Molecular Therapy
Commentary

Fresh versus Frozen: Effects of Cryopreservation on CAR T Cells

Patrick J. Hanley[1]

https://doi.org/10.1016/j.ymthe.2019.06.001

Chimeric antigen receptor T cell (CART) products have demonstrated promise as a treatment for relapsed or refractory pediatric acute lymphocytic leukemia (ALL) and adult relapsed or refractory diffuse large B cell lymphoma (DLBCL). Logistical complexities, requisite release testing, and other considerations currently favor a centralized manufacturing approach where the apheresis product is shipped fresh or frozen to a centralized manufacturing site and the final product is manufactured and cryopreserved, shipped frozen to the infusing center, and then infused. Data from Novartis and Kite have demonstrated a robust viability post-thaw of the cryopreserved products but it is largely unknown how these results compare to the product prior to cryopreservation. In this issue of *Molecular Therapy*, Panch et al.[1] evaluated fresh versus thawed peripheral blood mononuclear cell (PBMNC) products, as well as fresh versus thawed final CART cell products. They observed a decrease in cell viability within 2 days of thawing the cryopreserved PBMC but did not observe a difference in cell expansion, transduction efficiency, percentage of CD3% cells, or CD4:CD8 ratios. The study also reported elevated expression of mitochondrial dysfunction, cell cycle damage pathways, and apoptosis signaling in cryopreserved CART products, revealing potential differences in fresh versus cryopreserved products from healthy donors. Despite these differences, the authors reported similar *in vivo* persistence and clinical outcomes in patients treated with fresh, cryopreserved, and thawed CARTs.

The approval of Kymriah and Yescarta by the US Food and Drug Administration (FDA), as well as other regulatory agencies in Europe and Australia, indicate that cryopreserved CART products are efficacious, but differences between fresh and cryopreserved CART products have not been extensively studied in humans. Chong et al.[2] reported recently that in ALL and DLBCL patients receiving cryopreserved CARTs, there was no relationship between viability and clinical response, albeit with a favorable viability range between 73.7% and 98.4%. Previous cellular therapies—including antigen-specific T cells,[3] tumor-infiltrating lymphocytes (TILs),[4] and CARTs[5–7]—have required high viability in cryopreserved and thawed samples and thus clinical efficacy has not forced a widespread evaluation of whether fresh T cells would be more efficacious. However, this is not true for all cellular therapies. Initial reports in mice demonstrated that cryopreserved natural killer (NK) cells were inferior to fresh NK cells,[8] and this observation was later substantiated in a clinical trial testing the safety and efficacy of NK cells in multiple myeloma patients. Cryopreserved NK cell products exhibited poor potency and recovery post-thaw and robust NK cell expansion was only seen in patients infused with fresh NK cells.[9] Similarly, cryopreserved and thawed mesenchymal stromal cells (MSCs), which have been used extensively worldwide as a treatment for graft-versus-host disease and other inflammatory diseases with promising results, have demonstrated impaired ability to inhibit T cell proliferation, albeit in *in vitro* studies.[10]

Despite promising outcomes with CARTs from a diverse group of academic centers and companies using cryopreserved CARTs, recent data from centers manufacturing their own CARTs—particularly those using the Miltenyi Prodigy device—have reported viability as low as 47.2% (range: 47.2% - 68.9%) post-thaw, which is inconsistent with viability data and release criteria published by other centers and for other cellular immunotherapies (B.D. Johnson, 2019, Transplantation & Cellular Therapy Annual Meeting, conference).[2,5–7] Therefore, a formal assessment of the impact of cryopreservation on CART parameters and subsequent clinical outcomes would be highly valuable and could impact logistical considerations, clinical trial design, testing requirements, and infusion timelines of future studies.

Panch et al.[1] designed their elegant study to retrospectively analyze data from 6 of their single-center CART clinical trials. These CART products were manufactured at the Center for Cellular Engineering at the NIH and infused to adult and pediatric patients with hematologic malignancies or solid tumors who were enrolled in National Cancer Institute Institutional Review Board (IRB)-approved protocols. In addition to analyzing available data from patient-specific products, they also performed manufacturing runs from 3 healthy donors to compare cell surface markers and genetic signatures of fresh versus cryopreserved CART products. Overall, they addressed two key questions: (1) what is the effect of cryopreservation on PBMNCs that will be used to generate a final CART product, and (2) what is the effect of cryopreservation on the final CART product?

Of 147 infusates, the starting PBMNC fraction was cryopreserved for 70 infusions. Two days after thawing, the viable total nucleated cell percentage was significantly lower in the cryopreserved fraction. Despite the difference in viability, the cell yield was sufficient in all products to proceed with

[1]Program for Cell Enhancement and Technology for Immunotherapy, Center for Cancer and Immunology Research, Center for Cancer and Blood Disorders, Children's National Health System, and The George Washington University, Washington, DC 20010, USA

Correspondence: Patrick J. Hanley, PhD, Program for Cell Enhancement and Technology for Immunotherapy, Center for Cancer and Immunology Research, Center for Cancer and Blood Disorders, Children's National Health System, and The George Washington University, 111 Michigan Ave., Washington DC 20010, USA.
E-mail: phanley@childrensnational.org

manufacturing and ultimately the cryopreserved PBMNC fraction did not demonstrate a significant difference in fold-expansion, transduction efficiency, percentage of T cells, or CD4:CD8 ratio at the time of CART harvest.

Seventy-nine CART products were also cryopreserved (including 50 products where both the PBMNC and CART final product were both cryopreserved). When compared to the analysis of the same fresh CART products pre-cryopreservation, there were no significant differences in the percentage of T cells, transduction efficiency, and CD4:CD8 ratios pre- and post-cryopreservation and thaw. These results are similar to those published by Xu et al.[11] in a NOD/SCID model testing human B cell maturation antigen (BCMA)-specific CARTs that were fresh or cryopreserved and thawed. They also observed differences in cryopreserved T cells *in vitro*, but these differences were not observed in an *in vivo* NOD/SCID model.

In 2 CART protocols, Panch et al.[1] had a sufficient number of patients to statistically compare fresh and cryopreserved and thawed CART infusions. *In vivo*, peak levels of the CD22 CARTs were higher in the fresh CART group, but there was no difference in persistence.

To validate the previous findings and further evaluate the impact of cryopreservation at a gene-expression level, the authors performed manufacturing runs using 3 healthy donors and compared these products before and after cryopreservation and thaw. After analysis, 1,139 genes were identified that were overexpressed in the cryopreservation and thawed CARTs. These included genes related to apoptosis and cell-cycle damage.

One of the strengths of this manuscript is that it encompasses 6 different clinical protocols, a heterogeneous patient population, and a diverse range of CART constructs ranging from gamma retroviruses to lentiviruses and populations isolated by elutriation, (and/or) bead enrichment, or ficoll. Still, one caveat is that the manufacturing was all performed by the Center for Cellular Engineering: a large volume, highly skilled manufacturing center with years of experience with CARTs. How these data translate to centers with less experience that use more automated procedures such as CARTs manufactured on the Prodigy machine is a major caveat that requires further investigation.

The success of CARTs has been transformative for the field of cellular therapy, but the field itself is still in its infancy. Critical evaluations such as those presented in this issue of *Molecular Therapy* will play a pivotal role in influencing whether centralized manufacturing is a feasible, sustainable, and cost-effective model. Using fresh starting material may have recognized advantages, but the ability to cryopreserve the starting material also allows for a more efficient—and likely less expensive—manufacturing process that can be more easily managed and scheduled in industry and academic cellular therapy laboratories alike.

Based on this report by Panch et al.[1], cryopreservation does impact PBMNCs and CARTs, but in a way that seems to be compensated for by exposure to antigen and the appropriate microenvironment. All things considered, maybe frozen is good enough.

CONFLICTS OF INTEREST

P.J.H. holds equity and serves on the board of directors of Mana Therapeutics and has received honoraria from Dava Oncology.

REFERENCES

1. Panch, S.R., Srivastava, S.K., Elavia, N., McManus, A., Liu, S., Jin, P., Highfill, S.L., Li, X., Dagur, P., Kochenderfer, J., et al. (2019). Effect of Cryopreservation on Autologous Chimeric Antigen Receptor T Cell Characteristics. Mol. Ther. *27*, this issue, 1275–1285.

2. Chong, E.A., Levine, B., 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-Hodkin Lymphomas and B-Cell Acute Lymphoblastic Leukemia. Blood *132* (*Suppl 1*), 197.

3. Tanna, J.G., Ulrey, R., Williams, K.M., and Hanley, P.J. (2019). Critical testing and parameters for consideration when manufacturing and evaluating tumor-associated antigen-specific T cells. Cytotherapy *21*, 278–288.

4. Hopewell, E.L., Cox, C., Pilon-Thomas, S., and Kelley, L.L. (2019). Tumor-infiltrating lymphocytes: Streamlining a complex manufacturing process. Cytotherapy *21*, 307–314.

5. Roddie, C., O'Reilly, M., Dias Alves Pinto, J., Vispute, K., and Lowdell, M. (2019). Manufacturing chimeric antigen receptor T cells: issues and challenges. Cytotherapy *21*, 327–340.

6. Elavia, N., McManus, A., Highfill, S.L., Ren, J., Shah, N.N., Fry, T.J., Brudno, J., Kochenderfer, J.N., Stroncek, D., and Panch, S.R. (2017). The Post-Thaw Recovery of Cryopreserved Chimeric Antigen Receptor (CAR) T-cells during Manufacture Is Better Than That of Cryopreserved Peripheral Blood CD3+ Cells. Blood *130*, 4475.

7. Kalos, M., Levine, B.L., Porter, D.L., Katz, S., Grupp, S.A., Bagg, A., and June, C.H. (2011). T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. Sci. Transl. Med. *3*, 95ra73.

8. Miller, J.S., Rooney, C.M., Curtsinger, J., McElmurry, R., McCullar, V., Verneris, M.R., Lapteva, N., McKenna, D., Wagner, J.E., Blazar, B.R., and Tolar, J. (2014). Expansion and homing of adoptively transferred human natural killer cells in immunodeficient mice varies with product preparation and in vivo cytokine administration: implications for clinical therapy. Biol. Blood Marrow Transplant. *20*, 1252–1257.

9. Szmania, S., Lapteva, N., Garg, T., Greenway, A., Lingo, J., Nair, B., Stone, K., Woods, E., Khan, J., Stivers, J., et al. (2015). Ex vivo-expanded natural killer cells demonstrate robust proliferation in vivo in high-risk relapsed multiple myeloma patients. J. Immunother. *38*, 24–36.

10. François, M., Copland, I.B., Yuan, S., Romieu-Mourez, R., Waller, E.K., and Galipeau, J. (2012). Cryopreserved mesenchymal stromal cells display impaired immunosuppressive properties as a result of heat-shock response and impaired interferon-γ licensing. Cytotherapy *14*, 147–152.

11. 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.