YMTHE, Volume *27*

Supplemental Information

Effect of Cryopreservation on Autologous Chimeric Antigen Receptor T Cell Characteristics

Sandhya R. Panch, Sandeep K. Srivastava, Nasha Elavia, Andrew McManus, Shutong Liu, Ping Jin, Steven L. Highfill, Xiaobai Li, Pradeep Dagur, James N. Kochenderfer, Terry J. Fry, Crystal L. Mackall, Daniel Lee, Nirali N. Shah, and David F. Stroncek

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

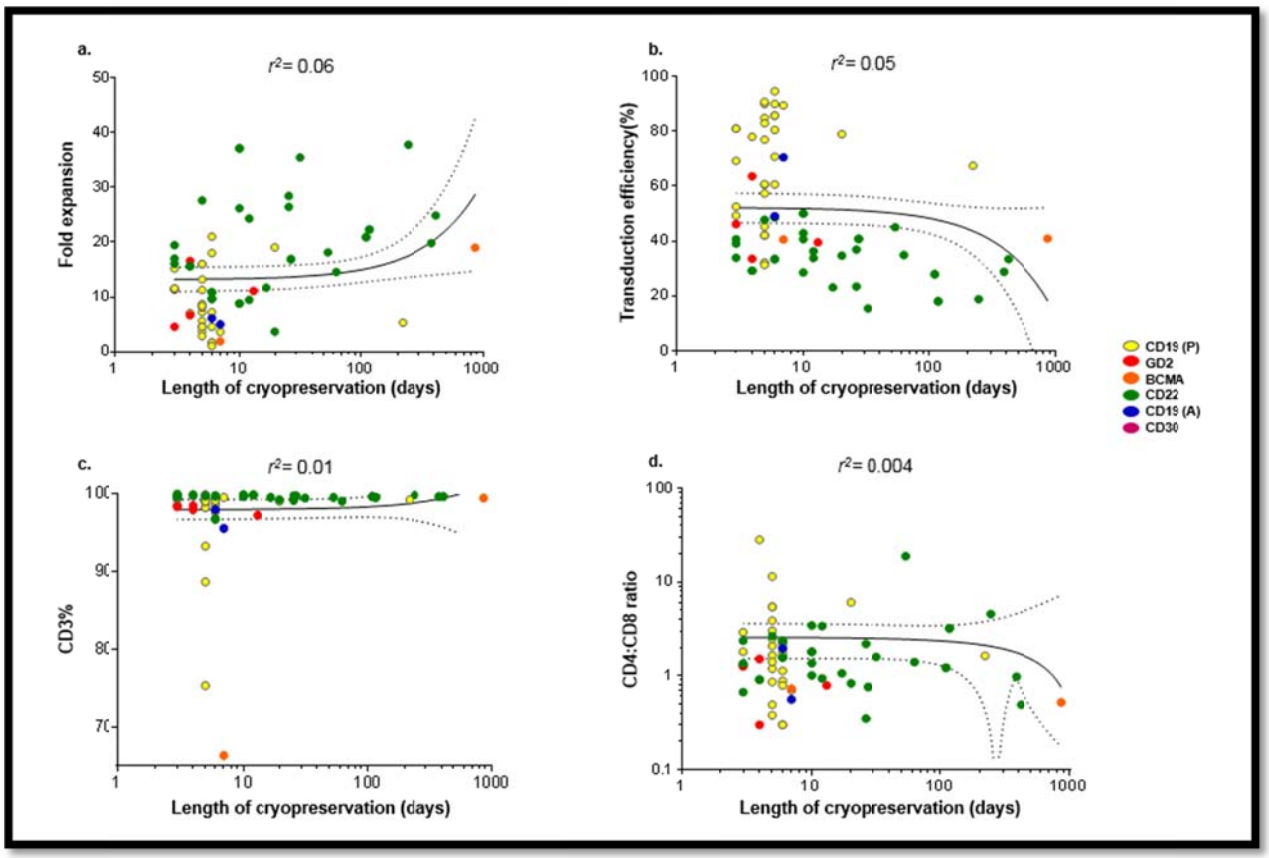

Figure S1. Results of a multivariate analyses demonstrated no impact of the duration of cryopreservation on the outcome variables: FE (a), TE (b), CD3% (c) and CD4:CD8 ratio (d).

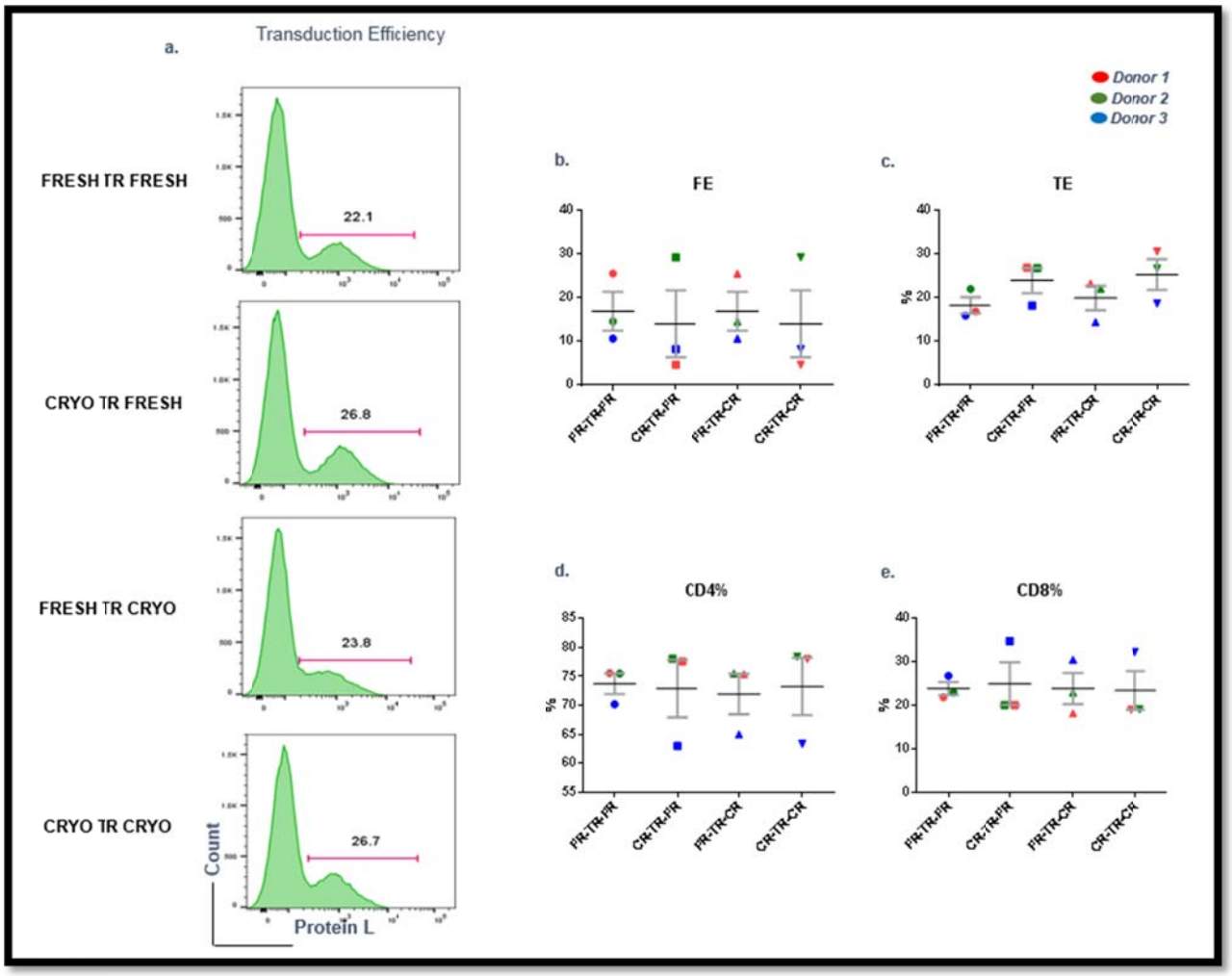

Figure S2. T-cell characteristics in 3 healthy volunteer donor CART cultures subjected to 4 manufacturing schemes- (i) Fresh-transduced-fresh, (ii) Cryo-transduced-fresh, (iii) Fresh-transduced-cryo, or (iv) Cryo-transduced-cryo. Panel a shows TE measured in the 4 culture arms for Donor 2. Panels b,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

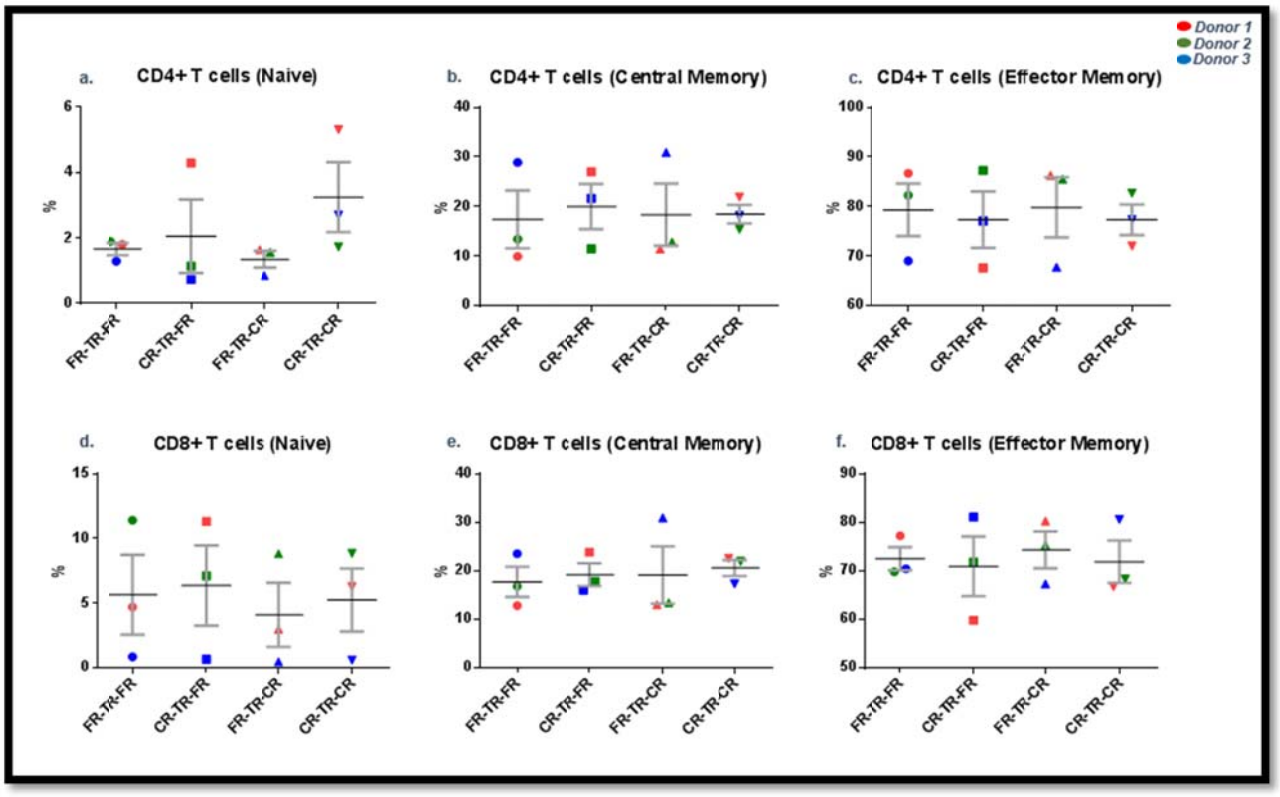

Figure S3. T-cell subsets, CD4 (a,b,c) and CD8 (d,e,f)) in the 3 healthy volunteer donor CART cultures using 4 combinations of fresh and cryo-thawed cell culture methods- (i) Fresh-transduced-fresh, (ii) Cryo-transduced-fresh, (iii) Fresh-transduced-cryo, or (iv) Cryo-transduced-cryo.