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Cryopreserved leukapheresis enables scalable and distributed CAR-T manufacturing: a multi-platform comparative study

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Cryopreserved leukapheresis is a scalable source for Chimeric antigen receptor T (CAR-T) manufacturing, yet it remains understudied (18.3% of 349 studies, 2010–2024). Current challenges include that cryopreserved leukapheresis lacks a standardized preparation process and has a low level of automation. Systematic validation of its clinical feasibility and platform compatibility is imperative. We carried out a series of optimizations, including the centrifugation procedure, the proportion of CS10, and the cryopreservation procedure. Eventually, a standardized cryopreserved leukapheresis process was established through a closed automated system. Subsequently, a systematic evaluation of the quality and functionality of cryopreserved leukapheresis was conducted. Results showed that cryopreserved leukapheresis achieved ≥ 90% post-thaw viability, with recovery and phenotypic profiles comparable to peripheral blood mononuclear cells (PBMCs). It exhibited a higher lymphocyte proportion than PBMCs (66.59% vs. 52.20%), correlating with enhanced CAR-T potential. Initial viability (91.0%) was lower than fresh samples (99.0%), but functional recovery post-electroporation and compatibility with both platforms were confirmed. In the non-viral CAR-T, lentiviral CAR-T, and Fast CAR-T platforms, cryopreserved leukapheresis and fresh leukapheresis were comparable in cell viability, expansion, cell phenotype, CAR + cell proportion, and cytotoxicity. This study comprehensively validated cryopreserved leukapheresis as a universal raw material for CAR-T manufacturing, preserving critical quality attributes (T-cell fitness, CAR functionality) without compromising consistency. Decoupling from fresh material logistics improves supply chain resilience. Protocol standardization and large-scale clinical validation remain critical next steps.

Keywords Cryopreserved leukapheresis, PBMCs, CAR-T, Fast CAR-T

Fresh leukapheresis exhibits time-sensitive viability decay (24-72-hour transport window)[1–3]. Current CAR-T production faces a paradoxical dilemma: While up to 33% of lymphoma patients are unable to receive treatment due to leukapheresis failure or rapid disease progression[4], the field still critically depends on cells sourced from these same immunocompromised patients for most cryopreservation protocols. These failure rates directly correlate with therapeutic outcomes: B-cell acute lymphoblastic leukemia (B-ALL) sees 15–40% treatment failures, rising to over 50% in B-cell lymphoma[5]. Intensive preconditioning regimens exacerbate T-cell deterioration. Furthermore, both advancing age and altered physiological conditions contribute to a progressive decline in immune cell quantity and function[6]. These studies indicate that cell quality impacts cell therapy outcomes. PBMCs cryopreservation demonstrates 20-30-year immune cell preservation[7]. Thus early storage is critical—driving our cryopreservation optimization.

Frozen PBMCs have a longer useful life and a wider application paradigm in cell therapy. However, with the continuous development of sorting technology, the selection of raw materials for cell therapy is more and more inclined to be treated directly with leukapheresis collection[8,9], rather than using PBMCs. Traditional PBMC isolation via density gradient centrifugation (e.g., Ficoll) is susceptible to temperature sensitivity and dilution ratio during processing[10], leading to significant cell loss, particularly of monocytes and stromal cells, which

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are critical for T-cell activation and immune function[11,12]. Direct cryopreservation of leukapheresis collections can significantly alleviate these bottlenecks, preserving cellular diversity and cell yields. Nevertheless, critical knowledge gaps persist in the systemic evaluation of cryopreserved leukapheresis for industrial applications. Most studies focus solely on T-cell function[13], neglecting non-T cell components and their cryotolerance[14].

This study demonstrates that the integration of standardized and automated closed production technologies for cryopreserved leukapheresis, when applied as starting materials for cutting-edge CAR-T platforms, constitutes a pivotal advancement toward distributed manufacturing. Implementing these innovations into future CAR-T therapeutic frameworks will not only provide novel raw materials for personalized treatments but also drive the transition of industrialized production toward distributed models, thereby enhancing therapeutic efficacy and safety.

Results

Systematic bibliometric analysis: research trends and key breakthroughs in cryopreserved leukocyte technology

A systematic search was conducted across PubMed using keywords: " ("PBMC" OR "peripheral blood mononuclear cells") AND ("cryopreservation" OR "freezing" OR "long-term storage"), ("leukapheresis" OR "leukopak") AND ("cryopreservation" OR "freezing") Inclusion criteria encompassed peer-reviewed studies (2010–2024) focusing on cryopreservation protocols, clinical outcomes, or process standardization. The search retrieved 285 studies on cryopreserved PBMCs and 64 studies on cryopreserved leukapheresis products, accounting for only 18.3% of total studies (Fig. 1A). From 2010 to 2024, annual publications on leukapheresis cryopreservation (2–8 papers) remained lower than those on PBMC cryopreservation (16–29 papers) (Fig. 1B). Notably, the surge in PBMC-related publications in 2016 coincided with the rising demand for CAR-T therapies. CiteSpace analysis of 349 publications revealed key research clusters, including "cellbanker", "flow cytometry", "biomonitoring", "T cells", and "CAR-T cells" (Fig. 1C-1D), highlighting major themes: cell banking, quality control, immune monitoring, T/B cell biology, cell expansion, and cancer therapeutics. Burst keyword analysis (Fig. 1E) demonstrated early focus on myeloid-derived suppressor cells and T cells, with later emphasis on PBMCs and whole blood, reflecting shifting research priorities. As a starting material for CAR-T products, leukapheresis offers advantages by reducing donor phlebotomy frequency and serving as a standardized, traceable raw material for off-the-shelf therapies. However, research on cryopreserved leukapheresis remains inadequate, lacking standardized protocols and post-thaw functional validation. Systematic evaluation of leukapheresis cryopreservation should address subpopulation stability, functionality, and integration with automated platforms to ensure reproducibility.

Breakthrough in closed automated process: standardization of CAR-T manufacturing using cryopreserved leukapheresis

The figure (Fig. 2) outlines the comprehensive process of developing a standardized cryopreservation protocol for leukapheresis products, followed by a comparability study, and finally, the different manufacturing processes for CAR-T cells. Initially, we embarked on developing the leukapheresis cryopreservation process to establish a standardized protocol for cryopreserved leukapheresis products. The primary objective of cryopreserved leukapheresis is to preserve leukocyte components, with current research focusing on optimizing T-cell quality post-thaw. While leukocyte cryopreservation technology is relatively mature, the critical challenge lies in mitigating the impact of non-target cellular impurities (e.g., residual red blood cells, platelets) in leukapheresis

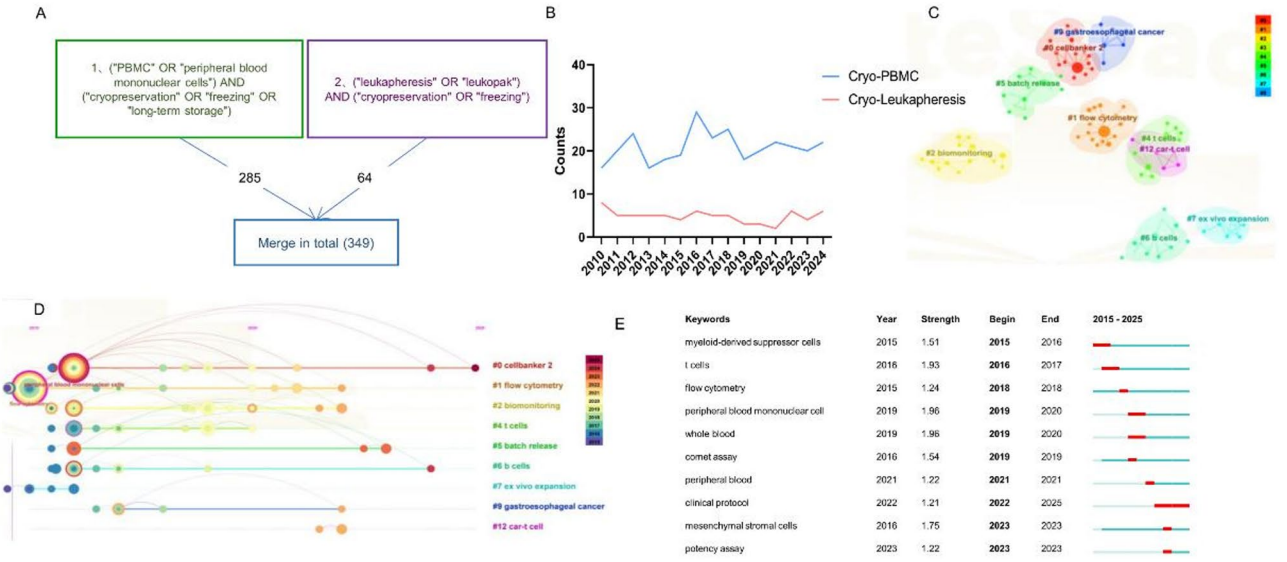


Fig. 1. Analysis of research trends in cryopreserved leukapheresis. (**A**) Literature search and screening workflow. (**B**) Annual publication trends (2010–2024). (**C**) Summary of literature keyword clustering analysis. (**D**) Research timeline of clustering. (**E**) Top 10 keywords with the strongest citation bursts.

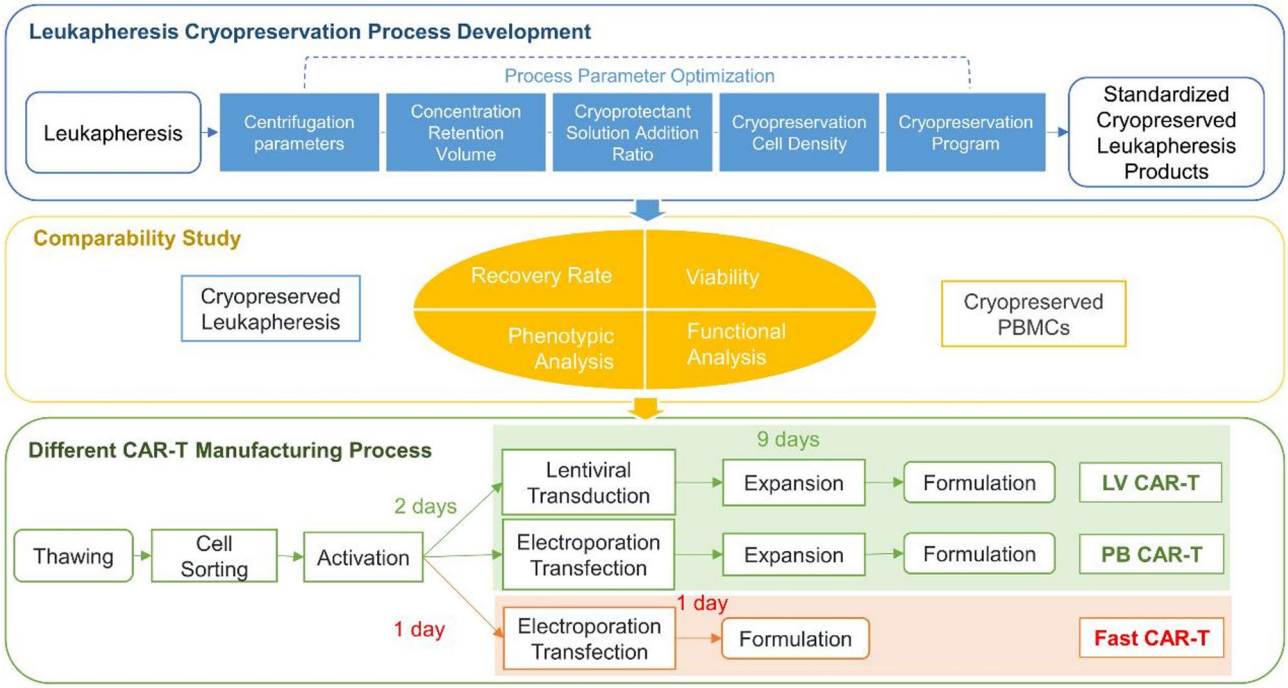


Fig. 2. Flowchart of the research process. This schematic illustrates the workflow for developing, validating, and applying cryopreserved leukapheresis products in CAR-T manufacturing. Part 1 details process optimization and standardization of leukapheresis cryopreservation. Part 2 validates product comparability between cryopreserved leukapheresis and PBMCs. Part 3 demonstrates diverse CAR-T manufacturing processes using cryopreserved products to ensure clinical readiness.

Parameter	Median (interquartile range)		
	Initial leukapheresis	Pre-cryopreservation	Post-cryopreservation
Cell concentration ($\times 10^7$cells/ml)	5.92 (5.09–9.71)	4.20 (4.06–5.12)	3.60 (3.49–4.67)
Viability (%)	99.5 (99.2–99.5)	95.3 (94.0-96.15)	93.4 (90.9–97.0)
CD3 + T lymphocyte proportion (%)	49.4 (43.82–56.31)	48.25 (41.19–56.45)	45.10 (42.01–51.21)
Formulation duration (min)	43 ~ 108		NA

Table 1. Key process attribution range for cryopreserved leukapheresis values.

products on post-thaw T-cell viability, recovery, and subsequent CAR-T product quality. To address this, a centrifugation-based strategy was systematically implemented to remove non-cellular impurities. Furthermore, clinical-grade cryoprotectant CS10 (10% DMSO) was selected to minimize erythrocyte volume interference with DMSO concentration, ensuring consistent cryoprotection efficacy.

Key parameters for leukocyte processing and cryopreservation are summarized in Table 1. The median cell concentration decreased progressively from $5.09–9.71 \times 10^7$ cells/ml at initial leukapheresis to $4.06–5.12 \times 10^7$ cells/ml pre-cryopreservation and further to $3.49–4.67 \times 10^7$ cells/ml post-cryopreservation. Viability remained high throughout, with initial values of 99.2–99.5%, declining slightly to 94.0–96.15% pre-cryopreservation and 90.9–97.0% post-thaw. The CD3 + T lymphocyte proportion showed minimal variation, ranging from 43.82 to 56.31% initially to 41.19–56.45% pre-cryopreservation and 42.01–51.21% post-thaw, indicating no significant loss of T cells during processing. Formulation efficiency was optimized using a closed-system automated platform, reducing processing times to 43–108 min. Cryopreservation parameters included a target cell concentration of $\sim 5 \times 10^7$ cells/ml to accommodate high-density requirements. Post-thaw viability (90.9–97.0%) and CD3 + purity (42.01–51.21%) confirmed effective cryoprotection and minimal cellular damage. Initial leukapheresis specifications included a leukocyte volume of ~ 1 ml per 1×10^9 cells, hematocrit levels of 5–10%, and residual volume retention (3 ml per 1×10^9 cells) to ensure ≥ 7.5% DMSO concentration in the cryomedium. These refinements underscored robust process reliability and consistency.

Optimized cryopreservation concentration: A target concentration range of 5×10^7–8×10^7 cells/ml was established, with a formulation volume of 20 ml/bag, ensuring ≥ 1×10^9 cells per bag as a critical quality attribute (CQA). Time-sensitive freezing protocol: The interval from cryoprotectant addition to controlled-rate freezing initiation was strictly limited to ≤ 120 min (Table 2), validated using the Thermo Profile 4 system to prevent ice crystal formation and ensure post-thaw viability ≥ 90%.

Process step	Parameter specification
Cryopreservation concentration ($\times 10^7$ cells/ml)	5–8
Final DMSO concentration (v/v)	7.5% – 10%
Formulation volume per bag (ml)	20
Freezing protocol	Controlled-rate freezing (Thermo Profile 4)
Formulation duration (min)	≤ 120

Table 2. Key process parameters for cryopreserved leukapheresis products.

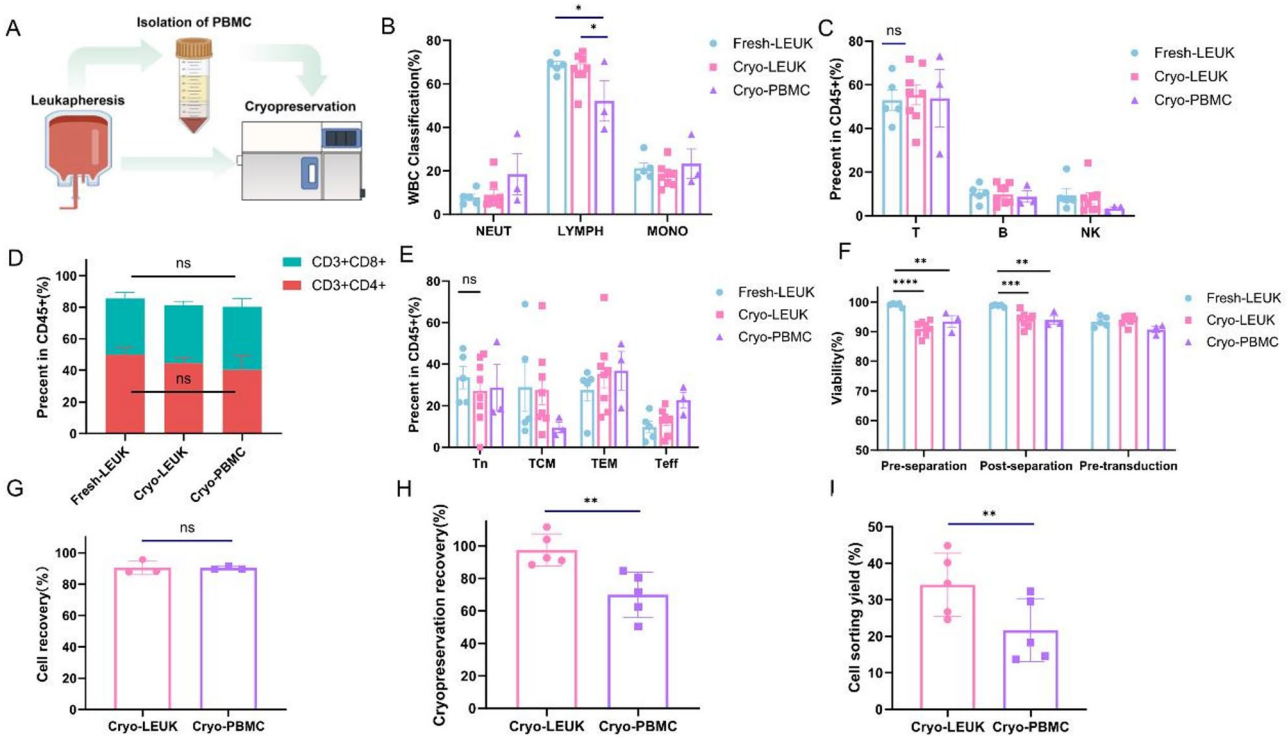


Fig. 3. Comparison of quality between fresh leukapheresis, cryopreserved leukapheresis, and cryopreserved PBMCs. (**A**) Flow chart of direct cryopreservation of leukapheresis versus cryopreservation of leukapheresis after isolation of PBMCs. (**B**) WBC Classification by Sysmex XS-1000i. NEUT = Neutrophil; LYMPH = Lymphocyte; MONO = Monocyte, Cryo = Cryopreserved. (**C**) T-, B-, and NK- cell subsets in Lymphocytes were analyzed by flow cytometry. (**D**) A flow cytometer analyzed CD4 and CD8 subsets in T-cells. (**E**) Different phenotypes of differentiated subpopulations were analyzed by flow cytometry. Tn = Naïve T cell; TCM = Central Memory T cell; TEM = Effector Memory T cell; Teff = Effector T cell. (**F**) Cell viability in different stages of the process. Pre-separation, Before T cell separation; Post-separation, After T cell separation; Pre-transduction, Before CAR transduction. The *p*-values for panels B-F were derived from two-way ANOVA (Fresh-LEUK $n = 5$, Cryo-LEUK $n = 8$, Cryo-PBMC $n = 3$). (**G**) Cell recovery from cryopreserved leukapheresis and cryopreserved PBMCs. Cell recovery = concentration of thawed cells/ concentration of frozen cells ($n = 3$). (**H**) Cell cryopreservation recovery from leukapheresis and PBMCs, Cell cryopreservation recovery = cell number of frozen cells/ cell number of leukapheresis. ($n = 5$). (**I**) Cell sorting yield from cryopreserved leukapheresis and cryopreserved PBMCs. ($n = 5$). Scatter dot plots with bars show the mean and SEM. The *p*-values for panels G-I were calculated using t-tests. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$)

Comprehensive functional profiling and comparative analysis of cryopreserved leukapheresis products versus PBMCs

To investigate the impact of the freezing process on the performance of cryopreserved leukapheresis products versus PBMCs, we conducted a comprehensive functional profiling and comparative analysis. Starting with an equivalent amount of leukapheresis material (Fig. 3A), we examined the phenotypic differences between fresh and cryopreserved samples.

Our analysis revealed a statistically significant difference in the proportion of major immune cells, specifically lymphocytes, between fresh and cryopreserved leukapheresis products compared to cryopreserved PBMCs. Fresh and cryopreserved leukapheresis products exhibited a higher percentage of lymphocytes (Fresh-LEUK: 68.68 ± 1.78%, Cryo-LEUK: 66.59 ± 2.64%) compared to cryopreserved PBMCs (Cryo-PBMC: 52.20 ± 9.29%,

$p < 0.05$) (Fig. 3B). In contrast, the proportions of neutrophils and monocytes were similar across all groups, with no statistically significant differences observed.

We further compared three key subsets within the lymphocyte population, including T, B, and NK cells. Our results indicated that there were no significant differences in the proportions of these subsets between the groups (Fig. 3C). The higher lymphocyte count in cryopreserved leukapheresis products was primarily attributed to an increased proportion of T cells, which may be advantageous for T cell-based therapies such as CAR-T. As a critical raw material for CAR-T manufacturing, we investigated the phenotypic and functional characteristics of T cells. The ratios of CD4 + and CD8 + T cells, as well as their functional subsets (Tn, TCM, TEM, and Teff), were comparable between thawed leukapheresis products and PBMCs (Fig. 3D and 3E). This suggests that the freezing process did not significantly alter the T cell composition or functional characteristics.

We compared the viability of cells at various stages of CAR-T manufacturing. Post-thaw recovery and T cell sorting resulted in lower viability for both cryopreserved leukapheresis products and PBMCs compared to fresh samples. Specifically, the viability of cryopreserved samples was significantly lower than fresh samples at the pre-separation stage (Fresh-LEUK: 99.04 ± 0.30% vs. Cryo-LEUK: 90.99 ± 0.86%, $p < 0.0001$; Fresh-LEUK: 99.04 ± 0.30% vs. Cryo-PBMC: 93.47 ± 1.95%, $p < 0.01$) and post-separation stage (Fresh-LEUK: 98.78 ± 0.17% vs. Cryo-LEUK: 94.06 ± 0.87%, $p < 0.001$; Fresh-LEUK: 98.78 ± 0.17% vs. Cryo-PBMC: 93.97 ± 1.42%, $p < 0.01$). However, before transduction, the viability of cryopreserved leukapheresis products was restored to levels comparable to fresh samples (Fresh-LEUK: 93.34 ± 0.78% vs. Cryo-LEUK: 93.88 ± 0.56%, $p > 0.05$; Fresh-LEUK: 93.34 ± 0.78% vs. Cryo-PBMC: 90.70 ± 1.01%, $p > 0.05$) (Fig. 3F).

We then focused on the recovery of cryopreserved leukapheresis products versus cryopreserved PBMCs after thawing. No statistically significant difference was observed in the recovery rates between the two groups (Fig. 3G). However, starting with an equivalent amount of leukapheresis material, cryopreserved leukapheresis products significantly preserved a greater number of leukocytes (97.5% ± 4.4%) compared to cryopreserved PBMCs (70.0% ± 6.2%, $p < 0.01$) (Fig. 3H). Consequently, this led to a higher yield of T cells in cryopreserved leukapheresis products (34.2% ± 8.6%) compared to cryopreserved PBMCs (21.7% ± 8.6%, $p < 0.01$) (Fig. 3I).

Cross-platform adaptability verification: cryopreserved leukapheresis as enablers of novel CAR-T production frameworks

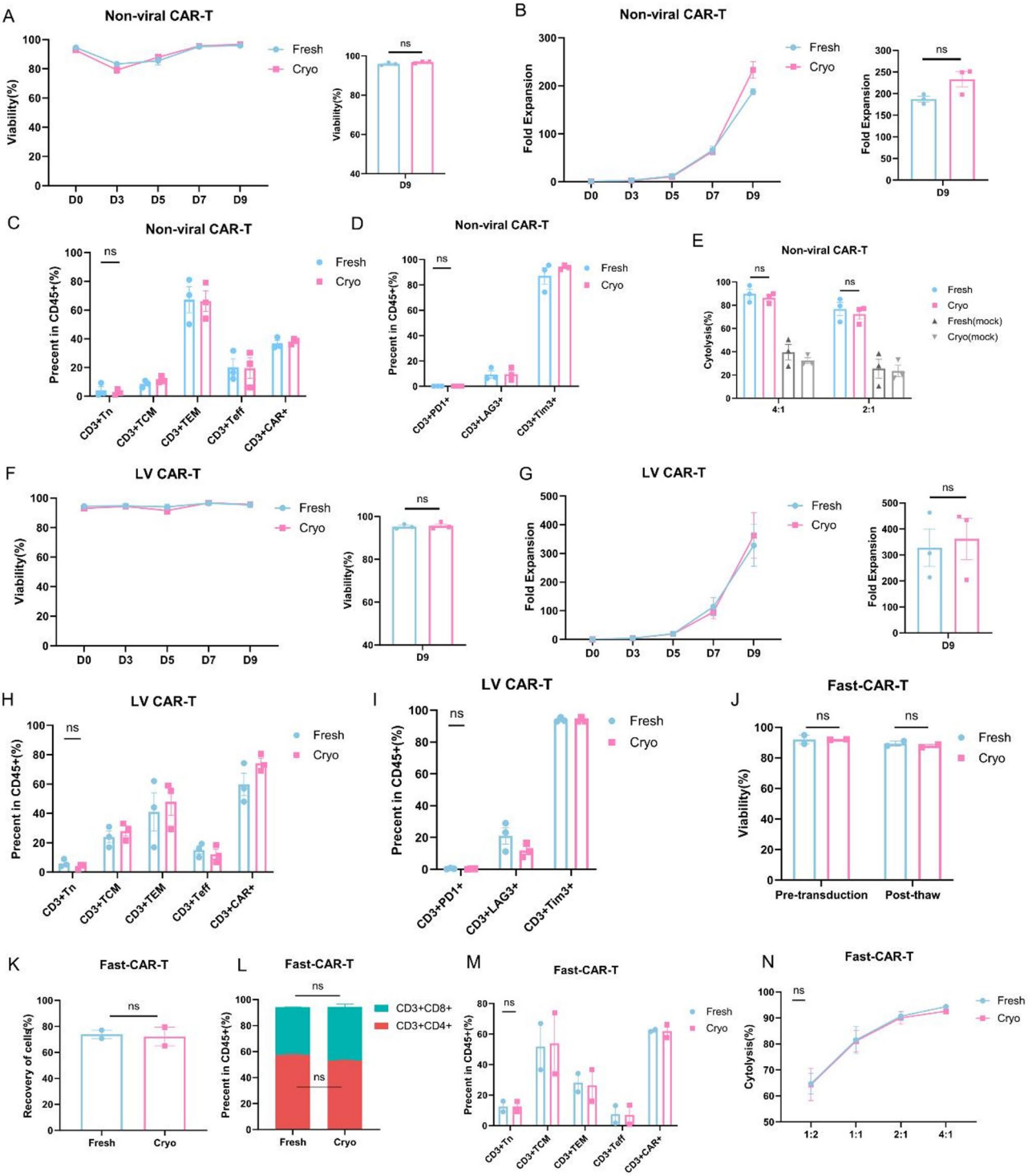
To investigate the feasibility of using cryopreserved leukapheresis as the initial raw material for conventional CAR-T manufacturing with the non-viral vector Piggy Bac transposon system, we compared the cell viability during the culture process (Fig. 4A) and continuous growth (Fig. 4B), finding no statistical difference between cryopreserved and fresh leukapheresis. Simultaneously, we explored the effects of cryopreserved and fresh leukapheresis on cell phenotype, cytotoxicity, and exhausted phenotype during CAR-T manufacturing. The study found no statistical difference in the proportions of Tn, TCM, TEM, and Teff between cryopreserved and fresh raw materials (Fig. 4C). For CAR-T, cryopreserved leukapheresis had no statistical difference from fresh one (Fig. 4C). Through the analysis of exhaustion functional markers (PD1+, LAG3+, and TIM3+) of CD3 + T cells on Day 9, we found no statistical difference between cryopreserved and fresh leukapheresis either (Fig. 4D). Finally, we conducted an assay of cellular cytotoxicity with effector: target (E: T) ratios of 2:1, 4:1, and found no statistical difference in the cytotoxicity effect between the two materials (Fig. 4E). Overall, cryopreserved leukapheresis is comparable to fresh one as the raw material for producing non-viral vector CAR-T.

To explore the extensive applicability of cryopreserved leukapheresis, apart from manufacturing CAR-T with non-viral vectors, we also used classical lentiviral vectors for CAR-T manufacturing. We compared the preparation of CAR-T using lentiviral vectors in T cells derived from cryopreserved and fresh leukapheresis and found no significant difference either in cell viability during the culture process (Fig. 4F) or in cell expansion (Fig. 4G). The study revealed that the proportions of Tn, TCM, TEM, and Teff (Fig. 4H) had no statistical difference between the cryopreserved and fresh leukapheresis. Certainly, when transfected at the same MOI = 1.5, there was also no significant difference in CD3 + CAR + between the cryopreserved and fresh raw materials (Fig. 4H). At the end-point of the culture, regarding the exhaustion phenotype analysis of PD1+, LAG3+, and TIM3 + cells, there was no statistical difference between the cryopreserved and fresh leukapheresis (Fig. 4I). In summary, cryopreserved leukapheresis is also on par with fresh as the raw material for producing lentiviral vector CAR-T.

To explore whether cryopreserved leukapheresis could be used as the initial raw material in the fast CAR-T process, which has more strict requirements for raw material. Our findings revealed no statistically significant differences between cryopreserved and fresh leukapheresis in terms of cell viability on the harvest day, even post-thaw (Fig. 4J). Similarly, the recovery rate after fast CAR-T preparation using the non-viral vector showed no significant difference between the cryopreserved and fresh ones (Fig. 4K). We also compared the impact of fast CAR-T preparation on cell phenotype and cytotoxicity. Post-thaw, there were no statistically significant differences in CD4 + T cell and CD8 + T cell phenotypes (Fig. 4L). Additionally, the proportions of Tn, TCM, TEM, and Teff cells were comparable, as well as between cryopreserved and fresh CAR-T (Fig. 4M). Furthermore, an assay of cellular cytotoxicity with an effector: target (E: T) ratio of 1:2 to 4:1 demonstrated no statistically significant difference in cytotoxicity efficacy between cryopreserved and fresh groups (Fig. 4N).

Cryopreserved leukapheresis as a functionally equivalent alternative to cryopreserved PBMCs for scalable immune cell therapy manufacturing

Our study compared the functional performance of cryopreserved leukapheresis and cryopreserved PBMCs as raw materials for immune cell therapies, focusing on T cells, NK cells, and NKT cells. No significant differences were observed between the two cryopreserved cell sources in multiple assays. Specifically, the proportions of CD4 + and CD8 + T cells within the CD3 + T cell population remained comparable (Fig. 5A), as did the distribution of T cell phenotypes in Tn, TCM, TEM, and Teff (Fig. 5B).



To assess functional viability, sorted T cells from both sources were cultured for 9 days. Cell viability exceeded 85% in all cases (Fig. 5C), and no differences in total T cell expansion were detected (Fig. 5D), indicating equivalent proliferative capacity. For NK/NKT cell evaluation, we employed a cytokine-based kit for NK cell expansion. However, this method concurrently amplified both NK and NKT cells, necessitating subsequent phenotypic analysis to distinguish these populations. Despite this co-expansion, viability (Fig. 5E) and total cellular expansion (Fig. 5F) during culture showed no significant divergence between cryopreserved leukapheresis and PBMCs. Further analysis of NK/NKT-specific expansion (Fig. 5G) and their proportional representation in culture up to Day 11 (Fig. 5H) also revealed comparable outcomes between the two cell sources.

These findings align with prior studies demonstrating that cryopreserved PBMCs and leukapheresis products retain functional equivalence in CAR-T manufacturing and immune cell applications, with no adverse impact on viability, expansion, or therapeutic efficacy. The observed co-amplification of NK and NKT cells highlights the importance of method-specific validation, as cytokine-driven protocols may inadvertently activate overlapping

◀**Fig. 4**. Generation of CAR-T cells by non-viral vector and lentiviral transduction. (**A**) Cell viability during the preparation of non-viral CAR-T cells from day 0 to day 9. ($n=3$). (**B**) Fold expansion during the generation of non-viral CAR-T cells from day 0 to day 9. ($n=3$). (**C**) Different phenotypes of differentiated subpopulations and CAR + within T cells from fresh and cryopreserved leukapheresis were analyzed by flow cytometry. ($n=3$). (**D**) Exhaustion markers PD-1, TIM-3, and LAG-3 in non-viral CAR-T cells were analyzed by Flow cytometry. ($n=3$). (**E**) Cytotoxicity of non-viral CAR T cells co-cultured with tumor cells in a 2:1 and 4:1 E: T ratio for 24 h. ($n=3$). (**F**) Cell viability in the process of LV CAR-T preparation through lentiviral technology. MOI = 1.5. ($n=3$). (**G**) Fold expansion during the generation of LV CAR-T cell cultures. ($n=3$). (**H**) Different phenotypes of differentiated subpopulations and CAR + within T cells from fresh and cryopreserved leukapheresis were analyzed by flow cytometry. ($n=3$). (**I**) Exhaustion markers PD-1, TIM-3, and LAG-3 in LV CAR-T cells were analyzed by Flow cytometry. ($n=3$). (**J**) Cell viability of the Fast CAR-T cell process. ($n=2$). (**K**) Recovery of Fast CAR-T cells from thawing. ($n=2$). (**L**) CD4 + and CD8 + T cell subsets of fast CAR-T cells 3 days post-thaw from fresh and cryopreserved leukapheresis. ($n=2$). (**M**) Different phenotypes of differentiated subpopulations and CAR + within T cells on 3 days post-thaw from fresh and cryopreserved leukapheresis. ($n=2$). (**N**) Cytotoxicity of fast CAR T cells co-cultured with tumor cells in a 1:2, 1:1, 2:1, and 4:1 E: T ratio for 24 h. ($n=2$). Scatter dot plots with bars show the mean and SEM. *P*-values were from t-tests.

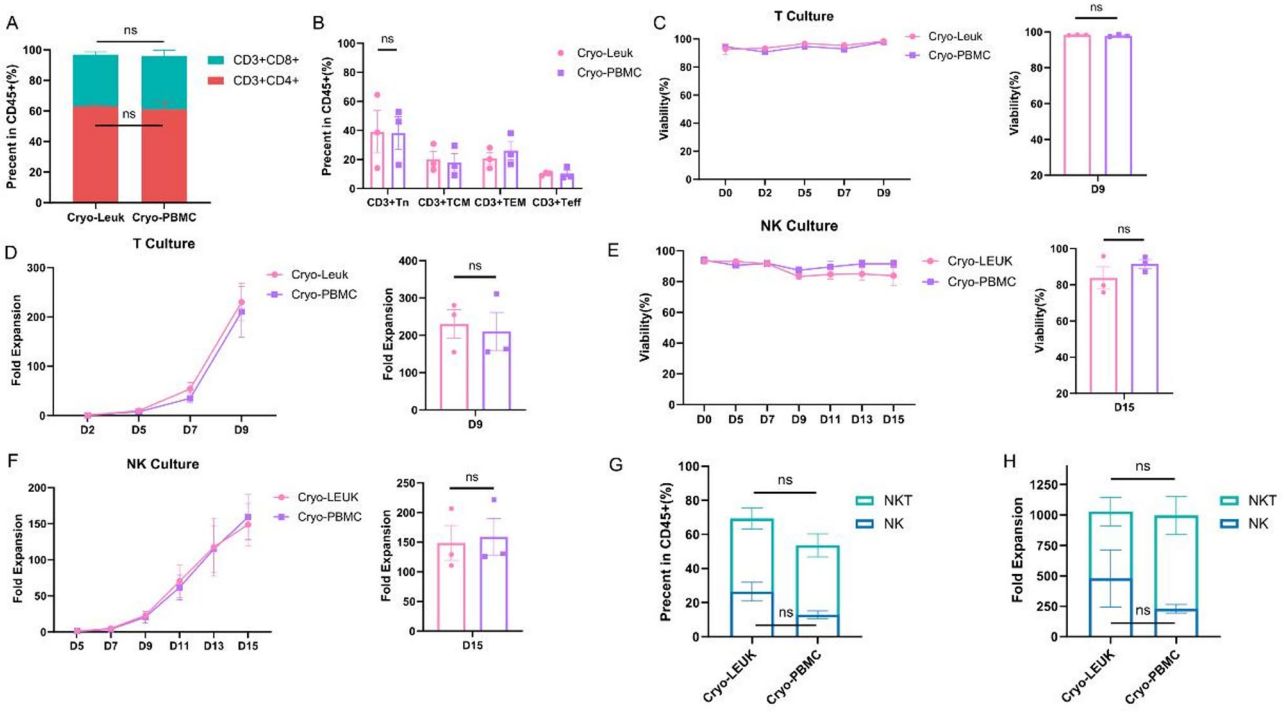


Fig. 5. Comparability study of immune cell culture in cryopreserved leukapheresis with cryopreserved PBMCs. (**A**) CD4 and CD8 subsets in Lymphocytes were analyzed by flow cytometry. ($n=3$). (**B**) Different phenotypes of differentiated subpopulations within T cells were analyzed by flow cytometry. ($n=3$). (**C**) Cell viability during the T cells from day 0 to day 9. ($n=3$). (**D**) Fold expansion of the T cells from day 0 to day 9. ($n=3$). (**E**) Cell viability during the NK and NKT cells from day 0 to day 15. ($n=3$). (**F**) Total fold expansion during the NK and NKT cells from day 0 to day 15. ($n=3$). (**G**) NK and NKT cell subsets in Lymphocytes were analyzed by flow cytometry on day 11 of the cell cultures. ($n=3$). (**H**) Specific fold expansion of NK cells and NKT cells at day 11 of cell cultures. ($n=3$). Scatter dot plots with bars show the mean and SEM. *P*-values were from t-tests.

subsets. Overall, our data support the interchangeable use of cryopreserved leukapheresis and PBMCs for scalable cell therapy production.

Discussion

Traditional CAR-T manufacturing uses a linear "patient-leukapheresis-immediate processing" workflow, creating geographically constrained supply chains vulnerable to cellular viability loss. Mature cryopreserved leukapheresis technology enhances flexibility by enabling centralized cryostorage combined with off-site manufacturing. Our study shows thawed products maintain sufficient viable cells (90% recovery rate, 90–99% T-cell purity) for CAR-T production. This centralized model involves cryopreserving patient materials for shipment to core facilities, genetic modification, and redistribution to clinics—distributing costs and

accommodating demand fluctuations[15]. It facilitates equitable access for remote patients while mitigating disease progression risks associated with manufacturing delays[16].

To our knowledge, this study demonstrates that cryopreserved leukapheresis products are compatible with lentiviral transduction, transposon-based systems, and rapid CAR-T manufacturing platforms, underscoring their adaptability for advancing next-generation immunotherapies. The long-term dynamics post-cryopreservation—such as in vivo expansion kinetics and the dynamics of memory subset proportions—require further elucidation through prospective clinical trials. Nevertheless, emerging clinical evidence supports the functional integrity of CAR-T products from frozen immune cells. In a multi-center trial of 115 patients receiving PBMC-derived CAR-T therapy, comparable objective response rates were observed between fresh (72%) and cryopreserved (80%) cohorts, with no statistically significant difference ($p = 0.727$)[17]. This suggests that cryopreservation protocols may preserve therapeutic efficacy. Cryopreserved leukapheresis advantages extend beyond supply chain optimization to broader clinical applications through compatibility with diverse CAR-T platforms. Studies have validated adaptability to lentiviral transduction[1] (e.g., Kymriah), transposon systems (e.g., Sleeping Beauty), and non-viral gene-editing platforms (e.g., CRISPR/Cas9)[18]. Cryopreserved cells show comparable therapeutic efficacy to fresh cells—demonstrated in B-cell leukemia trials with similar in vivo expansion, persistence, toxicity profiles, and disease response rates to fresh cells[19]. Our data reveal no significant differences in lentiviral transduction efficiency between cryopreserved leukapheresis-derived and PBMC-derived T cells. Cryopreserved umbilical cord blood-derived T cells displayed tumor-killing activity equivalent to autologous cells in preclinical models[20], supporting universal CAR-T development. These findings establish the cross-platform applicability, overcoming historical limitations of single-process studies.

Ultra-short cycle CAR-T manufacturing (< 3 days), exemplified by platforms like Ultra CAR-T[21]represents an emerging industry trend. Cryopreserved leukapheresis exhibits particular synergy with such technologies. Conventional ex vivo expansion (7–14 days[22] risks cryopreservation-induced metabolic and phenotypic T-cell alterations, whereas fast processes mitigate these effects by minimizing culture duration (e.g., completing transduction/activation within 72 h)[23]. Integration of non-viral technologies with automated closed systems (e.g., CliniMACS Prodigy) enables sub-3-day production cycles while leveraging cryopreserved leukapheresis samples for on-demand manufacturing. This "Freeze-and-Speed" strategy not only reduces cellular exhaustion risks through rapid thaw-to-manufacture workflows[24] but also enhances batch consistency by minimizing manual handling, thereby laying the groundwork for scalable production.

Standardized protocols for cryopreserved leukapheresis (e.g., automated apheresis devices and GMP-compliant cryopreservation) provide technical foundations for large-scale biobanking and mitigate patient-specific limitations. Critically, clinical heterogeneity is addressed through: (1) Industrial validation: Utilizing cryopreserved leukapheresis for CAR-T manufacturing achieves 82.2% release success rate across production batches for patient infusion[25]. The Kymriah® (tisagenlecleucel) manufacturing protocol accepts cells cryopreserved within 18 months under quality oversight[26]; (2) Proactive banking at diagnosis: In DLBCL patients, leukapheresis prior to ASCT therapy yielded significantly increased CD4+/CD8 + ratios ($p = 0.04$) and reduced absolute counts of CD8 + terminally differentiated T cells ($p = 0.05$)[27], preserving T-cell fitness. This strategy enables cross-population applicability (early/late-stage patients and healthy donors), where healthy donor banking serves future universal CAR-T development. Mechanistic optimization (e.g., cryoprotectant adjustments for memory Treg subsets[28]) will advance personalized strategies, steering CAR-T therapy from a "one-size-fits-all" toward precision medicine.

Cryopreserved leukapheresis has emerged as a cornerstone for CAR-T therapy scalability and accessibility by resolving supply chain bottlenecks, enabling cross-platform compatibility, synergizing with fast processes, and facilitating biobanking. Future research must address cryopreservation's epigenetic impacts on immune cells and leverage artificial intelligence to optimize the entire cryopreservation-manufacturing pipeline, ultimately realizing the vision of "one leukapheresis, multiple therapies."

Conclusion

In conclusion, there are no significant differences in the cell profiles (including differential of leukocyte, lymphocyte subset, and T cell subpopulation, as well as in the culture potency of T-cells and non-T-cells, such as NK and NKT cells, between cryopreserved leukapheresis and PBMCs. However, obtaining T cells from cryopreserved leukapheresis simplifies the process, reduces cell loss, and yields more sorted T cells. The main impact of cryopreserved leukapheresis is lower cell viability after recovery and sorting compared to fresh. Meanwhile, the cell profiles have no significant difference between fresh and cryopreserved leukapheresis. Cryopreserved leukapheresis was used to manufacture CAR-T using different processes with results comparable to fresh leukapheresis.

Materials and methods

Isolation and cryopreservation of PBMCs

PBMCs were isolated from leukopaks obtained from healthy donors (Milestone Biotechnologies, CN) using density gradient centrifugation. Briefly, leukapheresis samples were diluted 1:3 with normal saline and carefully layered over Ficoll-Paque Plus (Cytiva, 17-5442-03, USA). Centrifugation was performed at 800 × g for 20 min to separate PBMCs from other blood components. The PBMC layer was aspirated, washed three times with PBS, and resuspended in a freezing medium. For cryopreservation, PBMCs were adjusted to a concentration of 2×10^7 – 5×10^7 cells/ml in 2 ml cryovials using a cryoprotectant solution CS10 (STEMCELL, 07930, CA). The cryovials were placed in a controlled-rate freezing container and gradually cooled to − 80 °C for 24 h before long-term storage in vapor-phase liquid nitrogen tanks (Thermo). The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Shanghai Li Quan Hospital (Continuous

Review Number: 20212002-C221). Informed consent was obtained from all individual participants included in the study.

Culture of T and NK cells

T cells were magnetically sorted from PBMCs or leukapheresis products using the CliniMACS CD4/CD8 dual-positive selection system. Specifically, 2 µL of CliniMACS CD4 (Miltenyi, 200-070-213, DE) and CD8 (Miltenyi, 200-070-215, DE) microbeads were added per 1×10^7 cells for positive selection, with purity validated by flow cytometry. The sorted T cells were cultured in AIM-V medium (Thermo, 0870112DK, USA) supplemented with 5% human serum substitute (Thermo, A2596101, USA), recombinant human IL-7 (25 ng/ml) (PrimeGene, GMP-101-07, CN), and IL-15 (25 ng/ml) (PrimeGene, GMP-101-015, CN). Fresh cytokines were replenished every 48 h, and cells were passaged at a 5×10^5 cells/ml concentration. NK cells from PBMCs or Leukapheresis were cultured in NK expansion media (ExCell Bio, NE000-N032, CN) and as instructed.

Tumor cell line culture

The SKOV3 cell line (ATCC HTB-77) was routinely maintained in DMEM high-glucose medium (Corning, 10-013-CV, USA) containing 10% fetal bovine serum (Gibco, 10099–141 C, USA) at 37 °C and 5% CO2. Cells were passaged twice weekly using 0.25% trypsin.

CAR-T production by different techniques

T cell purification: T cells were isolated from leukapheresis-derived cells (1×10^7 cells) using CliniMACS CD4 and CD8 Microbeads (2 µL each) for magnetic separation, enriching $CD4^+$ and $CD8^+$ populations.

T cell activation: Purified T cells were activated with CD3/CD28 TransAct (Miltenyi, 200-076-202, DE) at a concentration of 2.86×10^6 cells/ml. For lentiviral vectors (LV) and PiggyBac transposon (PB) CAR-T methods, activation lasted 48 h, while fast CAR-T utilized a shorter 24-hour activation period.

Genetic modification

LV CAR-T: On day 3, T cells were transduced with lentiviral vectors (MOI 1.5) (Hillgene, HG-CT1901, CN) followed by a 9-day expansion phase before harvest.

PB CAR-T: On day 3, activated T cells were electroporated (MaxCyte GTx system) with the PB transposon vector and transposase mRNA, then cultured for 9 days.

Fast CAR-T: After 24-hour activation, T cells were electroporated in Opti-MEM using the JL transposon system and harvested immediately without further expansion.

Cell culture and harvest: LV and PB CAR-T cells underwent prolonged culture (9 days) to achieve expansion, whereas fast CAR-T bypassed amplification, emphasizing rapid production.

Cytotoxic assay

Cell toxicity was estimated by a 24-hour co-culture assay. Cell death was detected using the Real Time Cellular Analysis (ACEA XCELLIgene RTCA TP, Agilent, USA). T cells and target cells were co-cultured at an indicated E: T ratio. The day before the CAR T cytotoxicity assay, SKOV3 in good condition was inoculated into the E-Plate and set up according to the instrument instructions. The target cells were cultured overnight to achieve a cell index ≥ 1.0. Positive CAR T cells were added to the target cell culture wells for co-culture, and the experiment was completed after 24 h. Duplicate wells were performed for all samples. The percentage of the target cells killed was measured by the following method $= \frac{\text{target cell index} - \text{test cell index}}{\text{target cell index}} \times 100$.

Flow cytometry

The collection of in vitro cultured cells or isolated from leukocytes was washed twice with PBS, 1×10^6 cells were resuspended in 100µL PBS, and the corresponding antibodies were added and incubated for 15 min at 4 °C. Details of the antibodies used are given in Table 3. The lymphocyte subsets were analyzed: T (CD3+), B (CD3-CD19+), NK (CD3-CD56 + CD16+), and NKT (CD3 + CD56+). T cell differentiation phenotypes were analyzed: naïve T cell (CD3 + CD45RO-CCR7+, Tn); central memory T cell (CD3 + CD45RO + CCR7+, TCM); effector memory T cell (CD3 + CD45RO + CCR7-, TEM); effector T cell (CD3 + CD45RO-CCR7-, Teff). CAR positivity was assayed using recombinant proteins. 1µL PE was added as a secondary antibody per 1×10^6 cells and incubated at room temperature away from light for 15 min. Cell collection was performed using a flow cytometer (CytoFlex, Beckman, CA), and data analysis was performed using Kaluza Analysis.

Statistical analysis

The statistical analyses were performed using GraphPad Prism 8.0. Paired t-tests were used to compare one condition, while two-way ANOVA was used for comparisons involving more conditions. Error bars represent the standard errors of the mean (SEM). Statistically significant differences were indicated as follows: $p \geq 0.05$ (ns, no significant difference), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

Target	Fluorochrome	Supplier	Catalog number
Subsets of Lymphocytes			
CD45	PerCP/Cyanine5.5	Biolegend	304028
CD3	Alexa Fluor 488	Biolegend	317310
CD56	Brilliant Violet 421™	Biolegend	318328
CD16	PE	Thermo	12-0618-42
CD19	APC	Biolegend	302211
Different phenotypes of T cell			
CD45RO	Brilliant Violet 605™	Biolegend	304238
CD3	Alexa Fluor 700	BD	557943
CD4	Alexa Fluor 488	Biolegend	344618
CD8	PerCP-Cyanine5.5	Thermo	45-0088-42
CCR7	PE/Cyanine7	Biolegend	353226
CD95	APC	Biolegend	305635
Exhaustion			
LAG-3	PE/Cyanine7	Biolegend	369310
PD-1	BV421	BD	564323
TIM-3	BV650	BD	CD366
Others			
CD19	NA	ACRO	FM3-Y45
Mesothelin	NA	Self-produced	NA
Streptacidin-PE	PE	BD	554061

Table 3. The list of fluorescently labeled proteins/antibodies for flow cytometry.

Data availability

Data is provided within the manuscript.

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References

1. Ayala Ceja, M., Khericha, M., Harris, C. M., Puig-Saus, C. & Chen, Y. Y. CAR-T cell manufacturing: major process parameters and next-generation strategies. *J. Exp. Med.* **221**, e20230903 (2024).
2. Abraham-Miranda, J. et al. CAR-T manufactured from frozen PBMC yield efficient function with prolonged in vitro production. *Front. Immunol.* **13** (2022).
3. Abou-el-Enein, M. et al. Scalable manufacturing of CAR T cells for cancer immunotherapy. *Blood Cancer Discovery*. **2**, 408–422 (2021).
4. Agliardi, G., Dias, J., Rampotas, A., Garcia, J. & Roddie, C. Accelerating and optimising CAR T-cell manufacture to deliver better patient products. *Lancet Haematol.* **12**, e57–e67 (2025).
5. Baguet, C., Larghero, J. & Mebarki, M. Early predictive factors of failure in autologous CAR T-cell manufacturing and/or efficacy in hematologic malignancies. *Blood Adv.* **8**, 337 (2023).
6. Bailur, J. K. et al. Immune profiles of elderly breast cancer patients are altered by chemotherapy and relate to clinical frailty. *Breast Cancer Res.* **19**, 20 (2017).
7. Dyer, W. B. et al. Preservation of functionality, immunophenotype, and recovery of HIV RNA from PBMCs cryopreserved for more than 20 years. *Front. Immunol.* **15**, 1382711 (2024).
8. Qayed, M. et al. Leukapheresis guidance and best practices for optimal chimeric antigen receptor T-cell manufacturing. *Cytotherapy* **24**, 869–878 (2022).
9. Carbonell, D. et al. Advantages of high cell concentration prior to cryopreservation of initial leukapheresis in CAR-T cell therapy. *Blood Transfus.* **22**, 239–245 (2024).
10. Cui, C., Schoenfelt, K. Q., Becker, K. M. & Becker, L. Isolation of polymorphonuclear neutrophils and monocytes from a single sample of human peripheral blood. *STAR. Protocols*. **2**, 100845 (2021).
11. Grievink, H. W., Luisman, T., Kluft, C., Moerland, M. & Malone, K. E. Comparison of three isolation techniques for human peripheral blood mononuclear cells: cell recovery and viability, population composition, and cell functionality. *Biopreserv Biobank*. **14**, 410–415 (2016).
12. Li, B. et al. Comprehensive evaluation of the effects of long-term cryopreservation on peripheral blood mononuclear cells using flow cytometry. *BMC Immunol.* **23**, 30 (2022).
13. Cuffel, A. et al. Real-world characteristics of T-cell apheresis and clinical response to tisagenlecleucel in B-cell lymphoma. *Blood Adv.* **6**, 4657–4660 (2022).
14. Haider, P. et al. Quantitative and functional assessment of the influence of routinely used cryopreservation media on mononuclear leukocytes for medical research. *Int. J. Mol. Sci.* **23**, 1881 (2022).
15. Autologous CAR T-cell therapies supply chain: challenges and opportunities? | cancer gene therapy. https://www.nature.com/articles/s41417-019-0157-z.
16. Uncertainty quantification for gene delivery methods. A roadmap for pDNA manufacturing from phase I clinical trials to commercialization - Triantafyllou – 2024 - Biotechnology Journal - Wiley Online Library. https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/full/https://doi.org/10.1002/biot.202300103.
17. Brezinger-Dayan, K. et al. Impact of cryopreservation on CAR T production and clinical response. *Front. Oncol.* **12**, 1024362 (2022).

18. Kim, H., Kim, S., Lim, H. & Chung, A. J. Expanding CAR-T cell immunotherapy horizons through microfluidics. *Lab. Chip*. **24**, 1088–1120 (2024).
19. Dreyzin, A. et al. Cryopreserved anti-CD22 and bispecific anti-CD19/22 CAR T cells are as effective as freshly infused cells. *Mol. Ther. Methods Clin. Dev.* **28**, 51–61 (2023).
20. CD62L-selected umbilical cord. blood universal CAR T cells | bioRxiv. https://www.biorxiv.org/content/https://doi.org/10.1101/2024.01.18.576145v1.full.
21. Sallman, D. A. et al. A phase 1/1b safety study of Prgn-3006 Ultracar-T™ in patients with relapsed or refractory CD33-Positive acute myeloid leukemia and higher risk myelodysplastic syndrome. *Blood* **136**, 17–17 (2020).
22. Li, Y., Hu, Z., Li, Y. & Wu, X. Charting new paradigms for CAR-T cell therapy beyond current Achilles heels. *Front. Immunol.* **15**, 1409021 (2024).
23. Ahmadi, M. et al. Accelerating CAR T cell manufacturing with an automated next-day process. *Curr. Res. Transl Med.* **73**, 103489 (2024).
24. A digital platform for the design of. patient-centric supply chains | Scientific Reports. https://www.nature.com/articles/s41598-022-21290-5.
25. Tyagarajan, S., Schmitt, D., Acker, C. & Rutjens, E. Autologous cryopreserved leukapheresis cellular material for chimeric antigen receptor-T cell manufacture. *Cytotherapy* **21**, 1198–1205 (2019).
26. Yakoub-Agha, I. et al. Management of adults and children undergoing chimeric antigen receptor T-cell therapy: best practice recommendations of the European society for blood and marrow transplantation (EBMT) and the joint accreditation committee of ISCT and EBMT (JACIE). *Haematologica* **105**, 297–316 (2020).
27. Timely Leukapheresis May. Interfere with the fitness of lymphocytes collected for CAR-T treatment in high risk DLBCL patients. https://www.mdpi.com/2072-6694/14/21/5276.
28. Optimizing cryopreservation of hematopoietic stem cells collected for autologous stem cell transplantation in patients with multiple myeloma. *ResearchGate* https://doi.org/10.1016/j.jcyt.2019.03.375 (2024).

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Author contributions

M.R. wrote the manuscript. M.R., X.W., and J.C. designed and performed most of the experiments and data analysis. Y.X. assisted in project management and conducted the investigation. Y.S. and T.L. engaged in project administration and funding acquisition. Q.Q. and Z.X. provided funds, guidance, and conceptualized the research project. All authors have reviewed and approved the definitive version of this manuscript before submission.

Declarations

Competing interests

The authors declare no competing interests.

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