CELLULAR THERAPY AND REGENERATIVE MEDICINE

Review

Chimeric antigen receptor therapy for hematological malignancies: a pediatric perspective from leukapheresis to infusion

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Immunotherapy, particularly chimeric antigen receptor T cells (CAR T)s, has changed the landscape of B-cell malignancy treatment and represents a promising approach to cancer therapy. The use of CAR T-based therapy in pediatric patients presents several critical issues such as the quality of the leukapheresis process and the treatment-related toxicity. Nevertheless, the experience with anti-CD19 CAR Ts in treating pediatric B-cell precursor acute lymphoblastic leukemia (BCP-ALL) demonstrated its feasibility and efficacy with a complete response rate greater than 80%. Although CAR T therapy is still in its infancy, the growing clinical experience and expanding body of literature are gradually enhancing the management of complications and patient monitoring. Yet, in vivo, CAR T persistence issues emerged and highlighted the need for continuous investigations to improve the long-term efficacy of CAR T cell therapy. Concurrently, it is crucial to expand the use of CAR T cells in the treatment of pediatric tumors other than BCP-ALL and to extend access to such therapy. In this review, we outline the journey of treating pediatric patients with CAR T products, covering the process from referral to long-term monitoring, while also addressing key concerns and future perspectives.

Keywords: *chimeric antigen receptor, acute lymphoblastic leukemia, adoptive immunotherapy, pediatric.*

INTRODUCTION

Immunotherapy has recently changed the landscape of cancer treatment, especially for hematological malignancies. One of the most promising stories is adoptive cell therapy (ACT) and in particular chimeric antigen receptor (CAR) T cells (CAR Ts). In this review, we will briefly describe CAR Ts and we describe the process by which T cells are harvested and processed to obtain CAR T medicinal products. We will discuss some of the toxicities and clinical efficacy of CAR Ts in the setting of pediatric patients with precursor B-cell acute lymphoblastic leukemia (BCP-ALL) and mention future perspectives.

CAR Ts OVERVIEW

CAR Ts can be depicted as genetically manipulated T cells that combine the ability to recognize a target and to be activated through signal transduction domains.

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Most CAR T specificity has been conferred using antibody-derived single-chain proteins able to redirect T cells against a specific tumor antigen without MHC restriction. The original idea emerged from Eshaar's study in 1990, leading to the development of first-generation CAR, which consisted of only a CD3ζ intracellular domain. Unfortunately, first generation CAR did not work[1,2]. It was with the introduction of a co-stimulatory domain that resembled the physiological activation of the T cell receptor (TCR) that CAR T technology advanced. Since then, great efforts have been directed to improve and optimize the platform and further generation of CAR have been developed (**Figure 1**). Despite over 700 clinical trials with CAR T therapy have been registered at www.clinicaltrials.gov, only 6 CAR T products have been approved at the time of writing by U.S. Food and Drug Administration (FDA) for B-cell malignancies (**Table I**). All these products have been also approved by the European Medicine Agency (EMA).

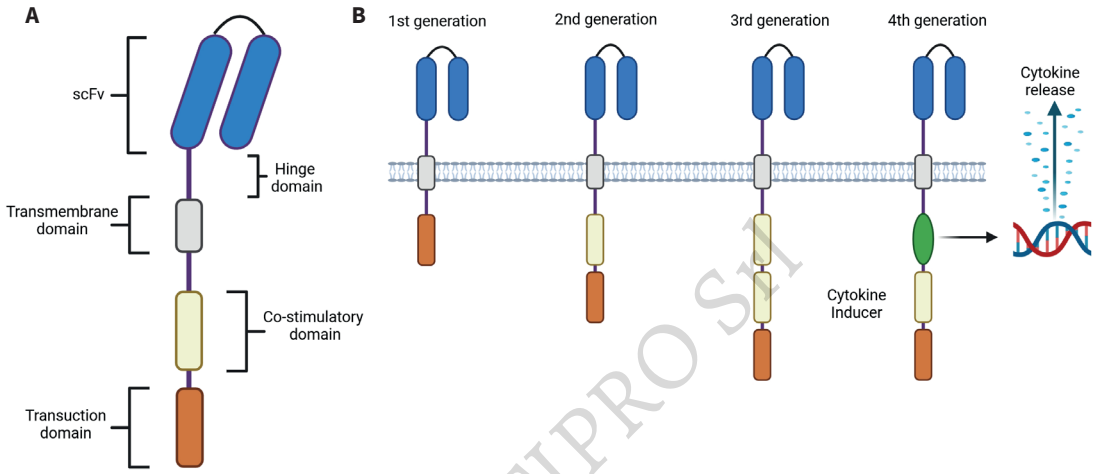

Figure 1 - CAR's structure overview
(**A**) CAR consisting of 1) the extracellular domain, usually formed by a single chain fragment variable (scFv) which confers the CAR specificity, or sometimes by a native protein or peptide that can bind to its receptor on target cells; 2) the hinge region derived from CD8, CD28 or IgG; 3) the transmembrane domain able to anchor the CAR to the immune cell membrane; 4) the intracellular signaling domain generally composed by a CD3ζ signal domain and one or more co-stimulatory domains (CD28, 4-1BB, OX40, ICOS). Any change in one of these regions can influence the activity, efficacy and persistence of CAR Ts. (**B**) CARs generation. First generation CAR was formed by a CD3z intracellular domain. Second generation CAR was characterized by the addiction of a costimulatory domain (CD28, 4-1BB, ICOS) to the CD3z domain. A second co-stimulatory domain is included in the third generation CAR. In the fourth generation CARs, incorporates a specific intracellular domain that enables the inducible expression of a transgenic product, such as a cytokine, to enhance CAR T-cell expansion, activity, and persistence. scFv: single chain fragment variable.

Table I - *FDA approved CAR T product and principal characteristics*

Pharmaceutical principle	Name	Manufacturer	Target	Indication
Tisagenlecleucel	Kymriah	Novartis	CD19	Pediatric and young adult R/R acute lymphoblastic leukemia; adult R/R DLBCL; R/R follicular lymphoma
Axicabtagene ciloleucel	Yescarta	Kite pharma and Gilead	CD19	Adult R/R Large B-cell lymphoma (DLBCL, PMBCL, high grade B-cell lymphoma)
Brexucabtagene autoleucel	Tecartus	Kite pharma and Gilead	CD19	Adult mantle cell lymphoma, adult B-acute lymphoblastic leukemia
Lisocabtagene maraleucel	Breyanzi	BMS and Juno Therapeutics	CD19	R/R large B-cell lymphoma
Idecabtagene vicleucel	Abecma	BMS and Blubird Bio	BCMA	Multiple myeloma
Ciltacabtagene autoleucel	Carvykti	Janssen and Johnson & Johnson	BCMA	Multiple myeloma

Kite pharma, Santa Monica, CA, USA; Gilead, Foster City, CA, USA; BMS, New York, NY, USA, Blubird Bio, Somerville, MA, USA; Juno Therapeutics, Seattle, WA, USA;Janssen, Beerse, Belgium; Johnson & Johnson, New Brunswick, NJ, USA.
R/R: relapsed/refractory; DLBCL: diffuse large B-cell lymphoma; PMBCL: primary mediastinal B-cell lymphoma.

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First clinical data from their use were impressive in adult patients with relapsed/refractory (r/r) B-cell non-Hodgkin lymphoma (B-NHL) and in children and young adults with r/r BCP-ALL[3]. All 6 commercial CAR T products target B cell surface antigens and in particular four out of six are directed against CD19, and two of them target the B cell maturation antigen (BCMA) for multiple myeloma. At the moment all approved CAR T cell medicinal products are produced using viral vectors (gamma-retroviral and lentiviral vectors) that guarantee an efficient gene transfer capacity, with a long-term history of application in adoptive cell therapy[4,5]. However viral vectors have some limitations and issues, such as the limited length of gene cassette, due to the small capsid dimension, the requirements of specific facility and staff training, and the complexities in viral supernatant production. All commercial products are based on centralized production, in which manufacturing is exploited and controlled by the pharmaceutical company. All these factors result in high costs that limit their accessibility and use. Moreover, the FDA's warning released in November 2023 following reports of T-cell lymphoma after CAR T infusion has shed light on the possible long-term side effects of the therapy. To overcome these limitations, in the last decade, non-viral vectors have been proposed and validated pre-clinically as well as in clinical trials[6]. To date the only CAR T cell product approved for pediatric patients is Tisagenleucleucel and in this review we mainly focus on this product.

CAR T JOURNEY

Considering the use of CAR T cells, patient eligibility is a primary issue. Eligible patients should be referred to a CAR T center where eligibility will be assessed by experts. The expression of the target antigen on all patient's leukemic cells has to be assessed first to any procedure. Moreover, the ability to successfully collect T cells and generate an effective product are also necessary elements. Among the multiple factors to be accounted for, disease characteristics are crucial, besides the patient conditions, which should be stable enough to allow CAR T manufacturing and shipping time. Screening tests may vary across different trials and aim at defining the eligibility of the patient for the specific treatment, according to clinical trial requirements or regulatory/commercial authorizations. Medical history, performance status, and the specific CAR T product should also be taken into account when assessing tolerability.

LEUKAPHERESIS: THE TRANSFUSION MEDICINE PERSPECTIVE

All approved CAR T cell products are obtained from patients' T cells, although several trials are investigating the use of allogeneic T cells[7]. In the autologous setting, the manufacturing of CAR T cells begins with a leukapheresis to obtain viable T cells from the patient peripheral blood. There are several devices to perform apheretical mononuclear cell (MNC) collection, including the COBE Spectra and Spectra Optia Apheresis systems (Terumo BCT Inc., Lakewood, CO, USA) and the Amicus Cell Separator (Fenwal Inc., Lake Zurich, IL, USA/Fresenius Kabi AG., Bad Homburg, Germany). The choice should be based on several factors, including the availability of the devices within the center, the quality and accessibility of reagents, and the expertise of trained staff. These factors influence the final quality of the apheresis product. Achieving the target number of CD3+ cells in the apheresis product is influenced by patient-specific factors, such as the proportion of circulating T cells within the total lymphocyte count, the patient's weight and age, and his/her circulating blood volume. Additionally, apheresis parameters, including the volume of peripheral blood processed by the device and the collection efficiency, play a significant role[8]. By applying centrifugal force to a continuous or semi-continuous flow of anticoagulated whole blood, the different blood components are separated into layers based on their density. Circulating lymphocytes contained within the MNC layer could be selected and harvested (**Figure 2**). The collection process is similar to the unstimulated leukapheresis usually performed to collect lymphocytes for donor lymphocyte infusion (DLI) after hematopoietic stem cell transplantation (HSCT). Patients' T cells have been exposed to multiple cycles of chemotherapy for treating the underlying hematological malignancy, which can result in lymphopenia and the presence of circulating blasts. These factors can impair both the collection of adequate numbers of CD3+ cells and the expansion of the T cells during manufacturing[9,10]. Moreover, inter-patient variability, differences between apheresis devices and operators, and different guidelines across

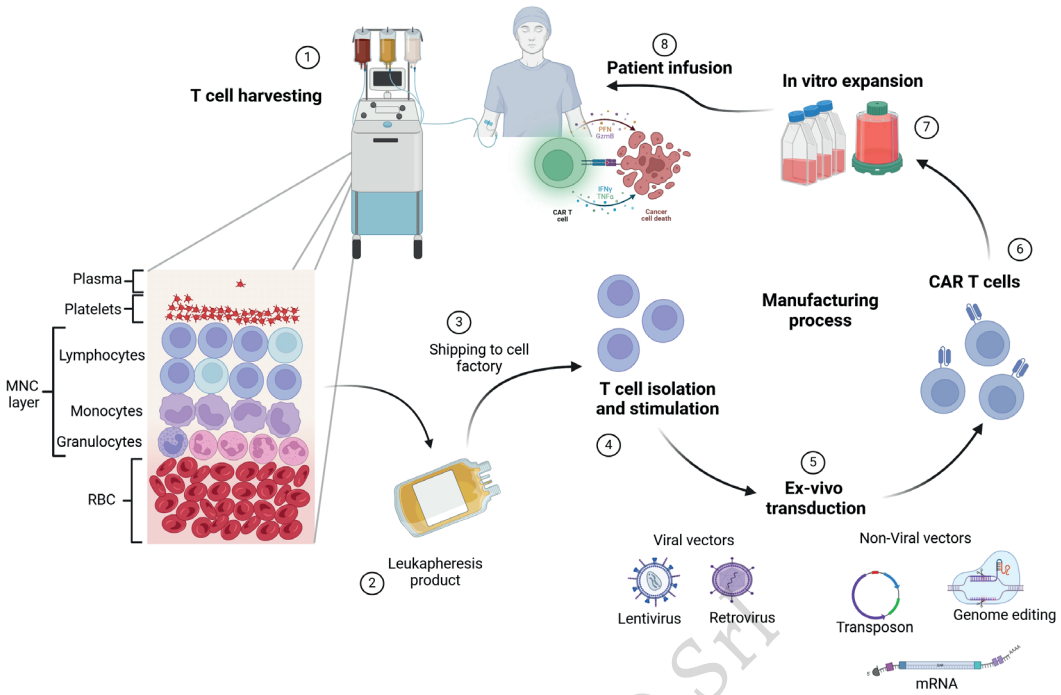

Figure 2- Eligible patients undergo lymphocytoapheresis
(**1**) The apheresis machine separates blood components and harvests the mononuclear cell layer. (**2**) The leukapheresis product is then shipped to a cell manufacturing facility (**3**), where T cells are isolated and purified (**4**). After stimulation, T cells are engineered using various mechanisms, including viral or non-viral vectors (**5**). The transduced T cells are expanded in vitro and undergo testing to ensure they meet release criteria, including number of cells, CAR expression and sterility (**6**). Upon completion, the CAR T-cell product is shipped back and infused into the patient following a lymphodepleting chemotherapy regimen (**8**). MNC: mononucleated cells; RBC: red blood cells.

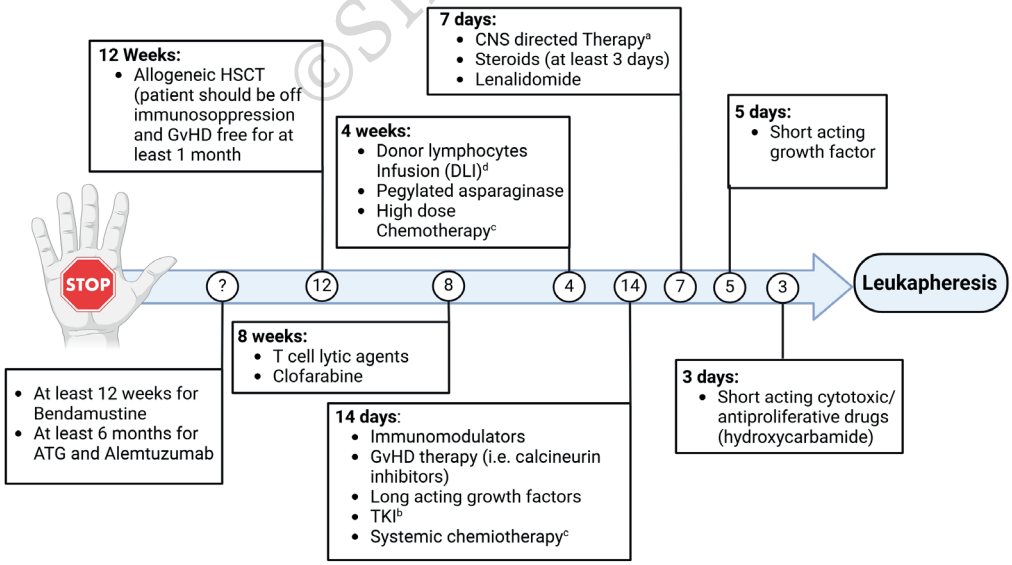

Figure 3 - Recommendations for drugs wash out prior to lymphocytes apheresis
[a]Intrathecal cytarabine can be given up to a day prior leukapheresis; [b]Except Nilotinib which can be administered until 5 days before apheresis; [c]Recovery from cytopenia is required; [d]6-8 weeks to avoid GvHD. CNS: central nervous system, ATG: anti-thymocyte globulin; HSCT: hematopoietic stem cell transplant; GvHD: Graft versus Host Disease; TKI: tyrosine kinase inhibitor.

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Table II - *Recommendations for optimizing leukapheresis in children and adolescents*

Prior to procedure	During the procedure
• Test for HCV, HIV, HBV, Syphilis and other infectious markers according to center guidelines	• Monitor ionized calcium, magnesium and blood gases and replace them as needed
• Check vascular accesses and plan a new one if necessary; involve the PICC team if appropriate	• Prevent hypothermia with warming blanket or inline blood warmer
• Maintain hemoglobin >10 g/dL and platelets >20,000/mcl	• Monitor flow rate
• Verify adequate CD3+ cell count	• Monitor collection volume
• Type&Screen to provide compatible irradiated RBC for blood priming of the device, if appropriate, in patients weighing <25 kg, to manage for extracorporeal volume	• Consider both CD3+ cell and WBC counts of the apheresis product during the procedure in order to optimize the timing to reach the target
• Guarantee a sufficient wash out period after drugs • Skip ACE inhibitors prior to apheresis	• Consider additional days of leukapheresis (>1) in order to meet acceptance criteria for leukapheresis product (TNC and CD3+)
• Involve an anesthesiology team if sedation is needed	• Collection efficiency evaluation

centers complicate the definition of the optimal MNC collection parameters for CAR T cell manufacturing[11]. Indeed, before starting the leukapheresis, patients should be evaluated for performance status, electrolyte balance, hemoglobin level (>80-100 g/dL) and platelets count (>30,000/mcl). Infectious disease markers, in particular for hepatitis B, hepatitis C, syphilis, HTLV, and HIV must be tested on peripheral blood within 30 days of the procedure. An absolute lymphocyte count (ALC) threshold of 0.3×10^9/L is generally recommended, but the decision to perform the procedure with lower counts depends on each center. To optimize T-cell fitness, an appropriate washout after chemotherapy, medications, and other agents is recommended before the leukapheresis. A washout period equal to five half-lives for each particular agent/drug is generally suggested for a sufficient clearance (**Figure 3**)[12,13]. The apheresis process is crucial because the quality of the leukapheresis product, in terms of number of CD3+ cells, T-cell subsets, and their activation/senescence status, is critical for the success of the manufacturing and the efficacy of CAR T products[14]. Pediatric population presents specific challenges for apheresis. These challenges are mainly determined by the patient's weight and blood volume, the frequent limited compliance with the apheresis procedure depending on the child's age, and poor venous access. These challenges require specific strategies as summarized in **Table II**[15]. One of the most crucial factors for a successful apheresis is the choice of an adequate vascular access and, generally, in pediatric patients, a central venous catheter is recommended to initiate and maintain a sufficient blood flow[16]. For smaller patients, the use of a single vascular access device increases the likelihood of reprocessing the same blood, thereby decreasing efficiency. However, having a second vascular access point, located in a different area from the primary device, can improve collection efficiency. This setup allows for more effective circulation and reduces the need to reprocess the same blood, enhancing the overall efficiency of the procedure in low-weight children. Real-world data have demonstrated the feasibility of collecting leukapheresis products from very young pediatric patients (<3 years of age) with r/r BCP-ALL. In small patients, it seems essential to optimize leukapheresis raising hematocrit to 40% with blood transfusion, prevention of hypothermia during collection, close monitoring of vital signs and electrolytes, and allowing >1 day of leukapheresis to meet acceptance criteria[17]. A blood prime of the apheresis device with irradiated packed red blood cells is generally recommended in patients weighing <25 kg to maintain patients isovolemic while a high proportion of their blood volume (>15%) circulates in the collection equipment. Due to the long time necessary for the procedures, to guarantee the collaboration of younger children, it may be necessary to perform apheresis in general anesthesia, requiring collaboration and coordination with anesthesiologists. Potential complications during and after the apheresis procedure include pneumothorax, bleeding, electrolyte disorder (calcium, magnesium, and potassium), and catheter site pain[18]. A retrospective study of pediatric patients enrolled in three different clinical trials, showed that 11/75 (15%) of patients experienced leukapheresis complications including paresthesia and nausea, which were easily managed in apheresis

clinic[19]. Well-trained apheresis staff is essential in the achievement a successful CAR T manufacturing process. Moreover, ensuring adequate venous access and properly preparing patients, particularly in terms of electrolyte balance, along with providing an environment where both the child and parents feel comfortable, could significantly improve the apheresis process in the pediatric population. Another important factor to consider is the timing of apheresis in relation to the patient's disease progression and treatment.This was demonstrated by Das KR and colleagues, who showed that chemotherapy cycles deplete T-cells in several pediatric tumors, thereby reducing the expansion potential necessary for successful adoptive cellular therapies[21]. An earlier collection and cryopreservation of patient's T cells can be considered based on the patient history, especially for high-risk diseases, to ensure better leukapheresis products timely. Many manufacturing companies, such as Novartis (Basel, Switzerland), would allow frozen leukapheresis products to be stored for 30 months[16]. Nevertheless, apheresis collections prior to eligibility might cause an undue procedure to yield a product which may not necessarily be used thereafter, moreover for some products a fresh apheresis is required.

MANUFACTURING OVERVIEW

Upon apheresis, patient's PBMCs must undergo transportation to and from the central manufacturing site, *ex vivo* modification and expansion, and stringent quality-control (QC) testing. Apheresis products are prepared and shipped to the specific facility for manufacturing. The pick-up and shipment represent further steps requiring the coordination between the treatment center and the manufacturing facility that increases in complexity when fresh leukapheresis is required. Leukapheresis products generally contain monocytes, lymphocytes, platelets, and especially in lymphopenic patients for whom MNC layers could be narrow, may contain red blood cells and granulocytes. As demonstrated by several authors, the contamination of the apheresis products with monocytes affected CAR T cell expansion and efficacy and, ultimately, the outcome of patients[22,23]. Another issue is represented by malignant cells in the apheresis product. The accidental transduction of leukemic cells with CAR can lead to the masking of CD19 on the cell surface, allowing these leukemic cells to evade CAR T cell immunosurveillance[24]. For this reason, all the undesired components might be removed through washing and selection methods based on density, cell size, or immunophenotypic characteristics, or through positive or negative selection using magnetic beads. Once selected starting cell population, T cells are activated and engineered to express CAR. The manufacturers provide the genetic manipulation of T cells with the introduction of CAR sequences through different approaches. Viral vectors are considered the "gold standard". As noted, all approved CAR T products rely on viral vectors for manufacturing. Several groups are developing and validating alternative strategies using no viral vectors and gene editing[6]. In particular our group has validated a platform using Sleeping Beauty (SB) transposon system to engineered Cytokine Induced Killer (CIK) cells for the treatment of adult and pediatric patients BCP-ALL (NCT03389035, NCT05252403) and B-NHL (NCT05869279)[25]. Each method is characterized by limitations and advantages such as immunogenicity, stability of transgene expression, delivery efficiency etc., that should be considered when choosing a specific gene delivery technology[26]. After transduction, CAR T cells should be expanded through *ex-vivo* culturing to obtain a number of cells suitable for clinical use. Once the medicinal product's release criteria (safety, potency, purity, identity, and stability) are fulfilled, the CAR T product is shipped back to the treatment center for infusion. Indeed, the optimization of the leukapheresis process is fundamental to improving CAR T manufacturing, guaranteeing a sufficient number of T cells, and reducing vein-to-vein time. Real-world data on CAR T cell therapy has highlighted the limitations of conventional cell-manufacturing processes, which are time-intensive and resource-demanding, ultimately restricting patient access to this advanced treatment. Oporto-Espuela *et al.* reported that in a UK cohort of children and young adults with r/r BCP-ALL, 12% of eligible patients did not receive Tisagenlecleucel infusion due to disease progression or sepsis. Notably, in 3.7% of cases, the final products failed to meet the manufacturer's release criteria, necessitating a second harvesting, an out-of-specification release, or a change in therapy[27]. Locke *et al.* showed how a vein-to-vein time exceeding 40 days affected overall survival (OS) in patients with

diffuse large B-cell lymphoma (DLBCL)[28]. These data emphasize the importance of reducing vein-to-vein time to ensure the timely infusion of the product and prevent disease progression. Various strategies have been developed to streamline the manufacturing process, reduce the risk of failure, and lower overall costs. Some protocols have achieved product generation within 24 hours by significantly minimizing *ex vivo* expansion. This approach not only shortens the vein-to-vein time but also enables the harvesting of a less differentiated T-cell population which is associated with a greater long-term proliferative potential after infusion, potentially enhancing anti-tumor efficacy and ensuring prolonged CAR T cell persistence as will be discussed in the next paragraph[29]. Several reduced-expansion or no-expansion CAR T cell manufacturing protocols are currently being evaluated in clinical trials. One example is the next-day manufacturing F-CAR platform developed by Gracell Therapeutics (Suzhou, China). The anti-CD19 F-CAR has been tested in a Phase I clinical trial (NCT03825718) in children and young adults with B-ALL, achieving a 92% MRD-negative response rate using relatively lower dose of CAR T cells per kilogram[30]. Similarly, platforms such as T-Charge (Novartis)[31,32], NEXT-T (Bristol Myers Squibb, New York, NY, USA)[33], UltraFast CAR (Kure.ai, Orlando, FL, USA)[34], and Ingenui (Kyverna Therapeutics, Emeryville, CA, USA)[35] being testing, highlight significant investments and advancements in rapid CAR T cell manufacturing. A detailed discussion of each platform exceeds the scope of this review[29,36]. Some issues must be faced in accelerating CAR T manufacturing as reported in **Table III**. Progress in the manufacturing process should be accompanied by advancements in quality control (QC) assessment to ensure the safety and efficacy of the product in a short time window. Tests for product identity, purity, safety, and potency are often time-consuming and can lead to delays in product release and infusion. To address these challenges, advancements in QC methodologies are essential to keep pace with the development of short manufacturing processes. For example, incorporating PCR-based methods for sterility assessment could significantly improve efficiency and reduce delays[37]. Another avenue for improving CAR T cell production lies in the automation of the manufacturing process, utilizing closed or semi-closed systems. Traditionally, the manufacturing process is based on manual systems open to a sterile environment susceptible to microbiologic contamination and human error. This system requires adequate facilities and trained staff, which means high costs. Closed and automated systems use platform devices that enable the manufacturing process within a single-use disposable kit. This approach potentially lowers production costs, minimizes the risk of manufacturing failures and contamination from repeated cell manipulations, and improves overall efficiency. Automation also supports the feasibility of on-site manufacturing, enabling the delivery of fresh, non-cryopreserved products directly to patients[38]. In fact, this automated system may be operated in facilities with less stringent classification and could permit the implementation of a de-centralized manufacturing model. Currently, available systems allow the processing of one product at a single time, requiring

Table III - *Main No/reduced expansion protocol evaluated in clinical trials*

	Strengths	Weaknesses	State of the art	Clinical trials	References
No/reduced expansion protocols	Shortened vein-to-vein time	Challange in product release testing	T-charge (Novartis)	NCT04318327 NCT03960840	Dickinson MJ. *et al*., 2023[31]
	Lower resource demand	Possible risk of malignant cell contamination	UltraFast CAR (Kure.ai)	NCT05400109 NCT06698744	Stadel R. *et al*., 2023[34]
	Increased manufacturing capacity	Activated phenotype with risk of severe toxicity	NEXT T (Bristol Meyers Squibb)	NCT04394650 NCT04231747 NCT05869955	Costa LJ. *et al*., 2022[33]
	Improve T cell phenotype in the final product		Ingenui (Kyverna Therapeutics)	NCT06451159 NCT06342960 NCT06588491 NCT06400303	Anaya D. *et al*., 2024[35]
	Reducing CAR T/kg per doses		Fast-CAR (Gracell Therapeutics)	NCT03825718	Yang J. *et al*., 2022[114]

Table IV - *Automated closed/semiclosed system for CAR T cell manufacturing*

	Strengths	Weaknesses	System	Description	References
Automated systems	Reduced personnel and infrastructure costs	Low flexibility in adapting protocols for individual patients	CliniMacs Prodigy (Miltenyi Biotec)	**Advantages**: Flexible closed system, performing all steps from cell preparation and harvest to final formulation with high cell output **Weakness**: complex software (training), high cost (maintenance)	Maschan M. *et al*., 2021[115] Shah BD. *et al*., 2021[38] Palani HK. *et al*., 2023[113] Kedmi M. *et al*., 2022[116] Del Bufalo F. *et al*., 2023[47]
	Reduced probability of human error	Challange in guarantee consistency across centers	Cocoon platform (Lonza)	**Advantages**: Flexibility using customize cassette, lower cost of maintenance **Weakness**: lower cell output, required other instruments for T-cell enrichment and transduction	Trainor M. *et al*. 2023[39] Anguille S. *et al*., 2022[117]
	Point of care manufacturing	Limited capacity to perform long-term release testing for fresh product	Ekko (Millipore Sigma)	GMP-compliant platform based on acoustophoresis for cell processing and production. Data is lacking for CAR T cell production	Li A. *et al*., 2021[40]
	Fresh product		G-rex bioreactor (Wilson Wolf)	**Advantages**: Simple, cost-effective. Allow the culture of different cells **Weakness**: required some manual step, risk of contamination, lower cell output per unit	Ludwig J. *et al*., 2020[118] Gotti E. *et al*., 2022[119]

more instruments (costly) to guarantee the satisfaction of products. At the moment, among the platform available for point-of-care production we find a) the CliniMACS Prodigy platform (Miltenyi Biotec, Bergisch Gladbach, Germany), a fully automated system that has been tested for CAR T manufacturing in several clinical trials; b) the Cocoon platform (Lonza, Basel, Switzerland) which presents some limitation due to the requirement of some manual steps and to perform T-cell enrichment with other methods[39]; c) ekko™ (MilliporeSigma, Burlington, MA, USA) a novel GMP-compliant platform relying on acoustophoresis for cell processing and production[40]; d) G-Rex® bioreactor M series (Wilson Wolf, St Paul, MN, USA); e) other developing systems: Cytiva (Marlborough, MA, USA) automated perfusion system, Gibco CTS rotea™ (Thermo Fisher Scientific, Waltham, MA, USA) and the Terumo Quantum® cell expansion system (**Table IV**). Discussion of the current advancement in automation is beyond the aim of this review and is discussed elsewhere[38,41,42].

THE ROLE OF CAR T PRODUCT COMPOSITION AND PHENOTYPE

Since early investigations, data suggested how the characteristics of the apheresis product and subsequently of the infused product are essential to generate a long-term response. Clinical evidence suggested that T cell differentiation negatively correlates with long-term antitumor activity, and patients responding to CD19 CAR were enriched in gene expression profiles involved in early memory compared to T cells from non-responder patients[14,43]. Recently, two independent groups demonstrated that an increase in CAR T regulatory cells in lymphoma patients was associated with clinical progression[44,45]. Increasing evidence supports an association between the presence of early T cells in patient leukapheresis and CAR T cell products with clinical response and persistence. In particular, less differentiated T cells (i.e., naive T cells, stem cell memory T cells [TSCM], and central memory T cells) showed higher proliferation and cytokine secretion potential compared to their effector counterparts (i.e., effector memory T cells, effector T cells, and effector memory CD45RA-re-expressing T cells). CAR T cell products manufactured from TSCM-selected starting material show enhanced anti-tumor activity at lower doses in preclinical models and better *in vivo* expansion and safety in the clinic else improvement in CAR T cell engraftment or persistence has not been yet demonstrated and requires further evaluation[46]. Several ongoing clinical trials are evaluating the impact of preselecting less differentiated T cells for CAR T cell manufacturing. Although the potential advantages of preselection are outlined earlier, drawbacks might include high cost, additional cell handling *ex vivo*, and the pursuit of low-frequency T cell populations, which can be particularly sparse in heavily pre-treated patients, and from which manufacturers might struggle

to reach the target CAR T cell dose. Nowadays, different groups are trying to optimize manufacturing protocols, shortening the manufacturing process and reducing cell manipulation to improve the stemness and to decrease exhaustion features of CAR T cells as discussed in the previous paragraph. In addition, the development of "off-the-shelf" allogeneic CAR T cells could represent a great opportunity. Using healthy donor cells could be a strategy to address some issues encountered with autologous products. In particular, allogeneic CAR T cells showed potentially greater fitness, avoided blast contamination, overcame the patient's lymphopenia, and the need for a drugs washout period to harvest T cells. Del Bufalo *et al*. reported a cohort of pediatric patients with r/r BCP-ALL treated with 2 non-edited allogeneic anti-CD19 CAR T products: a) a retroviral product including a suicide gene and obtained from cryopreserved apheresis, b) Lentiviral CAR T manufactured from fresh leukapheresis through CliniMACS Prodigy closed system and infused as a fresh product. All patients obtained CR (MRD negative) in the BM. With a median follow-up of 12 months 61.5% of patients were in CR. Allogeneic CAR T showed a safety profile similar to autologous products with only 1 case of graft versus host disease (GvHD) easily managed with corticosteroids and ruxolitinib[47]. This strategy could facilitate the development of a universal, off-the-shelf drug product bank, readily available to meet clinical needs. Additionally, it could reduce manufacturing costs by enabling the scalability of a single production process[48]. However, using therapeutic allogeneic T cells creates two challenges: (1) the native T cell receptor (TCR) can cause GvHD, and conversely, (2) alloreactive host T cells and NK cells can quickly reject the cells. Some groups have focused on developing gene-editing strategies to silence the expression of TCR and CD52 in CAR T cells, to both prevent alloreactivity and make CAR T cells resistant to the anti-CD52 antibody Alemtuzumab, allowing its inclusion in the lymphodepletion regimen to prevent CAR T cell rejection[49,50]. At the moment, the reduced persistence of allogeneic cells -due to both host rejection and the induction of an exhausted phenotype from prolonged manipulation during manufacturing- along with the higher risk of infection induced by Alemtuzumab, remain the limitations of this approach.

BRIDGING THERAPY

As described above, the vein-to-vein time is crucial interval that could make difference between to infuse or not a patient. In the literature, this interval ranging from 3 weeks to 3 months depending on the clinical trial. This period is crucial, as demonstrated in the ELIANA trial, where out of 97 patients successfully screened and enrolled, 18 were not infused due to disease progression or manufacturing failure[3]. A bridging therapy could be necessary to prevent disease progression while balancing the preservation of patients' good clinical condition to ensure they remain eligible for the CAR T cell infusion[51]. In a small analysis comprising 51 screened children and young adults with r/r BCP-ALL, only 35 proceeded to CAR T cell infusion. Analyzing the impact of bridging therapy on patient outcome authors showed how patients who received 2 or more cycles of bridging chemotherapy presented higher infection and lower OS. No difference emerged regarding cytokine release syndrome (CRS) and neurotoxicity[52]. Gupta *et al*. demonstrated similar findings when comparing pediatric patients who received low-intensity bridging chemotherapy with those who underwent high-intensity regimens. No significant difference was observed in the probability of reaching CAR T infusion between the two groups. However, the low-intensity regimen offered potential advantages, including lower toxicity rates and improved quality of life[53]. Other factors to consider when choosing bridging chemotherapy are the disease sensitivity to prior regimens and the associated side effects, such as cardiotoxicity for anthracycline or hepatic toxicities from Inotuzumab ozogamicin. Such precautions are essential to prevent potential side effects, including prolonged myelosuppression, infections, and organ toxicities, which could delay CAR T infusion[54]. Generally, immunotherapy, and in particular anti-CD19 and anti-CD22 targeted therapy (i.e., blinatumomab and inotuzumab), is not recommended before CAR Ts infusion despite the efficacy demonstrated in clinical trials[55,56], due to the possible down-regulation of the target antigen on leukemia cells and the depletion of target which can result in lack of CAR T cells expansion. Pillai *et al*., analyzing 166 patients treated with anti-CD19 CAR T cells showed how prior therapy

with blinatumomab was associated with a higher rate of failure to achieve minimal residual disease (MRD) remission or subsequent loss of remission with antigen escape[57]. Similar findings have been reported by Shah N and colleagues in patients with BCP-ALL who were referred to their center for anti-CD22 CAR T cell therapy[58]. In the presence of extramedullary relapse, radiotherapy might be a therapeutic option, but no studies have been performed yet and only a few cases are reported in the literature[59]. To date no standardized bridging therapy exists and the choice depends on healthcare providers and the patient's characteristics such as the disease burden, disease aggressiveness, manufacturing time, and patient's comorbidities.

LYMPHODEPLETION REGIMEN

Before CAR T cell infusion, the necessity of a lymphodepleting regimen is generally accepted. The aim of such procedure is: i) to increase the production of homeostatic cytokines such as IL-15 and IL-7 that enhance CAR T expansion and persistence[60]; ii) to reduce regulatory T cells and myeloid-derived suppressor cells[61,62]; iii) reduced tumor cells to avoid rapid CAR T cell exhaustion. Thus, the goal of lymphodepletion is to create a favorable microenvironment for CAR T cells to enhance their expansion, activity, and persistence while reducing the potential immunosuppressive responses. The most widely used regimen is composed of cyclophosphamide and fludarabine, however, doses and duration vary across institutions and trials and depend on the targeting disease as well as the T cell source (autologous or allogeneic). Furthermore, combinations with other agents such as cytarabine, etoposide, bendamustine, busulfan, or alemtuzumab (for allogeneic CAR T) were described[63]. The use of this lymphodepletion regimen is sustained by clinical data suggesting how cyclophosphamide dose intensity could improve CAR T therapy response in children and young adults with r/r BCP-ALL, further improved with the addition of fludarabine[64,65]. Moreover, Fabrizio and colleagues, analyzing 152 patients with r/r BCP-ALL infused with Tisagenlecleucel, demonstrated how a suboptimal fludarabine exposure was correlated with a higher risk of relapse or loss of B-cell aplasia compared to those with an optimal exposure[66].

CAR T CELL IN PEDIATRICS AND YOUNG ADULT BCP-ALL

As far as August 2024, two CAR Ts products had been approved by FDA and EMA for r/r precursor BCP-ALL: Brexucabtagene autoleucel and Tisagenlecleucel, but only the latter is approved for children and young adults ≤25 years old. Tisagenlecleucel (Kymriah; Novartis) is a second-generation autologous CAR T product directed against the CD19 antigen with 4-1BB as co-stimulatory domain. Tisagenleucleucel is indicated for patients with CD19+ BCP-ALL that is refractory to first-line therapy or with disease relapse after HSCT or for second or further relapses. In the registration ELIANA trial, 75 children and young adults were infused with Tisagenlecleucel and the MRD negative CR rate at 3 months was 81%. Event-free survival (EFS) and overall survival (OS) were 50% and 76% respectively at 1 month[3]. In the real-world setting, Tisagenlecleucel showed similar results to pivotal trials with a CR rate >80%, and EFS and OS rates were about 50% and 70% respectively at 12 months[67]. Interestingly, from clinical data, the association between high disease burden and inferior outcomes compared to low disease burden emerged in univariate and multivariate analyses[68].

It is important to note that generally the real-world setting had less restrictive eligibility criteria than pivotal trials and could bring out specific subgroup responses to treatment. It is the case of children <3 years old who were not included in the ELIANA pivotal trial. Ghorashian *et al.* reported in this population a CR rate of 86% and an EFS and OS of 69% and 84% respectively at 12 months, results confirmed by the report from the Pediatric Real-World CAR Consortium[69,70]. This data highlights how in this specific subgroup of patients often characterized by high-risk BCP-ALL (i.e., KMT2A-rearranged) and poor outcomes, CAR T therapy could be a feasible and encouraging option for treatment. Similar considerations should be made for patients with extramedullary disease (EM) excluded from initial trials. Moreover, a report describing some cases of severe neurotoxicity in early CD19-specific CAR T investigation in adults with CNS involvement posed caution in the use of CAR Ts in this setting[71]. More recently, emerging data from real-world life and clinical trials showed the feasibility of CD19-CAR T treatment in patients with CNS disease with similar outcomes compared to Bone Marrow (BM) involvement

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only and without an increased risk of severe neurotoxicity regardless of the type of CD19 CAR T product[72,73]. Interestingly, Jacoby and colleagues reported a higher risk of CNS relapse after Tisagenlecleucel compared to a CD28-based anti-CD19 CAR[74]. At the moment, CAR T cell therapy in patients with CNS involvement is possible and effective and could spare cranial radiation and its long-term adverse effects in children, but as a stand-alone therapy may not fully prevent subsequent CNS relapse. Regarding non-CNS extra-medullary BCP-ALL, a small number of patients (15) were analyzed, and although the CR rate was 66.6% only 4/15 patients maintained response without relapsing. CRS or neurotoxicity rates were not increased in this subset of patients[72]. Interestingly, a recent research letter highlighted how nearly 50% of patients referred for CD19 CAR T therapy had extramedullary disease when screened with positron emission tomography/computed tomography (PET/CT) even in the absence of clinical symptoms and suggested that the response rate to CD19 CAR T cell therapy might be lower in patients with EM compared to those with isolated BM disease[75]. One of the most promising uses of CAR T cell is related to Down syndrome associate-BCP-ALL. These patients presented a high vulnerability to conventional chemotherapy and the use of CAR T cells could spare chemotherapy-related toxicities and improve the outcome of these patients. Analyzing data from 3 clinical trials, Laetsch and colleagues demonstrated how Tisagenlecleucel is an effective treatment with manageable toxicities and a high response rate (88%) in down syndrome associate BCP-ALL and could be a valued alternative to HSCT[76]. Similarly, also highest fragile non-Down syndrome might benefit from a potentially less toxic practice compared with HSCT.

DETERMINANTS OF RESPONSE

The identification of response determinants is crucial for optimizing CAR T cell therapy and identifying high-risk patients who may benefit from additional treatment. However, product heterogeneity and disease characteristics make broad generalization challenging. Following infusion, monitoring CAR T cell expansion and the persistence of B-cell aplasia is crucial for assessing therapeutic response and guiding subsequent clinical decisions. CAR T cell expansion indicates the effectiveness and activity of the therapy, while sustained B-cell aplasia serves as a marker of ongoing CAR T cell function and tumor surveillance. Together, these metrics help clinicians evaluate treatment success and determine the need for additional interventions or supportive care. Researchers are investigating potential biomarkers and response determinants to identify which high-risk patients might benefit from a consolidative HSCT following CAR T cell therapy to prevent relapse. In a large cohort of patients infused with Tisagenlecleucel, the authors showed how a deep evaluation of BM minimal residual disease (MRD) through next-generation sequencing (NGS-MRD), was highly predictive of relapse and further therapy should be considered regardless of B cell aplasia status[77]. The role of consolidative allogeneic HSCT following CAR T therapy is still debated. Summers and colleagues, in a recent paper, showed how HSCT following CAR T cell therapy improves outcomes in terms of leukemia-free survival (LFS) compared to the group CAR T therapy stand-alone. In particular, these authors suggested a possible role of HSCT especially in patients with early CAR T function loss (p=0.01) and those without a previous history of HSCT (p=0.09)[78]. Among patients who received CAR T cell for relapse after HSCT, some studies highlighted how a second transplant as consolidative treatment (within 90 days from infusion) can be efficacious in improving long-term survival[79]. The role of CAR T cell dose in treatment response remains unclear. Some studies have reported improved outcomes in patients receiving higher doses of Tisagenlecleucel, while others have found no correlation between dose and efficacy. These discrepancies may be attributable to the unique characteristics of each autologous product, influenced by factors such as apheresis product heterogeneity, variations in transgene expression, T cell memory profiles, and exhaustion marker expression at the end of the manufacturing process[67,80]. An important factor affecting CAR T cell therapy response is disease burden as demonstrated by Schultz and colleagues. A high disease burden was associated with worse outcomes and emerged as a distinct high-risk population who may benefit from further interventional strategies to optimize CAR-mediated outcomes[68]. Building on the work of Fraietta and collaborators, which highlighted that patients achieving a complete response to CAR T therapy exhibited an enrichment of memory CAR T cells both in transcriptional signature and phenotype, the

impact of apheresis and CAR T cell products phenotype on therapy response becomes evident[14,44,45]. Moreover, disease characteristics, and its interaction with the tumor microenvironment could play a role in therapy resistance inducing the expression of an adaptive, therapy-induced, T-cell resistance program in tumor cells[81]. Only a subset of patients has been cured with CAR T cell therapy alone. Identifying response determinants would help guide patient management ultimately driving the optimization of CAR T cell therapy.

SAFETY

Early clinical trials showed that CAR T cell therapy is associated with specific toxicities related to the immune response. An expected on-target off-tumor toxicity is represented by B-cell aplasia and hypogammaglobulinemia, due to normal CD19-positive B cell depletion by CAR T cell activity. This toxicity may result in an increased risk of infection and required periodical gammaglobulin replacement. Monitoring B-cell aplasia provides, in addition to guiding Ig supplementation, information about functional CAR persistence and could guide often decisions in the management of patients (e.g., HSCT).

The two major and well-described toxicities are represented by the cytokine-releasing syndrome (CRS) and the immune effector-cell associated neurotoxicity syndrome (ICANS)[82]. A model of CRS and ICANS pathophysiology is depicted in **Figure 4**. CRS is characterized mainly by fever, which could be accompanied by symptoms such as malaise and anorexia. In the case of severe CRS, the manifestations of a systemic inflammatory response such as hypoxia, hypotension, and organ dysfunction could appear. The grading of clinical manifestations according to the American Society for Transplantation and Cellular Therapy (ASTCT) scoring is recommended and allow to staged CRS in four grades[83]. The management of CRS manifestations is based on intensive supportive care (fluid resuscitation, vasopressor, oxygen supplementation), the use of tocilizumab, an antibody blocking IL-6 receptor (IL-6R), with the possibility to add corticosteroids[12]. ICANS is staged in 4 grades according to ASTCT consensus and is characterized by tremors and myoclonus,

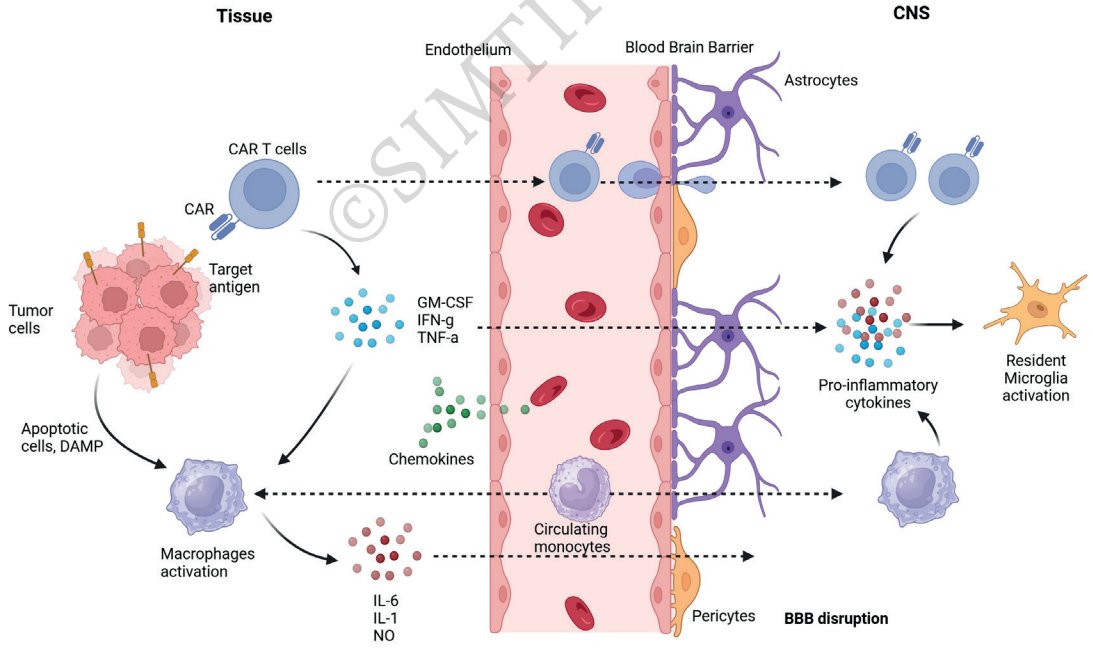

Figure 4 - CRS and ICANS overview
CRS is caused by the activation and expansion of CAR T cells after the recognition of the specific target and the recruitment and activation of host immune cells, in particular macrophages and monocytic line cells. The response is accompanied by a rise in cytokine levels (i.e., IL-6 and IL-1), that bring to a systemic inflammatory response with endothelial damage and the subsequent leakage in multiple organs and systems that lead to CRS symptoms. The transmigration of CAR T cells, cytokines, peripherally activated monocytes in the cerebrospinal fluid (CSF) and CNS could be accompanied by the disruption of the blood-brain barrier and related neurotoxicity. CRS: cytokine release syndrome; ICANS: immune effector-cell associated neurotoxicity syndrome; BBB: blood-brain barrier.

alterations in mental status, dysarthria or aphasia, deterioration in handwriting, or seizures. Interestingly, although tocilizumab is considered fundamental in the management of CRS, it has no impact on ICANS due to poor penetration through the blood-brain barrier[84,85]. Moreover, Tocilizumab, blocking the internalization of IL-6 through its receptor, may paradoxically expose the brain to high levels of IL-6 precipitating ICANS manifestation. Management of ICANS includes treatment of symptoms (i.e. anticonvulsants for seizures), followed by corticosteroids in cases of ICANS grade >1. Recently, accumulating data supported the use of anakinra as a second-line treatment[86]. Data on third-line agents used in the setting of refractory CRS and ICANS is increasing, including siltuximab, ruxolitinib, dasatinib, and etoposide[87].

Immune effector cell-associated hematotoxicity (ICAHT) is the most common CAR T cell-related adverse event across clinical trials and the real-world setting. Cytopenia in one or more lineages often occurs after CAR T cell therapy increasing morbidity (i.e., infection), hospitalization, and ultimate costs. A risk stratification score (CAR-HEMATOTOX) is available and enabling risk-based interventional strategies varies from growth factor support (i.e. GCSF) to HSC boost for refractory patients[88,89]. An emergent life-threatening toxicity after CAR T cell infusion is the immune effector cell-associated hemophagocytic lymphohistiocytosis (HLH)-like syndrome (IEC-HS) defined as a hyperinflammatory syndrome that is characterized by signs of macrophage activation/HLH, and is associated with progression or new onset of cytopenia, hyperferritinemia, coagulopathy with hypofibrinogenemia, and/or transaminitis. IEC-HS is not correlated with CRS and can have a delayed onset, emerging even after CRS has resolved or is resolving. It should not be used solely to describe manifestations of severe CRS[90]. More recently, several cases describing the use of Emapalumab, a monoclonal antibody targeting IFN-γ, have been published for the treatment of IEC-HS and severe CRS. These findings are supported by preclinical evidence indicating that Emapalumab does not impair the potency or efficacy of CAR T cells[91-93]. As reported above, the FDA in November 2023 releases a warning after the report of some cases of T cell lymphoma after CAR T cell infusion. Despite available data suggesting that the risk of a second cancer is not higher than expected among patients with substantial previous exposure to chemotherapy, further data are needed for conclusive evidence[94-96].

CAR T FAILURE AND RELAPSES

Despite the encouraging results in the treatment of children and young adults with BCP-ALL, about 50% of patients relapse after CAR T cells treatment. Interestingly, two pathways of relapse were observed: a) recurring CD19 positive disease, generally related to CAR T cell lack of persistence or low potency, b) CD19 negative relapse due to antigen down-modulation or loss. CD19 negative relapses, which account for almost half of the cases (from 39 to 68%)[3,97] is due to several possible mechanisms, such as lineage switch[47], accumulation of mutations affecting the CD19 gene expression and CD19 splicing variants[48], or selection of a pre-existent CD19 negative clone[98,99]. In the case of CD19 positive relapse a possible second CD19 CAR infusion was investigated, but results were not encouraging[100]. Mouse single chain fragment variable (scFv) could determine an immune response against the CAR that limited the efficacy and the persistence of CAR T cells in case of reinfusion. To mitigate or avoid anti-CAR immune response, some authors have investigated the use of humanized or fully human scFvs[100-102]. A possible strategy to face the CD19 negative BCP-ALL relapses is the CAR multi-targeting approach. One of the most studied antigens beyond CD19 is the CD22 molecule, expressed by both normal and leukemic B-cells. Clinical use of anti-CD22 CAR showed an efficacy comparable to anti-CD19 CAR T cells even in patients resistant to CD19-targeted immunotherapy[58]. Interestingly, in some patients a down-regulation of the CD22 expression on blasts was demonstrated suggesting the possible role of antigen escape in relapse, consistent with what we have seen for CD19[103]. Alternative strategies have been explored in clinical trials such as the simultaneous targeting of the two antigens using a bicistronic anti-CD19 and anti-CD22 CAR which have shown a good CR rate (86%) but poor persistence or the use of CD19/CD22 co-transduced CARs which may prevent antigen-negative relapse after CAR T cell therapy[104,105]. Another cause of therapy failure is represented by CAR T cell exhaustion.

T cell exhaustion is a state of dysfunction that results from chronic stimulation through the CAR in CAR T cells. Exhaustion is accompanied by a phenotypic, functional, transcriptional, and epigenetic modification that leads ultimately to T cell dysfunction. This dysfunction is represented by a decreased proliferative potential, a reduction in cytokine (e.g., IL-2, TNF-a, and IFN-g) release and production[106]. Exhausted T cells experienced metabolic impairment and showed the upregulation of multiple inhibitory receptors on the cell surface (such as PD-1, LAG-3, TIGIT, TIM-3, and CTLA-4). To prevent and/or reverse of CAR T exhaustion state, severe research aimed at improving the CAR T cell therapeutic response. Methods of modulating intrinsic T cell pathways include the blockade of exhaustion-promoting signaling, inhibition of downstream effectors, the negation of TME immunosuppression, the transformation of inhibitory signals to stimulatory signals, and modification of the CAR. Several of these strategies have demonstrated significant promise, and their safety and efficacy are currently being determined in clinical trials. One of the most studied pathways is the PD-1/PD-L1 axis and strategies include the use of antibody PD-1/PD-L1 blockade[107], CAR T cells modified to secrete PD-1 scFv, and cell intrinsic blockade of PD-1 signaling through gene editing. Xiaoqian L *et al.* showed the efficacy of CD19 CAR T cells modified to express dominant-negative PD-1 receptors in the treatment of refractory B cell lymphoma[108]. Other targeted pathways involved in CAR T cell exhaustion are TGF-β, TOX and NR4A. Several studies have used genetic engineering tools to overexpress or knock out individual molecules to prevent transcriptional and epigenetic changes associated with exhaustion. One example is represented by the overexpression of the AP-1 family member c-Jun[109] and the deletion of molecules such as the methyltransferase DNMT3A[110]. Emerging data suggested how CAR T cell exhaustion varies by CAR construct. Independent studies have demonstrated that CD28-costimulated CAR T cells are more susceptible to a state of exhaustion as compared to 4-1BB-costimulated CAR T cells. Moreover, CAR constructs associated to tonic signaling have also been positively associated with the development of CAR T cell exhaustion. Other groups have focused their attention on the detection of specific signatures in exhausted CAR T cells. Stewart CM and colleagues have identified IL-4 as a key regulator of CAR T cell exhaustion through three independent approaches and proposed the use of anti-IL-4 mAbs to prevent it[111]. An alternative strategy to prevent CAR T cell exhaustion can be the modulation of CAR-antigen interaction and in particular controlling CAR expression in T cells. Weber *et al.*, pre-clinically demonstrated the reversal of CAR T cells' exhaustive status inducing a "transient rest" through CAR degrading after the exposure of a particular small molecule[112]. A deeper knowledge of CAR T exhaustion mechanism will drive new strategies for a more precise and effective method of inhibiting T cell differentiation and hyporesponsiveness by targeting the intrinsic mechanisms of T cell exhaustion. A comprehensive analysis of the preclinical evaluation of strategies to overcome and prevent CAR T exhaustion is beyond the scope of this review and has been discussed in detail elsewhere.

FUTURE PERSPECTIVE

The initial impressive results with CAR T cells in treating hematological malignancies have generated significant enthusiasm in cell therapy research. However, the emergence of issues, such as relapses, and challenges in replicating this success in other malignancies have highlighted the need for technology improvements. Significant efforts are being made to identify biomarkers and response determinants to enhance patient stratification and enable earlier interventions to prevent relapse. Additionally, several research groups are working to enhance T-cell activation, such as by developing novel synthetic receptors, and extend their persistence to ensure sustained immune surveillance. Looking ahead, we aim to broaden CAR T cell therapy applications to encompass other neoplastic diseases, particularly solid tumors, as well as non-neoplastic conditions like autoimmune diseases. This expansion will also necessitate a rethinking of manufacturing processes, as centralized production may struggle to keep pace with rising demand and ensure vein-to-vein times that meet clinical needs. To address these challenges, shortening manufacturing timelines, or using off-the-shelf products could provide viable solutions in the coming decade. A promising strategy could be the implementation of a decentralized manufacturing model, enabled by the enhancement of the manufacturing

automation protocol. From this perspective, the story of ARI-001 has set the path. ARI-001 is the first approved academic anti-CD19 CAR, developed by the University of Barcelona. The manufacturing of ARI-001 relies on Miltenyi CliniMACS prodigy system and Lentiviral vector. The ARI platform has several point-of-care production sites in Spain allowing the manufacturing of the product near the site of infusion. This model enables the use of an academic product nationwide, reducing costs and expanding access to therapy. In the near future, international protocol of treatment for pediatric BCP-ALL could include treatment with anti-CD19 CAR T cells testing a de-centralized manufacturing model. Bringing the manufacturing near the infusion center could allow the use of fresh products and potentially improve response rates and persistence. However, several challenges must be addressed to fully implement this approach: a) Ensuring consistent product quality across all manufacturing centers; b) Navigating regulatory issues that vary between jurisdictions worldwide; c) Completing rigorous quality testing within reduced timelines; d) Overcoming logistical hurdles to facilitate the timely infusion of fresh products. Moreover, this model of production could reduce costs and ultimately expand access to therapy in low-middle-income countries as suggested by the work of Palani H.K. *et al*[113]. In addition, the development and optimization of non-viral methods and in particular genome editing will contribute to the reduction of costs and expand access to minority. After the initial impressive results and enthusiasm surrounding CAR T cell therapy for hematological malignancies, we have reached a crucial phase of research. With time, it will become clearer which direction this treatment will take.

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AUTHORS' CONTRIBUTIONS

AM conceptualized, wrote, and edited the manuscript. CB, SN, VB, and GG wrote the manuscript. AM and CB designed the figures and prepared the tables. AB and AB edited the manuscript. All Authors contributed to the article and approved the submitted version.

DISCLOSURE OF CONFLICTS OF INTEREST

BA: Speaker's Bureau Novartis, Medac (Wedel, Germany), Amgen (Thousand Oaks, CA, USA), Neovii (Rapperswil-Jona, Switzerland), Advisory Board Novartis.

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