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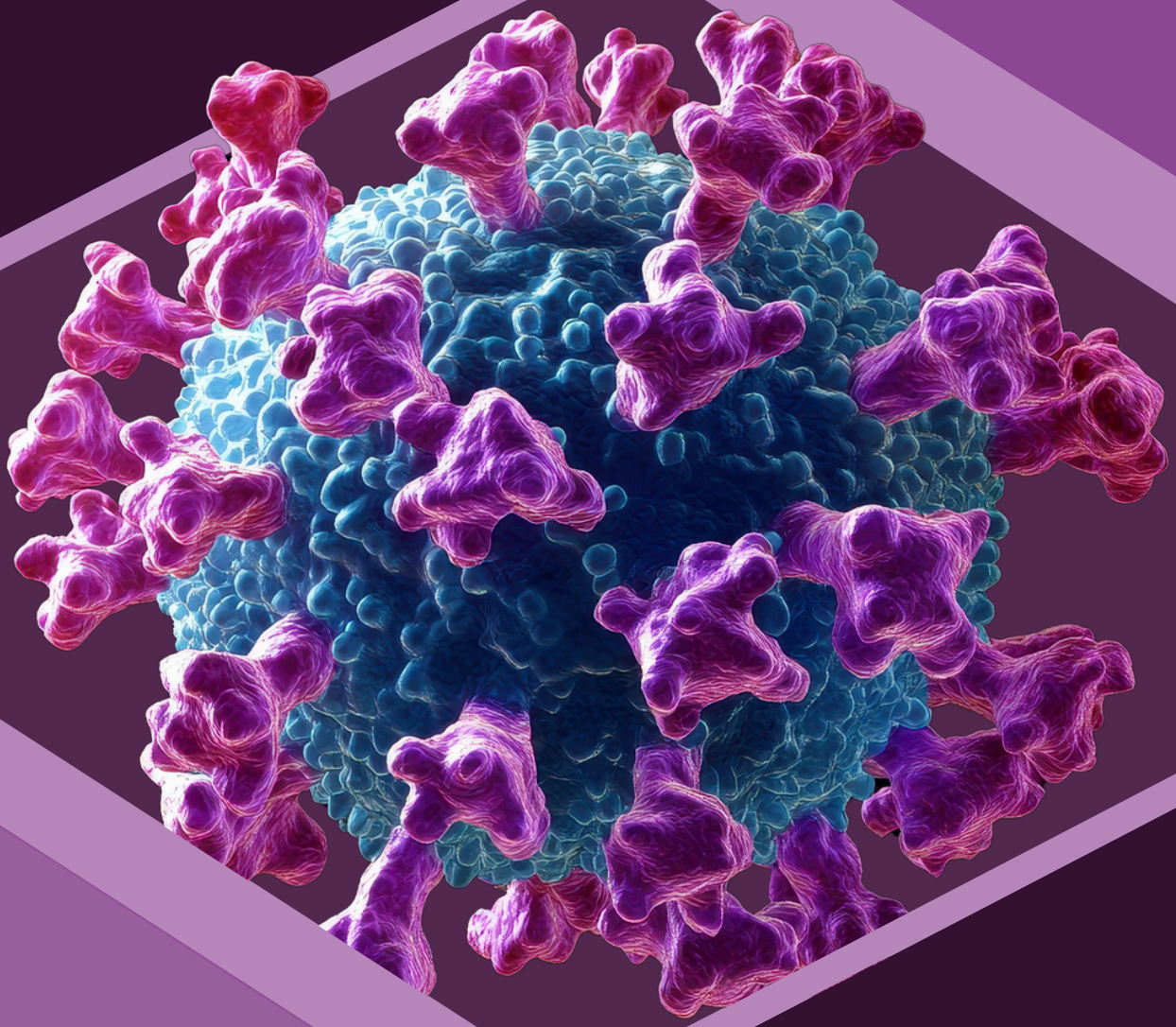

CAR-T therapy:
from laboratory optimization to clinical and commercial manufacturing

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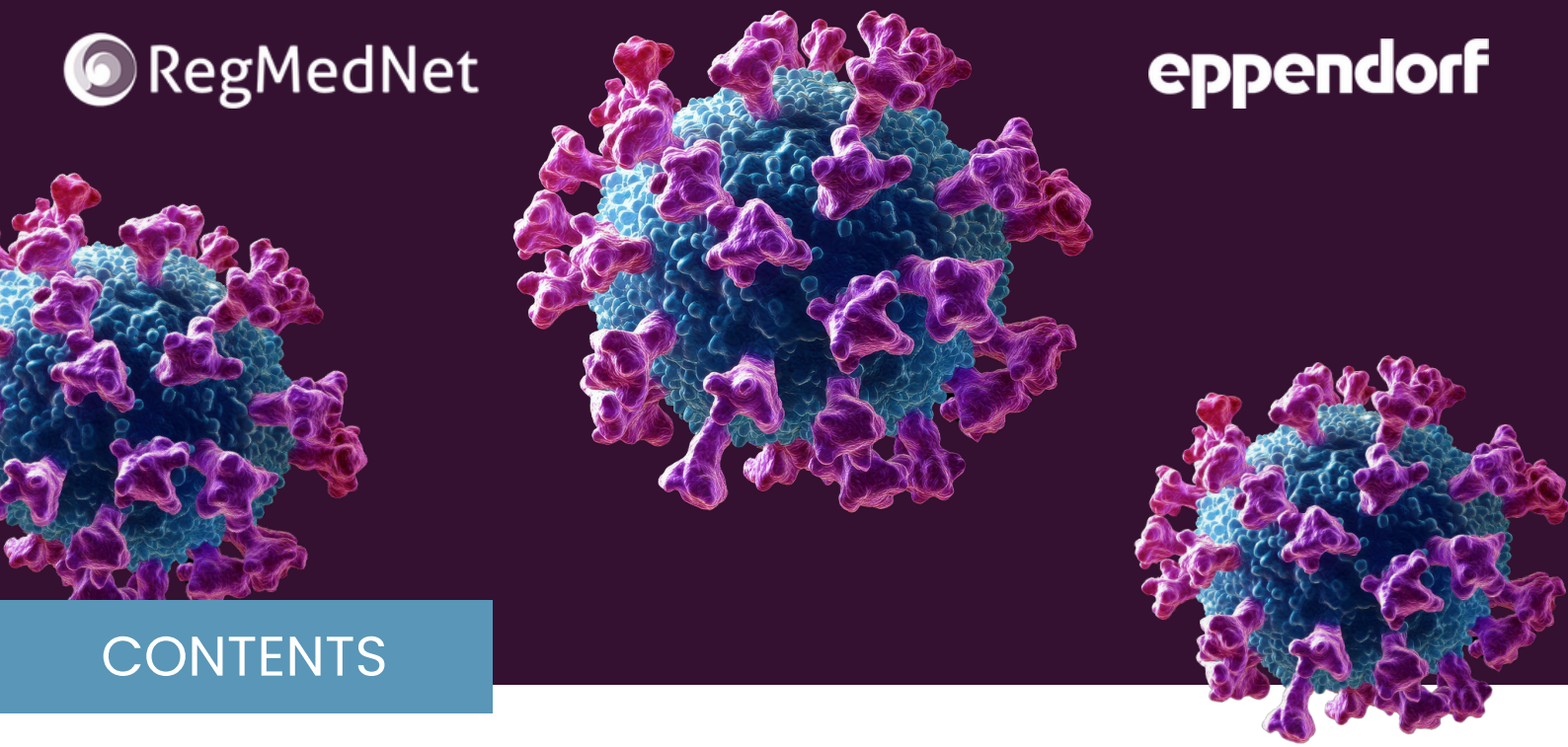

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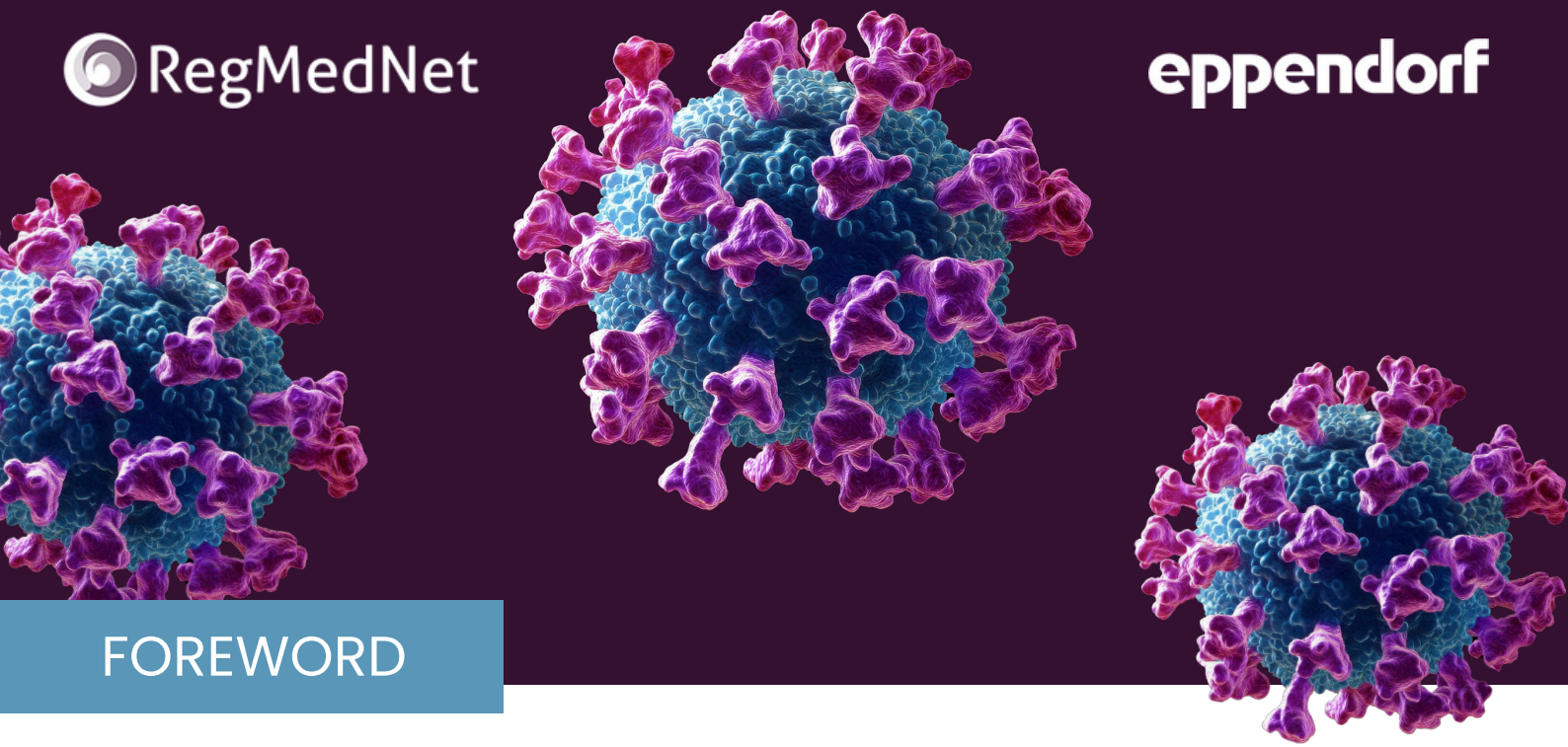

FOREWORD

We are pleased to present you with this eBook on CAR-T therapy, bringing together critical research and clinical perspectives on this groundbreaking immunotherapy approach.

CAR T-cell therapy has emerged as a revolutionary treatment modality, demonstrating remarkable efficacy in hematologic malignancies and showing promising potential for broader applications.

This eBook features a collection of essential articles spanning current clinical challenges, applications in T-cell malignancies, advances in solid tumor approaches, and technical innovations in cell manufacturing processes. Together, they provide a comprehensive overview of the field's current status and future directions.

We hope you enjoy reading these expert insights with us.

Megan Giboney
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WHITE PAPER No. 69

CAR T-Cell Research: Current Clinical Challenges and Outlook

Chimeric Antigen Receptor (CAR) T-cell therapy has revolutionized the field of oncology, producing exceptionally effective and durable clinical responses for some cancer types.

CARs are recombinant receptors engineered to redirect the specificity and function of immune cells. In CAR T-cell therapy, T-cells are genetically modified to express a CAR using genetic engineering tools such as viral transduction and CRISPR-Cas9. These modified T-cells target and bind to extracellular surface cancer antigens resulting in major T-cell activation and in turn, elimination of malignant cells through processes such as induced apoptosis and cytokine release[1].

The buzz surrounding CAR T-cell therapy has translated to a steep rise in the number of associated research papers, clinical trials, and approved drugs over the past decade. Nevertheless, major limitations to CAR T therapies prevent their widespread use in oncology treatment.

Here we review the main challenges, outline the research efforts underway to overcome them, and discuss the future direction of CAR T therapies.

Challenges associated with CAR T therapy starting materials

All current approved CAR T-cell therapies and the majority of published clinical trials have used autologous materials, which means the cells are derived from the patient for whom the therapy is being developed. However, therapies based on autologous T-cells are associated with several limitations. One major challenge, for example, is a dependence on the functional fitness of patient T-cells. And this is often diminished by the disease or prior therapies such as lymphodepleting chemotherapy and radiotherapy, which can make it difficult to obtain enough patient-derived, viable T-cells for development.

As an alternative, allogenic starting materials obtained from healthy donors yield high amounts of fully functional cells that can be used to generate multiple CAR T-cell products. While facilitating an 'off-the-shelf' therapy option, allogenic materials come with their own unique set of challenges.

The main limitation of allogenic CAR T-cell products is graft-versus-host disease (GvHD). This leads to host allorejection, which ultimately hinders anti-tumor activity and can potentially result in fatalities. GvHD arises when there is a mismatch between the donor and recipient human leukocyte antigens (HLA)[2]. In an attempt to overcome this issue, studies have worked on eliminating expression of T-cell receptors (TCR) in CAR T-cells using a range of gene editing tools; including Zinc Finger Nucleases (ZFN)[3–5], Transcription Activator-Like Effector Nucleases (TALEN)[6,7], and CRISPR-Cas9[8–10]. Although high gene-editing efficiencies have been achieved using these techniques, some TCR-bearing T-cells may persist that still have the potential to cause GvHD[11]. Alternative approaches to reduce the risk of GvHD and allorejection include the elimination of HLA molecules[12], and tumor site-specific activation strategies such as hypoxia-activated CAR T-cells[13].

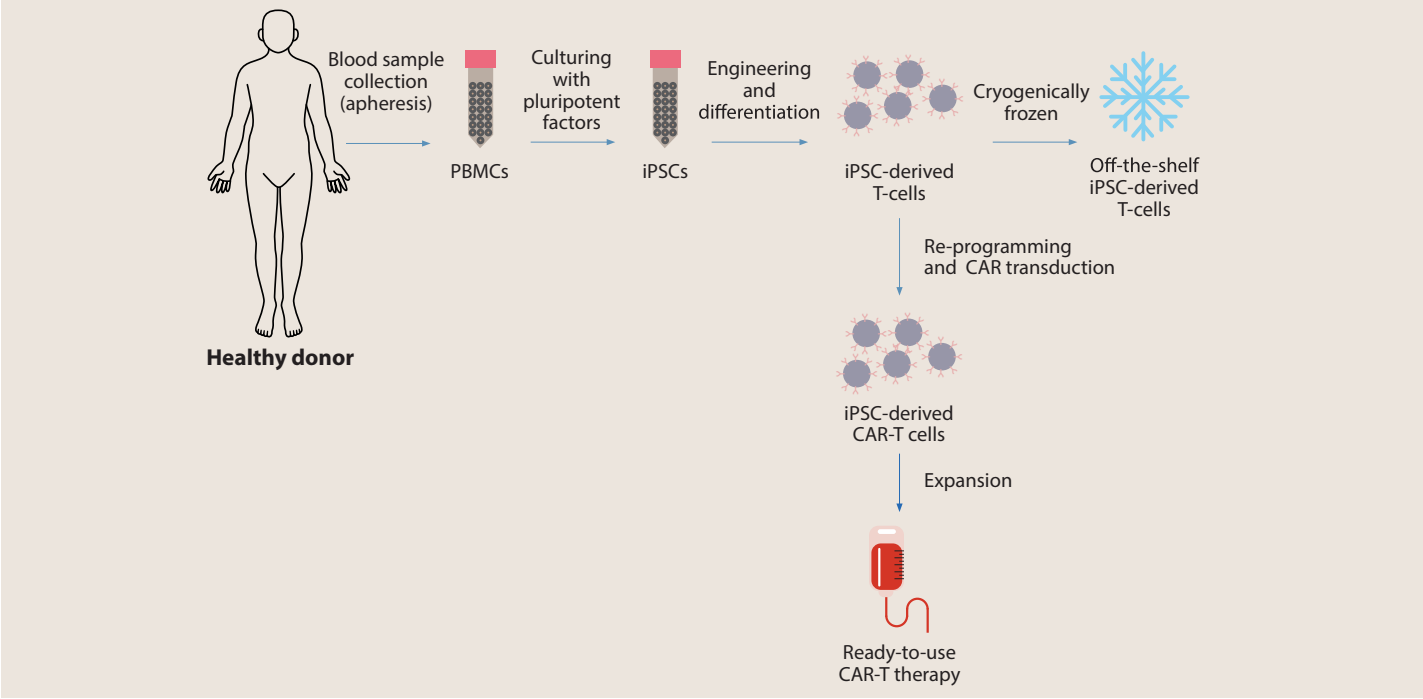

Fig. 1: Simplified workflow for the production of iPSC-derived T-lymphocytes. Briefly, peripheral blood mononuclear cells (PBMCs) are recovered from a healthy donor by apheresis. Culturing PBMCs with pluripotent factors yields iPSCs which can subsequently be engineered and differentiated to yield iPSC-derived T lymphocytes.

A general limitation to both autologous and allogenic CAR T therapies is the exhaustion of T-cell ability during their amplification – which is an essential step of CAR T therapy development[14]. Additionally, T-cells are notoriously difficult to edit, limiting their use with engineered antigen receptors[14]. T-cells differentiated from either hematopoietic stem and progenitor cells (HSPCs) and induced pluripotent stem cells (iPSCs) offer an opportunity to overcome these limitations (Fig. 1). Additionally, both HSPCs and iPSCs provide rapid access to unlimited, phenotypically defined, expandable and functional T-cells. This possibility is generating increasing interest in the use of HSPCs/iPSCs for CAR T therapies[15]. Engineered iPSC-derived T-cells expressing CARs have been generated, demonstrated therapeutic potential in model organisms[16], and recently, moved into Phase 1 clinical trials[17].

Lack of Efficacy for Treating Solid Tumors

The inability of CAR T-cells to traffic to disease sites, overcome physical barriers (e.g. tumor stroma) to infiltrate solid tumors, and maintain function in a hostile tumor microenvironment has so far limited their therapeutic potential to blood disorders. Indeed, all the current approved CAR T therapies are used to treat hematological malignancies.

One strategy to widen the scope of CAR T therapies is through engineering the expression of various proteins on CAR T-cells. For instance, CAR T-cells engineered to express heparanase, an enzyme that degrades the primary component of tumor stroma (heparin sulfate proteoglycan – HSPG), have been investigated as a method to improve tumor penetration[18].

Certain chemokines that are known to correlate with disease burden and prognosis in multiple solid tumors have recently been exploited to improve CAR T-cell trafficking. By engineering CAR T-cells to express chemokine receptors that recognize and respond to tumor-specific chemokines, various studies have shown enhanced trafficking and significantly improved antitumor efficacy both *in silico* and *in vitro*[19–22]. Whether these results translate *in vivo* is yet to be explored.

Even if CAR T-cells successfully traffic and infiltrate the tumor, the hostile and immunosuppressive environment of tumors can prevent T-cells carrying out their effector function. Weak or non-existent responses, poor T-cell expansion, and short-term T-cell persistence are all regularly observed when treating solid tumors with CAR T-cell therapy[22]. Again, genetically engineered CAR T-cells are at the center of research efforts. For example, so-called "armored CARs" secrete stimulatory cytokines that support the survival, proliferation, and antitumor activity of CAR T-cells[23].

Alternative methods to manage the tumor microenvironment include combining CAR T-cell therapy with checkpoint blockade (CPB) agents. Through this combination therapy, CAR T-cells are able to infiltrate tumors while CPB agents reverse CAR T-cell inhibition to aid sustained T-cell persistence and function[24]. Furthermore, the exploration of alternative antigen recognition domains to mitigate T-cell exhaustion is underway. Single-chain variable fragments (scFvs) are typically used to mediate antigen recognition but are linked to T-cell exhaustion. Engineered binding scaffolds, natural ligands, or receptors offer promising alternatives[25].

CHALLENGES	SOLUTIONS
Trafficking/migration Complex human physiology make it difficult for CAR T-cells to home in and migrate onto malignant sites	Engineering CAR T-cells to express proteins that aid migration, such as adhesion molecules and/or chemokine receptors that match and respond to tumor-derived chemokines
Infiltration Physical barriers can prevent or limit CAR T-cell infiltration of a solid tumor	Engineering CAR T-cells that express enzymes capable of degrading the tumor extracellular matrix
Maintenance of function Hostile and immunosuppressive environment of tumors can hinder T-cells carrying out their effector function	Engineering of CAR T-cells to secrete stimulatory cytokines that support the survival, proliferation, and antitumor activity as well as rebalance the tumor microenvironment. Combination therapy: combining CAR T-cell therapy with checkpoint blockade (CPB) agents.

Antigen Escape

Antigen escape is used to describe the partial or complete loss of target antigen expression on malignant T-cells in patients treated with CAR T therapy. It is a common mechanism of resistance that allows malignant cells to evade CAR T-cells. This phenomenon is responsible for the high rate of post-therapy relapse in patients treated with CAR-based therapies and is therefore an important focus for current research.

Engineering CAR T-cells that can recognize multiple targets is being explored as a solution to overcome antigen escape. These methods either use dual CAR constructs, tandem CARs, coadministration, or cotransduction (Fig. 2). Both dual CAR constructs and tandem CARs have shown promising efficacy in clinical trials, decreased antigen escape, and in some cases have even shown favorable safety profiles[22,26].

Most CAR T-cells for blood disorders are engineered to recognize CD19, an antigen expressed by malignant cells in leukemia patients[27]. However, the ability to recognize multiple targets hinges on the identification of novel antigens that are suitable targets for CAR T therapies. Moreover, suitable targets for solid tumors are scarce since antigen expression is highly heterogenous and many tumor antigens are also expressed on healthy cells, risking off target effects[28]. There are many potential novel targets under investigation including some which offer opportunities to develop new therapeutic options. For instance, targeting antigens on cancer stem cells that drive tumor growth offers a promising anti-tumor therapy[29].

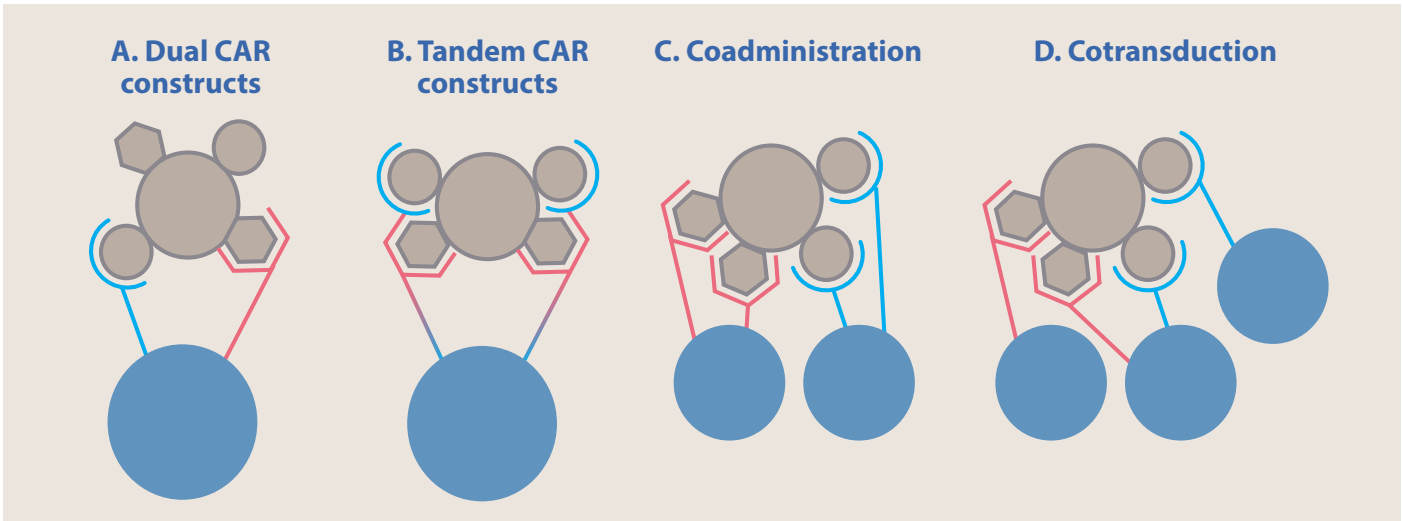

Fig. 3: . Strategies for engineering CAR T-cells to recognize multiple targets.

Summary

CAR T therapies are predominantly used after first-line treatment has failed. For instance, in the UK, CAR T therapies are vailable to children and young adults with B-cell acute lymphoblastic leukemia (ALL), whose first treatment has not worked[30]. However, there is considerable interest in utilizing CAR T-cell therapy earlier in the course of treatment.

While CAR T therapies have already revolutionized the treatment of certain hematological malignancies, there are still limitations to their implementation and therapeutic effect. Currently, approved therapies rely on autologous material that is often insufficient for CAR T-cell manufacture due to the health status of the patient. Even with successful CAR T-cell delivery, malignant T-cells can downregulate antigens due to selective pressures.

In solid tumors, getting CAR T-cells to traffic to and infiltrate solid tumors, penetrate physical tumor barriers and survive in the immunosuppressive tumor microenvironment is a significant challenge. While these challenges exist, CAR T-cell research is increasingly delivering novel strategies and potential solutions that drive forward more effective and safer therapies, as well as expanding the types of treatable malignancies.

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CAR-T therapy for T-cell malignancies

An off-the-shelf, CRISPR-edited CAR-T therapy has shown promise for T-cell malignancies.

MEGAN GIBONEY, DIGITAL EDITOR, REGMEDNET

Researchers at Washington University School of Medicine (MO, USA) have led an international clinical trial investigating a novel CAR-T cell therapy specifically designed to target aggressive T-cell blood cancers, including T-cell acute lymphoblastic leukemia and T-cell lymphoblastic lymphoma.

Patients with T-cell acute lymphoblastic leukemia or T-cell lymphoblastic lymphoma who fail to respond to standard therapies have an average survival of only six months and less than 7% surviving beyond five years. With approximately 1,000 new diagnoses annually in the US, these cancers represent a significant unmet medical need. CAR-T cell therapies have already shown remarkable success against B-cell cancers, but developing similar approaches for T-cell malignancies presents unique challenges, particularly the risk of "fratricide" where therapeutic T-cells might attack each other. This research aimed to overcome these obstacles through innovative CRISPR gene editing techniques, potentially offering hope to patients who previously had no viable treatment options after standard therapies failed.

The Phase I/II trial included 28 adult and adolescent patients with treatment-resistant or relapsed T-cell cancers across multiple sites in Australia, Europe and the United States. After determining optimal dosage through a dose-escalation phase, 13 patients received 900 million CAR-T cells following lymphodepletion, a procedure that clears patients' immune cells to make room for the therapeutic cells.

The results demonstrated remarkable efficacy. Among the 11 evaluable patients, the therapy achieved a 91% overall response rate, with 72.7% achieving complete remission. Six patients who subsequently underwent stem cell transplantation remained in remission 6-12 months later. These response rates of 70-90% significantly exceeded the 20-40% typically expected with standard treatments.

The therapy, called WU-CART-007, represents a significant innovation as a universal CAR-T cell treatment created using CRISPR gene editing technology. Unlike traditional CAR-T therapies that require harvesting a patient's own cells, this approach uses donor cells that can be prepared in advance as an "off-the-shelf" treatment, dramatically reducing wait times for critically ill patients.

What makes this therapy particularly groundbreaking is its novel approach to targeting T-cell cancers. The researchers used CRISPR gene editing to delete T-cell receptors from donor cells, reducing graft-versus-host disease risk, and removed specific antigens to prevent CAR-T cells from attacking each other. The cells were then engineered to target the CD7 protein on cancerous T cells. This overcomes the unique challenge of treating T-cell cancers with T-cell therapy, as previous CAR-T therapies only targeted B-cell cancers.

CAR-T therapy for T-cell malignancies

An off-the-shelf, CRISPR-edited CAR-T therapy has shown promise for T-cell malignancies.

MEGAN GIBONEY, DIGITAL EDITOR, REGMEDNET

The treatment showed manageable side effects, with 88.5% of patients experiencing cytokine release syndrome, mostly mild to moderate. Some patients developed more severe cytokine release syndrome, neurotoxicity syndrome, or low-grade graft-versus-host disease, but all adverse events were manageable with additional therapies.

Senior author John F. DiPersio, who first developed the therapy in his lab, described it as potentially "a transformative advance in the field" that could serve as a "bridge-to-transplant" therapy for patients previously ineligible for stem cell transplantation.

The research team has launched a larger international clinical trial to further evaluate the therapy. Longer follow-up periods will help determine if the therapy could be curative on its own.

Source: Ghobadi A, Aldoss I, Maude SL et al. Phase 1/2 trial of anti-CD7 allogeneic WU-CART-007 in patients with relapsed/refractory T-cell malignancies. Blood. doi:10.1182/blood.2025028387 (2025).

EXPERT REVIEW OF ANTICANCER THERAPY
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REVIEW

CAR-T cell therapy in T-Cell lymphoblastic leukemia/lymphoma: where do we stand now?

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ABSTRACT

Introduction: CAR-T therapies for relapsed or refractory (r/r) T-cell lymphoblastic leukemia/lymphoma (T-LL/L) faces challenges, with most clinical studies conducted in small, dispersed cohorts and often reviewed alongside preclinical studies. This review focuses exclusively on clinical studies, evaluating CAR constructs, safety and efficacy.

Methods: A systematic review was conducted of databases, clinical trial registries, and abstracts from conferences (June 2014 to June 2024). Preclinical studies and studies lacking clinical details were excluded. Data on patient demographics, CAR-T characteristics, response rates, survival, and adverse events were analyzed.

Results: Eleven CAR-T constructs targeting CD7 and two targeting CD5 were identified. Complete remission (CR/CRi) rates ranged from 55% to 100%, exceeding 80% in most studies. Relapse, often in extramedullary sites, ranged from 7% to 66%. Cytokine release syndrome and neurotoxicity were generally manageable. GVHD incidence varied (none to 60%), primarily in allogeneic CAR-T recipients. Infections contributed to 6–38% of treatment-related mortality.

Conclusions: CAR-T therapy achieves high response rates in r/r T-LL/L and may serve as a bridge to allogeneic transplantation. However, the short follow-up and the duration of responses remain concerns, and challenges endure, including GVHD, immune recovery and infection control. Standardized reporting is crucial to optimize therapy outcomes and safety in future trials.

Registration: PROSPERO (CRD420251024662).

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KEYWORDS
CAR-T cell therapy; T-cell lymphoblastic leukemia; T-cell lymphoblastic lymphoma; Immunotherapy; clinical trials

1. Introduction

Relapsed/refractory (r/r) T-cell lymphoblastic leukemia/lymphoma (T-LL/L) presents dismal outcomes for both pediatric [1] and adults patients [2], even with the introduction of new combinations, such as nelarabine and venetoclax [3], yielding overall survival (OS) rates inferior to 30%. Unlike its r/r B-cell counterpart, which has seen advancements through immunotherapy agents such as blinatumomab, inotuzumab, and chimeric antigen receptor T cells (CAR-T) [4,5], r/r T-LL/L remains a challenging unmet medical need. While several approaches, such as antithymocyte immunoglobulins [6], nanobody immunotoxins [7], and antibody-drug conjugates [8], show promise, none have yet achieved a breakthrough into clinical settings. In this context, CAR-T could represent a new hope for r/r T-LL/L adult and pediatric patients, and several clinical trials have been pursued in the last years exploring this innovative approach.

The development of CAR-T for r/r T-LL/L faces several problems, such as product contamination and CAR transduction of malignant T-cells, T-cell fratricide that precludes expansion, T-cell aplasia (especially when using pan-T-cell markers, such as CD3, CD5, CD7) which may even require an hematopoietic stem cell transplant (HSCT) salvage, and also the risk of inducing graft-versus-host disease [9]. Despite these setbacks, some CAR-T products managed to successfully transition from *in vitro* and animal studies to clinical trials in human subjects. However, these clinical studies are often conducted in small and scattered cohorts and are typically reviewed alongside preclinical studies.

Our study aims to review the available evidence on CAR-T therapies in this disease, analyzing the various investigational cellular products currently being tested in human clinical trials, along with their corresponding safety data and efficacy outcomes. Such a review could advance our understanding of the potential impact of CAR-T in treating real-life patients and its role in the current treatment algorithms for r/r T-LL/L.

2. Methods

2.1. Search strategy

This systematic review was conducted following the PRISMA 2020 guidelines [10] and registered in PROSPERO with the identification CRD420251024662. First, a structured search was performed using PubMed advanced search, using the following terms and Boolean operators, and encompassing publications within the last 10 years: ((T-cell lymphoblastic leukemia) AND ((CAR-T) OR (chimeric antigen receptor T-cell))) AND (('2014/06/01'[Date – Publication] : '2024/06/01'[Date – Publication])). Afterwards, a search was performed using clinicaltrials.gov, with 'T-cell lymphoblastic leukemia' as

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Article highlights

- CAR-T cell therapy in relapsed/refractory T-cell lymphoblastic leukemia/lymphoma shows high response rates.
- Responses are often short-lived, and consolidation with allogeneic hematopoietic stem cell transplantation may be required.
- Infections are the second leading cause of mortality, and there is a need for better understanding of GVHD.
- The current evidence base is limited in quality, emphasizing the need for prospective, controlled clinical studies.

condition or disease and 'chimeric antigen receptor T-cell' as intervention, as well as using euclinicaltrials.eu, with base criteria 'T-cell lymphoblastic leukemia' and 'chimeric antigen receptor T-cell,' in order to locate finished or ongoing human clinical trials with published results. Finally, abstract books from congresses organized by the following five major medical societies in the last 5 years (2020–2024) were reviewed for relevant studies: European Hematology Association, American Society of Hematology, European Society for Blood and Marrow Transplantation, American Society for Transplantation and Cellular Therapy and American Society of Clinical Oncology.

2.2. Inclusion and exclusion criteria

The inclusion criteria used were as follows: a) original research articles, abstracts or other trial/studies published results involving human subjects; b) results published in peer-reviewed journals or congresses with peer-reviewed abstract acceptance; c) texts available in English.

The exclusion criteria were as follows: a) preclinical studies either in vitro or using animal models; b) review articles, editorials, or comments; c) case reports, case series or abstract that were part of or later published within a bigger study or clinical trial.

2.3. Data extraction and synthesis

Data extraction was performed independently by two reviewers (P.C. and P.L.). PubMed search results were reviewed using Rayyan webapp [11]. Duplicates were automatically detected and were user-checked before definite exclusion. All other results from different sources were manually reviewed.

Discrepancies or disagreements were resolved through discussion between all the authors. Before information extraction and tabulation, all authors reviewed and agreed on the final article/abstract list. This workflow is depicted in Figure 1.

2.4. Variables selected and outcome measurement

We collected variables on the publication year and country. We reviewed demographic patient characteristics, T-LL/L characteristics (including genetics, phenotype, number, and types of lines of therapy, response to previous lines), CAR-T product (including target, apheresis, time to production), conditioning

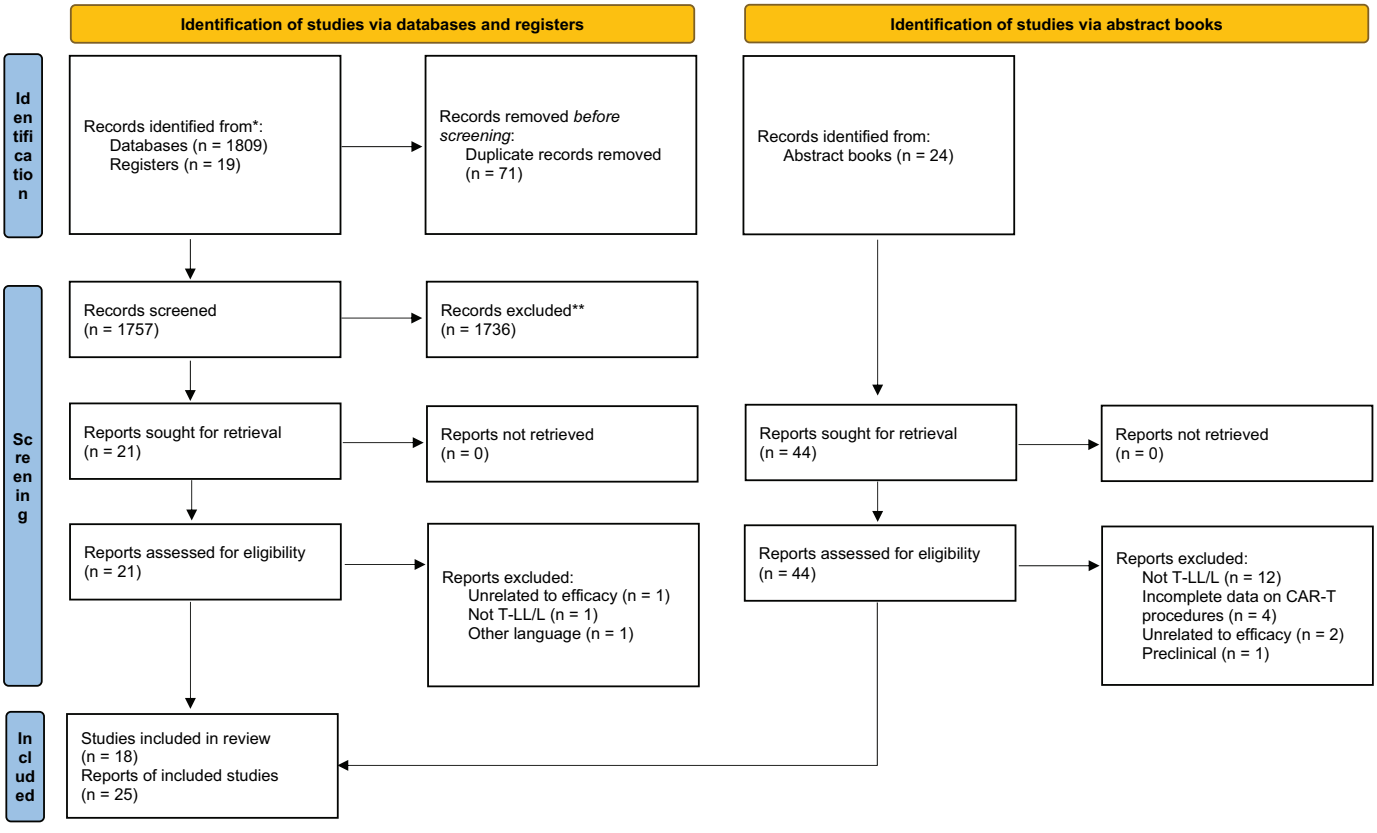

Figure 1. PRISMA flow diagram.

regimen, supportive therapy and bridging to allogeneic hematopoietic stem cell transplant (ASCT), whenever available.

The main outcomes selected were complete response, relapse, CAR-T related mortality, and incidence of main therapy outcomes, such as cytokine release syndrome (CRS), immune effector cell-associated neurotoxicity syndrome (ICANS), infections and graft-versus-host disease (GVHD). Secondary outcomes were OS and progression-free survival, reported as percent survival over a time of observation or the relative number of patients without an interest event at the maximum time span of observation.

Due to the scarce studies on this topic, meta-analysis could not be undertaken. Bias risk and certainty assessment for the selected outcomes was summarized following GRADE recommendations [12]. Missing data were reported as such. Unclear information was evaluated amongst authors before deciding whether to be reported or excluded from the review.

3. Results

3.1. Targets and constructs

The dominant epitope of human trials is CD7, which is an established marker in T-LL/L [13]. However, shared antigen expression of CD7 in normal T-cells poses the problem of fratricide. To avoid self-killing, many strategies can be applied during manufacturing, such as at the nuclei level, through genome editing to prevent CD7 expression [14–21], and at the intracellular domain, either by using protein expression blocker (PEBL) [22,23] or nanobody variable domain of heavy-chain antibody 6 (VHH6) [24], retaining CD7 in the cytoplasmatic organelles. Nevertheless, in vitro natural selection of CD7 fratricide-resistant CAR-T cells is also feasible, which would abrogate the need for further genetic manipulation [25,26]. The availability to use allogenic donors for production extends the use of this therapy as a universal and 'off-the-shelf' product. However, due to concerns about GVHD, some CAR-T products also include a step for the disruption of native T-cell receptor expression [14,19–21].

Another pan-T marker, CD5, has been reported in fewer studies, with data only in one of the constructs, which applies gene-editing of CD5 to avoid fratricide [27]. Regarding the T-cell origin for production, the same donor used for ASCT was also used for lymphocyte donation in some cases [15,17,25]. We identified 13 different CAR-T products used in clinical trials, summarized in Table 1, where we outline key characteristics of the constructs and manufacturing strategies, including production times ranging from 5 to 14 days.

Other potential antigens frequently present in T-LL/L (such as CD1a, CD2) who could be targeted by CAR-T have not been developed in human trials so far.

3.2. Patients and disease characteristics

We were able to identify clinical trials and reports on the use of CAR-T cells for the treatment of T-LL/L in more than 300 human subjects, all in phase 1/2 trials. Most treated patients are children and adolescents and young adults (AYA), although elderly (≥65 years old) have also been included. Median previous lines of treatment usually range from three to four, including a prior ASCT. At the time of infusion, most patients had bone marrow (BM) involvement, but extramedullary disease, including central nervous system (CNS) involvement, was also present, reflecting the frequent aggressiveness of the disease in these patient population. Table 2 summarizes the key features of the published human trials and results for r/r T-LL/L.

3.3. Bridging and Lymphodepletion

Bridging therapy to CAR-T was given in 95% for the NS7CAR dose escalation patient cohort ($n = 20$ patients) [25] and 92% in the expanded cohort ($n = 60$ patients) [26]. In another study, tucidinostat, an histone deacetylase inhibitor, was prescribed in 20% of patients [16]. In a cohort of 10 adults, all patients received chemotherapy prior to anti-CD7 CAR-T lymphodepletion [39]. Most studies failed to mention whether bridging therapy was used, though it was allowed.

Lymphodepletion regimens described usually consisted of cyclophosphamide and fludarabine given over three consecutive days, with dosages ranging from 250-1000 mg/m^2/day and 25-30 mg/m^2/day, respectively [15–18,21,23–26,28,30,31,42,47,51]. In some regimens, additional agents, such as alemtuzumab [14], steroids [20,43], methotrexate [44], idarubicin [44], melphalan [20,54], and etoposide [20,29], were included.

3.4. Toxicity profile

Regarding immune-mediated inflammatory adverse events (Table 2, Table 3), 60% to 100% of patients developed CRS, but in most reports grades 3–4 developed in ≤33%. ICANS was less frequent, ranging from 0% to 25% in bigger cohorts, with less of half of these episodes reaching grades 3–4. No deaths were attributed to CRS or to ICANS.

Grade 1–2 GVHD, primarily affecting the skin, was reported in 60% of a 20 patient cohort receiving anti-CD7 CAR-T [15], with one patient later progressing to grades 3–4 gastrointestinal GVHD [32]. In another cohort with the same construct, grades 1–2 GVHD were reported in 20% of patients [16], and it was only observed among the allogeneic CAR-T recipients. Other reports of GVHD with this construct were a grade 2 skin involvement in a T-LL/L patient without a previous transplant [44], a chronic oral and liver involvement in a patient with a previous ASCT [45], and one grade 4 gastrointestinal case in a cohort of 10 patients [40]. Again, all lymphocyte donors were of allogeneic origin. With the infusion of NS7CAR, 3 cases of mild GVHD were described in a cohort of 60 patients, all recipients of a previous ASCT [26]. In the KO7CAR cohort, one previously allotransplanted patient died of GVHD post-CAR-T infusion [18]. With CD5KO allogeneic CAR-T, there was one patient with grade 1 cutaneous GVHD [27]. Other trials either did not assess GVHD or failed to characterize this complication. Nevertheless, treatment of GVHD in the context of CAR-T, whether related to the CAR-T itself or to the previous ASCT, was reported to be successful in most cases, with one attributable death.

Table 1. CAR-T constructs tested in human trials.

CAR-T name	Target	Costimulatory domains	Source	Vector	Selection after apheresis	Fratricide mitigation	Other modifications	Manufacturing (days)	Ref
BE-CAR7	CD7	4-1BB-CD3ζ	Allogeneic	Lentivirus	None reported	Gene editing to ablate CD7	Gene editing to ablate TRBC and CD52	13	[14]
anti-CD7 CAR T	CD7	4-1BB-CD3ζ	Allogeneic Autologous	Lentivirus	None reported	Intrablock CD7 surface expression	No	5–11	[15–17]
NS7CAR	CD7	4-1BB-CD3ζ	Allogeneic Autologous	Lentivirus	CD3 purified	Naturally selected in culture	No	12–14	[25,26]
KO7CAR	CD7	4-1BB-CD3ζ	Allogeneic Autologous	Lentivirus	CD3 purified	Gene editing to ablate CD7	No	Not reported	[18]
CD7 VHH6	CD7	ICOS-4-1BB-CD3ζ	Autologous	Lentivirus	CD4 and CD8 selection	Nanobody VHH6 blockade of CD7	No	10–12	[24]
WU-007	CD7	CD28–4-1BB	Allogeneic	Lentivirus	None reported	Gene editing to ablate CD7	Gene editing of ablate TCRalpha	9	[19]
4SCAR7	CD7	CD28–4-1BB, CD27, OX40, ICOS, and IL-15 Rα.	Autologous	Lentivirus	None reported	Not reported	Not reported	Not reported	[28]
GC027	CD7	4-1BB-CD3ζ	Allogeneic	Lentivirus	None reported	Gene editing to ablate CD7	Gene editing of ablate TCRalpha	10	[20]
PEBL-CAR T	CD7	4-1BB-CD3ζ	Allogeneic	Lentivirus	None reported	Anti-CD7 PEBL	No	12	[22,23]
RD13–01	CD7	4-1BB-CD3ζ + CD28-Nkinhibitor	Allogeneic	Not reported	None reported	CRISPR/Cas9-edited anti-CD7	No	Not reported	[29]
UCD7-CAR T	CD7	Not reported	Allogeneic	Not reported	None reported	CRISPR/Cas9-CD7 ablation	CRISPR/Cas9-TCR ablation	Not reported	[21]
CD5KO CAR-T	CD5	4-1BB-CD3ζ	Allogeneic	Lentivirus	None reported	CRISPR-Cas9-mediated CD5 deletion	No	Not reported	[27]
CD5-IL15/IL15 sushi CAR	CD5	CD28-CD3ζ-IL15/IL5sushi	Allogeneic	Lentivirus	None reported	Not reported	Not reported	Not reported	[30]

CAR, chimeric antigen receptor; CRISPR, clustered regularly interspaced short palindromic repeats; PEBL, protein expression blocker; TCBC, T-cell receptor beta chain; TCR, T-cell receptor; VHH6, variable domain of heavy-chain antibody 6.

Table 2. Published human trials and results.

CAR-T	Phase	N	Age, median (range)	Previous lines, median (range)	Previous ASCT, %	BM/EM disease at infusion, %	CRS (≥3)[$], %	ICANS (≥3)[$], %	CR/CRi, %	ASCT post CAR, %	Relapse or survival	Trial	Ref
BE-CAR7	1	3	13 (12–15)	–	66	100/33	100 (33)	100 (0)	100	66	OS 66% at 1 m	EudraCT 2021 -004,312-25	[14]
anti-CD7 CAR-T	1	20	11 (2–43)	3 (2–4)	60	60/40	100 (10)	15 (0)	90	35	OS 73% at 1y PFS 52% at 1y (higher for ASCT)	ChiCTR 2,000,034,762	[15,31–34]
	1	10*	32 (16–69)	4 (2–10)	60	88/38	80 (10)	20 (0)	70	0	OS median ~8 m	NCT04823091	[16,35,36]
	–	12	14 (2–43)	10	8	–/67	–	–	90	100	OS 92% at 1y DFS 57% at 1y	ChiCTR 2,000,040,641	[17]
	–	18	–	–	–	–	–	–	94	100	OS 78% at 1y DFS 61% at 1 y	–	[37]
	1	48	–	–	–	–	–	–	–	100	OS 54% at 2y	–	[38]
	–	10*	32 (19–72)	–	70	100/–	90 (10)	10 (10)	90	–	OS 80% at 4.8 m DFS 70% at 4.8 m	–	[39]
	–	20*	35 (18–72)	–	45	–/60	– (15)	10 (0)	55	–	OS 66% at 1y PFS 57% at 1y	ChiCTR 2,200,058,969	[40]
	1	5	3 (1–13)	–	–	60/40	60 (20)	0	80	20	–	NCT04840875	[41]
	–	5	10 (3–13)	3 (2–5)	40	80/40	100 (0)	0	100	80	0% relapse at LFU	Case reports	[42–45]
NS7CAR	1	20	22 (3–47)	4 (2–8)	15	85/45	95 (5)	10 (0)	85	70	–	NCT04572308	[25]
	1	6	–	–	–	–	–	–	83	67	All ASCT patients alive at LFU	NCT04916860	[46]
	1/2	60	19 (2–47)	5 (2–15)	18	90/53	92 (12)	3 (2)	BM 94 EM 56 CNS 100	60	OS 64% at 2y PFS 54% at 2y (PFS higher ASCT)	NCT04572308 NCT04916860	[26,47,48]
	1/2	43	17	–	–	–	–	–	100	100	OS 64% at 2y LFS 64% at 2y	NCT04572308 NCT04916860	[49]
KO7CAR	1	15[#]	28 (8–46)	–	36	66/66	100 (33)	20 (7)	100	80	7% relapses at LFU	NCT04916860 NCT04938115	[18]
CD7 VHH6	1	8[#]	36 (15–68)	≥4	63	100/30	100 (13)	0	88	0	57% relapses at LFU	NCT04004637	[24,50]
WU-007	1/2	18	33 (20–68)	4 (2–7)	28	72/28	78 (6)	6 (0)	58	11	DOR 12.3 weeks	NCT04984356	[19,51,52]
4SCAR7	1/2	1	11	2	0	BM	100 (0)	0	100	100	Alive at 3 m	NCT04033302	[28]
GC027	1/2	12	28 (19–47)	4 (1–16)	17	92/33	83 (67)	0	92	8	36% relapse at LFU	ChiCTR 1900025311	[20,53]
	1/2	2	24, 39	2	50	100/50	100 (100)	0	100	0	50% relapse at LFU	ChiCTR 1900025311 ISRCTN19144142	[54]
PEBL-CAR T	–	1	11	4	100	100/100	100 (100)	0	100	0	Alive and in CR at 91 days	NCT04785833	[22]
	1	12	33 (20–60)	–	25	–	83 (25)	17 (0)	75	8	66% relapse at LFU	NCT05170568	[55]
	1/2	7	15 (5–18)	–	–	100/14	86 (0)	0	100	57	14% relapse at LFU	NCT06064903	[23]
RD13–01	1	10	16 (2–27)	4 (2–6)	20	100/70	100 (10)	10 (10)	70	70	29% relapse at LFU	NCT04620655	[29]

(*Continued*)

Table 2. (Continued).

CAR-T	Phase	N	Age, median (range)	Previous lines, median (range)	Previous ASCT, %	BM/EM disease at infusion, %	CRS (≥3)[$], %	ICANS (≥3)[$], %	CR/CRi, %	ASCT post CAR, %	Relapse or survival	Trial	Ref
UCD7-CAR T	2	5	5 (4–11)	3 (2–6)	20	80/60	80 (20)	0	80	60	25% relapse at LFU	NCT05454241	[21]
CD5KO CAR-T	1	16	–	–	69	–	75 (0)	25 (0)	100	25	ASCT: 0% relapse no-ASCT: 25% relapse at LFU	NCT05032599	[27,56]
CD5-L15/IL15 sushi CAR	1	1	22	3	100	100/100	0	0	100	0	No relapse at LFU	NCT04594135	[56]

*includes T-cell non-Hodgkin lymphoma; [#]includes mixed-phenotype acute leukemia; [$]values refer to total infused patients. ASCT, allogeneic stem cell transplant; BM, bone marrow; CNS, central nervous system; CAR, chimeric antigen receptor; CR, complete remission; CRi; complete remission with incomplete peripheral recovery; CRS, cytokine release syndrome; DOR, duration of response; EM, extramedullary involvement; ICANS, immune-effector cell associated neurologic syndrome; LFS, leukemia free survival; LFU, last follow-up; OS, overall survival; PEBL, protein expression blocker; PFS, progression free survival; VHH6, variable domain of heavy-chain antibody 6.

Several cohorts reported deaths from infections, accounting for 6% to 38% of all-cause mortality [14,16,20,24,29,31,37,39,52,56]. When reported, the most common agents were invasive mold infections [14–16,56] and Epstein–Barr related disease [16,27,52]. Additionally, several CMV reactivations were documented [14–16,23,25,26]. Although some studies fail to account for infectious complications, the data suggests infections to be the second leading cause of mortality, after relapse.

3.5. Efficacy data

The overall complete remission (CR) or complete remission with incomplete count recovery (CRi) rates after CAR-T infusion ranged from 55% to 100%, with most studies reporting rates > 80% (Table 2 , Table 3). Rates of extramedullary disease remission seem lower than for bone marrow involvement [25,26,29,31,35,47], except in the CNS, where blast clearance was very high [25,31,47]. Due to cohort heterogeneity and small sizes, a comparison between different CAR-T constructs is not possible.

Regarding response consolidation, a variable number of patients proceeded to ASCT following CAR-T therapy, with the infused genetically modified T cells serving as a bridging therapy to transplantation in some cases. However, the rationale behind these decisions was not always documented. Additionally, most trial designs lack specific transplant-related endpoints, as well as standardized conditioning regimens, intensity levels, or GVHD prophylaxis protocols, which were instead determined by trial sites. This variability complicates the interpretation of ASCT outcomes. Detailed data, including relapse and survival endpoints, are summarized in Table 2. Due to the short follow-up reported in studies, these findings should be interpreted with caution. Despite the high CR/CRi rates observed, responses appear to be short-lived, and relapses are frequent, suggesting that CAR-T therapy may not serve as a definitive treatment for all patients. Patients bridged to a first or second ASCT after CAR-T therapy could have improved progression-free and overall survival [17,31,37].

4. Discussion

In the context of relapse and refractory T-LL/L patients, which usually have a dismal outcome with conventional treatment, CAR-T therapy in human clinical trials, has shown high CR/CRi rates. Although responses may be short-lived for some patients, CAR-T may be used as a bridge for ASCT consolidation, which may further improve the outcome for these patients. Toxic events of CRS and ICANS seem manageable, however, further insights on GVHD are needed, and improvements in infection control are necessary. The potential use of healthy allogeneic stem cell donors could expand the application of CAR-T as a universal, 'off-the-shelf' therapy.

This review presents several limitations that merit consideration. The available studies have included a very limited number of patients, with heterogenous characteristics and short follow-up periods. Additionally, we report on 13 different constructs, which further complicates the summarization of evidence. Furthermore, given that the summarized

information is derived from single-arm trials, case reports, and conference abstracts, the quality of the available data is very low (Table 3). This warrants caution against overgeneralizing the efficacy and toxicity of CAR-T in r/r T-LL/L. Finally, no phase 3 studies randomizing CAR-T against standard care are available for inclusion, so patient selection bias cannot be excluded.

Results presented in this review are encouraging, suggesting that the CR/CRi rates obtained with CAR-T is at least as good as those offered by currently available agents for pediatric and adult r/r T-LL/L, such as conventional chemotherapy, nelarabine [3], bortezomib [57], BCL2 inhibitor [58–60], and daratumumab-based therapies [61]. Preliminary results with CD5 and CD7 targeted CAR-T should encourage further development of CAR-T trials in r/r T-LL/L targeting TRBC1 [62], CD1a [62], CD2 [63], CD38 [64], or other potential anti-leukemic epitopes. Although the duration of response and relapse remains a major challenge with these new therapies, head-to-head comparisons or even combination trials of CAR-T with other salvage agents will help us in the future to better sequence and tailor therapies for this difficult-to-treat patient group.

Whether CAR-T serves as a definitive treatment for r/r T-LL/L or if consolidation with a first or second ASCT is necessary to optimize outcomes remains an important open question. This uncertainty arises from the limited follow-up periods in clinical trials and will likely depend on the specific target and construct utilized. Data coming from B-cell lymphoblastic leukemia suggest that this approach may be tailored based on measurable residual disease after CAR-T and disease genetics [65,66], and this information should be considered in the design of future CAR-T trials for r/r T-LL/L. Another area of uncertainty is the risk of secondary malignancies, particularly myeloid neoplasms or CAR-T-derived lymphomas, which has garnered increasing attention for available products [67,68].

Infections were identified as a major concern, constituting the second leading cause of mortality in CAR-T recipients, who have typically undergone multiple lines of therapy and are deeply immunosuppressed. As we gain insights on immune reconstitution after CAR-T infusion [69], aggressive monitoring and infectious prophylaxis should be implemented. Due to the ubiquitous expression of CD7 and CD5 on T-cells, antigen-specific T-cell therapies, such as lymphocytes used to treat viral reactivations, may be less effective, as they could also be eliminated by the CAR-T. Considering the crucial role of T-cells in immunity, immune recovery after CAR-T for r/r T-LL/L is likely to be more challenging than that observed in CAR-T targeting B-cells, which is already associated with a significant infectious risk [70]. Recent studies in CAR-T therapy for B-cell malignancies have demonstrated that delayed immune recovery, including impaired T-cell reconstitution, is linked to increased relapse rates, infectious complications, and non-relapse mortality [71]. Therefore, patients undergoing CAR-T therapy for r/r T-LL/L may face an even higher risk of these adverse outcomes due to the prolonged immune suppression and delayed immune reconstitution. The incorporation of 'off switches' in constructs, such as the incorporation of rituximab-binding epitopes in the CD5-IL15/IL15sushi CAR [30], may prove valuable for patients with severe or recurrent infections who have achieved a CR/CRi, and CAR-T elimination via antibody infusion could be a potential strategy. Additionally, salvage ASCT may be required for patients who do not adequately recover immune function.

Another concern with CAR-T therapy is GVHD, a known risk associated with both autologous or allogeneic products [72]. In this review, the incidence of GVHD ranged from 0% to 60% among patients receiving allogeneic CAR-T therapy, with one reported related fatality. Notably, all reported cases of GVHD occurred with the 4-1BB costimulatory domain, which is known to increase the risk of GVHD [72], and no GVHD was reported when the expression of the native T-cell receptor was disrupted. These findings may provide insights into CAR-T design and the need for GVHD vigilance, although the complication appears to be manageable. Finally, as cellular therapy becomes more widely integrated into medical practice, CRS and ICANS are becoming more familiar to clinicians and easier to manage. The incidence of grade 3–4 events was low, and no fatal outcomes were reported.

In conclusion, CAR-T therapy as shown remarkable complete response rates in r/r T-LL/L and is likely to be incorporated in the therapeutic arsenal for this difficult-to-treat population. However, more follow-up and comprehensive

Table 3. Quality of evidence assessment for CAR-T in relapse/refractory T-cell lymphoblastic leukemia/lymphoma.

	Overall quality	Risk of bias	Inconsistency	Indirectness	Imprecision	Publication bias	Comments
Primary outcomes							
Complete response	Very low	High	Significant	No	High	High	Single-arm studies, very small sample sizes, and abstracts from scientific meetings.
Relapse	Very low	High	Significant	Present	High	High	
CAR-T related mortality	Very low	High	Significant	Present	High	High	
CAR-T complications	Very low	High	Significant	Present	High	High	
Secondary outcomes							
Overall survival	Very low	High	Significant	Present	High	High	Same as above
Progression free survival	Very low	High	Significant	Present	High	High	

CAR-T, chimeric antigen receptor T cells.

data are needed to determine the safety profile, the optimal bridging strategy, potential ability to induce long-term responses, and the role of bridging to ASCT. Given the lower frequency of r/r T-LL/L in pediatric patients, who may have a distinct safety profile and disease biology, future clinical trials should prioritize expanding the cohort of adult patients treated with CAR-T therapy. Additionally, infection control and a better understanding of immune recovery and GVHD complications are essential, as these factors will impact both survival and the quality of life of treated patients. Clinical trial reporting should consistently include a standardized set of variables, such as those discussed in this review, to improve the interpretation and comparison of results. Future trials should address current challenges and opportunities to better integrate CAR-T into the treatment algorithm for r/r T-LL/L.

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REVIEW

CAR-T Cell Therapy for Solid Tumors: Are we Still That Far? a Systematic Review of Literature

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ABSTRACT

This systematic review aims to assess all the prospective studies published to date on the efficacy of CAR-T cell therapy in solid tumors. Databases searched were PubMed and Google Scholar from inception through May 1st 2021. Search query was (Chimeric antigen receptor) or (CAR-T) or (T-CAR). Twenty-nine prospective studies (265 patients) were included. Most published clinical trials are phase I. Clinical benefit was 100% in epithelial ovarian cancer, 70–82% in gastrointestinal tumors, 79% in mesothelioma, 63% in small-cell lung cancer, 24–67% in sarcoma, 50–62% in prostate cancer, and 45–50% in central nervous system tumors. No serious CAR-T cell specific serious toxicities were noted.

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Introduction

Treating cancer has shifted over the years from drugs targeting cancer cells to molecules boosting the immune system in order to fight these cancer cells. One of the key elements of anti-tumoral immunity are T lymphocytes which are implicated in monitoring and killing malignant or potentially malignant cells. Many therapies have been established recently aiming at culturing, redirecting and/or enhancing anti-tumoral T-cells (1).

Chimeric Antigen Receptor T (CAR-T) cell therapy is one of these promising advances. A simple description of this technology consists of the extraction of T lymphocytes from the patient's own blood using leukapheresis or phlebotomy, engineering of these T-cells to attack specific cancer cells, and reinjection of those T lymphocytes into the patient (2). This engineering consists of (1) transfection of the separated T-cells by a CAR viral or non-viral vector (whose role is to insert artificially DNA genome) and (2) expansion and purification of those engineered T-cells (2,3).

Historically, first CAR T cells were developed in 1989–1993 by two immunologists; however, back then they were not clinically effective (4,5). Afterwards CARs were modernized and progressed through four generations, becoming more and more sophisticated, improving therefore their antitumoral activity, but also combining cytokine activity and costimulatory ligands that can degrade extracellular matrix in solid tumors with fourth generation CARs, called "TRUCKs" (6). Smart T cells are currently under investigation in order to enhance safety of CAR T cell therapy (7).

Initially, CAR T-cell therapy was developed for hematological malignancies and are commonly used nowadays in acute lymphoblastic leukemia, chronic lymphocytic leukemia, lymphoma, and multiple myeloma (8), with very promising results and survival outcomes (9). However, when it comes to solid tumors, treatment with CAR T-cell remains challenging for many reasons such as insufficient and atypical molecular

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targets, difficulty of controlling the hostile immunosuppressive tumor microenvironment (10), but also limited tumor infiltration of CAR-T cells due to T cell suppression at the tumor site (11). CAR T-cell failure is accompanied by upregulation of multiple tumor microenvironment-associated localized inhibitory molecules. Multi-pronged approaches against the tumor microenvironment and enhanced toxicity mitigation strategies are needed to further improve outcomes (11).

Numerous prospective studies have recently assessed the efficacy of CAR T-cell therapy in many solid tumors, with diverse results. We hereby perform a systematic review of all the prospective studies that have been published until now evaluating CAR T-cell therapy in solid tumors.

Materials and methods

Search strategy and eligibility criteria

A systematic literature search of PubMed/Medline and Google Scholar – including ASCO meetings – was conducted by two independent authors (A.K. and G.M.) to identify prospective trials evaluating CAR T-cell therapy for solid tumors, published in English or in French, from inception through May 1, 2021. Disparities between these two authors were solved by a third author (F.A.).

The following search query was used (Chimeric antigen receptor): or (CAR T-cell) or (CAR-T cell). Studies retrieved were deposited in the reference manager Zotero® and duplicates were removed. Two investigators (A.K. and G.M.) independently reviewed the titles and abstracts and assessed the eligibility of each study. Disparities were solved by a third investigator (F.A.). Studies that were vague from their title or abstract had their full text reviewed and were afterwards assessed for eligibility.

Only prospective studies evaluating CAR T-cell therapy in solid tumors were included in our systematic review. Studies evaluating CAR T-cell in hematological malignancies, systematic reviews, meta-analyses, retrospective studies, preclinical studies, and case reports were excluded. Abstracts of ongoing trials whose articles were published were also excluded.

Citation lists of included articles were screened manually to ensure sensitivity of the search strategy.

Data extraction

Two independent authors (A.K. and G.M.) completed data extraction from included studies. The variables which were retrieved for each study were: first author, publication year, type of study, trial status (completed-ongoing for abstracts; completed-published for published studies), type of cancer, CAR T-cell target used (product), pre-treatment depleting regimen, number of patients, radiological responses including complete response rate, partial response rate, stable disease rate and progressive disease rate, and CAR-T therapy specific toxicity. Overall clinical benefit was defined as complete response rate + partial response rate + stable disease rate.

Analysis

Due to the sparse and heterogenous literature, and a high risk of bias on risk-of-bias assessment (done by two independent reviewers), a meta-analysis was not done.

Ongoing trials

A search for ongoing trials was done on clinicaltrials.gov. Search query included: “Cancer”, “malignancy” and “neoplasm” for the condition, and “CAR T-cell” and “Chimeric Antigen Receptor T-cells” for the other terms. The same inclusion and exclusion criteria were applied.

Results

The PRISMA flow chart of our study is illustrated in Figure 1. Of 611,934 results, and after title, abstract and full-text screening, only 29 prospective studies, with a total of 265 patients, were included in our final analysis.

Prospective studies evaluating CAR T-cell therapy in solid tumors

CAR T-cell therapy boomed first in the treatment of hematological malignancies. The FDA

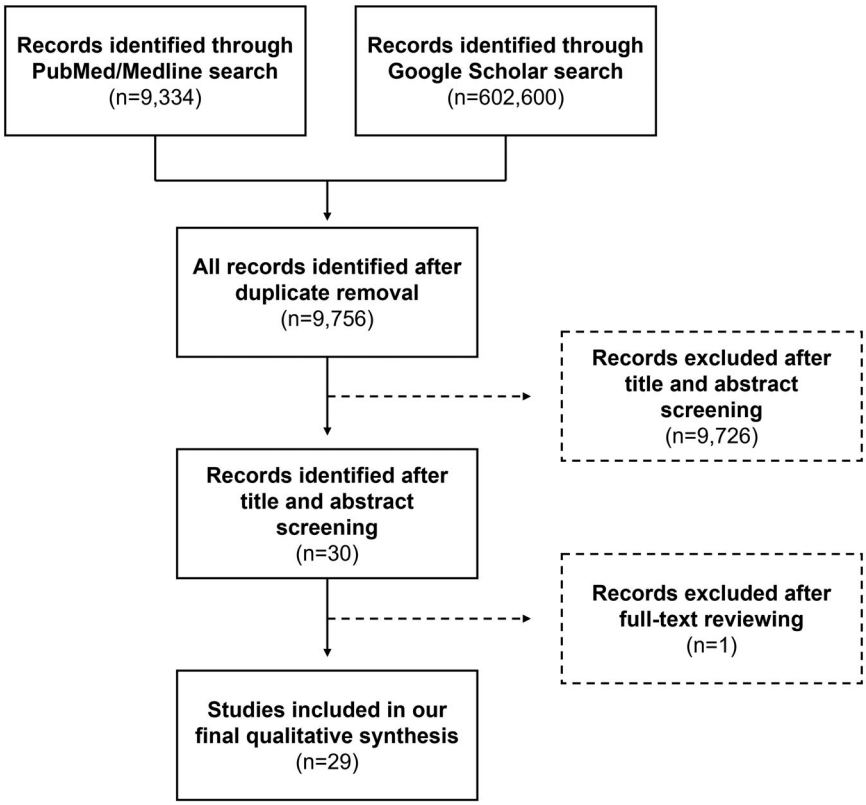

Figure 1. PRISMA flow chart of our systematic review.

approved CAR T-cells for the treatment of diffuse large B-cell lymphoma, acute lymphoblastic leukemia, mantle cell and follicular lymphoma, and multiple myeloma (12). Due to its success in hematological malignancies, this therapy was extrapolated to solid tumors. Table 1 summarizes the results of our systematic review, and Figure 2 resumes the summary of clinical benefit rates of all CAR T-cell therapy trials in solid tumors until now.

Gastrointestinal, liver, biliary tract and pancreatic tumors

Most studies were found on gastrointestinal, liver, biliary tract and pancreatic tumors. Different targets were used.

Carcinoembryonic antigen (CEA)-targeted CAR T-cell therapy was used in the treatment of metastatic liver and colorectal cancers. Katz et al. in a phase I clinical trial tested the percutaneous hepatic arterial infusion of CAR T-cells in 6 patients with CEA positive liver metastasis, without conditioning treatment, with and without systemic IL-2 support (13). One (17%) patient reached stable disease at 23 months, but 5 patients died of progressive disease. With IL-2 administration a decrease in CEA level by 37% was obtained. No grade 3 or 4 toxicity of cytokine release syndrome was reported (13). Zhang et al. also investigated the efficacy of CEA targeted CAR T-cell therapy in CEA-positive metastatic colorectal cancer (14). Five escalating dose levels (DLs) (1×10^5 to 1×10^8/CAR+/kg cells) of CAR-T were applied. Out of the 10 patients enrolled, 7 (70%) had stable disease after CAR-T therapy following lymphodepletion by cyclophosphamide and fludarabine with a significant decline in CEA level in long-term observation. Even with high doses, CEA CAR T-cell therapy was well tolerated in patients with metastatic colorectal cancer (14). Finally, Thistlethwaite et al. have shown in 14 patients with gastrointestinal tumors that a regimen of depleting chemotherapy, followed by CEA-targeted CAR T-cell infusion, and then by interleukin 2 infusion, achieved only a stable disease in 50%, with no complete or partial response noted (15).

The efficacy of CART-GP3, CAR-T targeted against GP3, a cell surface oncofetal proteoglycan, was assessed by Zhai et al. in patients with

Table 1. Summary of the results of our systematic review.

First author	Publication Year	Phase of trial	Trial status	Type of targeted cancer	CAR-T cell product used	Pre-treatment depleting regimen	Number of patients	Overall clinical benefit (%)	Complete response (%)	Partial response (%)	Stable disease (%)	Progressive disease (%)	CAR-T cell specific toxicity
Katz et al.	2015	I	Completed – Published	CEA positive liver metastasis	CEA (hepatic artery infusion)	No lymphodepletion	6	17%	0	0	17%	83%	No serious toxicity
Feng et al.	2017	I	Completed – Published	Biliary tract and pancreatic carcinoma	HER-2	Nab-paclitaxel and cyclophosphamide	11	55%	0	9%	45%	45%	1 reversible upper gastrointestinal hemorrhage; 2 grade 1–2 delayed fever
Guo et al.	2017	I	Completed – Published	Biliary tract cancers	EGFR	Nab-paclitaxel and cyclophosphamide	17	65%	6%	0	59%	35%	One acute respiratory distress syndrome requiring tocilizumab; oral mucositis, oral ulcer, gastrointestinal hemorrhage, desquamation, and pruritus
Zhai et al.	2017	I	Completed – Ongoing	Hepatocellular carcinoma	GP3	Fludarabine and cyclophosphamide	6	67%	0	17%	50%	33%	No serious toxicity
Zhang et al.	2017	I	Completed – Published	Colorectal cancer	CEA	Fludarabine and cyclophosphamide	10	70%	0%	0%	70%	30%	No serious toxicity
Thistlethwaite et al.	2017	I	Completed – Published	Gastrointestinal cancers	CEA	Fludarabine with or without cyclophosphamide	14	50%	0%	0%	50%	50%	Grade 1–2 toxicities in all patients Respiratory toxicities in 4 patients Other grade 3–4 toxicities linked to chemotherapy and/or interleukin 2
Beatty et al.	2018	I	Completed – Published	Pancreatic ductal carcinoma	Mesothelin	No lymphodepletion	6	33%	0	0	33%	67%	No serious toxicity
Zhan et al.	2019	I	Completed – Ongoing	Gastric and pancreatic carcinoma	CLDN 18.2	Fludarabine and cyclophosphamide with or without nab-paclitaxel	11	82%	9%	27%	45%	18%	No serious toxicity
Ahmed et al.	2015	I/II	Completed – Published	Sarcoma	HER-2	No lymphodepletion	17	24%	0	0	24%	76%	No serious toxicity
Hedge et al.	2017	I	Completed – Ongoing	Sarcoma	HER-2	Fludarabine with or without cyclophosphamide	6	67%	33%	0	33%	33%	No serious toxicity
Papa et al.	2018	I	Completed – Ongoing	Head and neck squamous cell cancer	(i) T1E28ζ, a CAR containing a promiscuous ErbB ligand coupled to a CD28 + CD3ζ endodomain; and (ii) 4$\alpha\beta$, an IL-4-responsive chimeric cytokine receptor	No lymphodepletion	13	69%	–	–	–	31%	Steroid-responsive tumor swelling, pain, fever, chills and fatigue. No serious toxicity.
Beatty et al.	2014	I	Completed – Published	Malignant pleural mesothelioma	Mesothelin	No lymphodepletion	2	100%	–	–	–	0%	One anaphylaxis (related) Jejunal obstruction (possibly related) Abdominal pain (possibly related)
Haas et al.	2019	I	Completed – Published	Malignant pleural disease either from malignant	Mesothelin	Cyclophosphamide	15	73%	0%	0%	73%	27%	Two serious adverse events (sepsis and anemia)

(*continued*)

Table 1. Continued.

First author	Publication Year	Phase of trial	Trial status	Type of targeted cancer	CAR-T cell product used	Pre-treatment depleting regimen	Number of patients	Overall clinical benefit (%)	Complete response (%)	Partial response (%)	Stable disease (%)	Progressive disease (%)	CAR-T cell specific toxicity
				pleural mesothelioma, or from metastatic ovarian carcinoma and pancreatic ductal adenocarcinoma									65 other grade 1–3 toxicities (fatigue, nausea, ascites, vomiting, confusion, diarrhea, abdominal pain, etc.)
Hiltbrunner et al.	2020	I	Completed – Published	Malignant pleural mesothelioma	Fibroblast activation protein (FAP)	No lymphodepletion	3	–	–	–	–	–	Two thromboembolic events labeled as not related to treatment
Adusumilli et al.	2021	I	Completed – Published	Malignant pleural disease either from malignant pleural mesothelioma or from metastatic lung and breast cancers	Mesothelin (in combination with anti-PD1 immune checkpoint inhibitor pembrolizumab)	Cyclophosphamide	18	83%	11%	28%	44%	17%	No serious toxicity
Feng et al.	2016	I	Completed – Published	Non small cell lung cancer	EGFR	With/without a combination of cyclophosphamide, pemetrexed or docetaxel, and cisplatin	11	63%	0	18%	45%	37%	No serious toxicity
Tchou et al.	2017	0	Completed – Published	Breast cancer	c-Met (intratumoral injection)	–	–	–	–	–	–	–	No serious toxicity
Tanyi et al.	2016	I	Completed – Ongoing	Epithelial ovarian cancer	Mesothelin	Cyclophosphamide	6	100%	0	0	100%	0	No serious toxicity
Louis et al.	2011	I	Completed – Published	Neuroblastoma	GD2	No lymphodepletion	11	45%	27	9%	9%	55%	No serious toxicity
Brown et al.	2015	I	Completed – Published	Glioblastoma	IL13Rα2	No lymphodepletion	3	–	–	–	–	–	No serious toxicity
O'Rourke et al.	2017	I	Completed – Published	Glioblastoma	EGFRvIII	No lymphodepletion	10	–	–	–	–	–	3 Neurological events (1 seizure, 2 neurologic decline)
Ahmed et al.	2017	I	Completed – Published	Glioblastoma	HER-2	No lymphodepletion	16	50%	0	6%	44%	50%	No serious toxicity
Goff et al.	2019	I	Completed – Published	Glioblastoma	EGFRvIII	Cyclophosphamide + Fludarabine	18	0%	0%	0%	0%	100%	Acute dyspnea in two patients (severe hypoxia pulmonary edema in one patient) Grade 2 neurologic symptoms or suspected seizure activity in 10 patients One deep vein thrombosis and pulmonary embolism Two liver toxicities
Slovin et al.	2013	I	Completed – Ongoing	Prostate cancer	PSMA	Cyclophosphamide	4	50%	0	0	50%	50%	No serious toxicity
Junghans et al.	2016	I	Completed – Published	Prostate cancer	PSMA	Cyclophosphamide with fludarabine	6	60%	0	40%	20%	40%	No serious toxicity
Becerra et al.	2019	I	Completed – Ongoing	PSCA positive pancreatic, gastric and prostate cancer	PSCA	Cyclophosphamide with or without fluadarabine	13	62%	0	0	62%	38%	1 encephalopathy resolved with steroids
You et al.	2016	I		Seminal vesicle cancer		Cyclophosphamide	–	–	–	–	–	–	No serious toxicity

(*continued*)

Table 1. Continued.

First author	Publication Year	Phase of trial	Trial status	Type of targeted cancer	CAR-T cell product used	Pre-treatment depleting regimen	Number of patients	Overall clinical benefit (%)	Complete response (%)	Partial response (%)	Stable disease (%)	Progressive disease (%)	CAR-T cell specific toxicity
			Completed – Published		MUC1 (intrametastatic injection)								
Lamers et al.	2013	I	Completed – Published	Renal cell carcinoma	CAIX	No lymphodepletion	–	0%	0%	0%	100%		High grade liver toxicity
Lamers et al.	2016	I/II	Completed – Published	Renal cell carcinoma	CAIX	No lymphodepletion	12	0%	0%	0%	100%		High grade liver toxicity

Overall clinical benefit (%) = complete response (%) + partial response (%) + stable disease (%). Published-ongoing = abstract with results (not yet peer-reviewed). Published-completed = published study (peer-reviewed). Abbreviations. CAR = Chimeric Antigen Receptor; CEA = Carcinoembryonic antigen; HER-2 = Human Epidermal-growth-factor Receptor – 2; EGFR = Epidermal Growth Factor Receptor; GP3 = glypican 3; CLDN 18.2 = Claudin 18.2; IL-4 = Interleukin-4; FAP = Fibroblast Activation Protein; PSMA = Prostate Specific Membrane Antigen; PSCA = Prostate Stem Cell Antigen; CAIX = carboxy-anhydrase-IX.

advanced glypican 3-positive hepatocellular carcinoma (16). Clinical outcomes were compared between patients who received lymphodepletion by a combination of fludarabine and cyclophosphamide and patients who received GP3 without pretreatment conditioning. GP3 CAR-T infusion was well tolerated without serious toxicity, at a dose of 0.92×10^7 to 8.72×10^7 cells/kg. Out of the 6 evaluable patients who received GP3 following lymphodepletion, one (17%) had partial response and three (50%) had stable disease (16).

Beatty et al. evaluated the efficacy of CAR T-cell therapy targeting mesothelin, an overexpressed protein by pancreatic ductal adenocarcinoma, without lymphodepletion (17). Among 6 patients with metastatic chemotherapy-resistant pancreatic ductal adenocarcinoma, 2 (33%) had stabilized disease lasting from 3.8 and 5.4 months.

Moreover, the efficacy of CAR T-cell therapy in patients with advanced biliary tract cancers refractory to Epidermal Growth Factor Receptor (EGFR) therapies was evaluated in a phase clinical trial conducted by Guo et al. after the same conditioning treatment with nab-paclitaxel and Cyclophosphamide (18). Of 17 evaluable patients, 1 (6%) achieved complete response and 10 (59%) achieved stable disease following CART-EGFR immunotherapy. The median PFS was 4 months (2.5–22 months) from the first cycle of treatment. Median administered dosage was 2.65×10^6 cells/kg. Acute respiratory distress syndrome with pulmonary edema occurred in one patient during infusion period but recovered after the administration of tocilizumab (18).

Human Epidermal growth factor Receptor (HER-2) targeted CAR T-cell immunotherapy was used in a phase 1 clinical trial in advanced biliary tract and pancreatic cancers. A study was conducted by Feng et al. after lymphodepletion using nab-paclitaxel and cyclophosphamide (19). Among 11 patients assessed for clinical response using CART-HER2 therapy, one patient (9%) achieved a 4.5-month partial response and five patients (45%) achieved stable disease. The median PFS was 4.8 months (1.5–8.3 months). No severe infusion related adverse events were identified, after a median dose of 2.1×10^6 cells/kg (19). A reversible upper gastrointestinal

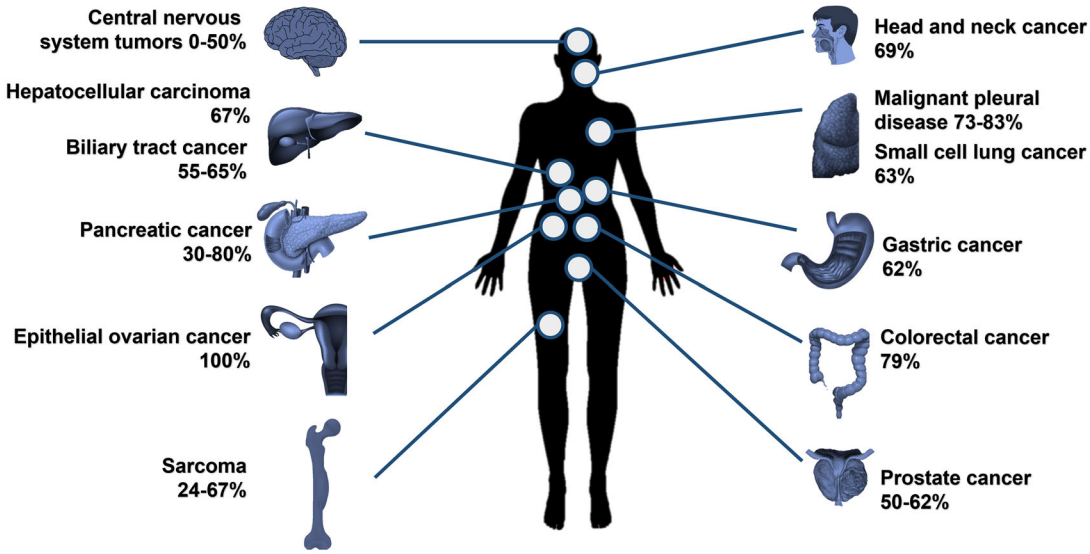

Figure 2. Summary of overall clinical benefit rates of all CAR-T cell therapy trials in solid tumors until now. Overall clinical benefit (%) = complete response (%) + partial response (%) + stable disease (%).

hemorrhage due to metastasis invasion occurred 11 days after infusion (19).

Finally, Zhan et al. used Claudin 18.2 (CLDN 18.2), which is a tight junction protein highly expressed in gastric and pancreatic adenocarcinoma, as a target in a CAR T-cell clinical trial (20). A lymphodepletion before treatment using fludarabine and cyclophosphamide with or without nab-paclitaxel preceded CART-CLDN 18.2 infusion in 12 subjects with metastatic adenocarcinoma (7 gastric and 5 pancreatic). The median PFS and OS were 130 days and 242 days respectively with a total objective response rate of 33% (20).

Therefore, so far, clinical studies on the treatment of gastrointestinal tumors by CAR T cell immunotherapy have shown a certain antitumoral effect, which is until now preliminary. Numerous targets have been studied including CEA, HER-2, EGFR, GP-3 and CLDN 18.2. Although some positive results were found for these studies, they were all phase I single-arm studies, surely limiting the drawing of genuine conclusions. Moreover, most of these studies used lymphodepleting regimens before CAR T therapy including chemotherapy with fludarabine and cyclophosphamide. Although good results were shown for these studies, a minimal overall clinical benefit was found for Katz et al. (13) and Beatty et al. (17). Interestingly, these were the only two trials in GI tumors that did not use lymphodepleting regimens before implementing CAR T therapy, and therefore the impressive positive results that were shown in the other studies can be maybe simply due to chemotherapy. Therefore, further prospective comparative studies are needed to confirm this efficacy shown in phase I trials.

Sarcoma

Ahmed et al. conducted the first clinical trial in order to evaluate the benefit of CAR T-cell immunotherapy in 19 patients with HER2-positive refractory sarcoma (osteosarcomas, Ewing sarcoma, primitive neuroectodermal tumor, and desmoplastic small round cell tumor). No dose limiting toxicity was identified (21). The median OS was 10.3 months (5.1–29.1 months). Out of 17 evaluable patients, 4 (24%) had stable disease for 12 weeks to 14 months, among which 3 had their tumor removed (21). Afterwards, Hegde et al. performed a phase I clinical trial to assess the role of lymphodepletion (fludarabine or fludarabine and cyclophosphamide) prior to HER2 targeted CAR T-cell therapy in 6 patients with advanced HER2-positive sarcoma (22). Four (67%) of these patients developed low grade cytokine release syndrome that resolved within 24 hours of supportive care after receiving $1 \times 10^8/m^2$ autologous HER2-CAR. Objective response was seen in 3 (50%) patients: complete response in one patient with rhabdomyosarcoma

metastatic to the bone marrow and stable disease in 2 other patients (22). The median overall survival was 14.2 months.

Similarly, an overall clinical benefit was shown in 67% of patients in Hegde et al.'s trial (22) where a lymphodepleting regimen was used, compared to only 24% of patients in Ahmed et al.'s trial (21) where no lymphodepleting chemotherapy was used. Although we know that the role of lymphodepleting chemotherapy is (1) debulking of the tumor, (2) reducing regulatory T-cells and changing the expression of costimulatory signals in the tumoral microenvironment, and (3) suppressing the immune system, therefore increasing theoretically the efficacy of CAR-T cell therapy, it can alone explain these positive results as we discussed in gastrointestinal tumors. Further prospective comparative studies are needed to confirm the efficacy of CAR-T cell therapy in sarcoma, especially that no serious toxicity was noted in phase I trials.

Head and neck squamous cell cancer

Only one study evaluated CAR-T cell therapy in head and neck squamous cell cancer. Papa et al. developed a T4-based immunotherapy using cells co-expressing a CAR which contains an ErbB ligand attached to a CD28 + CD3ζ endodomain, and an IL-4-responsive chimeric cytokine receptor (23). They tested it in a phase I clinical trial on 13 patients, by injecting intratumorally 1×10^7 to 1×10^9 T4+ T cells without lymphodepletion, showing an overall clinical benefit of 69% despite rapidly progressing tumors before entry to the trial, with no serious adverse events. Further studies are needed to confirm these results.

Pleural cancers

CAR-T cell therapy was also tested in pleural cancer. Beatty et al. introduced mesothelin-specific CAR mRNA T-cell therapy in two patients with malignant pleural mesothelioma (24). The aim of introducing T cells made with mRNA encoding CAR was to provide a safe alternative to those transduced with viral vectors by extenuating the side effects of constitutively active T cells, and their transient nature permits dose escalation through multiple injections, without the risk of permanent T-cell activity. An objective clinical response was seen in both patients. Adverse events were: one anaphylaxis in one patient (which was directly related to therapy), one jejunal obstruction and an episode of abdominal pain in another patient (these adverse events were labeled as possibly related to treatment).

Then, Haas et al. have tested mesothelin-targeted CAR T-cell therapy in 15 patients with malignant pleural disease and have concluded an overall clinical benefit of 73% (patients however only exhibited stable disease with no complete or partial response) (25). Numerous adverse events were noted among which two serious ones (sepsis and refractory anemia), and 65 other grade 1–3 toxicities, at a dosage of $1–3 \times 10^7$ or $1–3 \times 10^8$ of CART-meso cells/m^2 with or without 1.5 g/m^2 cyclophosphamide.

Hiltbrunner et al. have used a fibroblast activation protein (FAP)-targeted CAR T-cell therapy in three patients with mesothelioma (26). Two thromboembolic events were noted in two patients, labeled as not related to the treatment, but rather to cancer itself, active chemotherapy or indwelling catheter. Due to the low number of patients, efficacy outcomes could not be evaluated.

Mesothelin-specific CAR T-cell therapy was then administered intra-pleurally in combination with an anti-PD1 checkpoint inhibitor (pembrolizumab) in an updated phase I clinical trial by Adusumilli et al. in patients with malignant pleural disease (either malignant pleural mesothelioma or metastatic lung and breast cancers) following lymphodepletion by cyclophosphamide (27). Among the 18 patients with malignant mesothelioma who received a combination therapy, 15 (83%) had objective response: 2 (11%) of 18 patients had a complete response, 5 (28%) had partial response and 8 (44%) stable disease. One case of grade 3 pneumonitis that responded to steroids was identified (mainly attributed to immune checkpoint inhibitors rather than CAR T-cell therapy). No anaphylaxis nor digestive symptoms occurred.

Hence, intra-pleural administration reduced consequently toxic side effects seen with systemic administration and has shown promising results combined with immune checkpoint inhibitors

and a lymphodepleting cyclophosphamide pre-treatment regimen. This highlights the importance of combining CAR-T cell therapy with other treatment options, thus enabling a multimodal treatment approach, rather than using it as a unique treatment.

Lung cancer

Only one published study until now evaluated CAR T therapy in lung cancer. Feng et al. conducted a phase 1 clinical trial to study the efficacy of CAR T-cell therapy targeting EGFR with/without a combination of cyclophosphamide, pemetrexed or docetaxel, and cisplatin, in 11 patients with non-small cell lung cancer (28). Two (18%) patients obtained partial response and 5(45%) had stable disease for 2 to 8 months. The eradication of EGFR positive tumor cells in these patients was documented by targeted biopsies. The CAR T-cell infusion was well-tolerated without severe toxicity (28). This overall clinical benefit of 63% is comparable to that seen with EGFR targeted CAR T therapy in gastrointestinal tumors, specifically in biliary tract cancers (65%), which we have mentioned above (18). However, this study has shown no serious toxicity while that of biliary tract cancers showed oral toxic side effects such as mucositis and aphtosis, gastrointestinal hemorrhage and an acute respiratory distress syndrome (18).

Breast cancer

The safety and feasibility of CAR T-cell therapy targeting c-Met, a cell surface molecule expressed in approximately 50% of patients with metastatic breast cancer, was evaluated in a study performed by Tchou et al. (29). Intratumoral injection of CAR T-cells directed against c-Met positive tumors has resulted in extensive necrosis and inflammatory response within tumor microenvironment. Phase I and II trials are awaited and ongoing in this field and results are until now pre-clinical and preliminary.

Ovarian cancer

Tanyi et al. also used mesothelin targeted CAR T-cell therapy in a phase I clinical study in 6 patients with recurrent serous ovarian cancer patients with or without lymphodepletion (30). Higher CAR T-cells were detected in patients treated with high doses and with cyclophosphamide lymphodepletion. Stable disease was found in 100% of patients at one month after treatment. No severe adverse events were reported during or after infusion (30). No complete or partial response was noted. Other ongoing studies aim to target molecules other than mesothelin in ovarian carcinoma. We could mention targets such as MESO (NCT03916679, NCT03799913), B7-H3 (NCT05211557, NCT04670068), ALPP (NCT04627740) and many others.

Central nervous system tumors

CAR T-cell therapy was tested in the literature for glioblastoma and neuroblastoma.

A phase I study by Brown et al. has shown that intracranial administration of CAR T-cells targeted against IL13Rα2 is promising with transient anti-glioma response in two out of three patients (31). O'Rourke et al. have also demonstrated in a phase I study of 10 recurrent glioblastoma patients that treatment with CAR T-cells targeted at epidermal growth factor receptor variant III (CART-EGFRvIII) is feasible and safe (32). Median OS was 8 months with one patient out of 10 remaining alive after more than 18 months of a single infusion, requiring no further treatment (32). Another phase I clinical trial performed by Ahmed et al. used CAR-T modified virus specific T cells for the treatment of HER2-positive glioblastoma without lymphodepletion (33). The median overall survival was 11.1 months from the first infusion. Of the 16 evaluable patients, 1 (6%) had a partial response and 7 (44%) had stable disease (33). Finally, Goff et al. used CART-EGFRvIII which was demonstrated to be safe and feasible by O'Rourke et al. (32) and have shown that no clinical benefit was seen in glioblastoma patients for this type of CAR T-cell treatment, with however numerous serious adverse events (acute dyspnea, pulmonary edema, pulmonary embolism, liver toxicity, etc.) at a dose of $1.75 \times 10^8 - 5 \times 10^8$ CART-EGFRvIII+ (34).

CAR T-cell therapy was also used in a phase I clinical trial conducted by Louis et al. in the treatment of GD-2 positive relapsed/refractory neuroblastoma without pretreatment chemotherapy (35). Among 11 evaluable patients, 2 (18%) had

complete response, 1 (9%) partial response, and 2 (18%) had stable disease (35).

Consequently, minor anti-tumoral effect was found with CAR T cell therapy in glioblastoma, and this (1) regardless the targeted molecule, and (2) regardless the use or not of a lymphodepleting regimen (100% of progressive disease and 0% clinical benefit in 18 patients treated with EGFRvIII-CAR T cell therapy with cyclophosphamide and fludarabine as depleting regimen (34)).

Prostate cancer

A phase I clinical trial by Slovin et al. evaluated CAR T-cells targeting Prostate-Specific Membrane Antigen (PSMA) in metastatic castrate resistant prostate cancer (36). It was demonstrated to be safe with CAR T-cells persisting in the circulation for up to 2 weeks; however, no oncological outcomes were reported neither by PSA decline nor biopsies of the tumor sites. A second phase I trial by Junghans et al. of anti-PSMA CAR T-cells in five patients with prostate cancer has shown that two patients out of five achieved partial response, with PSA declines of 50% and 70%, and one patient showed a minor response (37).

Becerra et al. have then assessed ligand-inducible, prostate stem cell antigen (PSCA)-directed GoCAR T-cells in PSCA positive metastatic pancreatic, gastric and prostate cancers with measurable disease (38). Cyclophosphamide with or without fludarabine was used for lymphodepletion. Eight out of 13 patients (62%) had stable disease, while five of them (38%) had progressive disease. Progression-free and overall survivals were not reported.

Therefore, good results with an overall efficacy of 50–62% were observed in prostate cancer with PSMA and PSCA-targeted CAR T cell therapy. Although these remain phase I single arm studies, they have shown very limited toxicity, encouraging therefore phase II and III prospective comparative studies.

Seminal vesicle cancer

Specific CAR T-cells targeting MUC1 positive metastatic seminal vesicle cancer were developed and tested in a phase I clinical trial by You et al. (39). Exceptionally, in this study, CAR T-cells were injected intratumorally into metastatic lesions as part of an interventional treatment strategy. This resulted in tumor necrosis with no initial reported side effects. This has therefore presented another way of administration of CAR-T cell therapy, and studies on clinical benefit are awaited.

Renal cell carcinoma

Lamers et al. administered CAR T-cells targeted against carboxy-anhydrase-IX (CAIX) in order to evaluate the response of this technique in a phase 1 study, in 12 patients with CAIX-positive metastatic renal cell carcinoma (mRCC) (40). When receiving a maximum of 10 infusions of a total of 0.2 to 2.1×10^9 CAR T cells, no clinical responses were noted and cessation of the treatment was inevitable in 4 patients who had grade 2–4 liver enzymes disturbance with no clinical response documented. The same team has led another study Lamers et al. (2016) using CAIX-targeted CAR T-cells in 12 patients with mRCC, and confirmed the previous findings: no clinical benefit for mRCC with high grade liver toxicity (41). Therefore, no clinical benefit with CAIX-targeted CAR-T cell therapy was observed without however any lymphodepletion.

All current targets of CAR-T therapy are summarized in Table 2.

Ongoing trials

Due to the huge number of ongoing clinical trials on CAR T-cell therapy in solid tumors found on clinicaltrials.gov ($n = 595$ studies), these were not reported in this manuscript. Numerous types of solid tumors are being tested (prostate, breast, gastric, pancreatic, colorectal, biliary, central nervous system, etc.).

Discussion

Our review confirms that CAR T-cell therapy might be efficient in solid tumors, although response to treatment seems to be challenging to achieve compared to hematological malignancies. Even if the majority of published clinical trials until now remain phase I clinical trials, clinical benefit was found to be promising in some solid

Table 2. Current targets of CAR-T therapy and their corresponding applications in solid tumor types.

Target	Abbreviation	Type of tumors used in
CarcinoEmbryonic Antigen	CEA	Liver metastasis, colorectal cancer, gastrointestinal cancers
Human Epidermal-growth-factor Receptor – 2	HER-2	Biliary tract and pancreatic carcinoma, sarcoma, glioblastoma
Epidermal Growth Factor Receptor	EFGR	Biliary tract cancer, non-small cell lung cancer, glioblastoma
Glypican 3	GP3	Hepatocellular carcinoma
Claudin 18.2	CLDN 18.2	Gastric and pancreatic carcinoma
Mesothelin	Mes	Pancreatic ductal carcinoma, malignant pleural mesothelioma, metastatic ovarian carcinoma, metastatic lung cancer, metastatic breast cancer
Fibroblast activation protein	FAP	Malignant pleural mesothelioma
Tyrosine-protein kinase Met	c-Met	Breast cancer
Disialoganglioside	GD2	Neuroblastoma
Interleukin-13 receptor $\alpha 2$	IL13Rα2	Glioblastoma
Prostate Specific Membrane Antigen	PSMA	Prostate cancer
Prostate Stem Cell Antigen	PSCA	Prostate cancer
Mucin 1	MUC-1	Seminal vesicle cancer
Carboxy-Anhydrase-IX	CAIX	Renal cell carcinoma

tumors (100% of stable disease in epithelial ovarian cancer, 82% in gastric and pancreatic cancer, 73–83% in malignant pleural disease, 70% in colorectal cancer, 69% in head and neck squamous cell cancer, 67% in hepatocellular carcinoma, 55–65% in biliary tract cancer, 63% in small-cell lung cancer, 24–67% in sarcoma, 50–62% in prostate cancer, and 0–50% in central nervous system tumors). The majority have shown no serious CAR T-cell specific serious toxicities. Therefore, many clinical trials are currently ongoing in numerous solid tumors with different treatment optimization strategies to evaluate their efficacy and safety.

Hindrances and challenges

CAR T-cell therapy is still at its beginnings in solid tumors and there are many challenges to be stepped upon in order to optimize treatment response.

A first challenge is that the tumor antigens that are targeted by CAR T-cells lack optimal specificity and can be found in other normal tissues. For instance, CAIX targeted in renal cell carcinoma can be found in the bile ducts, explaining therefore high-grade liver toxicities in the study by Lamers et al. (40). Another example would be CEA-directed CAR T-cells that can cause lung toxicity due to the presence of CEA in the lung epithelium (15). The specificity of CAR T cell targets should be good enough to avoid serious damage to normal non-cancerous tissues and organs. Two main toxic side effects of CAR T therapy remain the cytokine release syndrome, but also the "off-tumor"effect (caused by damage of non-tumoral cells) (42). Thus, an ultimate target should have both high coverage and high specificity in order to ensure both effectiveness and safety. However, the "perfect" target does not exist practically.

A second challenge remains the escape mechanism by tumor antigen heterogeneity: an example would be the heterogeneous expression of EGFRvIII between different regions of the tumor after surgery in the study done by O'Rourke et al. (32). This is why, pharmaceutical approaches were developed in order to selectively upregulate target antigens on cancerous cells surface in order to sensitize sub-clones of tumoral cells that do not express this target antigen on their surface; the aim is to enhance recognition by CARs (43). New strategies such as dual and triple antigen targeting have also been developed progressively in order to decrease this antigen heterogeneity (43).

A third challenge is the inherent immunosuppressive characteristic of the tumor microenvironment discussed above, with CAR T-cells losing their functionality (differentiation and exhaustion) after reaching this microenvironment (44). This is why combinations are now used between CAR T cell therapy and immune checkpoint inhibitors, and suppression of other inhibitory factors of the microenvironment is actually being tested with promising results (45).

A fourth challenge remains also limited tumor infiltration of CAR-T cells due to poor infiltration and T cell suppression at the tumor site, through the expression of T regulatory cells that suppress the immune response by secreting inhibitory factors such as transforming growth

factor beta (TGF-β) (45). This is why some T cells are engineered with TGF-β shielding in order to improve treatment efficacy (11). Moreover, CAR-T cells are being also injected in some trials directly intratumorally or intrapleurally, in order to overcome this CAR-T delivery to tumoral site (26,29).

A final challenge is the time-consuming, costly, and complex manufacturing of CAR T-cells (46,47).

Limitations

Our study is not devoid of limitations. First, all the studies included are single arm phase I clinical trials limiting therefore drawing legitimate conclusions. However, some of these studies are promising and further prospective clinical studies will eventually confirm these findings. Second, the results obtained with CAR-T cell therapy could be only due to pre-treatment chemotherapy given for lymphodepletion, especially that overall clinical benefit was rather obtained in studies in which patients received lymphodepleting regimens. Nevertheless, multimodal treatment constitutes the new era in oncology and CAR-T cell therapy, if not used as monotherapy, could be used in combination with other treatments such as immunotherapy and chemotherapy, in order to enhance their activity (48); Ongoing trials will depict the efficacy of these combinations. Third, we only performed a qualitative analysis without a meta-analysis; however, due to the heterogeneity and the high risk-of-bias of the included studies, quantitative synthesis was not possible.

In the near future, CAR T-cell therapy may provide a more effective and safer future therapies in solid tumors.

Conclusion

CAR T-cell therapy is a promising strategy for the treatment of many solid tumors. However, different challenges need to be overcome in order to optimize this innovative treatment approach. Future prospective and comparative clinical trials will depict the true effectiveness and toxicity of CAR T-cell therapy in solid tumors and will help in selecting the right patient for this approach.

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Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its Supplementary materials.

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APPLICATION NOTE No. 453

Optimizing CD4+ T Cells Long-term Expansion Process in the DASbox® Mini Bioreactor System: Impact of the Dissolved Oxygen

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Abstract

T cell lymphocytes play a central role in the adaptive immune response. They are an essential tool of adoptive cell therapy for the treatment of chronic viral infections and malignant diseases. However, the development of cell-based therapy products generally requires the production of a large quantity of high-quality viable T cells in a controlled environment. Stirred-tank bioreactors can offer a suitable environment for the culture of T cells by providing homogeneous distribution of nutrients and gases, along with the maintenance of cells and molecules in suspension with a high process control capability. In this study, we tested the suitability of BioBLU® 0.3c Single-Use Bioreactors controlled by a DASbox Mini Bioreactor System in the long-term expansion of CD4+ T cells as well as the impact of different oxygen tensions on cell proliferation. The control of several growth parameters during cell expansion resulted in an efficient proliferation of highly viable and functional CD4+ T cells after 16 days of culture. Given that 100% dissolved oxygen (DO) in the bioreactor corresponds to 20.9% atmospheric oxygen level, incubation at two different oxygen tensions (70% or 20% DO) was carried out. The results suggested a tendency towards a positive impact of lower DO levels on CD4+ T cell proliferation rates. These results demonstrate the substantial potential of the DASbox Mini Bioreactor System used in combination with BioBLU 0.3c Single-Use Bioreactors to optimize T cell culture conditions.

Introduction

Adoptive cell therapy (ACT), a branch of immunotherapy, offers a promising treatment for chronic viral infections and malignant diseases such as cancer [1]. This innovative approach involves the autologous or allogenic transplant of immune cells into the patient's body. Specifically, T cells are extracted from the patient, modified genetically (if necessary), expanded *ex vivo* and reinfused into the patient to target viral or tumor antigens [2,3].

In clinical trials of various cancer treatments, adoptive cell therapy with tumor infiltrating lymphocytes (TIL), gene-modified T cells expressing novel T cell receptors (TCR), or chimeric antigen receptors (CAR) have shown favorable results in the eradication of different types of tumors. Other therapies in development include Cytokine-Induced Killer (CIK) cells, γδ T cells, Regulatory T (Treg) cells, and Natural Killer (NK) cells [4]. At this time, five adoptive cell

therapies have received approval by the US Food and Drug administration (FDA), all of them involving CAR T cells [5]. Such investigations have established that CAR T cell therapy is an important advance for children and young adults as well as for adult patients with resistant therapies, relapsed leukemia or other hematological malignancies [6].

In order to achieve successful therapeutic responses, the development of efficient and high-quality cell therapy products has become a challenge for clinical researchers and health care providers. As is true for every multistep process, the cell-based production workflow presents challenges of variability, complexity and other unresolved issues. Optimized protocols for the scalable manufacture of T cells are crucial, since T cells and other lymphocytes require meticulous culturing. They are highly sensitive to their culture environment and react easily by modifying their receptor/ligand repertoire, which can cause changes in cellular response to external substances and surfaces [7,8]. Additionally, T cells undergo frequent metabolic changes depending on the stimulation agents used for their activation *in vitro* [4]. They can enter into a quiescence or active state, move into the cell division cycle, go to apoptosis or differentiate [9]. Furthermore, due to the physiological and vascular structure of the bone marrow and lymphoid organs, immune cells experience a substantial gradient of oxygen tension *in vivo*, with oxygen concentrations reaching from 0.2 to 3% in the thymus and 0.5 to 4.5% in spleen and lymph nodes to 13% in the arterial blood [10-12]. Several studies have demonstrated that culture of T cells at different oxygen tensions (atmospheric (20%), physiological (2-12%) or hypoxic (< 2%) oxygen levels) can have a significant impact on cell proliferation, redox status and apoptosis activation [10,13-15]. Because of these complex characteristics, any small change in the culture environment can have profound consequences for the product quality.

One goal of preclinical research and process development is the determination of optimal cell culture conditions to achieve high-quality cells. Another key step towards the optimization of cell-based therapies is the production of sufficient cell quantities in a rapid time frame.

To this end, the use of 3D culture systems, such as stirred-tank bioreactors, presents several advantages compared to other culture systems, such as static culture in bags or flasks [4]. Bioreactors combine efficient mass transfer of oxygen and nutrients with high reliability of bioproduction and scalability. These advantages are ascribed to the accurate control of various critical parameters such as nutrient feeding, temperature-, DO-, pH-control, gas sparging, and agitation. In the present study, we used a DASbox Mini Bioreactor System equipped with BioBLU 0.3c Single-Use Bioreactors to evaluate the impact of different oxygen availabilities on T cell expansion efficiency.

Expansion of human $CD4^+$ T Cells was examined at two different oxygen tensions (70% or 20% of dissolved oxygen), given that 100% DO corresponds to 20.9% atmospheric oxygen level. This study shows that parallel bioreactor control systems are useful tools to optimize culture conditions before the scaling-up process, thus providing a solid basis for optimal yields in subsequent steps from benchtop to production scale.

Material and Methods

Culture of $CD4^+$ T cells in BioBLU 0.3c Single-Use Bioreactors

Lonza® human peripheral blood $CD4^+$ T cells (Lonza, 2W-200) were expanded on T75 CellBIND® flasks (Corning®, 3290). At day 0, cells were seeded at 1 x 10^6 cells/ml and activated with ImmunoCult® Human CD3/CD28/CD2 T Cell Activator (StemCell Technologies®, 10990) in ImmunoCult-XF Cell expansion medium (StemCell Technologies, 10981) supplemented with recombinant human interleukin 2 (rhIL-2) (StemCell Technologies, 78036). The cells were incubated at 37 °C, 5% CO_2 and 20% O_2 in a CellXpert® C170i Incubator (Eppendorf, 6731). Three days later, cells were counted using the Vi-CELL® automated cell counting device (Beckman Coulter®, 731050) and the culture volume was increased to adjust the viable cell density to ~ 1.0 to 2.5 x 10^5 cells/ml. After 5 days, cells were transferred to the bioreactor (DASbox Mini Bioreactor System, equipped with four BioBLU 0.3c Single-Use Bioreactors) at a cell density of ~ 1.0 to 3.0 x 10^5 cells/ml. Two protocols were evaluated in this study: in the first , the dissolved oxygen (DO) level was

set to 70%, while in the second the DO was set to 20%. All cultures were incubated at 37 °C. The agitation speed was adjusted to 70 rpm and the pH of the growth medium was maintained at 7.4 by automatic addition of CO_2 in the vessel headspace or addition of NaOH (1N) to the medium as summarized in Table 1. The culture was maintained for 21 days (5 days in T75 flasks + 16 days in the bioreactor) and the cell density was regularly adjusted according to the culture medium manufacturer's protocol by addition of fresh ImmunoCult-XF Cell expansion medium supplemented with rhIL-2 and partial harvesting. On day 7 and 14, the cells were re-activated with the ImmunoCult Human CD3/CD28/CD2 T Cell Activator in ImmunoCult-XF Cell expansion medium supplemented with rhIL-2 (Figure 1). Cell proliferation was evaluated at different time points during the cultivation with the Vi-CELL automated cell counting device. The lactate and glucose concentrations were monitored at different timepoints with the YSI® 2900 Biochemistry analyzer (YSI). The present study is based on three independent experiments with two different T cell donors.

Table 1: Overview of process parameters for the cultivation of $CD4^+$ T cells in BioBLU 0.3c Single-Use Bioreactors based on the protocol of Ou J. et al. [16].

Initial working volume	100 mL
Inoculation cell density (day 5)	1.0-3.0 × 10^5 cells/mL
Temperature	37 °C
pH	7.4
Agitation	70 rpm
Gas sparging rate	0.01 VVM
Dissolved oxygen (DO)	20 or 70 %

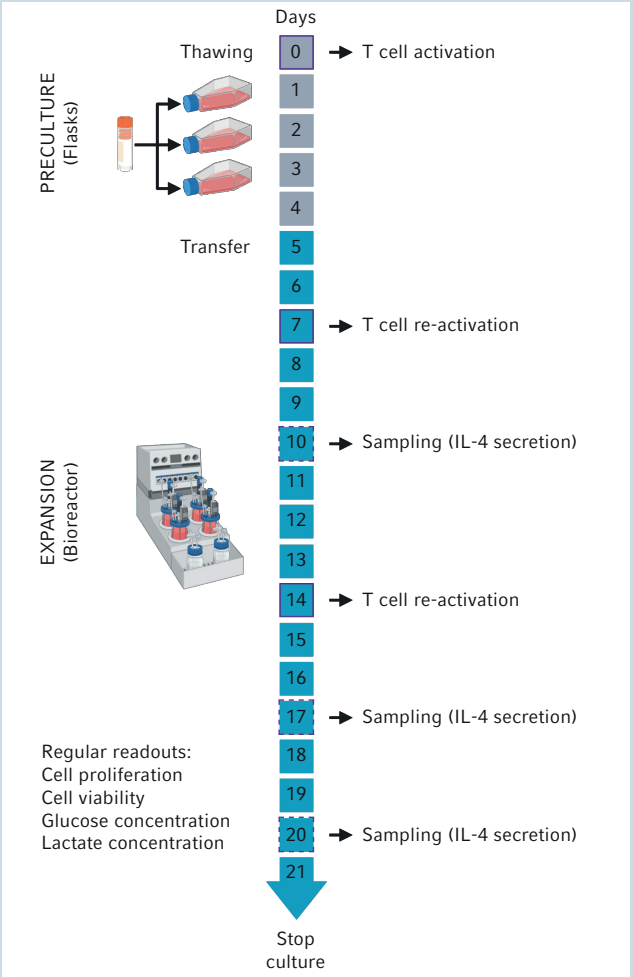

Fig. 1: Schematic representation of $CD4^+$ T cell expansion in BioBLU 0.3c Single-Use Bioreactors controlled by a DASbox Mini Bioreactor System for 21 days with a preculture of 5 days in flasks. Created with BioRender.com

To know more about the possibilities of configuring the DASbox system tailored to your needs, visit **www.eppendorf.group/dasbox**

Interleukin (IL)-4 production by $CD4^+$ T cells

On day 10, 17 and 20, cell culture samples were collected and seeded at a density of 1 x 10^6 cells/ml in a 24-well plate. $CD4^+$ T cells were stimulated with ionomycin at 2.5 µM (StemCell Technologies, 73722) and phorbol 12 -myristate 13 -acetate (PMA) at 10 ng/ml (StemCell Technologies, 74042) and incubated at 37 °C for 24 hours. At the end of the activation step, the cell suspension was collected and centrifuged at 200 x g for 5 min. The production of IL-4 by the $CD4^+$ T cells was measured in the clarified supernatant using the Invitrogen® IL-4 Human ELISA kit (Thermo Fisher Scientific®, BMS-225-2). Briefly, a combination of 50 µl of sample and 50 µl of biotin-conjugate anti-human IL-4 antibody was added to the anti-human IL-4 antibody coated plate and incubated at room temperature (RT) for 2 hours on a microplate shaker. At the end of the incubation, the plate was washed three times before the addition of 100 µl of diluted Streptavidin-HRP and incubated for 1 hour at RT. A total of 3 washes were also performed before the addition of 100 µl of the TMB substrate solution. The plate was incubated for 10 minutes at RT avoiding direct exposure to intense light before the addition of the stop buffer. Optical densities were recorded at 450 nm as a primary wavelength and 620 nm as the reference wavelength.

Results

CD4+ T cell expansion in BioBLU 0.3c Single-Use Bioreactors

Initially, CD4+ T cells were subjected to a static cultivation period of 5 days on T75-flasks to ensure appropriate cell numbers for the culture in the bioreactor. Next, the cells were seeded into two bioreactors at a density of ~ 1.0 to 3.0 x 10^5 cells/ml (~ 1.0 to 3.0 x 10^7 cells/vessel) and incubated at two different DO concentrations. In order to emulate the physiological oxygen levels that lymphocytes experience *in vivo* [10,17], DO in one culture was set to 20% oxygen saturation level whereas in the second protocol, the oxygen saturation level was set to 70%.

The culture was maintained in the bioreactor for 16 days (from day 5 to day 21). Cell density was adjusted every one to three days to maintain CD4+ T cells at lower cell density and improve cell proliferation and viability. Cell viability at each dilution point was comparable and remained stable under both conditions (between 97 to 87%) over the entire course of the experiment. Furthermore, high cell proliferation rates were achieved for both DO levels

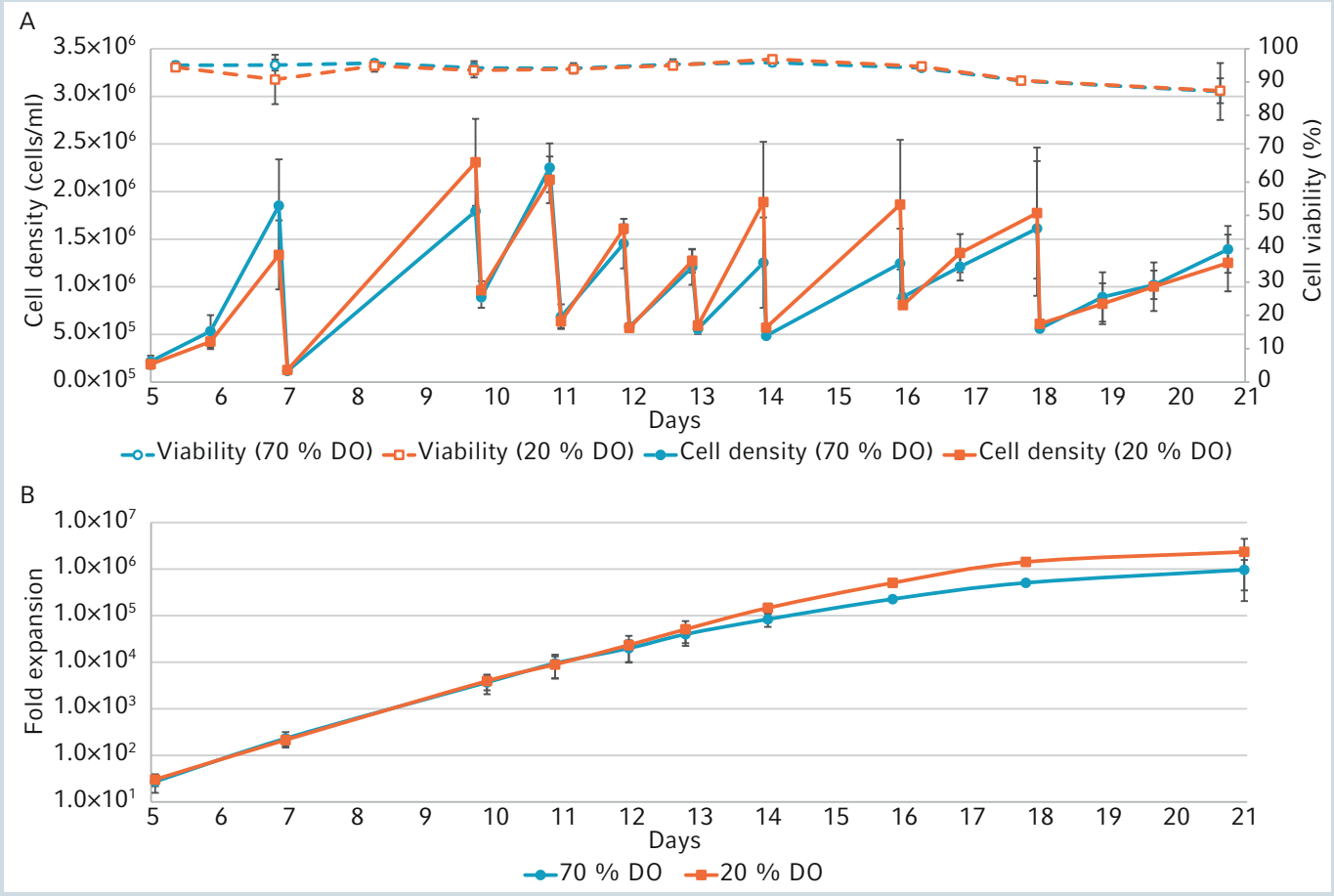

Fig. 2: CD4+ T cell proliferation in the BioBLU 0.3c Single-Use Bioreactor controlled by a DASbox Mini Bioreactor System during a 16 day-expansion phase (from day 5 to day 21). (A) Cell density and viability of CD4+ T cells under both conditions (70 or 20% DO) were monitored at different time points and cell density was adjusted accordingly to the manufacturer's protocol. (B) Average fold expansion of CD4+ T cultured at 70 or 20% DO. Results are compiled from three independent culture replicates.

during the incubation period. Taking into account the cell expansion in flasks prior to bioreactor inoculation (from day 0 to day 5) and the cell expansion in the bioreactor (from day 5 to day 21), the average fold-expansion of $CD4^+$ T cells cultured at 70% DO and 20% DO was of 9.63 x 10^5-fold and 2.35 x 10^6-fold on day 21, respectively (Figure 2B). These results indicate a possible positive impact of 20% DO on $CD4^+$ T cell numbers compared to 70% DO. This tendency is supported by a similar bioreactor study by Bohnenkamp *et al.* [18] which identified enhanced T cell expansion at lower air saturation (25% and 50% over 75%). Moreover, in a static study (using T-flaks) Carswell *et al.* [14] found increased proliferation rates of stimulated T cells grown at 5% atmospheric O_2 levels compared to cells grown at 20% atmospheric O_2 level.

In addition to growth factors, the proliferation of T cells in an *in vitro* environment is also regulated by substrate and metabolite concentrations. Metabolite monitoring during the whole culture process revealed comparable glucose consumption under both conditions (Figure 3). Cell density adjustment every 1 to 3 days by dilution allowed maintaining glucose levels within a narrow range, avoiding large concentration fluctuations that could adversely affect cellular metabolism.

Additionally, lactate production was monitored at each dilution point. As expected, lactate levels increased as the available glucose in the culture was consumed. However, as previously observed, cell density adjustment reduced fluctuation of lactate levels during the culture process. The fine control of these parameters is essential to maintain cells in a robust state and improve their proliferation.

$CD4^+$ T cell quality assessment by Interleukin (IL)-4 production

To ensure a high-quality product, a series of validation steps are required to establish the maintenance of T cell quality. In the present study the functional capability of $CD4^+$ T cells was evaluated by measuring the production of interleukin 4 (IL-4). This signature cytokine is involved in several immunological processes. It promotes the differentiation of

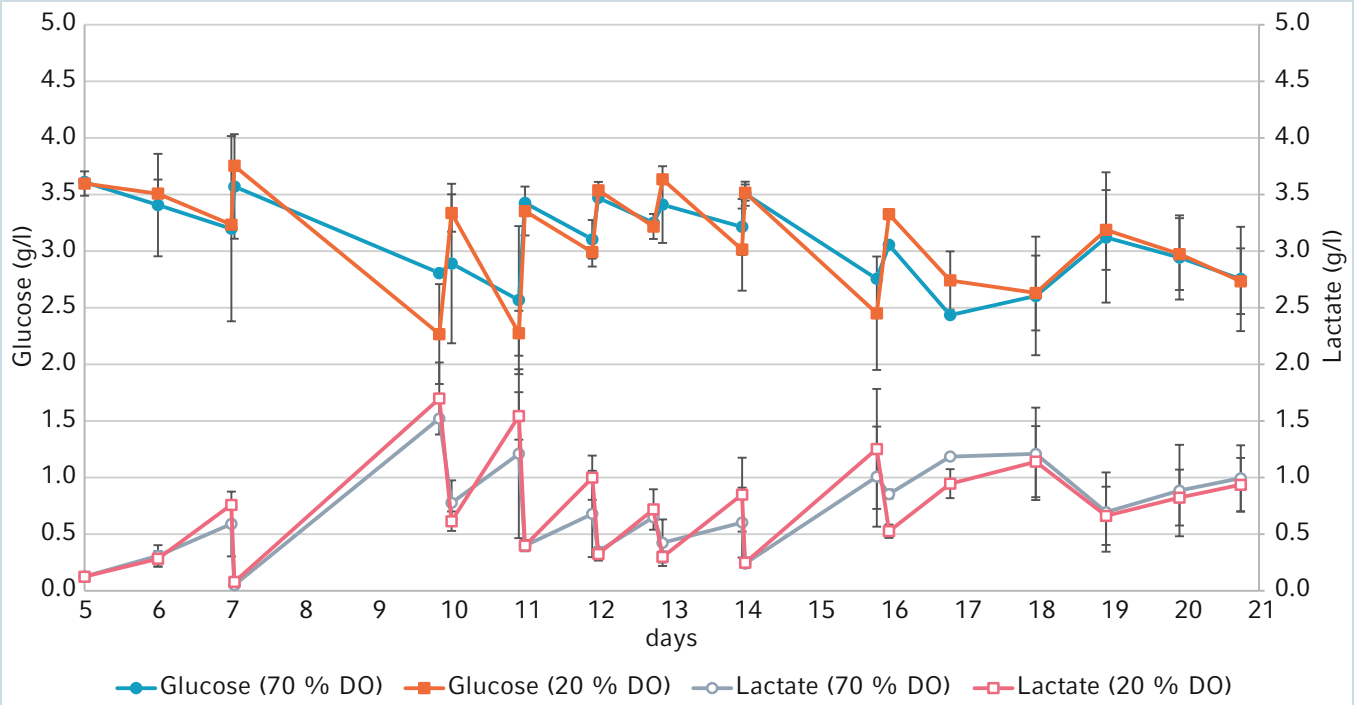

Fig. 3: Glucose consumption and lactate production profile of $CD4^+$ T cells cultured for 16 days in the BioBLU 0.3c Single-Use Bioreactor controlled by a DASbox Mini Bioreactor System. Results are an average of three independent culture replicates.

Th2 cells, a subset of T cells, it regulates immunoglobulin (Ig) class switch to IgG1 and IgE in the antibody response of B cells and induces alternative activation of macrophages in concurrence with IL-13 [19].

Bioreactor cultures were sampled at different time points (day 10, 17 and 20) and T cells were either left untreated (NS) or stimulated (ST) with PMA and ionomycin for 24 hours. Thereafter, the cell suspension was collected and centrifuged and the levels of secreted IL-4 were determined (see Material and Methods). As shown in figure 4, IL-4 inducibility was maintained in stimulated but not non-stimulated cells.

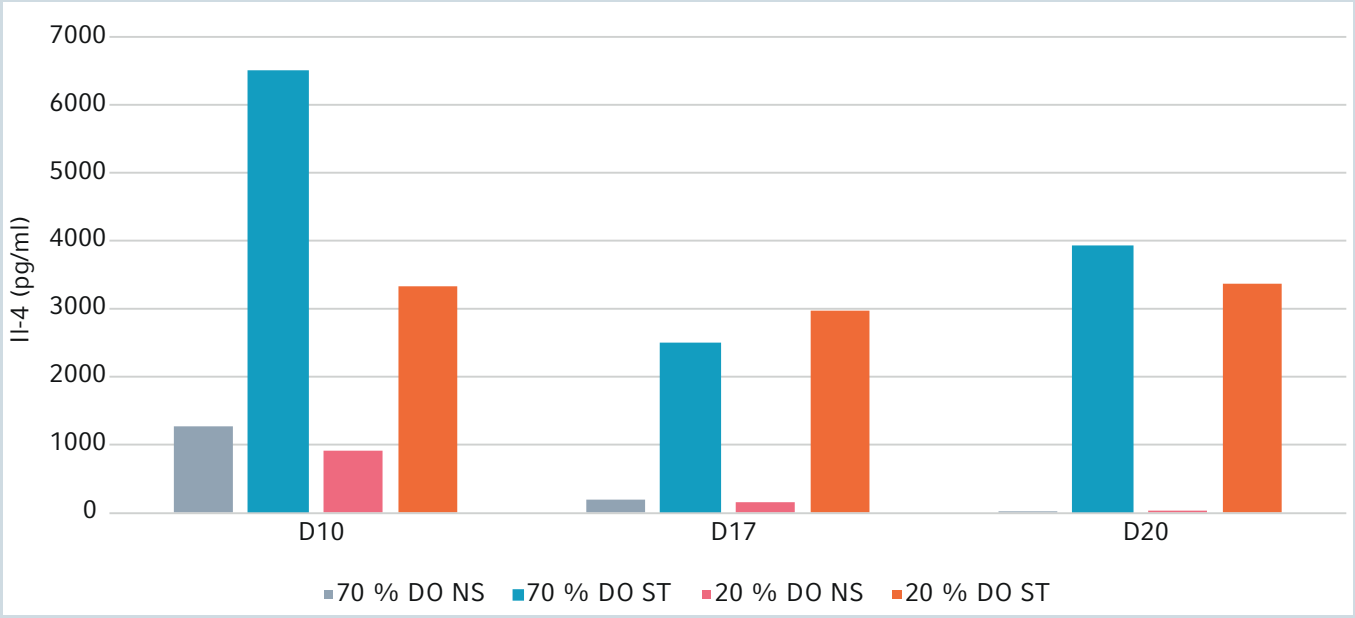

Fig. 4: Determination of Interleukin (IL)-4 production by $CD4^+$ T Cells cultured at 70 or 20% DO. Culture samples were harvested at day 10, 17 and 20 and seeded at a density of 1 x10^6 cells/ml in a 24-well plate. IL-4 production of stimulated (PMA and Ionomycin treatment for 24 hours at 37 °C) (ST) or non-stimulated (NS) cells, was assessed in the supernatant using an ELISA assay. Representative results are expressed in pg/ml of IL-4.

Conclusion

The importance of controlling oxygen levels in T cell culture has been emphasized in several studies [4, 9, 10, 16, 18]. However, culturing methods (flasks, bags or bioreactors) and the level of fine tuning control of the critical parameters (temperature, pH, DO, etc.) differs from one study to another, which may lead to disparate conclusions reached in the current literature [13,14, 20]. The DASbox Mini Bioreactor System offers a fully controllable culture system appropriate to generate high-quality viable and functional T cells throughout the entire process, which will impact the quality of the data. The present study illustrates the successful long-term expansion of $CD4^+$T cells in a DASbox Mini Bioreactor System equipped with BioBLU 0.3 Single-Use Bioreactors. A possible positive impact of a lower oxygen tension (20% instead of 70% DO level) was suggested on $CD4^+$ T cell proliferation rates without impacting cell functionality

in terms of IL-4 secretion. Finally, reproducibility, a key requirement for the optimization of scalable bioprocessing, was confirmed by testing T cells from different donors. Thus, the approach presented here illustrates the advantages of a controllable bioreactor-based culturing systems for the development of scalable T Cell production processes.

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ALA-CART: zeroing in on cancer cells that evade detection

CAR-T cells have been redesigned to improve their ability to target cancer cells that would previously fly under their radar.

MEGAN GIBONEY, DIGITAL EDITOR, REGMEDNET

Scientists at the University of Colorado Anschutz Medical Campus (CO, USA) have created ALA-CART, an advanced CAR-T therapy aimed at improving therapeutic persistence and effectiveness, especially against cancer cells that evade traditional CAR-T treatments.

CAR T-cells are not sensitive enough to detect and eliminate antigen-low tumor cells — cancer cells that express lower levels of target antigens. This problem has been observed in multiple cancers, including B-cell acute lymphoblastic leukemia, lymphoma and multiple myeloma. The inability to target antigen-low cells not only reduces CAR T-cell efficacy but also limits the range of cancers that can be treated with this therapy.

To address this issue, the researchers focused on understanding antigen sensitivity mechanisms in traditional CAR T-cell therapy by studying a clinically active CD22-targeting CAR (CD22BBz CAR). They analyzed the signaling pathways that are activated when CAR T-cells encounter antigen-low tumor cells and found that antigen-low stimulation led to inefficient LAT (linker for activation of T cells) activation.

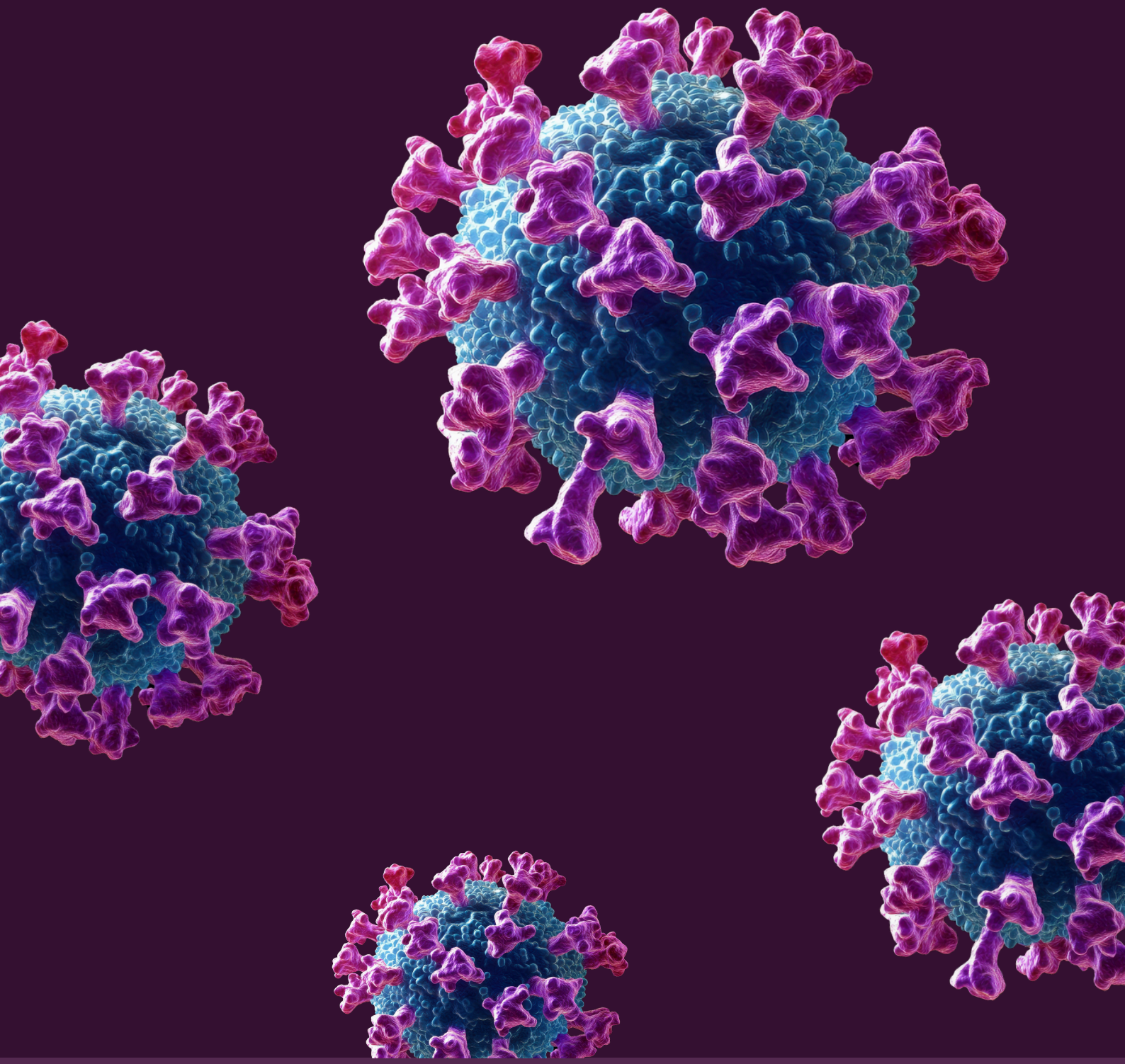
Based on these findings, the team designed a bicistronic CAR platform called ALA-CART. This system co-expresses a second-generation CAR alongside an adjunctive LAT-activating CAR, which incorporates the intracellular domain of LAT. This modification enhances LAT activation and strengthens downstream signaling, enabling ALA-CART cells to more effectively target and eliminate cancer cells, even those with low antigen expression.

When tested in leukemia patient-derived xenografts with extremely low CD22 antigen levels (~300 molecules per cell), ALA-CART demonstrated superior efficacy and persistence compared to traditional CAR T-cells. Since ALA-CART can target antigen-low tumor cells that evade traditional CAR-T therapy, it is likely to have a lower risk of relapse.

Additionally, ALA-CART increased the proportion of T stem cell memory cells, which are associated with long-term survival and resistance to exhaustion. This enhancement could lead to more durable responses, ultimately improving treatment outcomes and reducing the likelihood of therapy failure.

By overcoming the challenge of targeting antigen-low cells and improving durability, the ALA-CART platform shows great promise for enhancing long-term outcomes. The next step for the researchers is to advance ALA-CART into clinical trials to evaluate its safety and efficacy in human patients, with plans to begin within the next two years. In the meantime, they are also testing the therapy on other cancer types, including acute myeloid leukemia, multiple myeloma and solid tumors.

Source: Pham-Danis C, Novak AJ, Danis E *et al*. Restoration of LAT activity improves CAR T cell sensitivity and persistence in response to antigen-low acute lymphoblastic leukemia. Cancer Cell 43(3), 482-502 (2025).



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