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PACT Project:

Multiple Transient Warming Events Did Not Affect Stability of Gene Modified Virus Specific T cells and Hematopoietic Progenitor Cell Products Manufactured in the Center for Cell and Gene Therapy, Baylor College of Medicine

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Cryopreserved cellular therapy products are stored in vapor phase liquid nitrogen (LN_2) tanks to ensure acceptable recovery and potency of cells after the preservation. Short exposure to ambient temperatures, or transient warming (TW), may decrease the recovery and potency of cells; therefore, TW should be minimized during cellular product storage, transportation and administration. In this study we have enumerated the number of transient warming events (TWE) for cryopreserved Hematopoietic Progenitor Cells (HPC) and T-cell products during long-term storage in Good Manufacturing Practices (GMP) Facility at the Center for Cell and Gene Therapy (CAGT) at Baylor College of Medicine (BCM). In the second part of this report we have subjected three chimeric antigen receptor-expressing T cells to multiple TWEs and assessed cell viability, phenotype and function after thaw. We found that multiple cycles of TW did not affect viability, phenotype and function of cryopreserved T-cell and HPC products.

Methods

Retrospective analysis of TWEs for T-cell products and HPC

Using the CryoTRAK electronic records database (BCM, Houston, TX) used in CAGT GMP facility we analyzed the number of TWEs for the T cell and HPC products during their storage period in vapor phase in a LN_2 freezer (MVE Cryo-Preservation Freezers, Burnsville MN).

For T-cell products a TWE was considered as a removal of the rack in which the cells were located during storage in LN_2. For HPC products a TWE occurred when the section of LN_2 freezer was opened for the addition or removal of any of cell products into that section.

T-cell product specimens

To determine the number of TWEs that may be experienced by T-cell products we performed a retrospective analysis of CryoTRAK records for three varicella zoster virus (VZV)-specific T-lymphocyte products genetically-modified to express chimeric-antigen receptor specific to disialoganglioside (GD2) tumor antigen (GD2CAR-VZVSTs) (manufactured for NCT01953900);

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and two Epstein-Barr Virus-specific T-cell (EBVST) products (manufactured for NCT01555892). These T-cell products were stored for up to 31 months.

To examine the effects of TWEs on cell phenotype and function, three GD2CAR-VZVST products were subjected to multiple cycles of TW. Two vials of each cryopreserved product were analyzed. One vial was subjected to six TW cycles (5 minutes at ambient temperature and 10 minutes in LN_2 (**Figure 1**). The second vial was analyzed immediately after thaw as a control.

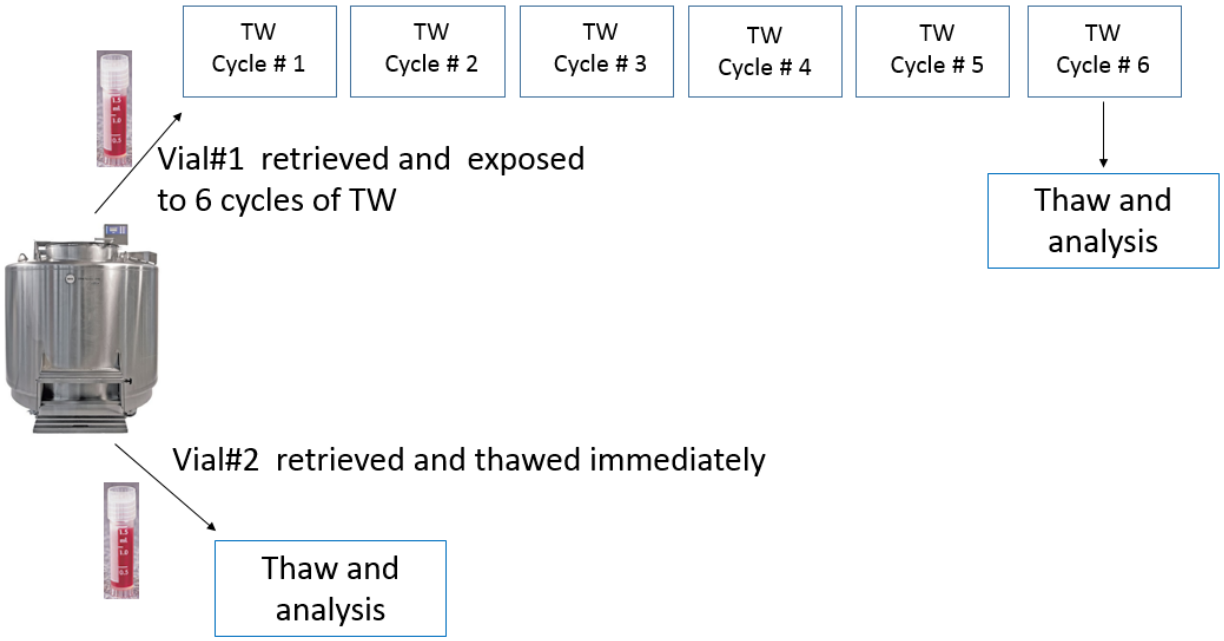

Figure 1. Experimental design for analysis of transient warming effects on T-cell products. Two vials of each of three GD2.CAR-VZVSTs were thawed at the same time. One vial was exposed to six cycles of transient warming (5 minutes at ambient temperature and 10 minutes in liquid nitrogen) prior to thaw. Cells were analyzed for viability with 7-AAD staining, CD3/4/8 T-cell markers, GD2.CAR transgene expression by flow cytometry and IFN-g ELISPOT for function and specificity for VZV antigens.

T cell analysis

Phenotypic, subset and transgene

The GD2.CAR transgene expression was analyzed using an idiotypic antibody to the ScFv (1A7) and rat anti-mouse IgG PE antibody. Transgene expression, viability (7-AAD staining) and CD3/4/8 expression were analyzed by flow cytometry.

Functional, IFN production in response to antigen stimulation

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Interferon-g (IFN-g) production in response to antigenic challenge was analyzed by enzyme-linked immunospot (ELISPOT) assay with overlapping pepmix libraries spanning VZV antigens (gE, IE62, IE63, ORF10 and ORF68) as previously described (Sun *et al*, 2016)

HPC products

We analyzed the numbers of TWEs for three cryopreserved HPC products (plasma-reduced apheresis cells, donor lymphocyte infusion (DLI) cells from plasma-reduced apheresis product and buffy coat-enriched marrow). These products had been stored in the vapor phase of LN_2 freezers for 17 to 66 months and their viability, phenotype and recovery were assessed in an annual stability study.

Data analysis

Data was analyzed in GraphPad Prism Version 6.02. The average ± standard deviation is shown. Data were analyzed by the two-way ANOVA or *t*-test.

Results

Retrospective analysis of TWEs for T-cell products and HPC products

We analyzed the number of TWE during storage in LN_2 freezers for each cryopreserved T-cell sample and HPC product evaluated. This was performed retrospectively by examining the freezer inventory database (CryoTRAK) to determine the numbers of products added and removed from the freezers in which the test products were stored.

For T-cell products every time a sample(s) was added or removed from the rack in which test cells were located was considered as one TWE. Removal or addition of multiple samples of the same component was also considered as one event. We have analyzed the number of TWE for three GD2.CAR-VZVST cell products and two EBVST products, during their storage in LN_2 freezers (**Table 1**). T cell products are typically stored in cryovial vials located in boxes stacked in inventory racks. **Table 1** summarizes LN_2 storage periods for each product and number of times the rack (in which they were stored) was subjected to TW. We found that during storage periods ranging from 16 to 31 months, these five products were exposed to a mean of 218±149 (n=5) TWE. Cells maintained their viability and potency (data summary is presented in Tables 3-6 for vial #2 of each GD2.CAR-VZVST sample tested and not shown for EBVSTs).

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Table 1. Number of TWE during Storage of T-cell Products.

T cell product	Storage time period	Total number of times rack removed
Product 1 GD2.CAR-VZVST	20 months	277
Product 2 GD2.CAR-VZVST	20 months	46
Product 3* GD2.CAR-VZVST	18 months	350
Product 4 EBVSTs	31 months	71
Product 5 EBVSTs	16 months	348

*This sample was used in multiple transient warming cycles experiment only.

For three HPC products, (plasma-reduced apheresis cells, donor lymphocyte infusion (DLI) cells from a plasma-reduced apheresis product, and buffy coat-enriched marrow cell product) (**Table 2**) we analyzed TWE. These cells were stored in LN_2 for up to 66 months and were subjected on average to 105±69 TWE (calculated for the section in which the test cells were stored).

Table 2. TWEs analysis for HPC products.

HPC product	Storage time period	Total number of times rack removed or added into the section with the product
Buffy coat enriched marrow	66 months	211
Plasma reduced apheresis	55 months	86
T cells apheresis plasma reduced	17 months	17

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Transient warming cycles

Although TW of T-cell products in storage appeared to have minimal impact, additional TWEs of longer duration occur during verification of product identity before infusion. We have timed TWEs for T-cell products retrieved from the LN_2 freezer for infusions on five different protocols. T-cell products were exposed to ambient temperature (1) during product retrieval and transfer from LN_2 storage into LN_2 Dewar (average 3.8 ± 1.3 minutes, n=5), and (2) immediately before thaw in a water bath at the clinical site (average 1.6±0.4 minutes, n=5). Total average time for all products at room temperature was 5.4 ± 1.4 minutes, n=5.

Additional TWEs for a product may occur due to infusion cancellations and transfer between LN_2 freezers. To simulate possible multiple TWEs we have exposed three GD2.CAR-VZVST products to 6 cycles of TW, *i.e.,* 5 minutes at ambient temperature followed by 10 min in LN_2. A second vial of each product was used as a control and thawed and analyzed immediately after removal from LN_2 freezer. Both vials were analyzed in parallel. The experimental design is outlined in **Figure 1**.

The viability of fresh (prior to cryopreservation) GD2.CAR-VZVSTs was 90.0±3.0% (n=3). After thaw the viability was 88.0±3.1% (n=3) and 84.4±1.0% (n=3) without and with 6 TW cycles respectively. Overall the viability was not significantly affected by 6 cycles of TW (p=0.05 by two-way ANOVA analysis) and stayed above 70% release criterion for T-cell products.

The surface expression of T-cell markers is summarized in **Tables 3 and 4** and was not significantly affected by TW (p=0.3 for % $CD3^+CD4^+$ T-helper cells and p>0.9 for $CD3^+CD8^+$ cytotoxic T lymphocytes).

Table 3. Frequencies of $CD3^+CD4^+$ T Cells in three GD2.CAR-VZVST samples.

	% $CD3^+CD4^+$ Product #1	**% $CD3^+CD4^+$ Product #2**	**% $CD3^+CD4^+$ Product #3**
Prior to cryopreservation	24.8	57.5	70.5
vial #1 (thawed after 6 TW cycles)	24.7	54.7	66.8
vial #2 (thawed without TW)	26.3	58.3	66.8

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Table 4. Frequencies of $CD3^+CD8^+$ T cells in three GD2.CAR-VZVST samples.

	% $CD3^+CD8^+$ Product #1	**% $CD3^+CD8^+$ Product #2**	**% $CD3^+CD8^+$ Product #3**
Prior to cryopreservation	68.0	27.7	17.7
vial #1 (thawed after 6 TW cycles)	65.0	26.5	16.1
vial #2 (thawed without TW)	63.7	25.7	16.1

We next analyzed the effects of multiple TW cycles on transgene expression by the GD2.CAR-VZVSTs. Expression of GD2.CAR was lower in thawed cells with (62.3±20.8%, n=3) or without TW (61.1±21.9%, n=3) compared to fresh cells (71.1±18.8%, n=3). However, there was no significant difference (p=0.28) between thawed cells with or without cycles of TW and percentage of transgene-positive T cells stayed above the release criterion of 20%. Overall cryopreservation diminished the expression of transgene, however TW did not affect frequency of GD2.CAR-positive cells.

We also tested whether multiple cycles of TW would affect the antigen-specific function of GD2.CAR-VZVSTs in ELISPOT assays that measured their ability to secrete IFN-g in response to viral antigens (**Table 5**). TW did not significantly (p=0.45) affect the VZV-specific responses of GD2.CAR-VZVSTs and they stayed above the release criterion, *i.e.,* 15 spot forming cells (SFC)/10^5 T cells.

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Table 5. VZV specificities for Three Analyzed Products (SFC/10^5 cells).

	# IFNg SFC/10^5 T cells in Product #1	**# IFNg SFC/10^5 T cells in Product #2**	**# IFNg SFC/10^5 T cells in Product #3**	**Average±StDev**
Prior cryopreservation	229	604	3457	1430±1765
vial #1 (thawed after 6 TW cycles)	177	597	2961	1245±1501
vial #2 (thawed without TW)	263	497	3692	1484±1916

The effects of TWE on the three HPC products were evaluated during the 2016 annual stability evaluation. The results acceptable recoveries for viable total nucleated cells (mean 72%), viable CD34 (mean 76.4%) (for bone marrow and apheresis cells) and $CD3^+$ T cell (42.8%) for the DLI product.

Overall, multiple cycles of TWEs did not affect the viability, phenotype and function of cryopreserved genetically-modified virus-specific T-cell products and HPCs.

References

1. Sun J., Huye L.E., Lapteva N., Mamonkin M., Hiregange M., Ballard B., Dakhova O., Raghavan D., Durett A.G., Perna S.K., Omer B., Rollins L.A., Leen A.M., Vera J,F., Dotti G., Gee A.P., Brenner M.K., Myers G.D., Rooney C.M. Early Transduction Produces Highly Functional Chimeric Antigen Receptor-Modified Virus-Specific Cells with Central Memory Markers: A Production Assistant for Cell Therapy (PACT) Translational Application, *Journal of Immunotherapy for Cancer*. 2015 Feb 18;**3**:5.