CAR T cell generation from cryopreserved leukapheresis products in a 7-day process

CliniMACS Prodigy® T Cell Transduction

Background

Chimeric antigen receptor (CAR) T cell therapy is revolutionizing cancer treatment, particularly hematologic malignancies, generating significant interest in immunotherapy.[1,2] However, the manufacturing process of engineered T cells remains complex and labor-intensive, with additional challenges in hospital logistics and economic feasibility. The use of frozen starting materials and the development of more flexible, shortened manufacturing processes offer promising solutions to these challenges.[3,4]

In this study, CAR T cells were successfully generated from cryopreserved leukapheresis products within a 7-day manufacturing process (fig. 1). T cells were isolated, activated, transduced, expanded, rebuffered, and harvested using the CliniMACS Prodigy T Cell Transduction (TCT). Throughout the process, flow cytometry–based in-process controls, evaluated with the MACSQuant® Analyzer, ensured reliable quality control of the cells.

Two different CAR constructs were used to evaluate the feasibility of the method: a CD19 CAR and a CD19/CD22 tandem CAR (TanCAR). The results for both constructs were consistent in terms of transduction efficiency, expansion rate, and viability. Cytotoxicity was further confirmed by *in vitro* and *in vivo* testing.

Materials and methods

Note: Using frozen leukapheresis products as the starting material for the TCT process is not within the official specifications for this application.

Thawing and characterization of frozen leukapheresis products

Frozen leukapheresis product was thawed in a 36 °C water bath. After thawing, the cells were diluted at a 1:3 ratio with TexMACS™ GMP Medium supplemented with 3% AB serum and 12.5 ng/mL MACS® GMP Recombinant Human IL-7 and IL-15.

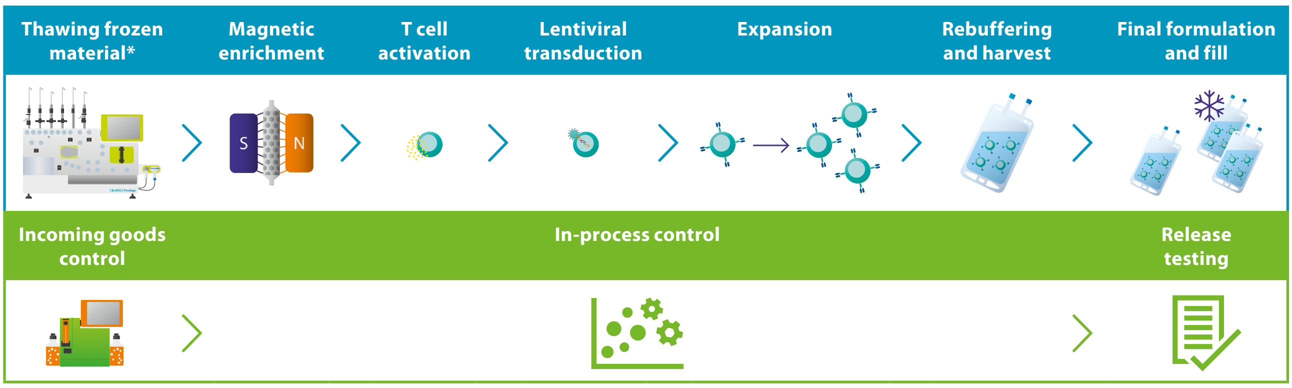

Figure 1: Integrated manufacturing and in-process control and release testing (IPC/RT) workflows enable efficient generation of CAR T cells from cryopreserved leukapheresis products within 7 days.
* Please note that thawing of frozen starting material is not included in the TCT process.

Samples were taken and stained with individual liquid antibodies corresponding to the dried StainExpress™ Immune Cell Composition Cocktail and analyzed on the MACSQuant Analyzer, using the Immune Cell Composition Express Mode (part of the CAR T Cell Express Mode Package).

CAR T cell manufacturing

Thawed and diluted leukapheresis product was loaded onto the CliniMACS Prodigy Instrument and processed using the TCT process, which encompasses all key steps of CAR T cell manufacturing: T cell enrichment, activation, transduction, expansion, rebuffering, and harvest.

The process began with $CD4^+$ and $CD8^+$ T cell enrichment via dual-labeling and magnetic cell separation using CliniMACS® CD4 and CD8 Reagents. The isolated $CD4^+$ and $CD8^+$ T cells were eluted in TexMACS GMP Medium supplemented with 3% AB serum and 12.5 ng/mL MACS GMP Recombinant Human IL-7 and IL-15, followed by activation with MACS GMP T Cell TransAct™ Reagent.

After activation, the T cells were transduced with a proprietary CAR lentiviral vector and expanded in culture for seven days. The TCT process also automatically performed a culture wash on day 3, and media exchanges on days 5 and 6 (table 1). On day 7, the cells were rebuffered in CliniMACS Formulation Solution and harvested.

Day	Activity	Note
0	Magnetic separation	$CD4^+$ and $CD8^+$ cell enrichment
0	Activation	Part of culture setup
1	Lentiviral transduction	
3	Culture wash	2 cycles
3	Activate shaker	Type 2
5	Medium exchange	Remove 50 mL/add 50 mL
6	Medium exchange	Remove 130 mL/add 130 mL
6	Activate shaker	Type 3
6	Medium exchange	Remove 130 mL/add 130 mL
7	Harvest	Rebuffer in CliniMACS Formulation Solution and harvest

Table 1. Overview of the different steps in the TCT process used in this study.

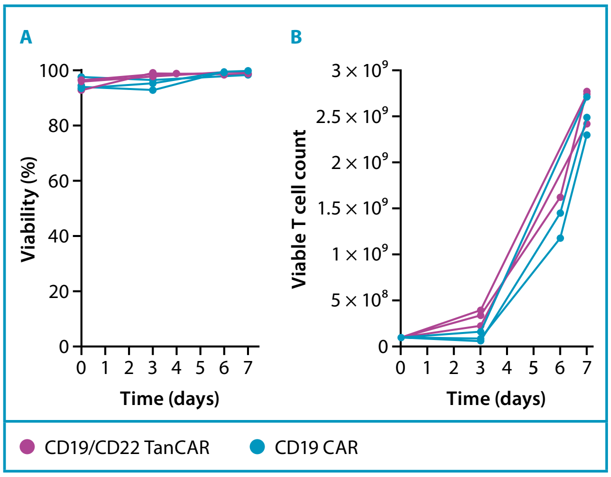

Figure 2: Viability (A) and cell expansion (B) of the cells at different time points.

Flow cytometric analysis of key parameters

Throughout the CAR T cell manufacturing process, the purity, transduction efficiency, and CAR T cell count was assessed by flow cytometry. Cells were stained with the CD19 CAR Detection Reagent (Miltenyi Biotec) alongside the antibody panel of the StainExpress Immune Cell Composition and CAR T Transduction Cocktails.

For standardized and automated acquisition and analysis, the MACSQuant Analyzer was used with corresponding Express Modes (Immune Cell Composition and CAR T Cell Transduction) to evaluate these parameters.

Vector copy number (VCN) analysis

To quantify the number of integrated transgene copies per transduced cell, genomic DNA was extracted using the DNeasy® Blood & Tissue Kit (QIAGEN®). The MACS COPYcheck Kit was then used to amplify an amplicon of the lentiviral gag gene and the human reference gene PTBP2 via quantitative PCR. The VCN per transduced cell was calculated using the following formula:

$$\text{VCN per transduced cell} = \frac{\text{qPCR copy no.} / \mu\text{L (gag)}}{\text{qPCR copy no.} / \mu\text{L (PTBP2)}} \div \frac{\text{transduction freq. (\%)}}{100\%} \times 2$$

In vitro cytotoxicity assay

To assess tumor-killing activity, CAR T cells were cocultured with GFP-positive target cells at different effector-to-target (E:T) ratios for 16 hours at 37 °C and 5% CO_2 in TexMACS GMP medium. GFP-positive target cells with a knockout of the CAR-targeted antigen were used as a negative control. Cytotoxicity was determined by counting the remaining GFP-positive target cells using flow cytometry.

Cytokine secretion assay

After 16 hours of coculture supernatants were collected and analyzed for IL-2, IFN-γ, and TNF-α using the MACSPlex Cytotoxic T/NK Cell Kit, human, on the MACSQuant Analyzer.

In vivo tumor targeting

For preclinical evaluation of CAR T cell functionality, luciferase-positive target cells were transplanted into NSG mice. After one week of tumor cell engraftment with 5×10^5 tumor cells, 1×10^{10} CAR T cells were injected intravenously into each mouse. Tumor burden was continuously measured as whole-body luminescence (p/s) at different time points using the IVIS system.

Results

Enabling 7-day CAR T cell manufacturing with frozen starting material

Viability of thawed cell products is a critical parameter during the manufacturing of CAR T cells. In this experimental setup, the thawed leukapheresis products (n=6) showed an initial viability of 93–97%. During the expansion phase, the viability increased and remained consistent, reaching 98–99% at harvest on day 7 (fig. 2A).

The engineered T cells expanded as expected, with an average of 2.5×10^9 viable T cells per sample at day 7 (fig. 2B). Importantly, the viability and expansion rates were consistent across both CAR constructs used, demonstrating that these factors were independent of the construct.

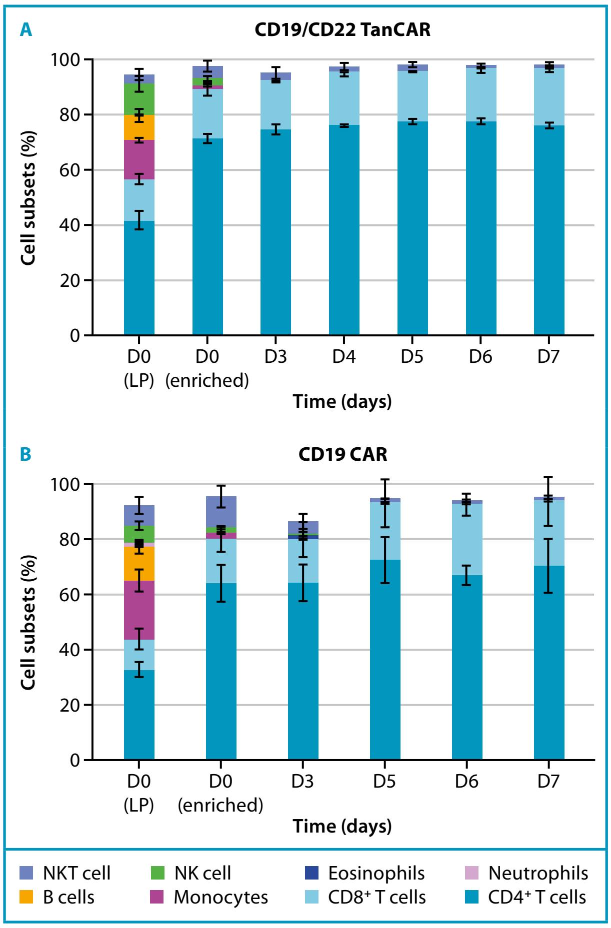

Figure 3: The average percentage of CD4⁺ and CD8⁺ cells within viable CD45⁺ cells was evaluated before (leukapheresis product, LP) and after enrichment, and throughout the expansion phase.

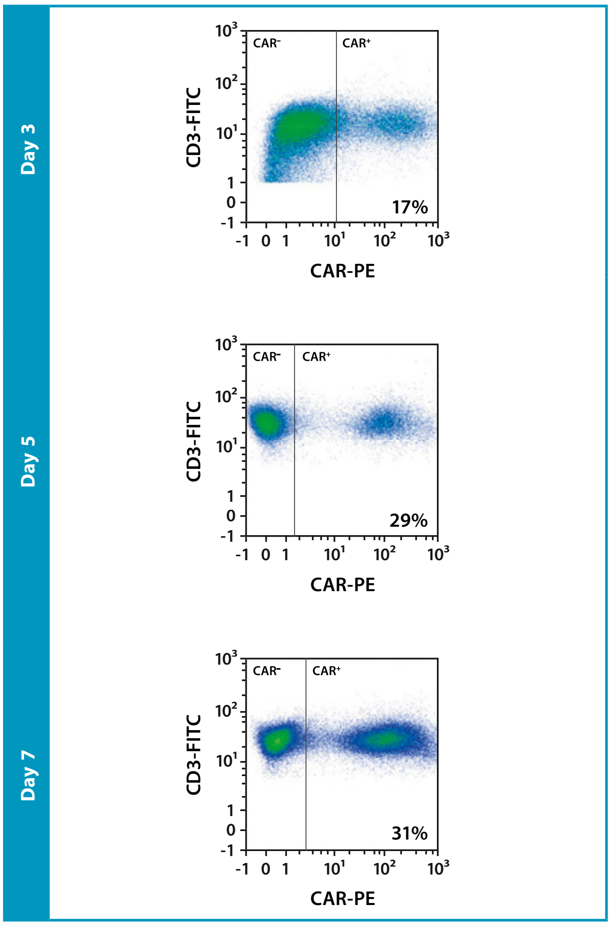

Figure 4: Flow cytometry analysis showing the frequency of CD19 CAR⁺ T cells on days 3, 5, and 7.

Efficient CD4⁺ and CD8⁺ cell enrichment via magnetic separation

Starting from frozen leukapheresis products, CD4⁺ and CD8⁺ T cells were enriched, achieving an average combined purity of 90% among viable CD45⁺ cells. A minor fraction of NK cells and NKT cells was also present due to their lower CD8 expression. Culture conditions were optimized to favor T cell outgrowth, resulting in a final CD4⁺ and CD8⁺ T cells purity of 97.4 ± 0.97% at day 7 (fig. 3).

Cryopreserved leukapheresis product as a reliable source for T cell transduction

Enriched T cells were successfully transduced with CAR constructs (fig. 4), yielding consistent transduction levels across samples, with an average frequency of 38% for the CD19/CD22 TanCAR and 35% for the CD19 CAR at day 7 (table 2). The VCN per transduced cell was below 2.5 for each donor's final product (table 2), suggesting a safe profile for the final cell product.

Days	% CAR T cells	CAR T cell count	VCN
CD19/CD22 TanCAR			
Day 3 (n=5)	Ø 30.7% ± 5.0%	Ø 0.1 × 10^9 ± 0.02 × 10^9	Ø 2.8 ± 1.0
Day 5 (n=2)	Ø 39.8% ± 10.1%	Ø 0.3 × 10^9 ± 0.01 × 10^9	Ø 1.4 ± 0.7
Day 7 (n=6)	Ø 37.9% ± 6.1%	Ø 0.9 × 10^9 ± 0.2 × 10^9	Ø 1.6 ± 0.5
CD19 CAR			
Day 3 (n=3)	Ø 17.6% ± 2.5%	Ø 0.02 × 10^9 ± 0.01 × 10^9	4.8
Day 5 (n=2)	Ø 35.4% ± 7.2%	Ø 0.3 × 10^9 ± 0.06 × 10^9	Ø 1.6 ± 0.6
Day 7 (n=6)	Ø 35.3% ± 6.1%	Ø 1.0 × 10^9 ± 0.3 × 10^9	Ø 2.2 ± 1.3

Table 2: Measured transduction efficiency, CAR T cell counts, and VCN on days 3, 5, and 7. Values are presented as mean ± SD unless otherwise noted. For CD19 CAR day 3, the VCN value reflects a single measurement (n=1).

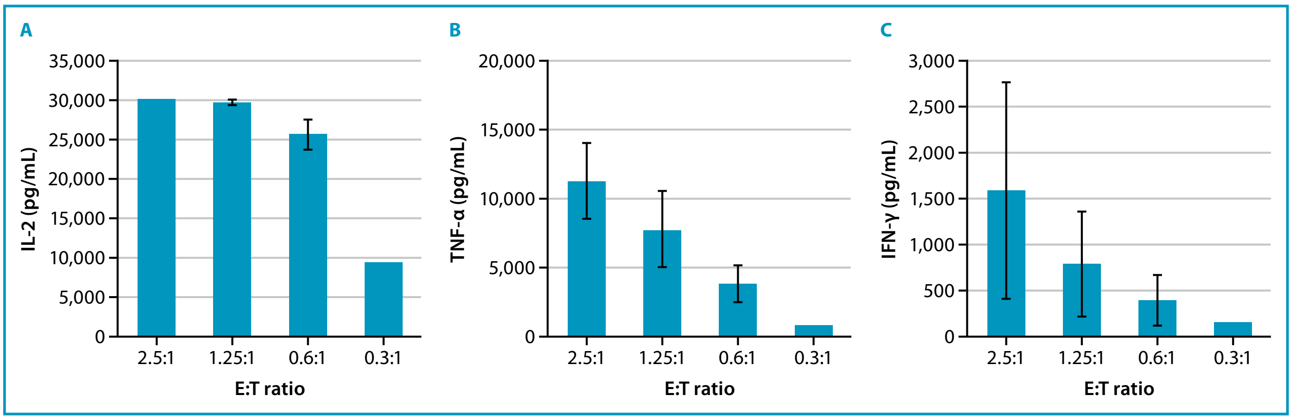

Figure 5: Cytokine secretion by CD19/CD22 TanCAR cells after co-incubation with target cells at different E:T ratios.

Cytokine secretion triggered by target cell stimulation
The engineered CAR T cells showed robust cytokine secretion upon recognition of the specific antigen. After 16 hours of coculture with their respective target cells, high levels of IL-2, IFN-γ, and TNF-α were detected in the supernatants, indicating strong CAR T cell *in vitro* functionality (fig. 5).

Strong cytotoxic function of CAR T cells from cryopreserved starting material
The potency of gene-engineered T cells was assessed through *in vitro* and *in vivo* cytotoxicity assays.

In vitro, CAR T cells showed 26% cytolytic activity at an E:T ratio of 0.3:1 and an average of 85% at 2.5:1, demonstrating a significantly higher killing rate than that observed against the knockout control (fig. 6A).

For *in vivo* studies, functionality was analyzed by monitoring tumor growth in NSG mice. CAR T cells demonstrated complete tumor clearance starting from day 15, while untreated controls showed continuous tumor growth (fig. 6B). These results highlight the potent antitumor activity of CAR T cells generated from cryopreserved leukapheresis products in a 7-day manufacturing process.

Conclusions

This study demonstrates that cryopreserved leukapheresis product is a suitable starting material for CAR T cell generation within a 7-day manufacturing process using the CliniMACS Prodigy TCT Process. The successful generation of functional CAR T cells, as assessed by viability, expansion rate, cytokine secretion, and cytotoxic activity, confirms the feasibility of this approach. These findings address key challenges in CAR T cell manufacturing, particularly the use of frozen starting materials to mitigate logistic challenges and the need for more efficient time-saving processes.

These advancements have the potential to optimize and streamline gene-engineered T cell manufacturing, reducing costs and improving scalability for future applications.

References

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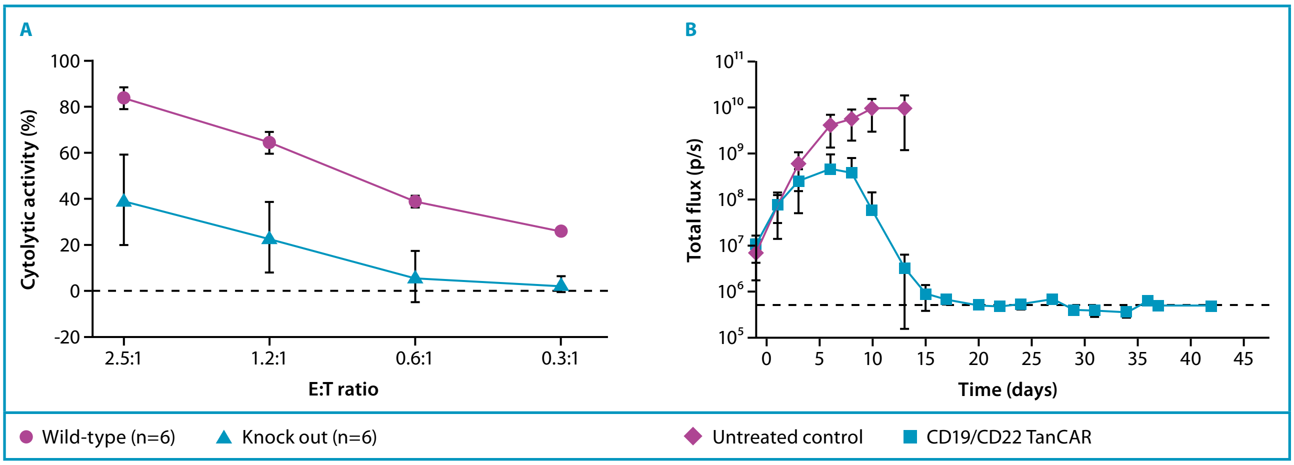

Figure 6: *In vitro* (A) and *in vivo* (B) cytolytic activity of the generated CD19/CD22 TanCAR cells.

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