi)(N=17). Cells were transduced at 0.5 x 10^6 cells/mL on day 2 using a lentiviral vector EF1a-BBZ-CD22-CAR with protamine sulfate (1mg/mL). Culture bag spinoculation was performed to enhance lentiviral transduction and cells were centrifuged at 1000xg at 32C for 2 hours. Media was replaced at 24 hours (day 3) and cells were de-beaded and diluted to 0.4 x 10^6 cells/mL on day 4. On day 7, cells were diluted to 0.6 – 1 x 10^6 cells/mL and on day 9 cells were harvested for infusion or cryopreservation.

BCMA CART, CD30 CART and CD19 CART (A)

In the BCMA and CD19 adult protocols, Fresh PBMNC underwent automated density gradient separation on a COBE 2991 cell processor (TerumoBCT) and were either cultured fresh or cryopreserved and later thawed for culture. On day 0, fresh or thawed mononuclear cells were placed in complete medium containing AIM-V CTS™ medium (Life Technologies, Grand Island NY), 5% heat-inactivated pooled human AB serum (Valley Biomedical, Winchester VA), 2milliMolar GlutaMax™ (Gibco/Life Technologies, Carlsbad CA) and 40 IU/mL interleukin-2 (IL-2) (Proleukin; Prometheus Laboratories, San Diego CA), 50ng/mL anti-CD3 (MAC® GMP CD3 pure, Miltenyi Biotech, Gladbach Germany) and incubated in Permalife FEP culture bags (Origen Biomedical, Austin TX) for 48 hours in a 37□C, 5% CO2 humidified incubator. On day 2, anti-BCMA vector supernatant (MSGV-11D-5-3-CD828Z) was thawed and diluted 1:1 with AIM V media and incubated in Retronectin® (Takara Bio Inc, Japan). Coated Permalife bags incubated for 2 hours in a 37□C, 5% CO2 humidified incubator. Cells were concentrated, culture supernatant removed, and cells were re-suspended in complete media as described above with the exceptions of IL2 concentration which was increased to 300IU/mL and the omission of the

anti-CD3 antibody. The suspended cells were added to the pre-incubated bags containing vector for a final vector dilution of 1:4 and final cell concentration of 0.5 x 10^6 CD3+ cells/mL. The transduction process was repeated on day 3. Transduction was stopped on day 4 when cells were re-suspended in fresh complete media containing 300 IU/mL IL2 in polyolefin culture bags (Charter Medical, Winston-Salem NC). Culture was continued at a concentration from 0.4 – 1 x10^6 cells/mL until day 7-9 when cells were harvested, concentrated, and washed on a COBE 2991 cell processor and infused in Plasmalyte A (Baxter Healthcare, Deerfield IL) with 4% human serum albumin. Cell doses were determined by the predetermined dose escalation plan, and were based on a number of CAR expressing viable CD3+ cells per kg of patient bodyweight.

Supplementary Methods II

Cell Counts and Flow Cytometry

An aliquot of the product was diluted 5-fold in Plasma-Lyte A immediately after thawing. Nucleated cell counts were performed using an automated cell counter (Abbott CellDyn 3500) and the cells were analyzed by flow cytometry (BD Biosciences, San Jose, CA) with anti-CD3 and CD45 (BD Biosciences). Viability was assessed by trypan blue and/or by flow cytometry (7-AAD staining, BD biosciences). Each product was tested once.

Cell-surface CAR expression was detected by Biotin-labeled protein L (GenScript, Piscataway, NJ) followed by flow cytometry. The percentage of CAR-expressing (CAR+) T cells was calculated as the percentage of T cells in CAR-transduced cultures that stained with protein L minus the percentage of identically cultured untransduced T cells from the same donor that stained with protein L. Post infusion, staining for CART in peripheral blood and bone marrow were performed as described previously in each protocol[12,13].

On healthy volunteer donor samples, in addition to the tests above, pre-apoptotic markers were tested using Annexin-V (BD biosciences) and Helix-NP (Bio legend). T-cell subset analysis used CCR7 and CD45RA (BD biosciences).

Viable post-thaw TNC recovery (%) was calculated using the formula:

$$\frac{\text{Post-thaw TNC X post-thaw viability X 100}}{\text{Pre-cryopreservation TNC X pre-cryopreservation viability}}$$

As previously shown, post-thaw viable cell recovery correlated well with post-thaw cell viability in our study[39]. Although no formal studies are available in this regard, in our experience, viable post-thaw cell recovery, as a composite of total cell count and cell survival, represents a better outcome measure than cell viability alone. Cell count increases in the post-thaw samples (as a result of manual and/or automated cell counter discrepancies), resulted in calculated viable cell recovery (%) of greater than 100% on occasion (recovery range: 38% - 155%).

Acceptance Criteria for Cell Infusion

Acceptance criteria for cell infusion included the following. Appearance is milky white cell suspension. Viable transduced CD3+ cells: ± 20% of dose level, Trypan Blue Viability: ≥70%, CD3 of viable cell %: ≥80%, TE: ≥15%, Endotoxin: < 5EU/mL, Gram stain: No organisms seen (NOS), 48 hour sterility: No growth, Sterility: No growth, RCR-PCR: Negative.

Total RNA Isolation, Amplification, Hybridization and Slide Processing

Total RNA from 30 healthy volunteer donor samples were isolated and purified using a miRNeasyKit (Qiagen, Germantown, MD, USA). The RNA concentration was determined using a Nano Drop ND-1000 Spectrophotometer (Nano Drop Technologies, Wilmington, DE, USA) and RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa

Clara, CA, USA). RNA was amplified and labeled using an Agilent LowInput QuickAmp Labeling Kit and subsequently mixed with Universal Human Reference RNA (Stratagene, Santa Clara, CA, USA) and co-hybridized to Agilent Chip Whole Human genome, 4 × 44 k slides according to the protocol provided by Agilent. The slides were incubated for 17 h at 65 °C and then the scanned using an Agilent B Scanner.

Microarray Data Analysis.

Raw images were obtained by scanning the slides with an Agilent Scan G2505B and Agilent Scan Control software (version 9.5). The images were extracted using the Feature Extraction Software (Agilent Technologies). Partek Genomic Suite 6.4 (Partek Inc., St. Louis, MO, USA) was used for data visualization, identification of differentially expressed transcripts and hierarchical cluster analysis. We transformed the fluorescence intensity data to log2 ratios of each sample versus the universal human RNA reference (Stratagene, Santa Clara, CA, USA). Then t-tests were used to identify differentially expressed genes (both p value and FDR less than 0.05). The Ingenuity Pathway Analysis (IPA) tool (http://www.ingenuity.com, Ingenuity System Inc., Redwood City, CA, USA) was used for analysis of functional pathways. The microarray data was deposited in GEO (GSE77814).

Statistical analysis: Descriptive statistics (counts and percentage for categorical variables; mean ±SD, median and range for non-categorical variables) were provided. Differences in results and their statistical significance between fresh and cryopreserved groups was determined using non-parametric tests (Wilcoxon signed-rank test). Multiple linear Regression analysis was performed to examine the effect of cryopreservation on the CAR-T cell production adjusting for other factors such as different protocols, cell manipulation methods, and infusion dose levels. All statistical analyses were performed using SAS version 9.4 statistical software (SAS Institute,

Cary NC) and creating plots on in Prism (GraphPad Software, LaHolla, CA). Statistical significance was established at $p<0.05$ (2-tailed) for all tests given the exploratory nature of the analyses. Results for post-thaw outcomes measured after outcome variables demonstrated a dominant effect of protocol ID in multiple linear regression analyses, masking any changes that may be attributable to other confounding variables (cell selection/enrichment methods, protocol specific manipulations, disease risk stratification). Hence data points in the figures were color coded by protocol.

Supplementary Figures

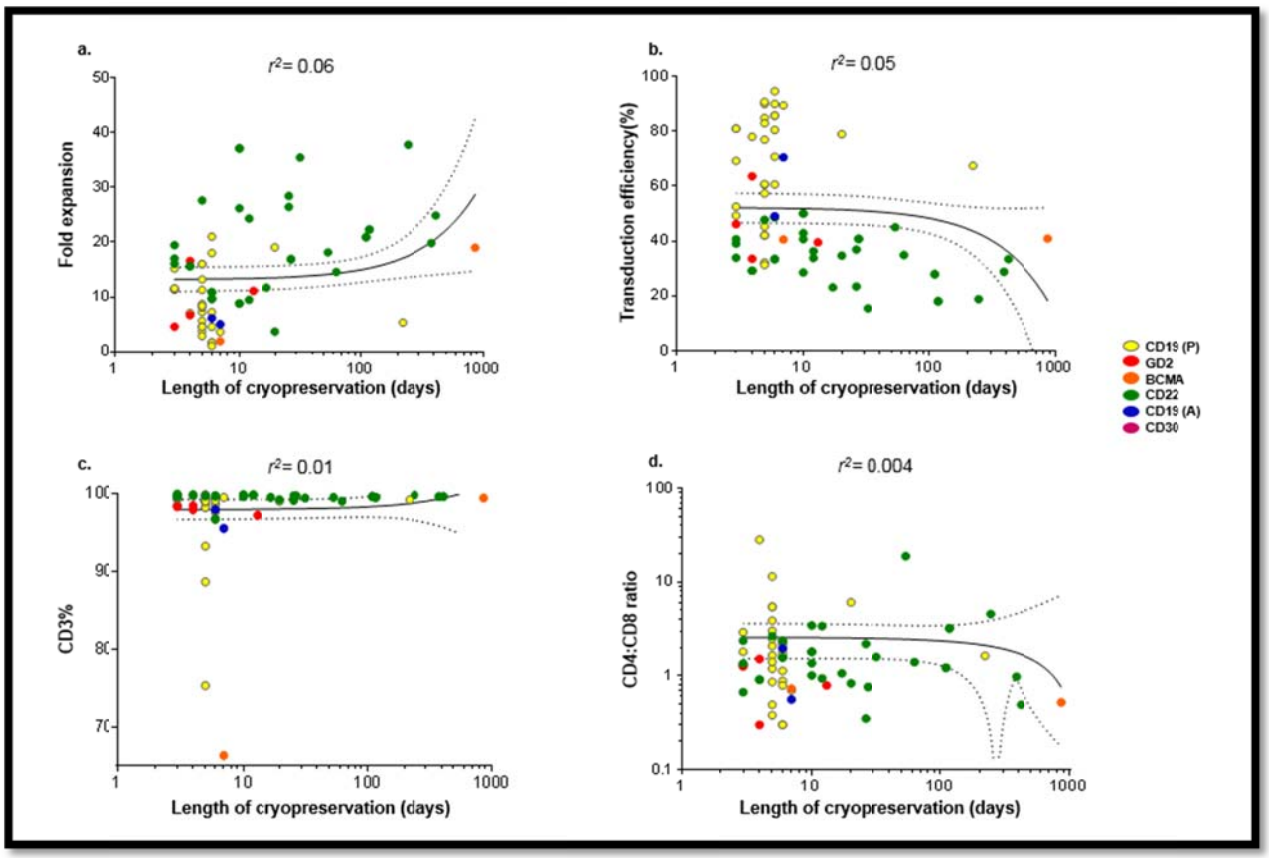

Figure S1. Results of a multivariate analyses demonstrated no impact of the duration of cryopreservation on the outcome variables: FE (a), TE (b), CD3% (c) and CD4:CD8 ratio (d).

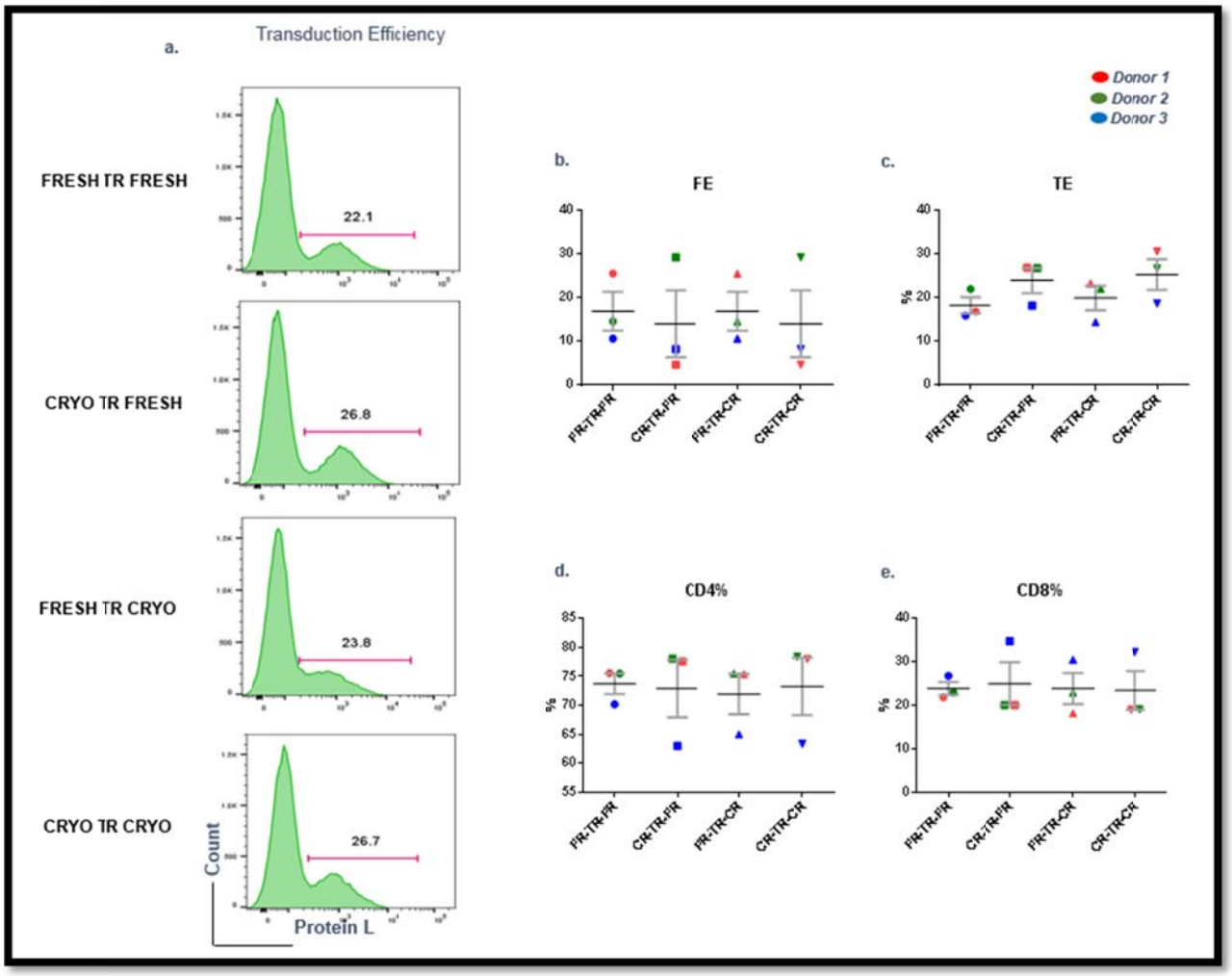

Figure S2. T-cell characteristics in 3 healthy volunteer donor CART cultures subjected to 4 manufacturing schemes- (i) Fresh-transduced-fresh, (ii) Cryo-transduced-fresh, (iii) Fresh-transduced-cryo, or (iv) Cryo-transduced-cryo. Panel a shows TE measured in the 4 culture arms for Donor 2. Panels b, c, d, e show scatter plots of FE, TE, CD4% and CD8%, in the 3 donors respectively.

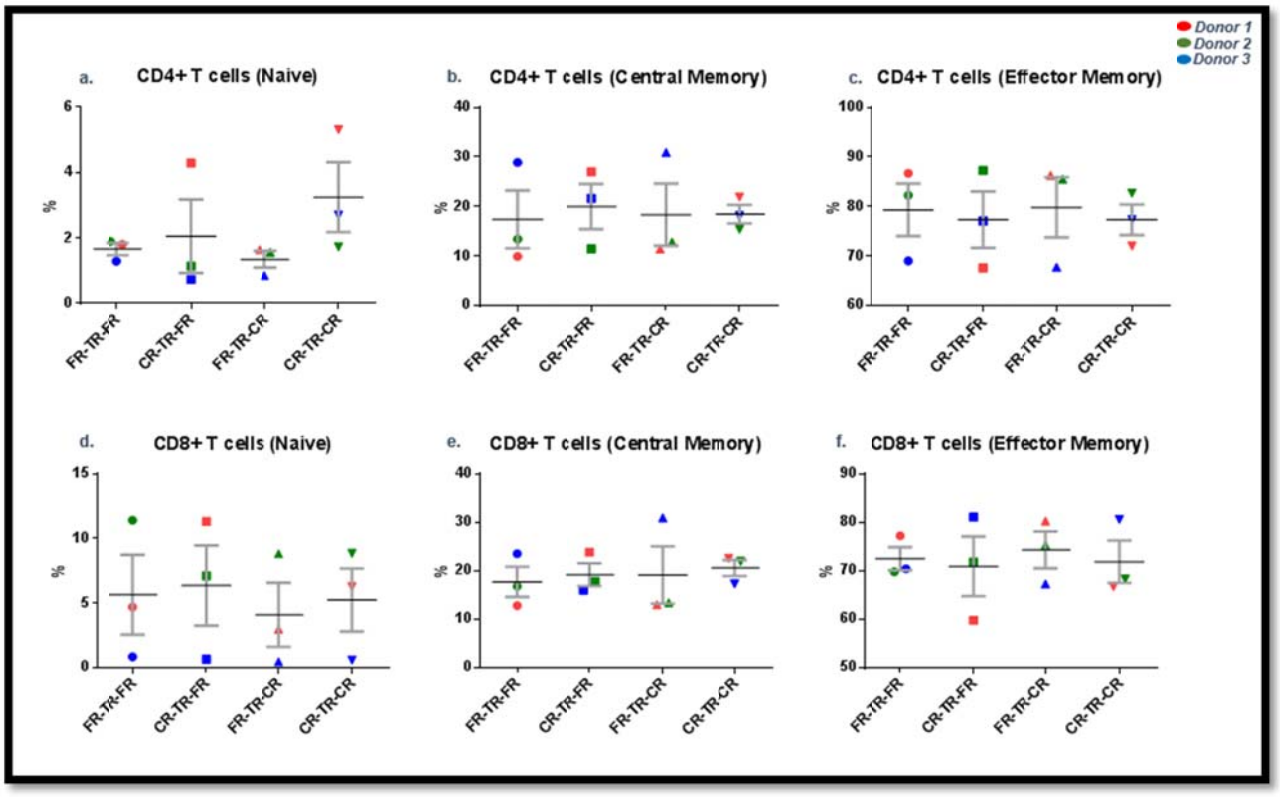

Figure S3. T-cell subsets, CD4 (a,b,c) and CD8 (d,e,f)) in the 3 healthy volunteer donor CART cultures using 4 combinations of fresh and cryo-thawed cell culture methods- (i) Fresh-transduced-fresh, (ii) Cryo-transduced-fresh, (iii) Fresh-transduced-cryo, or (iv) Cryo-transduced-cryo.