Advancing CAR T Therapies:

The Importance of Closed Processes in Manufacturing and Future Innovations

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Introduction

In addition to advances in cancer treatments [1-2], T-cell therapy also represents a transformative approach to the treatment of autoimmune and rare diseases [3-5]. This form of immunotherapy involves isolating and modifying T-cells, a type of white blood cell integral to the immune response, to target diseased cells more effectively.

Two innovative forms of T-cell therapy are at the forefront of this research: Chimeric Antigen Receptor T-cell (CAR-T) therapy and T-cell Receptor (TCR) therapy. Historically, CAR-T therapy was designed to engineer T-cells to express a synthetic receptor that can directly recognize and bind to antigens on the surface of cancer cells. Once reintroduced into the patient's body, this allows the modified T-cells to attack and destroy the diseased cells. In recent years, the field has seen an increasing number of studies leveraging CAR-T cells to treat autoimmune diseases, utilizing a similar concept to identify hyperactive B cells [6-8]. TCR therapy takes a slightly different approach by modifying the natural T-cell receptors to recognize specific cancer antigens presented on the surface of tumour cells by the major histocompatibility complex (MHC). This enables the T-cells to detect and target the internal proteins of cancer cells, which can be particularly useful for solid tumours that are less accessible to CAR-T cells.

These therapies are designed to offer tailored treatments by harnessing and enhancing the immune system's ability to combat malignancies, autoimmune conditions, and rare disorders with greater efficacy. They have shown remarkable success in treating certain blood cancers and hold great potential for the treatment of solid tumours [1]. While the potential extends beyond oncology, their therapeutic capacity is impeded by significant manufacturing challenges. The biotechnological pipeline for T-cell therapies, which encompasses cell collection, genetic modification, expansion, and reinfusion, faces critical hurdles such as high therapeutic costs, patient safety concerns, and the need for standardization and automation. Additionally, issues with scalability and the pace of innovation are significant barriers to overcome. Doing so is essential for the advancement of T-cell therapies, requiring the implementation of optimized processes and workflows. To streamline production and reduce lead times, it is crucial to focus on improving process efficiency. This includes the development of products designed for Current Good Manufacturing Practice (CGMP) in cell therapy, the use of closed and automatable instrumentation, and the provision of support and scalable solutions. Furthermore, investing in innovative products that address these manufacturing challenges is critical. By tackling these issues, we can enhance the accessibility of these treatments and fulfill the potential of T-cell therapies for a wider array of health conditions, ultimately benefiting a larger patient demographic.

This Expert Insights eBook begins with a study by Liu *et al*. [9] that explores a closed, autologous bioprocess optimized for TCR-T cell therapies. The process effectively yields a high number of TCR-expressing T-cells with enriched memory phenotypes and metabolic efficiency, crucial for treating solid tumours. By integrating activation, transduction, and expansion within a single bioreactor system, this innovative approach streamlines production, reduces contamination risks, and supports scale-out feasibility. This process has the potential to transform the manufacturing landscape of personalized T-cell therapies.

The second paper is a study on a novel CAR-T cell therapy that targets DLL3, an antigen prevalent in small-cell lung cancer (SCLC). The study by Nie *et al*. [10] unveils a new multiple-chain CAR-T cell design incorporating TREM1 and DAP12 signaling domains, which shows strong potential in selectively eradicating SCLC cells. This advancement represents a significant step in precision oncology, offering a promising new treatment avenue for a particularly aggressive form of cancer.

Overall, research in T-cell therapy is an evolving landscape disease treatment, utilizing the body's immune system to enhance its ability to combat various health conditions more effectively. Breakthroughs in CAR-T and TCR-T cell engineering demonstrate remarkable potential in addressing challenging cancers, autoimmune diseases, and other rare diseases, paving the way for a new era of personalized medicine.

Through the methods and applications presented in this Experts Insights eBook, we hope to educate researchers on new technologies and techniques for advancing T-cell therapies.

To broaden your knowledge of cell therapy manufacturing advancements and explore options that can significantly improve your research outcomes, visit Thermo Fisher Scientific for access to their extensive resources and expert guidance. For more information on the Thermo Fisher content included in this eBook, please visit Thermo Fisher Cell Therapy Solutions.

Andrew Dickinson, Ph.D.
Content Strategist at Wiley.

References

[1] med.stanford.edu/news/all-news/2024/05/car-t-melanoma.html

[2] fda.gov/news-events/press-announcements/fda-approves-first-cellular-therapy-treat-patients-unresectable-or-metastatic-melanoma

[3] Schett, G. *et al*. (2024). Advancements and challenges in Car T cell therapy in autoimmune diseases. *Nat Rev Rheumatol*. DOI: 10.1038/s41584-024-01139-z

[4] Arnold, C. (2024). Autoimmune disease is the next frontier for Car T cell therapy. *Nat Med*. DOI: 10.1038/s41591-023-02716-7

[5] Ghobadinezhad, F., *et al*. (2022). The emerging role of regulatory cell-based therapy in autoimmune disease. *Frontiers in Immunology*. DOI: 10.3389/fimmu.2022.1075813

[6] Müller, F., *et al*. (2024). CD19 Car T cell therapy in autoimmune disease—a case series with follow-up. *New England Journal of Medicine*. DOI: 10.1056/NEJMoa2308917

[7] Kansal, R., *et al*. (2019). Sustained B cell depletion by CD19-targeted Car T cells is a highly effective treatment for murine lupus. *Science translational medicine*. DOI: 10.1126/scitranslmed.aav1648

[8] Arnold, C., (2024). Autoimmune disease is the next frontier for Car T cell therapy. *Nature medicine*. DOI: 10.1038/s41591-023-02716-7

[9] Liu, Y., *et al*. (2023). A closed, autologous bioprocess optimized for TCR-T cell therapies. *Biotechnology and Bioengineering*. DOI: 10.1002/bit.28389

[10] Nie, F., *et al*. (2024). TREM1/DAP12 based novel multiple chain CAR-T cells targeting DLL3 show robust anti-tumour efficacy for smal- cell lung cancer. *Immunology*. DOI: 10.1111/imm.13776

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A Closed, Autologous Bioprocess Optimized for TCR-T Cell Therapies

Adapted from Liu, Y., *et al.* 2023

A fully closed, autologous bioprocess for generating engineered T-cell receptor (TCR)-T cells is described. This process yields 5-12 × 10^9 TCR-expressing T-cells within 7-10 days, using low multiplicity of infection transduction. The resulting cells exhibit an enriched memory T-cell phenotype and enhanced metabolic fitness. The process involves activating, transducing, and expanding leukapheresed cells in a bioreactor without T-cell or peripheral blood mononuclear cell enrichment steps and achieves high T-cell purity (~97%). Several critical process parameters were investigated, including high cell density culturing (7 × 10^6 cells/mL), adjusted rocking agitations during scale-up, lowering glycolysis through 2-deoxy-D-glucose addition, and modulating interleukin-2 levels. These parameters were studied for their effects on transduction efficiency, cell growth, and T-cell fitness, including memory phenotype and resistance to activation-induced cell death. This bioprocess supports scale-out feasibility by enabling parallel processing of multiple patients' batches within a Grade C cleanroom.

Introduction

Autologous T-cell therapy has shown promise in treating hematological malignancies, with six FDA-approved CAR-T therapies currently available. However, developing effective therapies for solid tumours remains challenging. Manufacturing these personalized therapies is complex and costly, with issues including high costs of goods manufactured (COGM), inability to scale, complex logistics, long processing times, and limited patient access. Treating solid tumours requires higher doses and T-cells equipped to function within the tumour microenvironment.

Typical manufacturing processes involve multiple open steps, requiring manual cell transfers in biosafety cabinets. This increases contamination risks, affects cell viability and phenotype, and requires ISO 7 cleanrooms. Reported here is a fully closed bioprocess for autologous T-cell therapies that eliminates the need for syringes, T-cell selection steps, and static incubators. By integrating activation, transduction, and expansion in a rocking bioreactor, high transgene expression and desired T-cell yield was achieved for solid-tumour indications.

Results

Establishing a fully closed bioprocess to generate autologous TCR-T cells

Figure 1 shows key aspects of the bioprocess that yields an average of 10 × 10^9 T-cells from ~1 × 10^9 leukapheresis starting material within 7-8 days.

Key features include:

- Activation with soluble ImmunoCult CD3/28/2 activator
- Transduction at optimized multiplicity of infection (MOI) of 1 in the bioreactor
- Expansion in a single bioreactor with semicontinuous perfusion
- No T-cell or PBMC enrichment step

The process maintained glucose levels above 2 g/L and lactate below 2 g/L. TCR expression on CD8+ T-cells ranged from 30-70%. The final product purity was 97% CD3+ T-cells, regardless of the starting material composition. Harvested cells showed potent and specific cytotoxicity against target cells.

Eliminating the T-cell enrichment step resulted in comparable T-cell purity and differentiation to enriched cells, with higher transduction efficiency for various transgenes (CAR, GFP, TCR) across multiple donors.

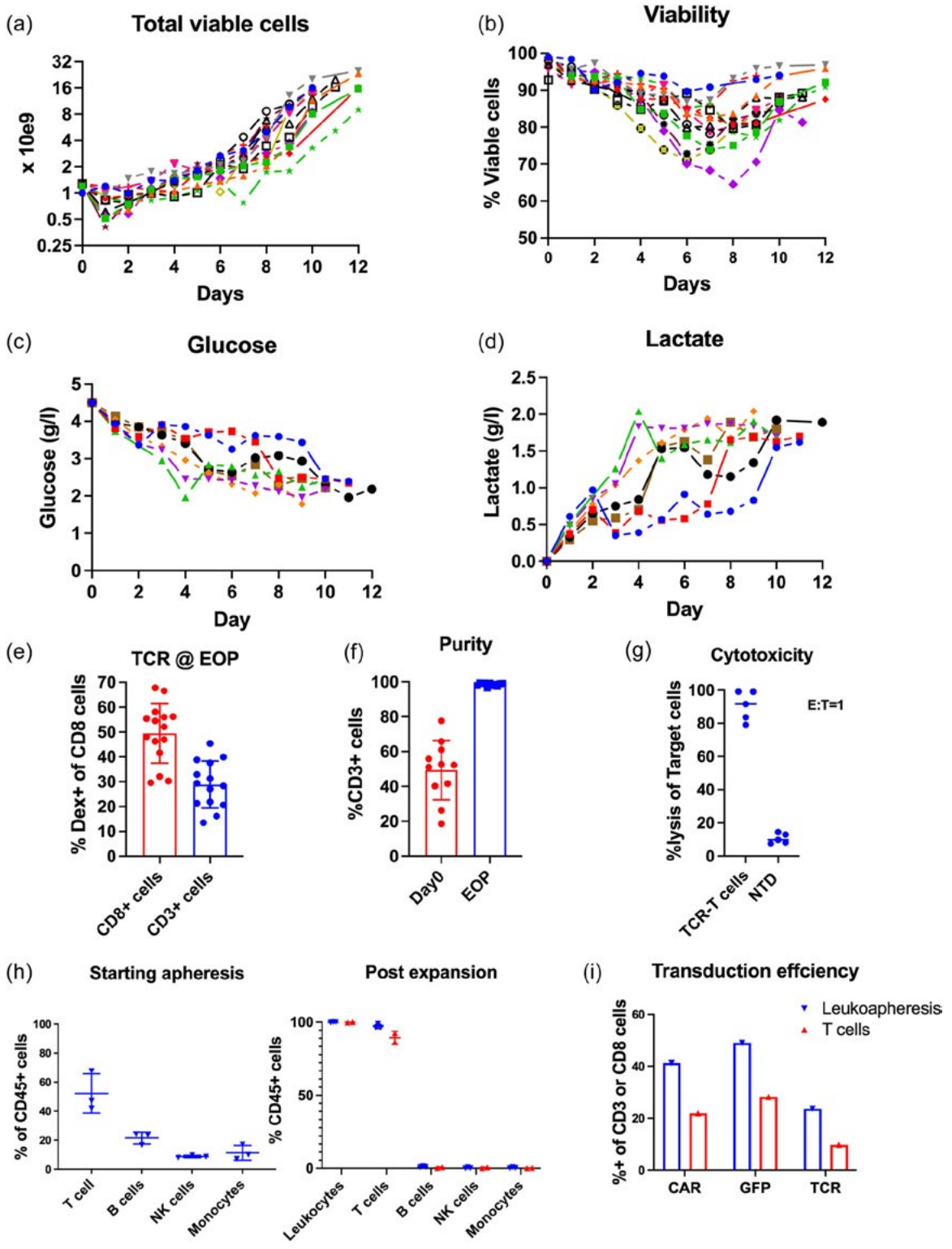


Figure 1: Process and final T-cell product characterization of a closed, autologous bioprocess. In-process measurements of total viable cells (a), percentage of viable cells **(b)**, glucose concentration **(c)**, and lactate concentration **(d)**. Each colored line represents a different donor. End-of-process (EOP) TCR expression of CD8 and CD3 T-cells detected by PE dextramer **(e)**. Donor leukapheresis and EOP CD3 T-cell purity **(f)** and cytotoxicity against MAGE-B2 target cells **(g)**. T-cell purity of leukapheresis at the start and following activation and expansion **(h, left panel)** compared with T-cell purity at EOP following CD4 and CD8 antibody positive enrichment **(h, right panel)**. Comparison of transduction efficiency of lentivirus coding for CAR, TCR, and GFP, measured at day 8 by flow cytometry, of T-cells and PBMCs derived from three healthy donors (n = 3) (i). PBMC, peripheral blood mononuclear cell.

Optimizing bioreactor parameters

Bioreactor parameters were evaluated to accelerate cell growth, including seeding density, dissolved oxygen (DO) level, and rocking agitation.

Key findings included:

- Higher agitation or direct oxygen gassing promoted cell growth and viability
- Higher inoculation density (7 × 10^6 cells/mL) led to ~15% increase in transduction efficiency
- DO feedback control from the start compromised transduction efficiency
- Enhanced oxygen mixing through rocking agitation at 500 mL culture volume maintained TCR expression levels similar to standard conditions

Cells grown with higher agitation contained significantly higher percentages of central memory T-cells (Tcm) when compared at equal yields. Overall, higher inoculation density and greater rocking agitation at 0.5 L culture volume enabled shorter expansion time with optimal T-cell phenotype and potency.

Inhibiting glycolysis skews T-cells towards a more memory phenotype

To test whether limiting glucose intake could sustain the memory phenotype of T-cells and potentially lead to increased efficacy and persistence, 2-deoxy-D-glucose (2-DG), a glycolysis inhibitor, was added with the following effects (Fig. 2):

- No inhibition of cell growth was observed with 2-DG

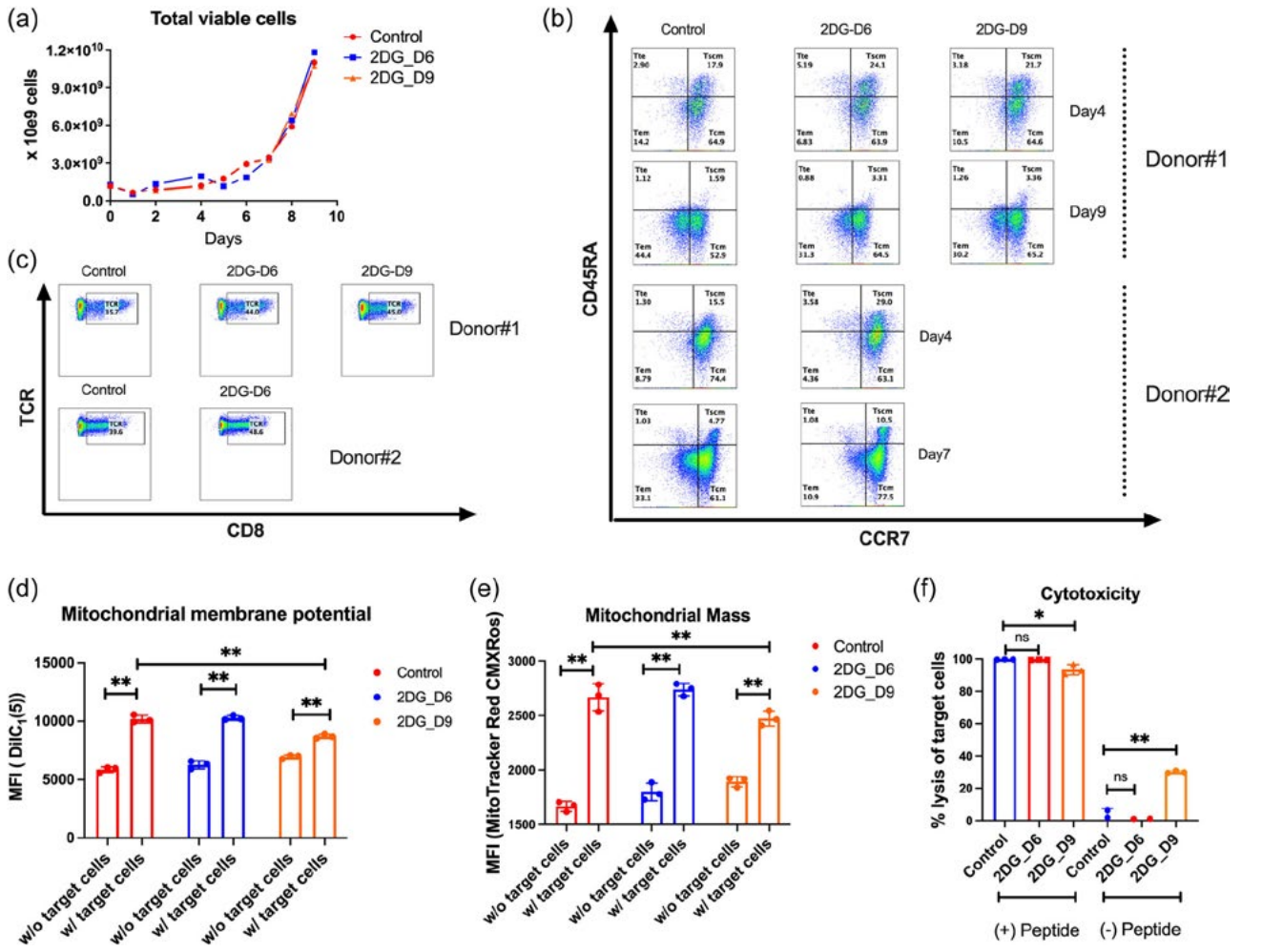


Figure 2: Enrichment of memory T-cell subset in final TCR-T cell product via glycolysis inhibition. Glycolysis inhibitor 2-DG at 2mM was added in culture media starting from inoculation until day 6 or 9. In-process cell growth (a) was monitored. CD8+ T-cell differentiation was compared for their percentage of Tscm, Tcm, and Tem subsets in-process at day 4 and at harvest for two representative donors **(b)**. EOP TCR-T cells of CD8+ cells are shown with two representative donors **(c)**. MMP **(d)** and mitochondrial mass **(e)** at basal and activated state, and cytotoxicity against MAGE-B2 peptide (+) and (–) T2 cells **(f)**. EOP, end-of-process; MMP, mitochondrial membrane potential.

- 2-DG increased the percentage of stem cell memory (Tscm) and Tcm T-cells
- TCR expression increased by 10% with 2-DG treatment
- 2-DG-treated cells showed lower mitochondrial membrane potential (MMP) and mitochondrial mass after target cell encounter, suggesting greater mitochondrial capacity and fitness

Removing interleukin-2 (IL-2) in-process promotes metabolic fitness and reduced background killing

IL-2 (e.g., see here for IL-2-related reagents) was withdrawn in-process to investigate whether the withdrawal could attenuate activation signaling and generate T-cells with lower background activity and enhanced fitness (Fig. 3).

Key findings included:

- IL-2 withdrawal on day 5 or 6 resulted in <5% CD25+ T-cells at harvest, compared to 50-80% with continuous IL-2
- Cells with lower CD25 expression exhibited reduced non-specific killing while maintaining specific target cell lysis
- IL-2 withdrawal differentially affected TCR expression (compared with control conditions) between donors, but the differences were not significant
- T-cells from IL-2 withdrawal conditions had lower levels of MMP and reactive oxygen species (ROS) at both resting and effector states

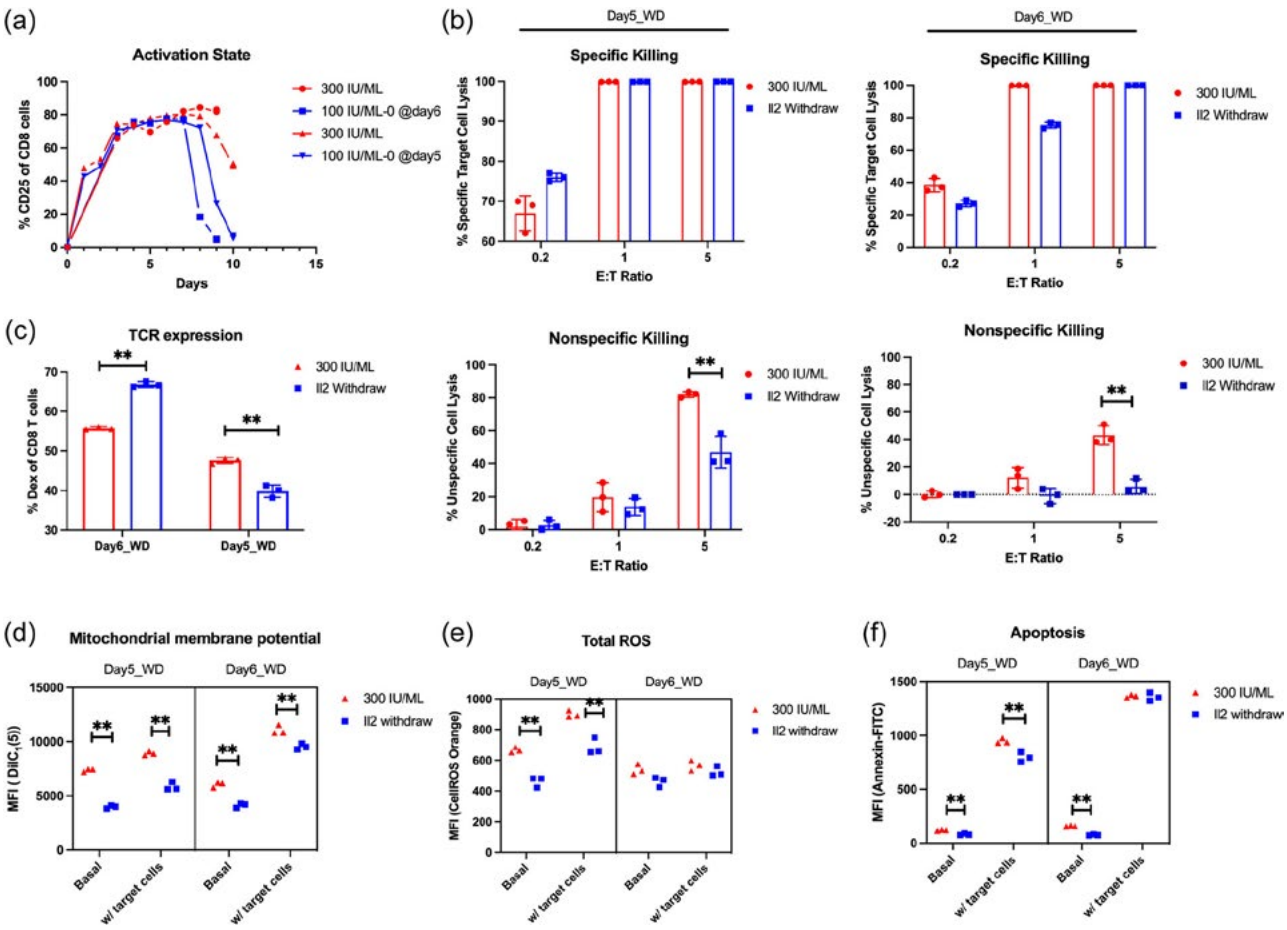


Figure 3: IL2 withdrawal from culture medium at day 5 or 6 reduced background activation of EOP TCR-T cells with improved metabolic fitness. Impact of IL-2 level on CD25 expression (a), cytotoxicity of TCR-T cells against MAGE-B2 peptide-loaded **(b, top panels)** and no peptide-loaded T2-Luc cells **(b, bottom panels)** at an E:T ratio 0.2, 1, and 5, EOP TCR expression detected by PE dextramer **(c)**, levels of mitochondrial membrane potential **(d)**, ROS **(e)**, and Annexin **(f)** of TCR-T cells. EOP, end-of-process; IL-2, interleukin-2; ROS, reactive oxygen species.

Discussion

A fully closed, autologous T-cell bioprocess is described that eliminates the need for T-cell enrichment, reduces manual steps, and integrates activation, transduction, and expansion in a single bioreactor. This approach offers several advantages:

1. Increased transduction efficiency and comparable T-cell purity to enriched cells
2. Reduced processing time and enhanced key critical quality attributes (CQA) of engineered T-cells
3. Improved manufacturing efficiency by enabling parallel processing of multiple lots in a Grade C cleanroom

The integrated operation resulted in superior CQA of engineered T-cells compared to a representative CAR-T cell bioprocess, including higher TCR-T cell yield, TCR expression, memory subset percentage, and cytotoxicity.

Several process parameters were optimized to enhance T-cell fitness and function:

1. Bioreactor parameters: Higher inoculation density and rocking agitation promoted cell growth, viability, and transduction efficiency while maintaining central memory phenotype.
2. Glycolysis inhibition: Addition of 2-DG increased the percentage of memory T-cells and TCR expression, potentially improving therapeutic efficacy and persistence.
3. IL-2 withdrawal: Removing IL-2 during the process resulted in lower background activity and enhanced metabolic fitness of T-cells.

These optimizations enabled a robust manufacturing bioprocess that generates a high yield of quality TCR-T cells with a memory phenotype, which has been shown to correlate with increased clinical efficacy. The process also reduces COGM and eliminates many manual touchpoints necessary in typical autologous manufacturing processes.

This fully closed, autologous T-cell bioprocess offers a promising approach for manufacturing TCR-T cell therapies for solid tumours, addressing key challenges in the field and potentially improving clinical outcomes.

TREM1/DAP12 Based Novel Multiple Chain CAR-T Cells Targeting DLL3 Show Robust Anti-tumour Efficacy for Small Cell Lung Cancer

Adapted from Nie, F., *et al*. 2024

Small cell lung cancer (SCLC) is an aggressive cancer with poor prognosis and limited treatment options after first-line therapy failure. While immunotherapy has shown promise in non-small cell lung cancer, its impact on SCLC remains unclear. DLL3, highly expressed in SCLC but not normal cells, has emerged as a promising target. This study developed a novel DLL3-TREM1/DAP12 CAR-T (DLL3-DT CAR-T) therapy, building on previous work with TREM1 and DAP12. DLL3-DT CAR-T cells showed comparable *in vitro* efficacy to conventional Car T cells but demonstrated superior tumour eradication in murine xenograft and patient-derived xenograft models. The therapy also exhibited elevated memory phenotypes, durable responses, and activation under antigen-presenting cells. These findings suggest DLL3-DT CAR-T cells could be a promising therapeutic strategy for DLL3-expressing SCLC and other solid tumours.

Introduction

Small cell lung cancer (SCLC) is an aggressive subtype of lung cancer with poor prognosis and limited effective treatment options, especially after first-line therapy failure. While immunotherapy has shown promise in non-small cell lung cancer (NSCLC), its impact on SCLC prognosis remains limited. Delta Like Ligand 3 (DLL3), an inhibitory Notch pathway ligand, has emerged as a compelling target for SCLC therapy due to its high expression on SCLC cells and minimal expression in normal tissues.

The authors previously developed a novel multiple-chain chimeric antigen receptor (CAR) using TREM1 receptor and DAP12, which efficiently activated T-cells and demonstrated potent cytotoxicity. In this study, they developed a DLL3- DAP12/TREM1 CAR-T (DLL3-DT CAR-T) therapy and evaluated its efficacy against SCLC *in vitro* and *in vivo*.

Results

DLL3 is highly expressed in SCLC tissues and cell lines

The authors examined DLL3 expression in 50 SCLC tissues and various normal tissues from Chinese subjects. Moderate to high DLL3 expression was identified in 80% of SCLC tissues, while expression was virtually undetectable in most normal tissues cultured in DMEM or RPMI 1640 medium (Thermo Fisher Scientific) (Fig. 1). Analysis of RNA seq data confirmed negligible DLL3 mRNA levels in normal tissues and predominant expression in SCLC tissues and malignant cells. Flow cytometry and immunofluorescence assays also showed high DLL3 expression on SCLC cell membranes.

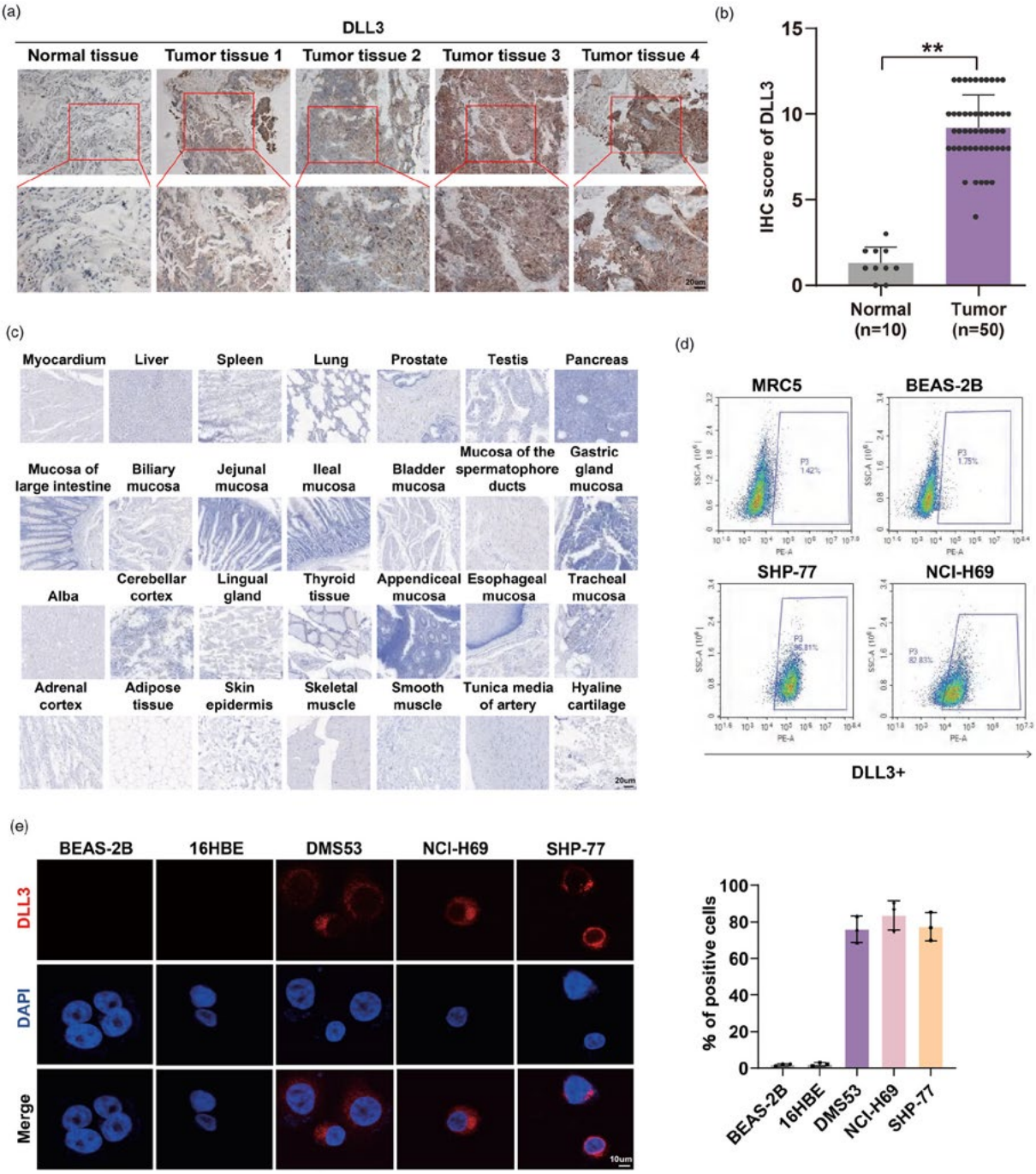


Figure 1: DLL3 is highly expressed in SCLC tissues and cell lines. (a, b) Immunohistochemical analysis of DLL3 protein expression in SCLC tissues. **(c)** Immunohistochemical assessment of DLL3 expression in various normal human tissues and organs. **(d)** Flow cytometric assessment of DLL3 expression in SCLC cells. **(e)** Flow cytometry analysis was used to determine the localization of DLL3 in membrane of SCLC cells. Statistical significance: $^{**}p < 0.01$.

DLL3-TREM1/DAP12 CAR-T cells demonstrate robust anti-tumour efficacy and safety *in vitro*

A novel, multiple-chain DLL3-targeting TREM1/DAP12 (DLL3-DT) CAR structure was developed using a humanized DLL3 nanobody. It was compared to a second-generation DLL3- BBZ CAR and both CARs showed similar transduction efficiency (~40%) in T-cells (Fig. 2).

In vitro cytotoxicity assays demonstrated that DLL3-DT CAR-T cells effectively killed SCLC cells at different effector-to-target ratios, comparable to DLL3-BBZ CAR-T cells. However, DLL3-DT CAR-T cells showed no off-target cytotoxicity against DLL3-negative cells.

DLL3-DT CAR-T cells secreted higher levels of cytokines (IFN-γ and IL-2) upon SCLC cell stimulation compared to DLL3-BBZ CAR-T cells. They also showed comparable antigen-stimulated proliferative capacity. Flow cytometry analysis revealed a higher proportion of central memory T-cells (Tcm) in DLL3-DT CAR-T cells compared to DLL3-BBZ CAR-T cells (58.76% vs 38.6%).

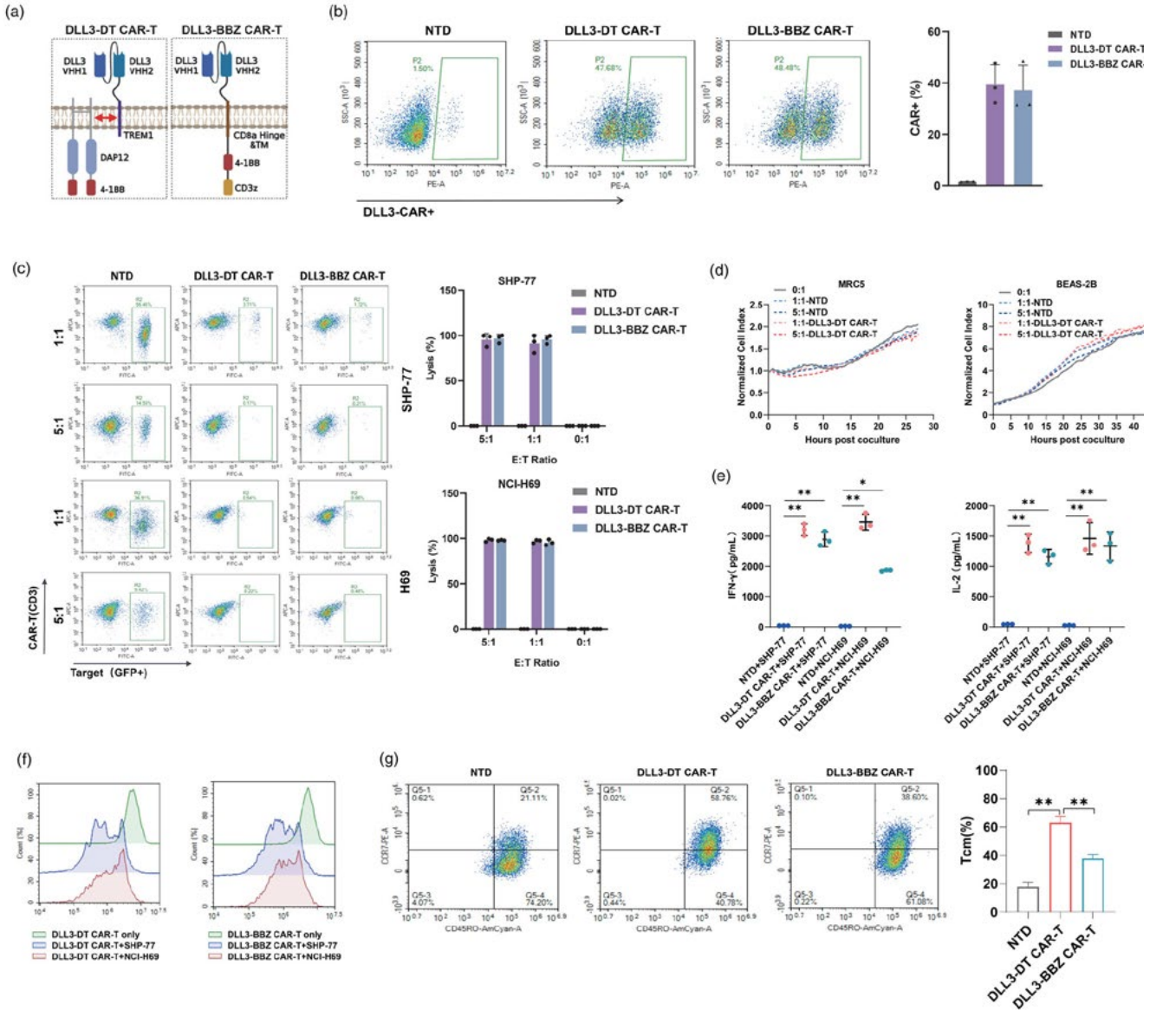


Figure 2: DLL3-TREM1/DAP12 CAR-T cells show robust anti-tumour efficacy and safety *in vitro*. **(a)** Design of DLL3-TREM1/DAP12 CAR-T and DLL3-BBZ CAR-T constructs. **(b)** Flow cytometric analysis of CAR-positive T-cell percentage 7 days post lentiviral transduction. **(c)** Flow cytometry-based assessment of *in vitro* cytotoxicity of DLL3 CAR-T cells against SHP-77 and NCI-H69 cells at various effector-to-target ratios. **(d)** xCELLigence RTCA system evaluation of *in vitro* cytotoxicity of DLL3 CAR-T and NTD cells against MRC5 and BEAS-2B cells. **(e)** ELISA analysis of IFN-γ and IL-2 secretion by DLL3 CAR-T cells following co-culture with SHP-77 and NCI-H69 cells. **(f)** CFSE Staining assay for assessing the proliferation of DLL3-DT CAR-T and DLL3-BBZ CAR-T cells. **(g)** Flow cytometry-based gating strategy for identifying Tn, Tcm, Tem and Tef subsets in NTD and DLL3 CAR-T cells. Statistical significance: *p < 0.05; **p < 0.01.

DLL3-DT CAR-T cells significantly eradicate SCLC xenograft tumours *in vivo*

To further validate the *in vivo* anti-tumour efficacy of DLL3-DT CAR-T cells, subcutaneous xenograft mouse models using luciferase-expressing SHP-77 and NCI-H69 SCLC cells were established. In both models, DLL3-DT CAR-T cells efficiently suppressed tumour growth and completely eradicated tumours by day 40, with durable responses lasting until day 72. Consequently, DLL3-DT CAR-T treatment significantly prolonged survival compared to DLL3- BBZ CAR-T or control treatments.

Importantly, DLL3-DT CAR-T cells did not cause weight loss or other adverse physiological responses in mice. Histological analysis showed no off-target organ toxicity.

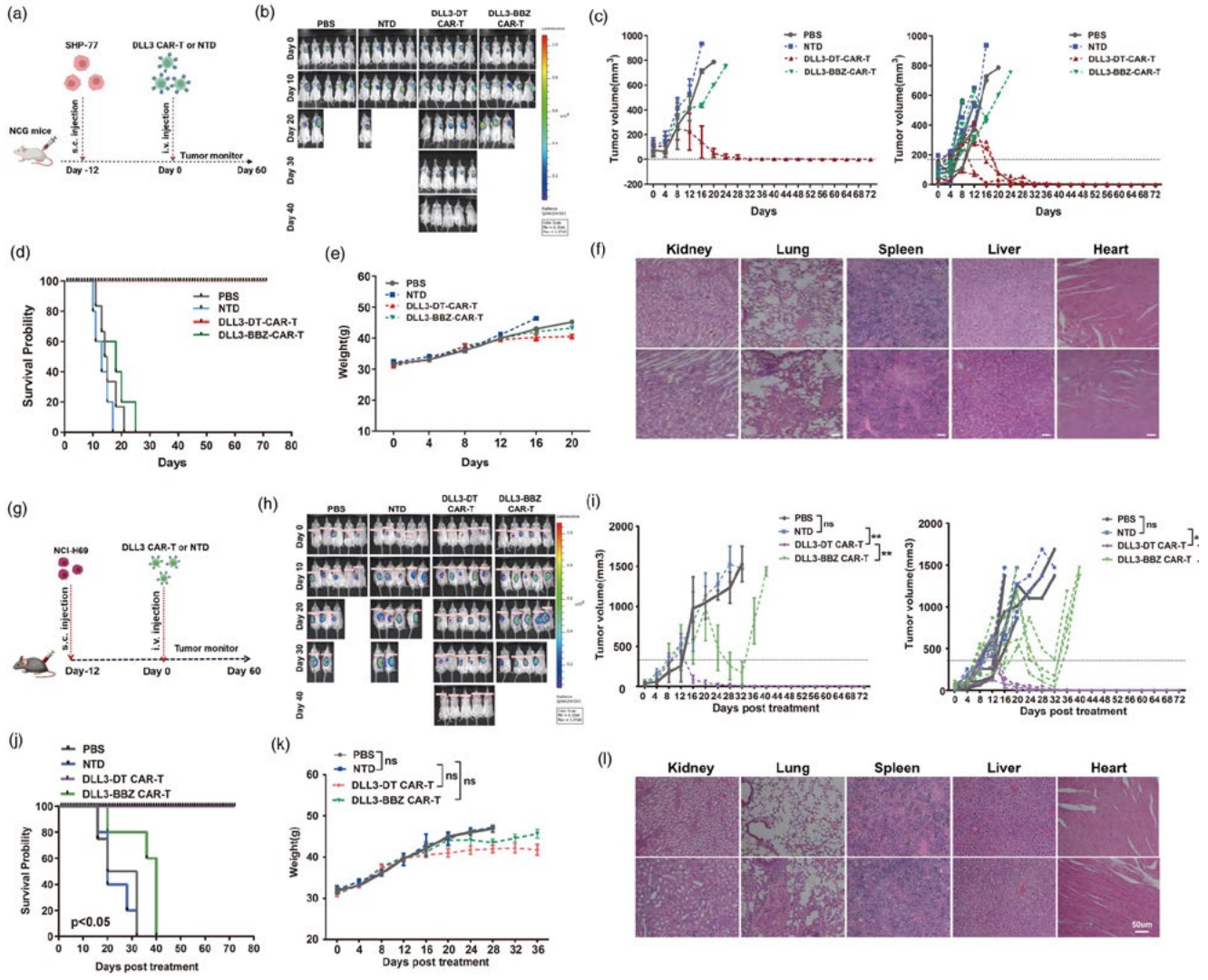


Figure 3: DLL3-DT CAR-T significantly eradicates SCLC xenograft tumours in vivo. (a, g) Schematic representation of the SHP-77 and NCI-H69 xenograft models infused with NTD or DLL3 CAR-T cells on day 12 post tumour inoculation. **(b, h)** Bioluminescence imaging of the SHP-77 and NCI-H69 xenograft models on Days 0, 10, 20, 30, and 40 following injection of NTD or DLL3 CAR-T cells. **(c, i)** Tumour growth curves demonstrating the anti-tumour efficacy of NTD or DLL3 CAR-T cells in SCLC. **(d, j)** Survival analysis of SHP-77 and NCI-H69 xenograft model mice treated with NTD or DLL3 CAR-T cells. **(e, k)** Monitoring of body weight in SHP-77 and NCI-H69 xenograft model mice infused with NTD or DLL3 CAR-T cells. **(f, l)** H&E staining for the assessment of organ toxicity in SHP-77 and NCI-H69 xenograft model mice treated with NTD or DLL3 CAR-T cells. Statistical significance: $^{**}p < 0.01$.

DLL3-DT CAR-T cells are effective in preventing SCLC recurrence in a mouse model

To assess the ability of DLL3-DT CAR-T cells to prevent SCLC recurrence, a challenging subcutaneous model with SHP-77 cells was employed. After initial tumour eradication by DLL3-DT CAR-T cells by day 10, mice were rechallenged with SHP-77 cells on day 42. DLL3-DT CAR-T-treated mice showed no detectable residual tumour and prolonged survival compared to control groups (Fig. 4). Rechallenge led to dramatic DLL3-DT CAR-T expansion and response against SCLC cells. These results indicate that DLL3-DT CAR-T is not only effective in eradicating SCLC tumours but also in preventing the recurrence of SCLC.

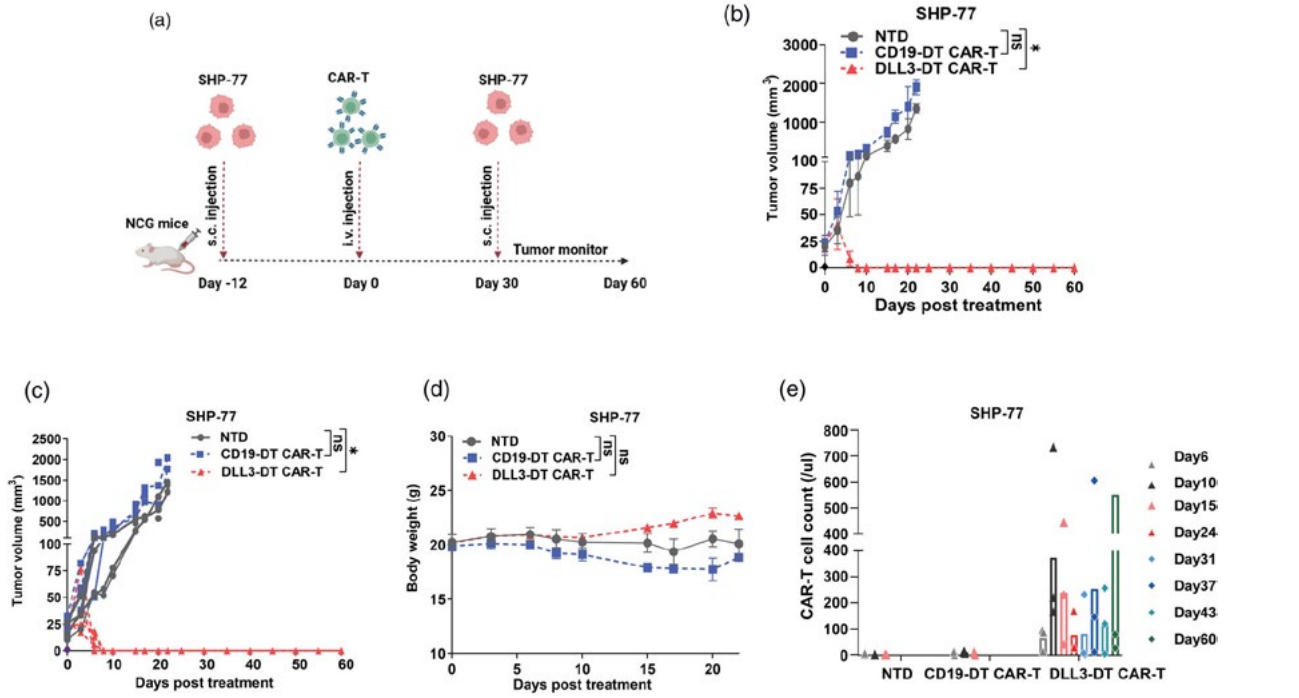


Figure 4: DLL3-DT CAR-T cells are effective in preventing SCLC recurrence in mouse model. (a) Schematic of the SHP-77 cells xenograft tumour model re-injected with SHP-77 cells on day 30 post DLL3 CAR-T cell therapy. **(b, c)** Tumour volume changes over time in mice. **(d)** Body weight changes in mice. **(e)** Flow cytometric analysis for assessing the persistence of DLL3 CAR-T cells in peripheral blood of mice. Statistical significance: *p < 0.05.

Anti-tumour efficacy of DLL3-DT CAR-T cells in SCLC PDX subcutaneous models

A patient-derived xenograft (PDX) model using SCLC patient tissue samples was constructed to simulate the tumour microenvironment. In this model, DLL3-DT CAR-T cells achieved complete regression of PDX tumours by day 36 post-infusion, with no relapse observed up to day 72 (Fig. 5). In contrast, DLL3-BBZ CAR-T cells only delayed tumour growth. DLL3-DT CAR-T cells induced long-term survival without causing weight loss or organ toxicity.

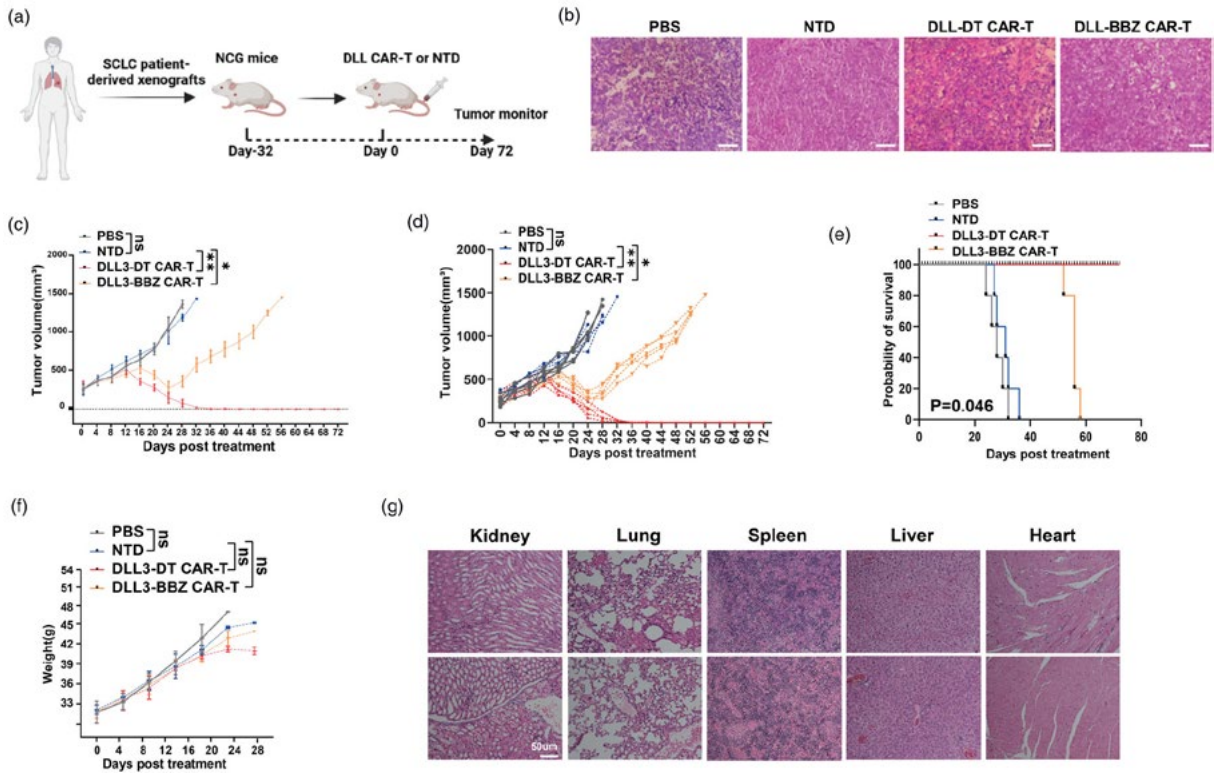


Figure 5: Anti-tumour efficacy of DLL3-DT CAR-T cells in SCLC PDX subcutaneous models. (a) Schematic of the PDX model construction from SCLC patient. **(b)** H&E staining for assessing tumour morphology in the SCLC PDX model. **(c, d)** Tumour volume changes in SCLC PDX model mice treated with NTD or DLL3 CAR-T cells. **(e)** Survival analysis of SCLC PDX model mice treated with NTD or DLL3 CAR-T cells. **(f)** Body weight changes in SCLC PDX model mice treated with NTD or DLL3 CAR-T cells. **(g)** H&E staining for the assessment of organ toxicity in SCLC PDX model mice treated with NTD or DLL3 CAR-T cells. Statistical significance: *p < 0.05; **p < 0.01.

DAP12/TREM1 CAR triggers a transcription profile distinct from the BBZ CAR

Gene expression analysis of DLL3-DT CAR-T and DLL3-BBZ CAR-T cells after SCLC cell stimulation revealed both common and unique gene expression patterns. Pathway analysis showed enrichment of differentially expressed genes in DLL3-DT CAR-T cells in cytokine-cytokine receptor interaction, NF-kappa B, Jak-STAT signaling, natural killer cell-mediated cytotoxicity, and T-cell receptor signaling pathways (Fig. 6).

Western blot analysis of proximal signaling events showed that DLL3-DT CAR-T cells mainly transduced signals through ZAP70 and SYK phosphorylation, similar to myeloid and NK cells. Both CAR types activated NF-κB signaling, but a stronger nuclear factor of activated T-cells (NFAT) activation was observed in DLL3-DT CAR-T cells.

In sum, DT CAR-activated T-cells more closely phenocopied MCR or NK cells and differed from BBZ CAR-T cells in terms of gene expression pattern and activation signaling, which accounts for their different performance *in vivo*.

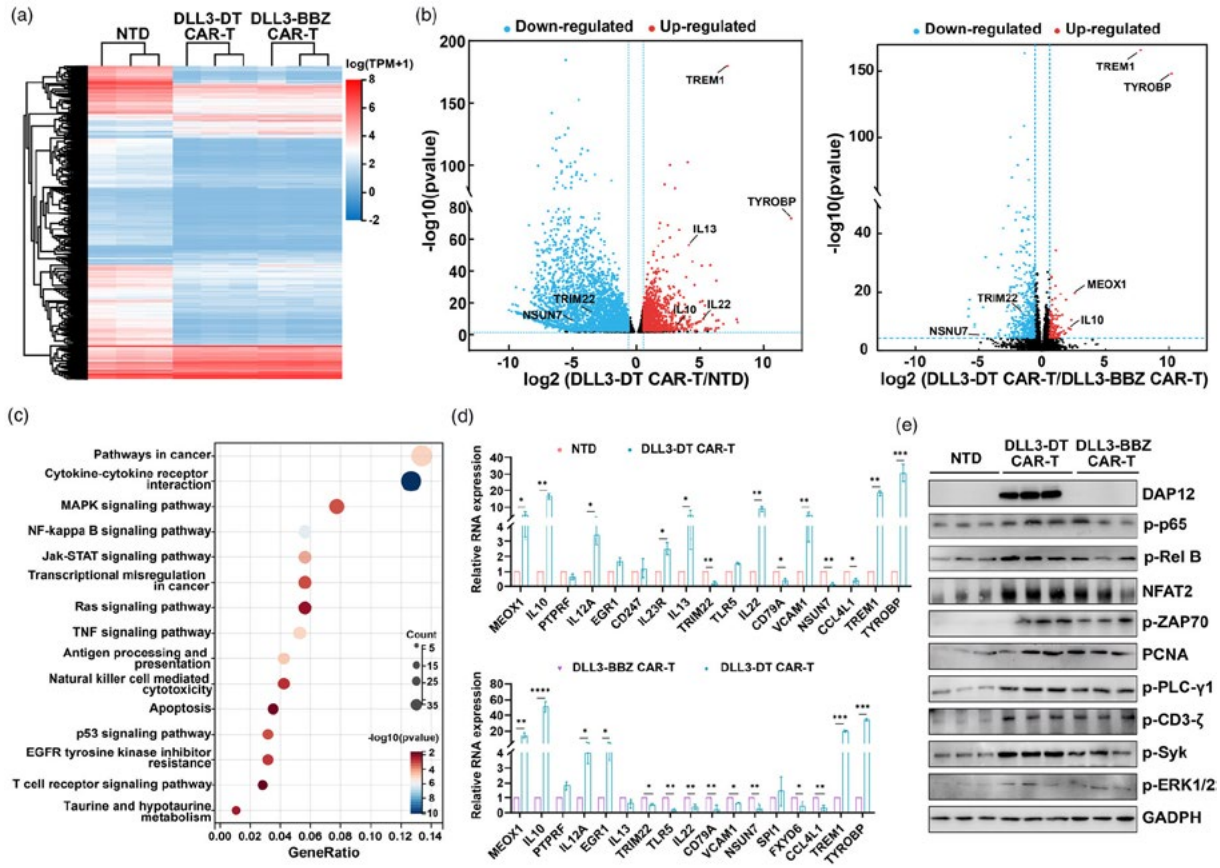


Figure 6. DAP12/TREM1 CAR triggers transcription profile distinct from the BBZ CAR. (a) Heatmaps of RNA sequencing data from NTD or DLL3 CAR-T cells co-cultured with SCLC cells. **(b)** Volcano plot illustrating differentially expressed genes in DLL3-DT CAR-T cells. **(c)** KEGG pathway analysis revealing activated signaling pathways in DLL3-DT CAR-T cells. **(d)** Validation of differentially expressed genes in DLL3-DT CAR-T cells using qRT-PCR. **(e)** Western blot analysis for assessing the expression of key proteins in enriched signaling pathways in NTD or DLL3 CAR-T cells.

Discussion

CAR-T cell therapies have achieved remarkable success in the treatment of patients with hematological malignancies but have not proven to be as efficient in treating patients with solid tumours. Potential reasons for the therapeutic failure of CAR-T cells in solid tumours are primarily attributed to the heterogeneity of tumour cell antigens and the immunosuppressive tumour micro-environment, which induces CAR-T cell exhaustion and anergy.

A novel multi-chain DLL3-targeting DAP12/TREM1 CAR-T cell therapy was developed in this study for SCLC treatment. While DLL3-DT CAR-T cells showed comparable *in vitro* cytotoxicity to second-generation BBZ CAR-T cells, they demonstrated significantly enhanced anti-tumour efficacy in SCLC xenograft and PDX models.

The DT CAR design described here was inspired by the myeloid cell activation receptor machinery, consisting of TREM1 and DAP12. This design may explain the ability of DLL3-DT CAR-T cells to overcome the immunosuppressive tumour microenvironment and retain anti-tumour activity.

A PDX model was successfully established using SCLC patient tissue, demonstrating the superiority of DLL3-DT CAR-T cells over DLL3-BBZ CAR-T cells in tumour eradication and prevention of relapse. No off-target organ toxicity was observed in any of the mouse models.

The study's findings underscore the promise of DLL3 as a safe and effective target for SCLC treatment. The novel multi-chain DLL3-targeting DAP12/TREM1 CAR-T cells represent a potential therapeutic candidate for SCLC and other DLL3-positive cancers, offering new hope for patients with these challenging diseases.

Application note | CTS DynaCellect, Rotea, and Xenon systems

Cell therapy manufacturing

Efficient CAR T cell manufacturing using a closed and integrated instrument workflow

Introduction

Chimeric antigen receptor (CAR) T cell therapeutics continue to achieve extraordinary results in treating once-fatal cancers like large B cell lymphoma [1] and acute lymphoblastic leukemia [2]. These therapeutics also show promising results for long-term antineoplastic effects, helping keep patients in remission longer [3].

Considering this, there is an increased focus on developing tools and technologies to help support standardization of cell therapy manufacturing using closed, automated workflows that reduce manufacturing failure rates, reinforce high-quality production standards, and ultimately help lead to positive clinical outcomes.

Manual processing of biotherapeutics is prone to contamination, inconsistency, and significant lot-to-lot variability. Using a closed, automated CAR T cell therapy manufacturing workflow could help overcome these challenges while reducing their cost and increasing their safety profile. Eliminating factors such as lot-to-lot variability and contamination also helps ensure that the therapies reach the patients who need them the most, quickly. To provide closed and automated workflows that support CAR T cell manufacturing, we continue to study and optimize varying unit operations for cell therapy manufacturing.

The Gibco™ CTS™ DynaCellect™ Magnetic Separation System allows for the isolation of specific cell populations based on the use of Gibco™ CTS™ Dynabeads™ CD3/CD28 magnetic beads that attach to surface-bound T cell markers.

The multipurpose Gibco™ CTS™ Rotea™ Counterflow Centrifugation System is a versatile cell processing tool that helps enable GMP manufacturing. The CTS Rotea system applies proven counterflow centrifugation to a broad range of cell processing applications. It is gentle on cells and enables over 95% cell recovery while maintaining cell viability and high throughput.

The Gibco™ CTS™ Xenon™ Electroporation System reliably delivers DNA, RNA, and protein payloads into cells, with high cell viability and recovery. This enables efficient transfection even in hard-to-transfect cell types.

The above systems can be digitally and physically integrated to create a closed workflow that can be controlled by process automation software to help minimize drawbacks associated with manual processes. Here we demonstrate the closed and integrated use of the CTS Rotea system with the CTS Xenon system to edit T cells isolated with the CTS DynaCellect system.

Methods

T cell isolation

Frozen leukopak bags containing white blood cells (WBC) from healthy donors were thawed. T cells were isolated using the CTS DynaCellect system with the isolation protocol shown in Table 1 and CTS Dynabeads CD3/CD28 magnetic beads. Isolated T cells were cultured in G-Rex™100 bioreactor vessels (Wilson Wolf Manufacturing) containing Gibco™ CTS™ OpTmizer™ Pro Serum-Free Medium (SFM) supplemented with 100 U/mL of IL-2.

Table 1. T cell isolation protocol using the CTS DynaCellect system.

Port	
A	Isolation bag
B	1,000 mL bag for 550 mL DPBS with 1% HSA
C	1,000 mL bag for output (positive fraction)
D	Empty
E	1,000 mL bag for 550 mL DPBS with 1% HSA (same bag as B)
F	600 mL bag for CTS OpTmizer Pro SFM
G	Conical input bag with thawed leukopak
H	Conical bag with CTS Dynabeads CD3/CD28 magnetic beads (3:1 bead-to-cell ratio)
I	1,000 mL bag for supernatant (negative fraction)

After 3 days of culture, cells were debeaded using the CTS DynaCellect system as outlined in Table 2.

Table 2. Removal of Dynabeads magnetic beads on day 3, following isolation and activation using the CTS DynaCellect system.

Port	
A	Bead removal bag in port
B	Bead removal bag out port
C	Empty
D	Empty
E	1 x 1,000 mL bag for export/harvest
F	Empty
G	250 mL DPBS with 0.1% HSA in 300 mL bag
H	1,000 mL input bag with cell/bead input
I	Empty

Closed integration of the CTS Rotea system with the CTS Xenon system

Once the CTS Dynabeads CD3/CD28 magnetic beads were removed, cells were washed, and electroporation buffer exchange was carried out using the CTS Rotea system. (Download the protocol here.) Both the payload (Invitrogen™ TrueCut™ Cas9 Protein v2, Invitrogen™ TrueGuide™ Synthetic gRNA, and target DNA) and the concentrated T cells were transferred to the CTS Xenon system via welded PVC tubing (connected to line H on the Gibco™ CTS™ Rotea™ Single-Use Kit), to be electroporated using the setup outlined in Table 3. T cells were genetically modified using the payload strategy outlined in Table 4 and the Gibco™ CTS™ Xenon™ Multishot Electroporation Cartridge with the parameters noted in Table 5, and with the setup as shown in Figure 1. The total electroporation volume was adjusted based on cell count and desired cell density as listed in Table 6.

Table 3. Day 3—setup for integration of the CTS Rotea system to the CTS Xenon system.

Port	
A	1,000 mL bag for waste
B	300 mL bag for 200mL DPBS with 1% HSA
C	Gibco™ CTS™ Xenon™ Genome Editing Buffer, bag
D	Cell input loop
E	2 mL payload in 10 mL squeeze pouch sample port
F	Empty
G	Cell input loop
H	Direct weld PVC line to CTS Xenon system

Table 4. Payload component concentrations.

Payload	Concentration
TrueCut Cas9 Protein v2	120 µg/mL
TrueGuide synthetic gRNA	30 µg/mL
CD19 CAR (2.4 kb)	240 µg/mL

Table 5. Electroporation conditions on the CTS Xenon system.

Voltage	Pulse width	Number of pulses	Pulse interval
2,300 V	3 ms	4	500 ms

Table 6. Electroporation volumes.

Run	Electroporation volume per 5 x 10^7 cells
Run 1	13 mL
Run 2	17 mL
Run 3	16 mL

A

B

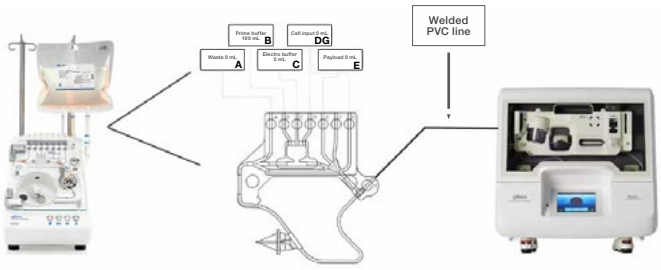


Figure 1. T cell isolation process summary. T cells were isolated using the CTS DynaCellect system and then cultured in CTS OpTmizer Pro SFM with 100 U/mL IL-2 for 3 days. Following bead removal on day 3, T cells were washed and then transferred from the CTS Rotea system to the CTS Xenon system via a closed integration. **(A)** The CTS DynaCellect system, the CTS Rotea system, and the CTS Xenon system (from left to right) are shown. **(B)** Schematic representation of the physical integration via a welded PVC line between the CTS Rotea system and CTS Xenon system used for T cell electroporation on day 3.

Post-electroporation, the edited T cells were cultured and expanded in CTS OpTmizer Pro SFM for 10 days. The cell characterization parameters measured included cell viability, immune cell phenotypes, and T cell activation marker expression on day 0 and day 3 post-activation. The viability of T cells post-electroporation and the knockout/knock-in (KO/KI) efficiency of edited T cells were analyzed as well. The data shown here represent three independent experiments. Flow cytometric analysis was done using the Invitrogen™ Attune™ CytPix™ Flow Cytometer.

Results

Three days after T cell isolation and following removal of the Dynabeads magnetic beads, we observed high (>90%) T cell viability (Figure 2A). Additionally, on day 3, debeaded cell cultures exhibited an average of 95% T cell content, with the major contaminating cell type being NK/NKT cells (1.71%, Figure 2B). As shown in Figure 2B, the percentage of all non–T cell types (NK/NKT cells, B cells, and monocytes) consistently decreased between day 0 (pre-isolation) and day 3.

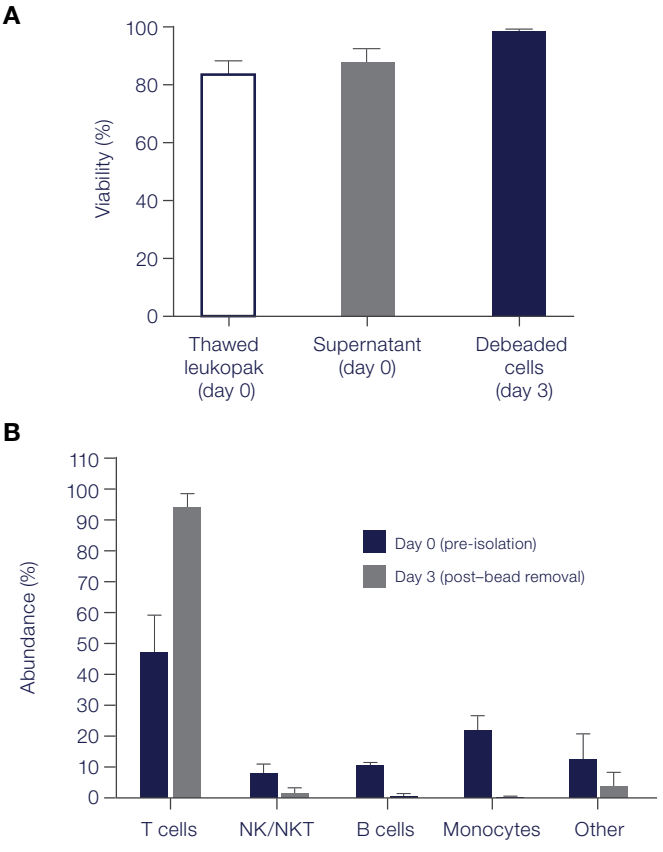


Figure 2. Viability and cell type distribution. (A) Cell viability from day 0 to day 3. **(B)** Cell type distribution for thawed leukopak cells on day 0 and on day 3 post–bead removal.

Flow cytometric analysis using the Attune CytPix Flow Cytometer, on day 0 and on day 3 following T cell activation and culture in CTS OpTmizer Pro SFM supplemented with IL-2, showed effective induction of T cell activation markers CD69, CD25, and HLA-DR by day 3 (Figure 3).

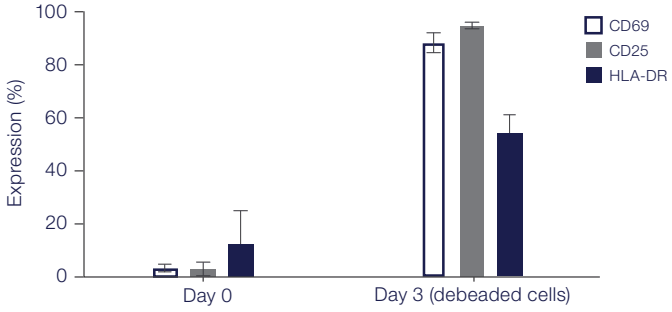


Figure 3. T cell activation marker expression on day 0 and post-activation on day 3.

Taken together, Figures 2 and 3 demonstrate that viable, activated T cells were used in subsequent processing and electroporation using the CTS Rotea and CTS Xenon systems.

Following the transfer of T cells from the CTS Rotea system to the CTS Xenon system using a closed process, cells were electroporated to deliver the gene editing payload mix of TrueCut Cas9 Protein,* TrueGuide Synthetic gRNA, and dsDNA. Then the edited T cells were grown in stirred-tank bioreactor vessels. By day 10 of culture post-electroporation, T cell viability averaged approximately 85% across the three independent runs. This demonstrates that a high percentage of T cells survived the electroporation process (Figure 4).

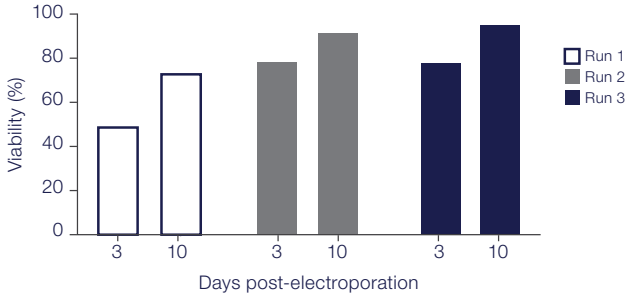


Figure 4. Viability of edited T cells on days 3 and 10 following electroporation using the CTS Xenon system.

* Gibco™ CTS™ TrueCut™ Cas9 Protein is also available. Learn more.

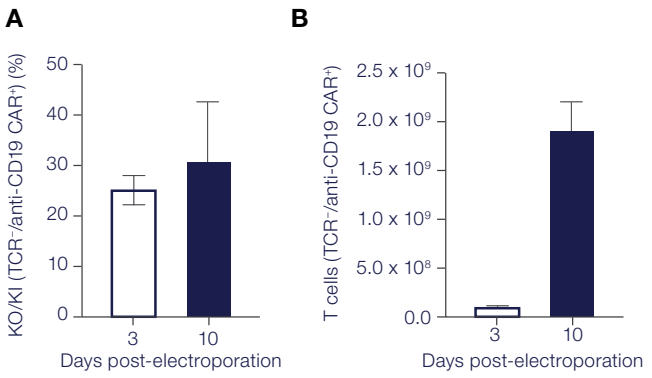


Figure 5. Post-electroporation analysis of (A) gene editing efficiency (KO/KI) of T cells and (B) total number of edited T cells.

Gene editing efficiency in the T cells was analyzed on days 3 and 10 post-electroporation. On average, 25% and 30% of T cells expressed anti-CD19 CAR with no T cell receptor expression (Figure 5A) on days 3 and 10, respectively. These percentages represented an average of 10^8 cells at day 3 with an expansion to 2 x 10^9 edited T cells by day 10 following electroporation (Figure 5B).

Conclusions

In this application note, we sought to highlight a functional, closed physical integration between the CTS Rotea and CTS Xenon systems in manufacturing CAR T cells. We successfully isolated T cells using the CTS DynaCellect system. The cells were activated and cultured for 3 days in medium supplemented with IL-2. On day 3, T cells were washed using the CTS Rotea system. Both the payload and T cells were then delivered via a welded PVC line to the CTS Xenon system, where T cells were electroporated using the CTS Xenon Multishot cartridge. T cells electroporated using this closed and integrated method resulted in an average of 2 x 10^9 anti-CD19 CAR T cells, with high viability.

Here we have demonstrated the use of our portfolio of modular CTS instruments, and effective physical integration of the CTS Rotea and CTS Xenon systems in a closed manner. The results noted here also provide evidence that the closed integration between the CTS Rotea and CTS Xenon systems can be used to help maintain consistency throughout the cell therapy manufacturing process.

The modular nature of our instruments, coupled with their digital and physical integration compatibility, enables countless configurations for a wide array of applications. An example of a future study may include a complete physical connection between the CTS DynaCellect, CTS Rotea, and CTS Xenon systems on day 3 using welded tubing, which would ideally be controlled by automation software. This closed system integrating multiple instruments could be used in the processing of various kinds of cell therapies while reducing contamination and helping to ensure consistency during the cell therapy manufacturing process.

References

1. Roschewski M, Longo DL, and Wilson WH (2022) CAR T-cell therapy for large B-cell lymphoma—who, when, and how? *N Engl J Med* 386(7):692–696. **doi.org/10.1056/NEJMe2118899**
2. Pehlivan KC, Duncan BB, and Lee DW (2018) CAR-T cell therapy for acute lymphoblastic leukemia: transforming the treatment of relapsed and refractory disease. *Curr Hematol Malig Rep* 13(5):396–406. **doi.org/10.1007/s11899-018-0470-x**
3. Lin H, Cheng J, Mu W *et al.* (2021). Advances in universal CAR-T cell therapy. *Front Immunol* 12:744823. **doi.org/10.3389/fimmu.2021.744823**
4. **CTS DynaCellect Magnetic Separation System** (2022).
5. **Flexibility and modularity in cell therapy manufacturing-incorporating the CTS Rotea and Xenon systems** (2022).

Ordering information

Product	Cat. No.
CTS Rotea Counterflow Centrifugation System	A44769
CTS Rotea Single-Use Kit	A49313
CTS DynaCellect Magnetic Separation System	A55867
CTS DynaCellect Isolation Kit	A52300
CTS DynaCellect Bead Removal Kit	A52301
CTS Xenon Electroporation System	A50301
CTS DPBS (without calcium and magnesium)	A1285602
CTS OpTmizer Pro SFM	A4966101
CTS GlutaMAX-I Supplement	A1286001
L-Glutamine (200 mM)	25030081
IL-2	PHC0021
TrueCut Cas9 Protein v2*	A36498 (100 mg)
TrueGuide synthetic gRNA	Multiple catalog numbers (custom)
0.5 M EDTA (pH 8.0)	15575020
CTS Dynabeads CD3/CD28	40203D
5 mL luer-lock syringe	15869152
60 mL luer-lock syringe	14955455
CTS Xenon Genome Editing Buffer	A4998001
CTS Xenon Multishot Electroporation Cartridge	A50306
CD19 CAR DNA (2.4 kb)	LineaRx (Custom)**
BioBLU™ c Single-Use Bioreactor Stirred Tank	Eppendorf (1386100600)
Recombinant Human Serum Albumin (HSA 25%)	Octapharma (ALB064302)
Vial spike with needle-free valve	OriGen Biomedical (VSV)
Spike to male luer	OriGen Biomedical (NC1313150)
Spike adapter with needle-free valve	OriGen Biomedical (NC1768513)
Spike to female luer with 50cm SCD tubing and pinch clamp	OriGen Biomedical (S-F50)
Spike to female luer with 10cm tubing and pinch clamp	OriGen Biomedical (S-F10)
T-SEAL™ III Sterile tubing sealer	Terumo (T3828)
TSCD™ II Sterile tubing welder	Terumo (3ME-SC203A)
Teruflex™ 150 mL blood bag	Terumo (1BBT015CB70)
Teruflex™ 600 mL–1 L blood bags	Terumo (1BBT060CB71)
Clamps	Terumo (T100BM)
G-Rex™100 Gas Permeable Cell Culture System	Wilson Wolf (P/N 80500)
Leukopak	Multiple donors (AllCells or HemaCare)
C-Flex™ tubing to PVC adapter	CharterMedical (CT-014-CVT)
Sterile sampling pouch	Biosafe SA (AK-101)
T-SEAL™ Mobile	Terumo (T5460)

* "CD19 CAR DNA" was a custom-designed sequence.

** CTS TrueCut Cas9 Protein is available. Learn more.

Learn more at **thermofisher.com/ctxmanufacturing**

Case study

EVOLVING

THE FUTURE OF CELL THERAPY MANUFACTURING

Cell therapy

Streamlining cell therapy manufacturing with rapid and reliable bead removal

Situation

The successful development of a cell therapy can have a life-changing impact on patients with diseases such as cancer and immune-mediated conditions. In this growing industry, rapid manufacture of a safe and effective therapeutic is vital.

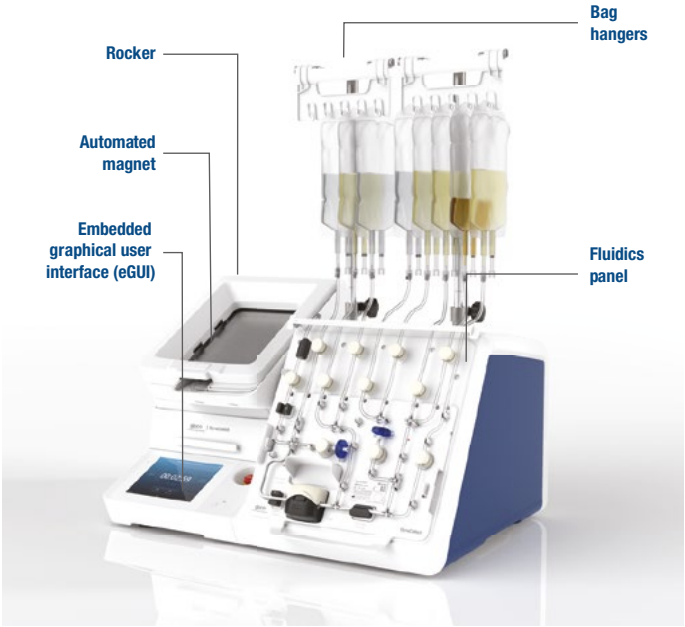
An important step of cell therapy manufacturing is isolating target T cells using magnetic beads, which must then be removed. The T cells can then be transformed into a highly targeted therapeutic product.

The challenge is to remove the paramagnetic beads quickly while ensuring recovery and viability of the target T cells, so they are ready for downstream applications. Manual bead removal solutions are often labor-intensive and vulnerable to human error and long throughput times. With a manual system, it is also difficult to achieve reproducible results with different operators. With a limited number of cells available to work with and variable cell quality, failure to correctly remove the beads while maintaining high cell recovery could directly impact a patient's treatment. Consequently, the success of bead removal is vital in the manufacturing process and essential for the delivery of effective therapies. Here we outline a customer's transition from a manual to an automated bead removal system, and how this helped to accelerate the process while maintaining cell recovery and viability.

Solution

The customer had an established workflow utilizing the Gibco™ CTS™ DynaMag™ Magnet—a manual cell isolation and bead removal device—and was interested in trialling an automated approach. The Gibco™ CTS™ DynaCellect™ Magnetic Separation System was designed to enable closed, fully automated, and rapid cell isolation and bead removal, while decreasing variability in cell therapy manufacturing.

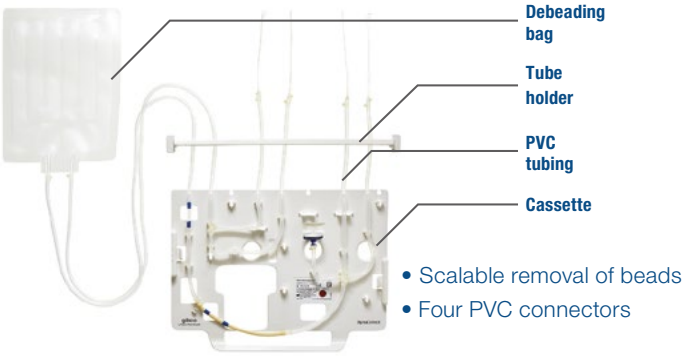
CTS DynaCellect Magnetic Separation System



gibco

When used with the dedicated single-use Gibco™ CTS™ DynaCellect™ Bead Removal Kit, the system reduces the manual processes needed for the bead removal step of cell therapy production. The CTS DynaCellect instrument is a flexible and modular system that can run as a stand-alone device or be integrated into an existing workflow for easy scale-up. The customer wanted to see if the system could support a consistent and reliable workflow, with operator-independent results. Replacing the CTS DynaMag Magnet with the CTS DynaCellect system was a simple process requiring little optimization. In addition, reproducible results could be achieved whether the person operating the instrument had used it many times or had little experience.

CTS DynaCellect Bead Removal Kit



The Gibco CTS DynaCellect Bead Removal Kit is specifically designed for bead removal from immune cells for cell processing applications.

Results

As the existing workflow was already optimized around Gibco™ products, the CTS DynaCellect system was easily incorporated with no challenges around technology transfer. Using the CTS DynaCellect system, the customer was able to achieve comparable cell recovery to that obtained with the CTS DynaMag Magnet, at around 90%. The customer was also able to reach around 95% cell viability, exceeding their criteria of 90% (Figure 1). Achieving sufficient cell recovery and viability is vital to rapidly develop a successful therapy.

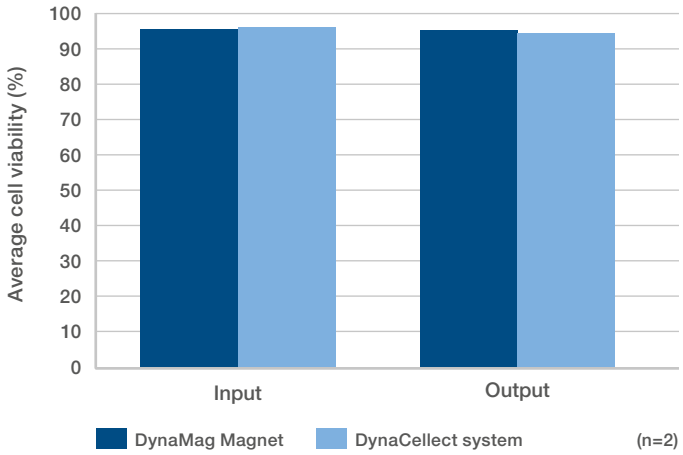


Figure 1. Cell viability is maintained using the CTS DynaMag Magnet and the CTS DynaCellect Magnetic Separation System.

Crucially, bead removal time when using the CTS DynaCellect system was significantly reduced compared to the CTS DynaMag Magnet workflow. The CTS DynaCellect system accelerated the debeading process from 2 hours to 29 minutes, representing a 76% reduction, without impacting cell recovery (Figure 2). Shortening this process and minimizing handling enables developers to accelerate throughput, maximize consistency, and streamline the cell therapy manufacturing workflow.

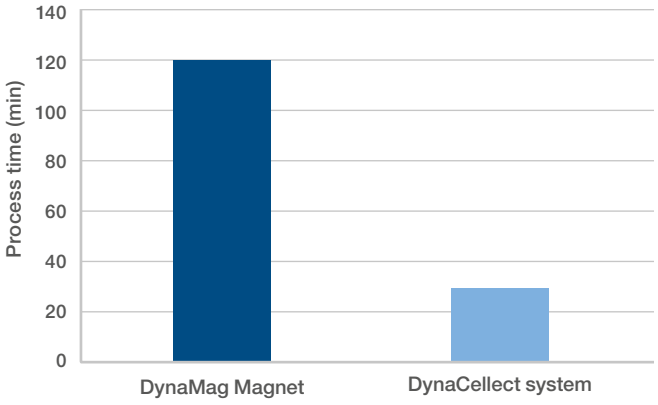


Figure 2. Cell recovery time savings when using the CTS DynaCellect system. Process time was reduced from 120 minutes with the CTS DynaMag Magnet to 29 minutes with the CTS DynaCellect system, representing time savings of 91 minutes.

The ease of use, speed, and consistent performance was evident, with the system demonstrating the same impressive results as part of an existing workflow as the results obtained during development. Furthermore, seamless transition from the CTS DynaMag Magnet to the CTS DynaCellect system meant the customer could experience excellent results without disruption.

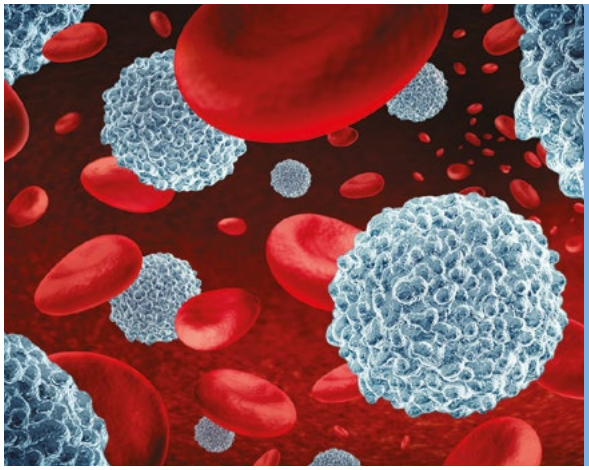
Summary

The CTS DynaCellect system enhanced bead removal by:

- Achieving 90% cell recovery and 95% viability
- Reducing bead removal time by >75%
- Eliminating the need for manual handling

In addition, because the CTS DynaCellect system uses a continuous flow process for bead removal, the processing volume is potentially unlimited, making it well suited for scaling up. By taking advantage of this, users can easily and successfully scale up their process, transitioning from producing a single treatment for one patient to producing for many.

Combining scalability, flexibility, and automation with high speed, proven performance, and modularity, the CTS DynaCellect system can help developers streamline and optimize their workflows. By staying ahead in the rapidly growing cell therapy industry, manufacturers can quickly deliver their essential therapies to patients who urgently need them.



Combining scalability, flexibility, and automation with high speed, proven performance, and modularity, the CTS DynaCellect system can help developers streamline and optimize their workflows.

CELL & GENE THERAPY INSIGHTS

CELL THERAPY UPSTREAM PROCESSING AND MATERIALS

SPOTLIGHT

INNOVATOR INSIGHT

An automated 24-hour CAR-T manufacturing process

Mina Ahmadi

As the cell therapy field progresses, manufacturing challenges like patient safety, cost, automation, closed operation, and scalability must be addressed with improved capabilities and workflows. Leveraging new cell therapy technologies and tools to develop innovative end-to-end workflows can enable more cost-effective cell therapy processes and workflows. This article presents an automated and shortened lentiviral-based CAR-T workflow using the Gibco™ CTS™ Detachable Dynabeads™ CD3/CD28 magnetic beads, Gibco™ CTS™ Detachable Dynabeads™ Release Buffer, and Gibco™ CTS™ DynaCellect™ Magnetic Separation System.

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AUTOLOGOUS CAR-T WORKFLOW CHALLENGES AND SOLUTIONS

A typical autologous CAR-T workflow is a complex, multi-day GMP manufacturing process, which is often characterized by several labor-intensive and open steps that are prone to errors. These lengthy processes also require costly and extensive QC steps prior to product release. The need for CAR-T cell therapies, however, continues to grow, meaning there is a crucial need to address these challenges so that life-saving therapies can reach patients in a timely manner.

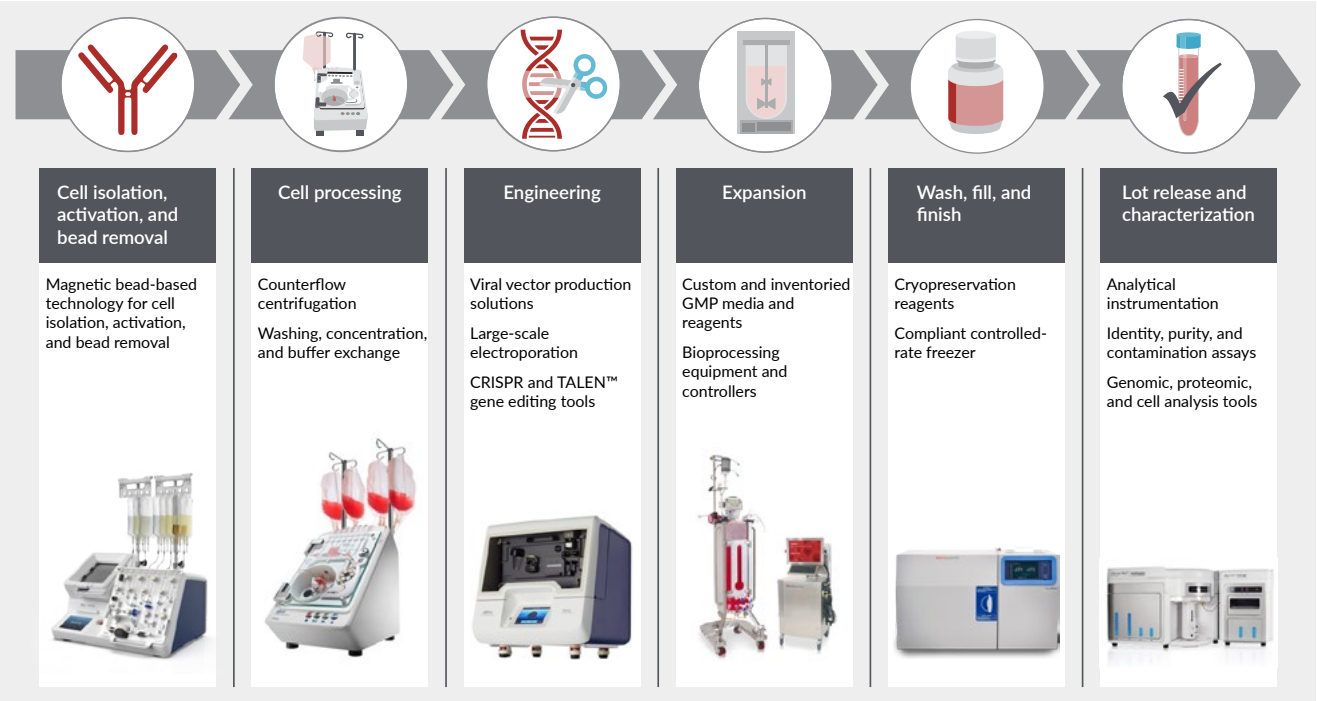
Several of these challenges could be addressed by scaling up the process, closing and automating the manufacturing process, and including in-line analytics. Figure 1 shows Cell Therapy Systems (CTS) solutions developed by Thermo Fisher Scientific, which help provide manufacturers with closed, automated, and modular technologies that can be combined with reagents specially formulated for cell therapy manufacturing to enable end-to-end cell therapy workflows.

The cell therapy instruments in Figure 1 are flexible, fast, and have intuitive touchscreen interfaces that enable users to easily scale their

CELL & GENE THERAPY INSIGHTS

FIGURE 1

Closed, modular CAR-T cell therapy manufacturing process workflow.



cell therapy processes from research through to clinical manufacturing. Additionally, manual touchpoints in this workflow can be reduced with the use of CTS Cellmation™ software, an off-the-shelf automation solution powered by Emerson's DeltaV™ Distributed Control System (DCS). Each of the CTS instruments comes equipped with an open platform communication unified architecture (OPC-UA) that is compatible with Emerson's DeltaV software.

CTS Cellmation software allows manufacturers and users to digitally connect these closed and modular systems, allowing for data traceability and consistency (using batch recipes), while enabling increased efficiency and scalability.

CTS DETACHABLE DYNABEADS CD3/CD28 MAGNETIC BEADS

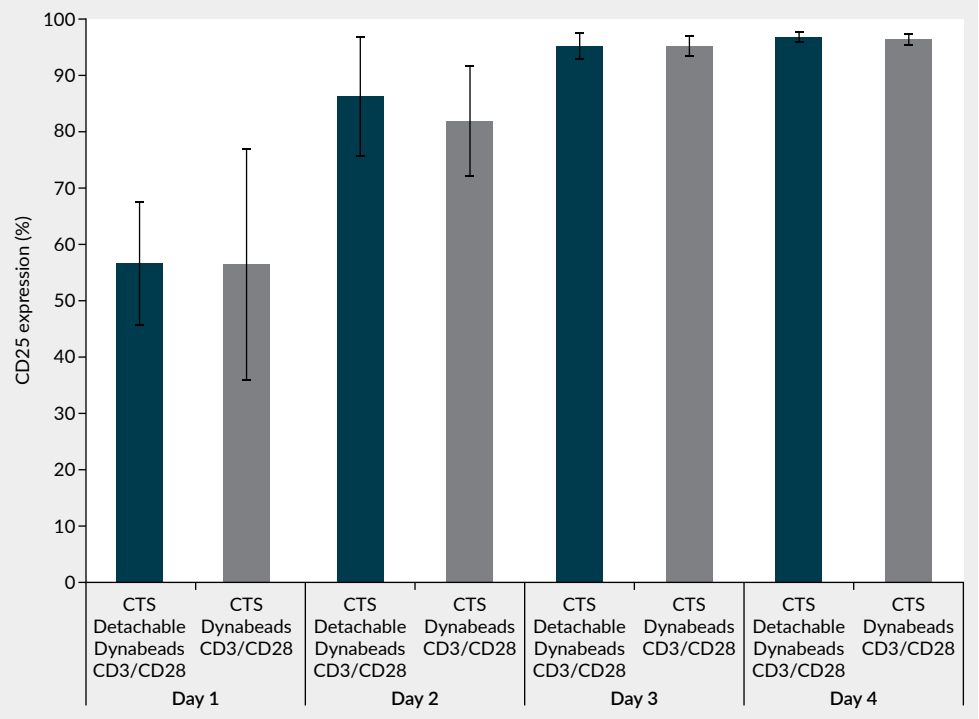
In addition to the closed and modular instruments, significant efforts have been made to create reagents that can offer increased flexibility and that can be customized and leveraged for different cell therapy workflows. One example is the CTS Detachable Dynabeads CD3/CD28 beads, which combines CaptureSelect™ and Dynabeads™ technology. The CaptureSelect ligands are variable domain of heavy chain (VHH) fragments. The VHH antibodies are 12–15 kDa in size with tunable specificity and affinity to efficiently isolate or activate target cells of interest. These VHH antibodies are free of animal-derived components, highly stable, and suitable for use in GMP environments.

When used with the CTS Detachable Dynabeads Release Buffer, the CTS Detachable Dynabeads CD3/CD28 magnetic beads are actively released, enabling greater control of the T cell activation signal. The same Dynabead magnetic core is currently being used in over 200 active clinical trials, as well as several approved cell therapy drugs. In combination with the CTS DynaCellect system, the Detachable Dynabeads provide a powerful tool that allows for customization,

CELL & GENE THERAPY INSIGHTS

FIGURE 2

Average CD25 expression for active- and passive-release Dynabeads CD3/CD28 magnetic beads.



which can contribute to high drug efficacy, safety, and cost efficiency downstream of cell isolation.

With an emphasis on automation, flexibility, and scalability, the CTS DynaCellect system includes fit-for-purpose, single-use consumables for cell isolation. It has a touchscreen user interface that allows for the customization of protocols for cell isolation, cell activation, depletion, and magnetic separation.

CTS DETACHABLE DYNABEADS CD3/CD28 VERSUS CTS DYNABEADS CD3/CD28 BEADS

While the passive release Gibco CTS Dynabeads CD3/CD28 (SKU: 40203D) magnetic beads are a robust product which has been used in over 200 active clinical trials and several commercialized drugs, the active release CTS Detachable Dynabeads CD3/CD28 beads help provide manufacturers with better control of the T cell activation signal. Unlike the passive release Dynabeads magnetic beads, which rely on passive dissociation of beads from target cells over time, the CTS Detachable Dynabeads magnetic beads are designed so that users can actively detach at any point, post-isolation, using the CTS Detachable Dynabeads Release Buffer. Furthermore, because inefficient bead removal may result in hyper-activated and exhausted T cells, the ability for users to actively remove the CTS Detachable Dynabeads has the potential to reduce these effects while delivering comparable or improved results compared to the passive release beads.

Figure 2 shows data from a recent study that measured the average expression of T cell activation marker, CD25, for active- and passive-release Dynabeads magnetic beads. T cell expression of CD25 was comparable for both Dynabeads magnetic beads across the time points studied.

CAR-T cells with a less differentiated T memory cell phenotype show higher effectiveness in treating patients with blood

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FIGURE 3

T cell memory phenotypes for active-and passive-release CTS Dynabeads magnetic beads.

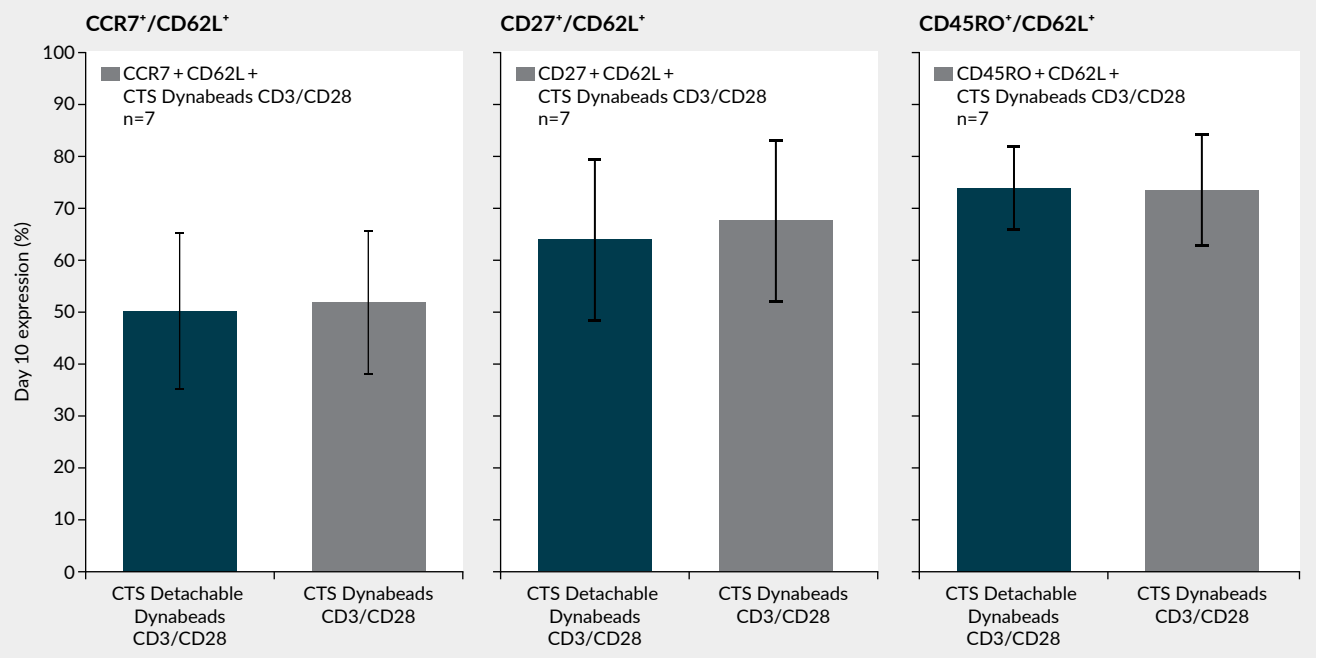
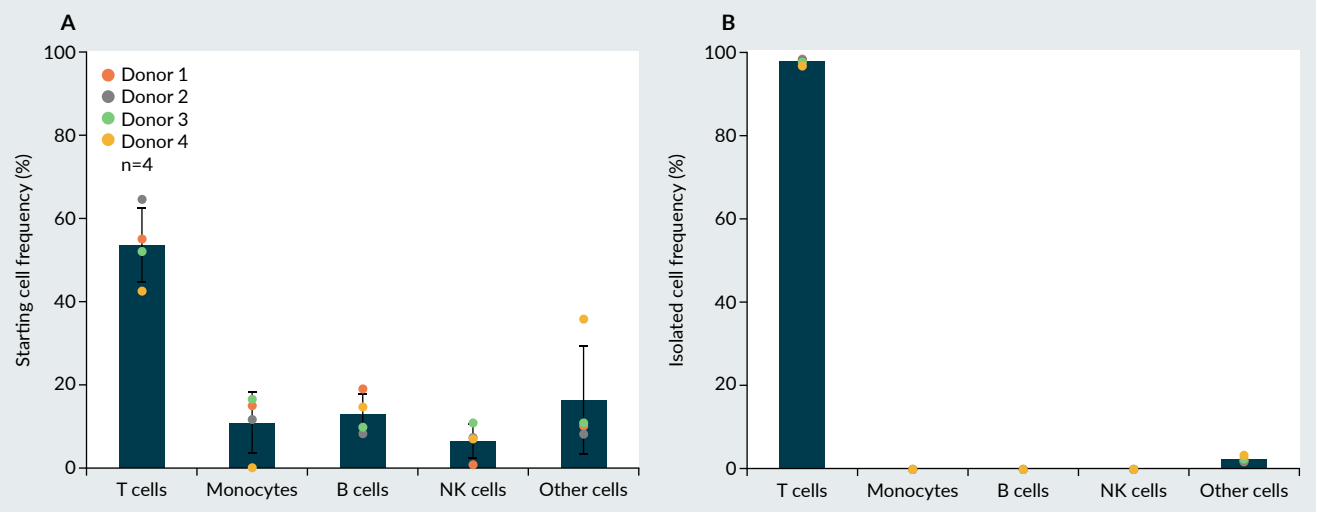


FIGURE 4

T cell purity with CTS Detachable Dynabeads CD3/CD28 beads.



malignancies. Therefore, this attribute was also investigated with both active- and passive-release Dynabeads magnetic beads. CCR7, CD62L, and CD27 were examined to assess the phenotype of early memory cells. Figure 3 shows comparable levels of naïve central memory phenotypes observed on day 10 post-isolation for both passive release and active release CTS Detachable Dynabeads.

Figure 4B shows that T cell purity levels following isolation using CTS Detachable CD3/CD28 beads of >98% was achieved with

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samples from four donors. This consistent performance is necessary when starting material is characterized by great biological variability, as shown in Figure 4A. Apheresis profiles differ from one individual to another and fluctuate further based on the patient's indication and stage of disease. CTS Detachable Dynabeads CD3/CD28 magnetic beads can help overcome that challenge by consistently delivering high T cell purity post-isolation.

CASE STUDY: ADDRESSING THE CURRENT CHALLENGES IN CELL THERAPY MANUFACTURING USING A SHORTENED LENTIVIRAL-BASED CAR-T CELL WORKFLOW

The high cost of commercially available CAR-T cell products creates a major access barrier and limits its broad application for

FIGURE 5

Workflow for a 24-hour CAR-T process.

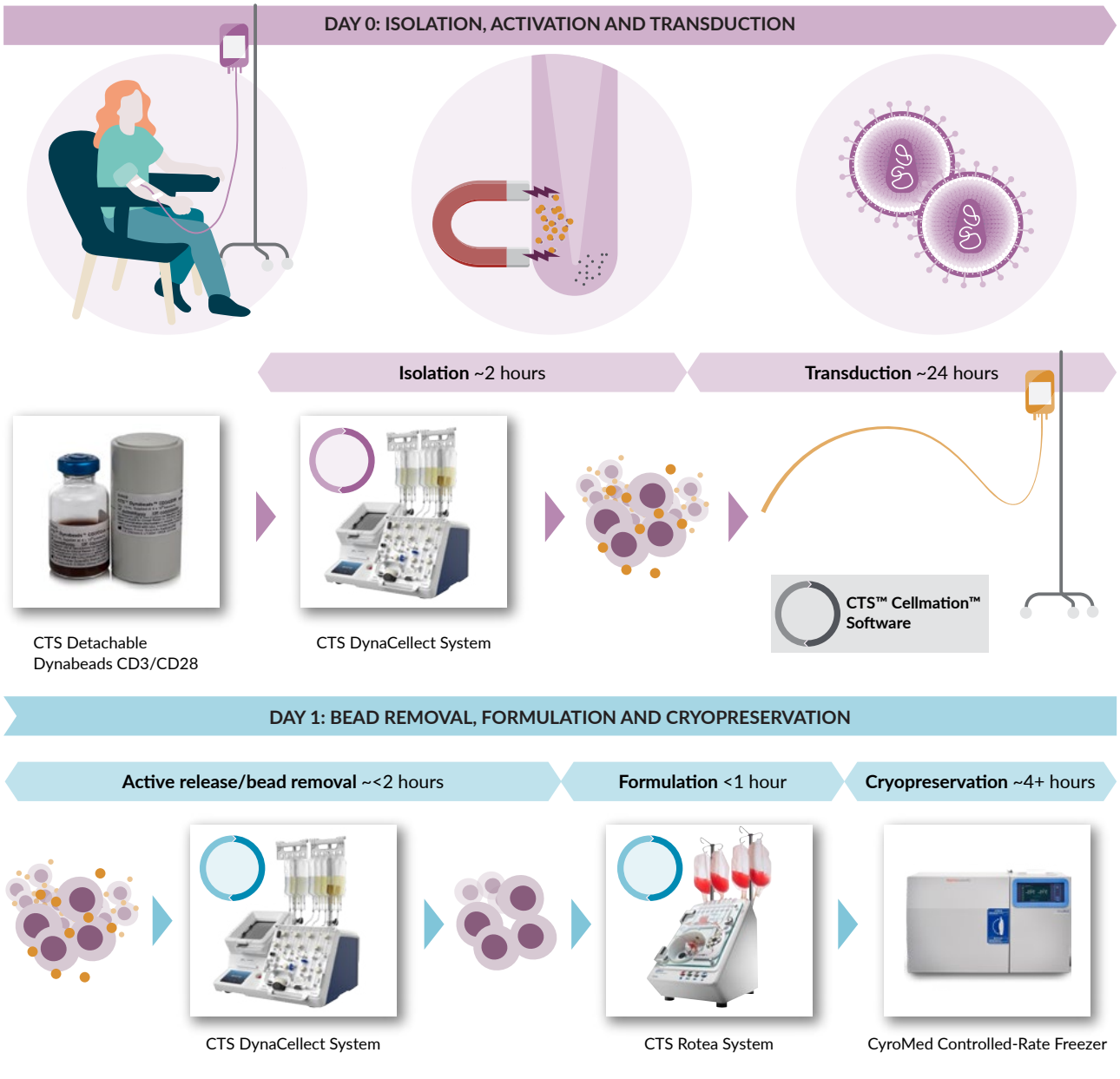

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FIGURE 6

LV-CAR expression and total anti-CD19 CAR positive T cells.

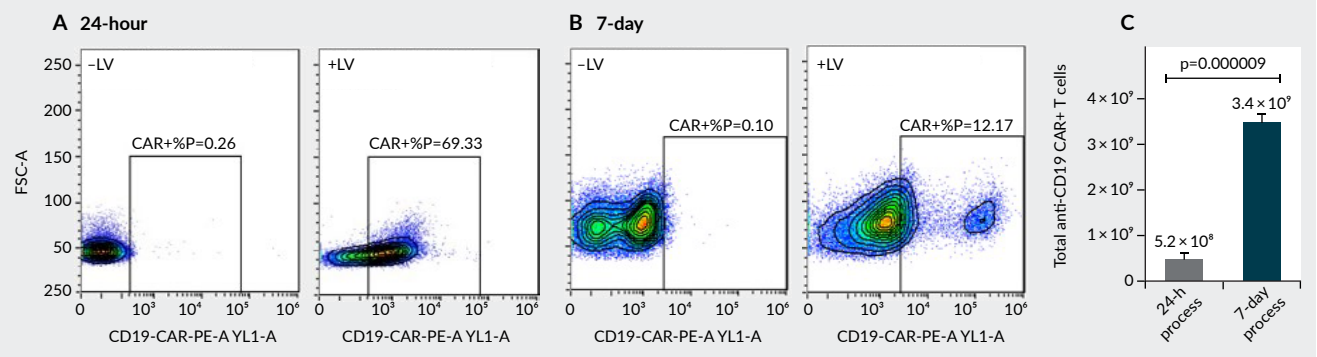
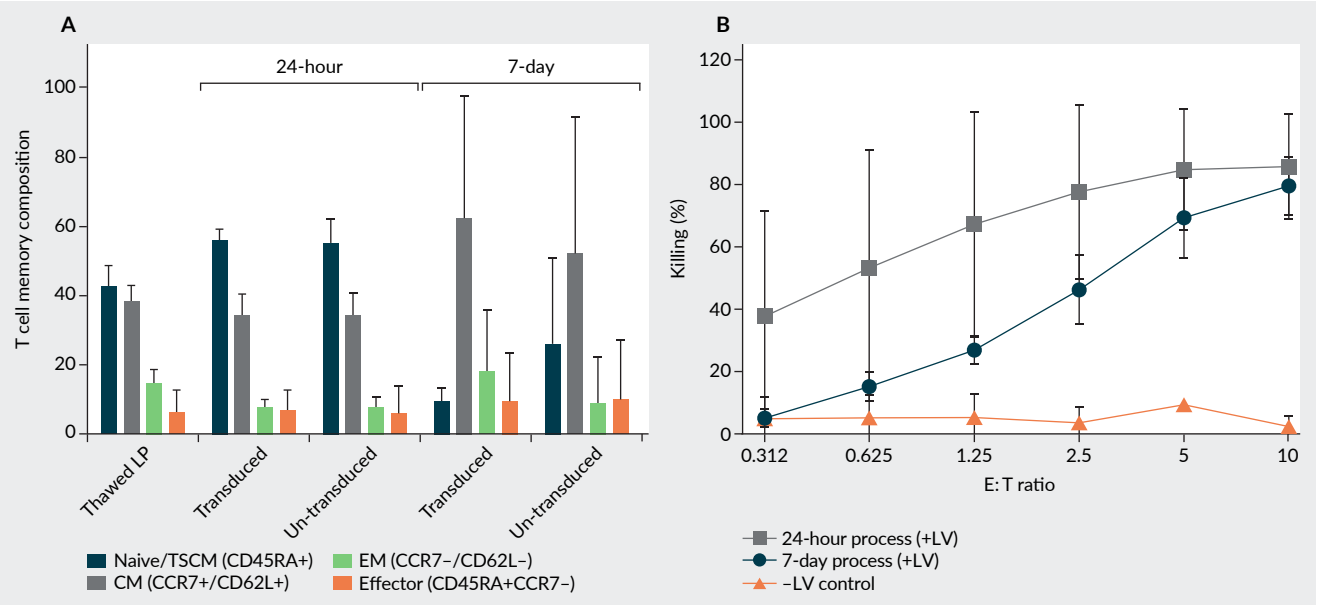


FIGURE 7

CAR-T cell memory phenotypes and cytotoxic activity.



patients who could benefit from these modalities. Recent reports indicate that a significant portion (20%–30%) of patients with B-cell acute lymphoblastic leukemia (B-ALL) who were scheduled to receive CAR-T cell therapy did not end up receiving it due to either rapid disease progression or manufacturing failure [1,2]. Therefore, speeding up the time between apheresis and CAR-T cell therapy infusion is crucial for patients with relapsed refractory B-ALL, as well as other fast-progressing cancers. Additionally, streamlining and automating these processes can help reduce the risk of treatment delays.

Furthermore, traditional CAR-T cell manufacturing often involves a cell expansion step, which can lead to a more differentiated CAR-T cell profile. These highly differentiated cells may exhibit lower cytotoxic activity and cytokine activity compared to less differentiated T cell phenotypes, such as naïve central memory cells or stem cell-like memory T cells.

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Shortening these manufacturing workflows not only results in a cost-effective process that helps provide patients with quicker access to CAR-T cell therapies, but importantly, it could result in a product with higher potency [1,2].

Recently, CTS Detachable Dynabeads CD3/CD28 beads were used with the CTS DynaCellect system for one-step isolation and activation of T cells in a 24-hour lentiviral (LV)-based CAR-T workflow. The goal of this study was to eliminate the expansion step in regular autologous CAR-T workflows and shorten the process from a standard 7- to 14-day process to a 24-hour process. Figure 5 highlights a general overview of this workflow.

Under the control of CTS Cellmation software, T cells were isolated with the CTS Detachable Dynabeads CD3/CD28 and the CTS DynaCellect system from quarter Leukopaks. Post-isolation, cells were transduced with a lentiviral vector encoding a CD19-targeted CAR gene with a multiplicity of infection (MOI) of 2. 24 hours later, the CAR-T cells were separated from the CTS Detachable Dynabeads using the CTS DynaCellect system and the CTS Detachable Dynabeads Release Buffer. These CAR-T cells were then washed and concentrated using the Gibco CTS Rotea™ Counterflow Centrifugation System, while the separated CTS Detachable Dynabeads CD3/CD28 beads were captured by the CTS DynaCellect system.

One portion of the washed and concentrated CAR-T cells was cryopreserved using the Thermo Scientific CryoMed™ Controlled-Rate Freezer while a second was cultured for an additional seven days for comparison.

This method resulted in CAR-expressing T cells just 24 hours after lentiviral transduction, as shown in Figure 6.

Figures 6A and 6B illustrate anti-CD19-CAR expression levels on transduced cells at 24 hours and following culture for 7 days.

While a high CAR expression 24 hours post-transduction was observed, there was a drop in CAR-expressing T cell percentage by day 7 post-transduction. This is likely due to high levels of pseudotransduction at earlier time points post-transduction. This observation is not surprising, as it is well known that membrane proteins expressed in packaging cells could be incorporated into an HIV envelope, which can then passively transfer to both activated and naïve T cell membranes.

The lower-than-expected levels of transduction efficiency on day 7 may also be a result of using an MOI of 2 for these experiments, which is on the lower end of what has been routinely used for these types of processes. The percentage of CAR-expressing T cells remained above 40% on day 7 when higher MOIs, (e.g. MOI=5) were used in small scale experiments (data not shown).

Figure 6C shows that the total number of CAR-expressing T cells increased from day 1 to day 7. Although there was a lower percentage of CAR-expressing T cells on day 7 (Figure 6B), there was still effective expansion of CAR T cells resulting in approximately 3.4 billion CAR-T cells by day 7 post-transduction.

As mentioned previously, a key factor in determining the ability of CAR-T cells to engraft following adoptive transfer is their state of differentiation. Preclinical studies show that less differentiated and naïve stem cell-like memory T cell (TSCM) populations show greater potency [1–2].

As shown in Figure 7A, a higher naïve TSCM memory phenotype (CD45RA+/CCR7+) was observed in the 24-hour CAR-T cells, while the 7-day CAR-T cells had an increased number of the more differentiated central memory phenotype (CD45RA-/CCR7+). Both CAR-T cell products had lower numbers of the more differentiated T cell phenotypes-effector memory cells and effector T cells.

The CAR-T cell potency for each process was measured by exposing the two different CAR-T cell products to CD19-expressing NALM6 cells. 24-hour CAR-T cells exhibited higher cytotoxicity, especially at the lower effector-to-target (E:T) ratios (Figure 7B).

Overall, this data suggests that the 24-hour process results in a highly functional CAR-T product with an enrichment of early T memory phenotype cells.

SUMMARY AND FUTURE DIRECTIONS

In this study, a simplified and automated 24-hour LV-based CAR-T cell manufacturing workflow produced CAR-T cells with improved cytotoxicity compared to a 7-day process.

Future efforts will be made to expand optimization for these shorter workflows. It is also key that the potency of such products is tested with animal models. There are important ongoing efforts to implement proper assays to better characterize cell therapy products generated through shorter workflows such as the 24-hour LV-based workflow. For example, one concern regarding these shorter LV-based workflows is the pseudotransduction that appears to happen at earlier time points post-transduction.

Besides using flow cytometry to check the CAR expression on the cell surface, a more direct approach must be implemented to allow more input regarding CAR integration at earlier time points. This can help the users of such workflows to better determine the dosage of the final drug product.

Q&A

MINA AHMADI AND FABIO FACHIN

Q Have you observed any difference in CD4 versus CD8 transduction efficiency in the shorter process?

MA: We did not see any differences between the transduction efficiency in our CD4 and CD8 populations. The transduction efficiency was similar in both early time points as well as later time points.

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Q Does this shorter CAR-T workflow fit a centralized or a decentralized model?

FF: Both of these models can and will coexist. This is an area that will generate a lot of excitement in terms of the future and some of these new approaches. When it comes to start-ups, biotechs, and academic centers, I do feel that the decentralized approach represents a novel manufacturing space where these players can now demonstrate their drugs.

That approach was not available at the beginning of the industry. The ability to be able to manufacture close to the clinical centers and be able to leverage this broader network, especially with some of these 'GMP on demand' and movable GMP pods that are starting to become available, is quite exciting. It also fits nicely into this shorter manufacturing span where there is more turnaround of the plots.

At the same time though, as I said, these models will have to coexist. It is naïve to think that manufacturing infrastructure will be available everywhere. That balance will need to play out, especially as products move towards the commercial side.

That is perhaps where the more centralized approach can be leveraged. There are benefits there in terms of economies of scale, which is something to consider. I am very curious to see how it pans out.

So, to answer the question: I do believe that this rapid process does lend itself more to a decentralized approach than the more traditional CAR-T cell therapy manufacturing processes.

Q What are the pros and cons of all-in-one versus modular automation?

FF: It depends on the maturity of your process and product. Simplistically, the more you move towards, for example, Phase II clinical trials or commercialization, the more your process and product become concrete.

In that regard, there are many benefits of an all-in-one solution because you have a locked-in process to pursue. The downside of having an all-in-one device is that you are allocating that instrument to your lot for the entire duration of the manufacturing. That is where I see, again, the rapid manufacturing approaches being a little more amenable to the solution.

They provide a shorter timeframe, so you are not utilizing an instrument for 12 days, but rather you are dedicating only 3 days or so to that instrument. However, there are also the downsides of being locked in to one partner as opposed to having the ability to derisk your supply chain. To rectify this, there are solutions in progress with a little bit more flexibility. On the other side, the modular approach is more dynamic. It gives you the ability to mix and match, to some extent.

New instrumentation is coming all the time, so another downside to an all-in-one approach is that if a great technology comes out, you would need to essentially get out of your all-in-one and do a process in this other modular component and then go back, which could introduce error. The modular approach is easier to use, especially earlier in your development, but this approach also has more potential failure points. There are different connections, different disposables, and different data systems that need to be considered. There are also more vendors that you need to manage and support.

The balance between both approaches will continue to evolve, and it is important that each continues to be implemented and pursued.

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REFERENCES

1. Ghassemi S, Durgin JS, Nunez-Cruz S, *et al.* Rapid manufacturing of non-activated potent CAR T cells. *Nat. Biomed. Eng.* 2022; 6, 118–128.
2. Yang J, He J, Zhang X, *et al.* Next-day manufacture of a novel anti-CD19 CAR-T therapy for B-cell acute lymphoblastic leukemia: first-in-human clinical study. *Blood Cancer J.* 2022; 12(7), 104.

BIOGRAPHIES

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AUTHORSHIP & CONFLICT OF INTEREST

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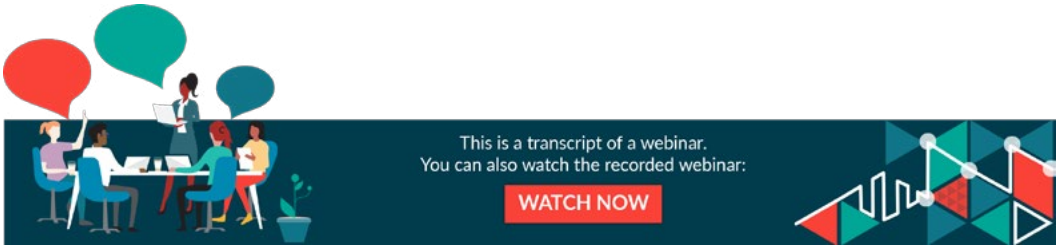

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Further reading and resources

- **White paper:** Enhancing the efficiency of Car T cell therapy manufacturing through automated bioprocessing
- **Article: Enhancing** CAR-T Cell Manufacturing Efficiency through Automation
- **Brochure:** Cell Therapy: From development to delivery

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