



Universal CAR cell therapy: Challenges and expanding applications

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ABSTRACT

Chimeric Antigen Receptor (CAR) T cell therapy has gained success in adoptive cell therapy for hematological malignancies. Although most CAR cell therapies in clinical trials or markets remain autologous, their acceptance has been limited due to issues like lengthy manufacturing, poor cell quality, and demanding cost. Consequently, “Off-the-shelf”, universal CAR (UCAR) cell therapy has emerged. Current concerns with UCAR therapies revolve around side effects such as graft versus host disease (GVHD) and host versus graft response (HVGR). Preclinical research on UCAR cell therapies aims to enhance efficacy and minimize these side effects. Common approaches involve gene editing techniques to knock out T cell receptor (TCR), human leukocyte antigen (HLA), and CD52 expression to mitigate GVHD and HVGR risks. However, these methods carry drawbacks including potential genotoxicity of the edited cells. Most recently, novel editing techniques, such as epigenetic editing and RNA writer systems, have been developed to reduce the risk of GVHD and HVGR, allowing for multiplex editing at different sites. Additionally, incorporating more cell types into UCAR cell therapies, like T-cell subtypes (DNT, $\gamma\delta$ T, virus-specific T cells) and NK cells, can efficiently target tumors without triggering side effects. In addition, the limited efficacy of T cells and NK cells against solid tumors is being addressed through CAR-Macrophages. In summary, CAR cell therapy has evolved to accommodate multiple cell types while expanding applications to various diseases, including hematologic malignancies and solid tumors, which holds tremendous growth potential and is promised to improve the lives of more patients in the future.

Introduction

Immunotherapy has emerged as a transformative innovation in cancer treatment, positioning itself as the fourth pillar alongside surgery, radiotherapy, and chemotherapy [1]. In comparison to conventional anti-cancer modalities, immunotherapy demonstrates superior precision in targeting antigens and extends disease control over a longer timeframe. Particularly, chimeric antigen receptor (CAR) cell therapy is a frontrunner in this field. Following advancements in second-generation CAR-T cell therapies, it has shown notable anti-tumor effects, especially against hematological malignancies [2]. CAR-T cell therapies against CD19⁺ B cell malignancies and BCMA⁺ multiple myeloma have been successfully introduced to the market, displaying significant clinical potential [3]. Existing commercially available CAR-T therapies rely on autologous treatments, utilizing cells derived from patients themselves. Currently, most data on long-term outcomes of autologous CAR T-cell therapy originate from patients with B-cell

lymphoma or chronic lymphocytic leukemia (CLL), with overall response rates (ORRs) achieving 44–91 %, complete response (CR) rates of 28–68 % [3]. Despite their high efficacy, autologous CAR cell therapy faces limited application due to its high cost, intricate manufacturing process, severe side effects, and restricted cell availability [2]. Consequently, the concept of “Off-the-shelf”, third-party, healthy donor-derived universal CAR therapy has gained incremental attention [4].

Conventional autologous CAR-T therapy involves several steps, such as patient plasmapheresis, cell sorting, CAR virus transfection, CAR-T cell expansion, and transfusion back to the patient post-quality check [5]. The universal CAR-T cell therapy shares a similar process (Fig. 1). This process is intricate and time-consuming, demanding stringent laboratory conditions and operations. The prolonged manufacturing process poses challenges for autologous CAR therapy, where a lot of patients may not survive until the product is ready. In contrast, “off-the-shelf” CAR T-cell products offer the opportunity for the treatment of patients

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with rapidly progressing diseases. Moreover, patients undergoing CAR-T cell therapy after chemotherapy often exhibit reduced T-cell quantity and quality, rendering autologous CAR-cell therapy less effective [3]. On the contrary, healthy donor cells provide a more homogeneous starting material, ensuring a more predictable product quality. In short, allogeneic CARs offer numerous advantages over autologous CAR therapies (Table 1).

Although numerous UCAR therapies targeting hematologic malignancies have entered clinical trials, no market approvals for UCAR therapies have been obtained, potentially due to safety and efficacy concerns. Consequently, recent studies have been devoted to ameliorating these issues. This review first provides an overview of the current stage of UCAR cell therapy and then encapsulates the latest advancements while exploring the future trajectory for CAR-based therapies.

Current challenges and conventional solutions for UCAR cell therapy

In recent years, UCAR therapy has demonstrated promising success in hematological malignancies, including aggressive B cell lymphomas, follicular lymphomas, mantle cell lymphomas, and chronic lymphocytic leukemia [3]. However, complications such as graft-versus-host disease (GVHD), host versus graft response (HVGR), and treatment regimen-related immunotoxicity remain the major obstacles [6–8]. A variety of solutions have been developed and become widely adopted as conventional strategies to enhance the safety and efficacy of UCAR cell therapy.

GVHD

GVHD arises from grafts attacking immunocompromised recipient tissues due to histocompatibility differences [2]. Its pathophysiology primarily involves donor T cells recognizing host tissue as foreign, leading to severe organ damage. To address this challenge, a multitude of strategies are rapidly under investigation, encompassing the application of gene editing techniques and the selection of more suitable cell

Table 1

The comparison between allogeneic CAR-T cell therapy and autologous CAR-T cell therapy.

	Allogeneic CAR-T therapy	Autologous CAR-T therapy
Manufacturing process		
Cell Source	Healthy donor	Patient (recipient)
Expansion capacity	High	Limited
Off-the-Shelf	Standardized product	Customized for each patient, limited off-the-shelf availability
Availability		Limited
Possibility of T-cell phenotype selection	High	Low
Quality of original T cells	High	Low
Time of accessibility	Readily available	Time-consuming, possible for manufacturing failure
Treatment		
Requirement for lymphodepletion	Enhanced lymphodepletion regimen, or standard lymphodepletion regimen (with additional HLA depletion)	Standard lymphodepletion regimen
Flexibility in dosing	Flexible for redosing or changes of target	Limited flexibility as the dose is dependent on the patient's ability to yield sufficient T cells
In vivo persistence	Short (days to months)	Long (months to years)
Side effects		
GVHD	High risk	Low risk
Immunogenicity (HVGR)	Possible	Lower risk
CRS and ICANS	Yes	Yes
Cost	Lower	Expensive, upwards to US \$350,000

sources to mitigate GVHD risk.

$\alpha\beta$ -type T cell receptor ($\alpha\beta$ TCR) is instrumental in mediating the occurrence of GVHD. The TCR protein complex on the surface of $\alpha\beta$ -type T cells consists of α and β chains, with only one gene encoding the constant regions of the α chain [9]. Therefore, disrupting the gene encoding the T cell receptor α -chain constant region (TRAC) emerges as

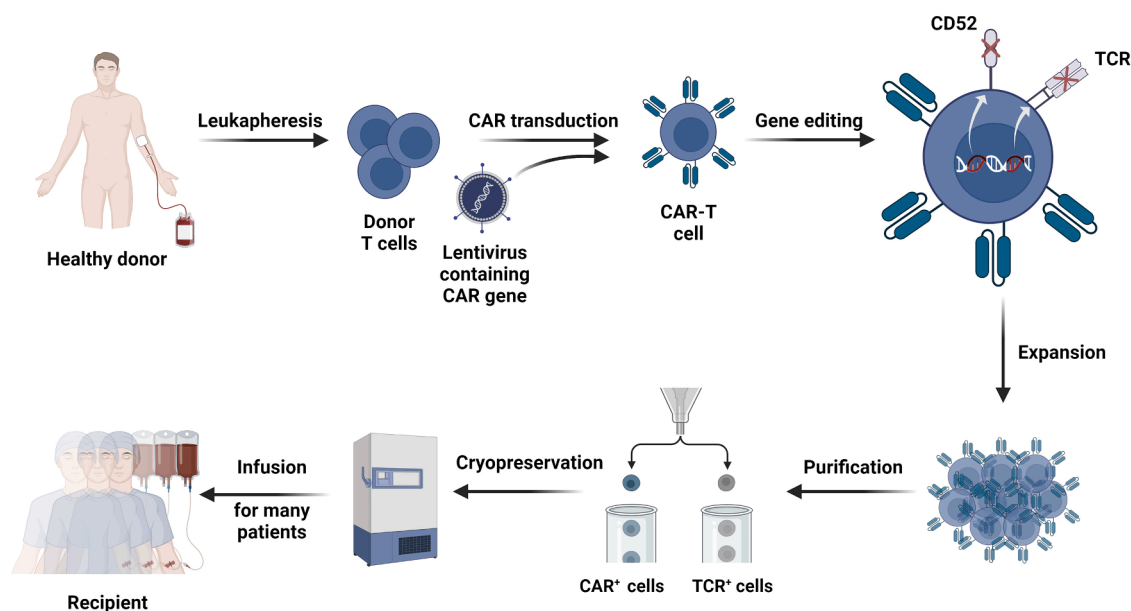


Fig. 1. The conventional production route of the UCAR-T therapy. The first step in the manufacturing of universal CAR-T cells is the collection of donor T cells from peripheral blood mononuclear cells (PBMC), which are extracted from the donor through leukapheresis. The T cells are then isolated via magnetic bead technology. The CAR gene is then transduced into the collected T cells by lentivirus. To limit the occurrence of GVHD and HVGR, gene editing techniques are employed to knock down the expression of alloreactive genes (e.g., TCR and CD52). After acquiring stable CAR-T cells, large-scale in vitro expansion is required to obtain the desired dose. The expanded CAR-T cells are further purified to ensure safety. Eventually, the CAR-T cells are cryopreserved and readily available for infusion into multiple patients.

a direct and effective approach to block $\alpha\beta$ -type TCR expression. Researchers have developed several methods to prevent TCR expression on the surface of CAR-T cells, with gene editing techniques being one of the most rapidly advancing methods. Frequently employed gene editing tools include Zinc Finger Nucleases (ZFN), Transcription Activator-Like Effector Nucleases (TALEN), and Clustered Regularly Interspaced Short Palindromic Repeat Correlation 9 (CRISPR-Cas9) [10]. Additionally, the use of short hairpin RNA (shRNA) to degrade mRNA proves to be effective in reducing the adverse effects of GVHD [11] (Fig. 2). Another approach leverages the homologous recombination mechanism of cells to introduce CAR-expressing transgenes directly into the TRAC gene locus, allowing CAR transgene expression while disrupting natural TCR expression [9,12]. Another intriguing strategy to disable the TCR involves adopting an anti-TCR ScFv in combination with the KDEL endoplasmic reticulum (ER) retention domain, resulting in intracellular retention of the TCR [13,14].

Furthermore, the utilization of other cells that do not perform anti-tumor function through $\alpha\beta$ TCR is under hot research to reduce the risk of GVHD (Figs. 2 and 3). These include natural killer cells (NK cells), $\gamma\delta$ T cells, virus-specific T cells, and NKT cells, which will be discussed in detail later.

HVGR

Following UCAR-T treatment, the host's immune system may recognize and eliminate allogeneic CAR-T cells, thereby limiting their therapeutic effect [15]. Therefore, overcoming the challenge of host rejection is a significant hurdle in allogeneic CAR-T therapies. Typically, before the allogeneic CAR therapy, patients will receive an enhanced lymphodepletion regimen, as host T cells may swiftly attack "foreign" CAR-T cells. Commonly used lymphodepletion agents include

conventional chemotherapy drugs and monoclonal antibodies such as alemtuzumab [13]. In order to protect allogeneic CAR cells from lymphodepletion, gene editing techniques are often employed to knock out CD52 on these cells, preventing recognition by the alemtuzumab [15] (Fig. 2). Combining alemtuzumab with chemotherapy allows for more effective clearance of the host T cells, albeit with an increased risk of infection [16–18]. Therefore, specific targeting of the alloimmune components may be preferable. For example, bispecific-CAR-T cells (biCAR-T) have been designed to prevent HVGR, with one CAR targeting tumor antigens and the other aiming at the host immune cells [13] (Fig. 2). One clinical study has adopted CD19/CD7 dual-targeted CAR for tumor killing and elimination of the alloreactive T and NK cells [19]. Moreover, a chimeric receptor, alloimmune defense receptor (ADR), has been developed to target the co-stimulatory receptor 4-1BB (CD137), which is transiently upregulated in the activated lymphocytes but not resting and non-alloreactive immune cells [17,20]. In this way, the CAR-T cells loaded with ADR can be resistant to host rejection while reducing the risk of infection.

Furthermore, gene editing techniques could also be used to eliminate HLA proteins on the surface of allogeneic CAR-T cells. Deletion of the β 2-microglobulin gene, which is required for normal expression of HLA proteins, is a strategy to reduce the immunogenicity of CAR-T cells [9, 21]. However, decreased HLA expression may be recognized by NK cells, leading to NK-mediated CAR-T cell elimination. To address this, countless NK cell inhibiting strategies have been introduced. For instance, targeted insertion of universal MHC I constructs (e.g., monomorphic HLA-E loaded with decoy peptides) into the B2 M gene prevents NK cells from eliminating the allogeneic cells [22] (Fig. 2). Introducing other inhibitory ligands for NK cells, such as siglec 7/9 ligands and E-cadherin, can also reduce the risk of HVGR [13]. Moreover, recent research has shown that overexpressing CD47 in allogeneic CAR T cells

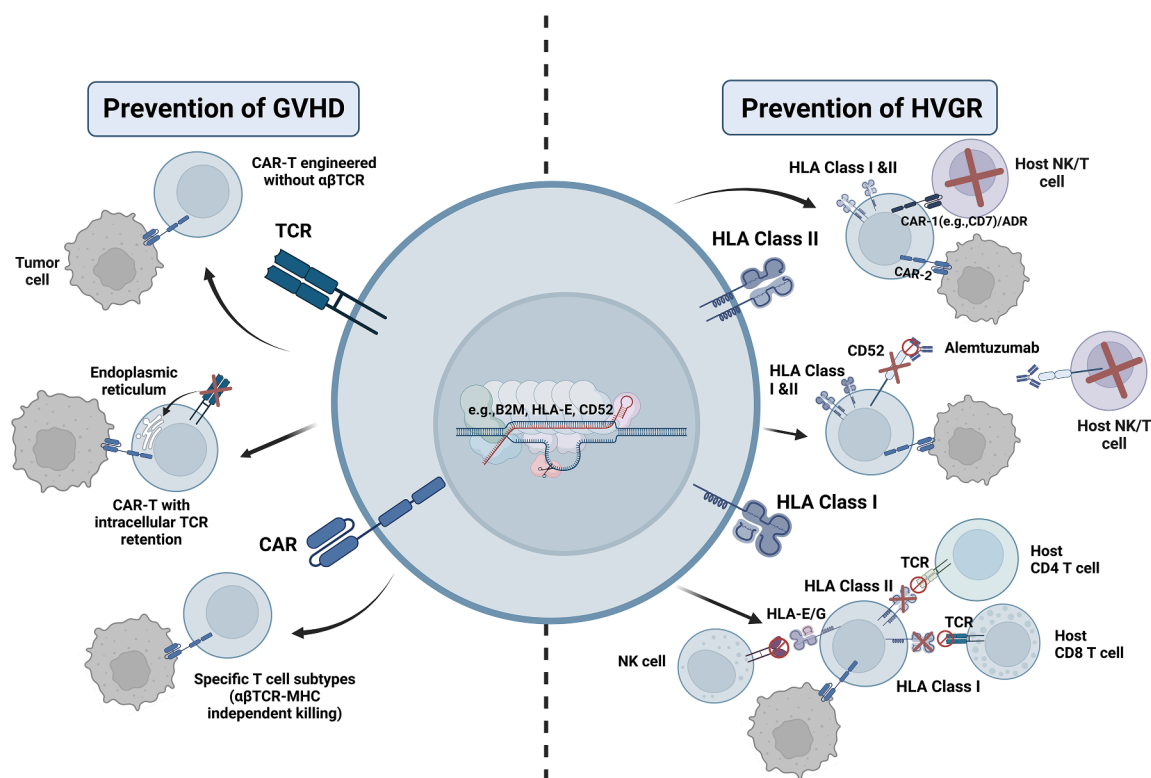


Fig. 2. Currently developed strategies to prevent GVHD and HVGR. In terms of preventing GVHD, most strategies aim to target the $\alpha\beta$ TCR by knocking out the relevant gene or inactivating the $\alpha\beta$ TCR protein. In addition, certain specific T-cell subsets can exert a killing effect independent of $\alpha\beta$ TCR and HLA, which could also be used to prevent GVHD. For the prevention of HVG, available approaches include bispecific CAR, ADR, or monoclonal antibodies to eliminate allo-rejecting host NK and T cells. Knocking out the HLA gene while adopting NK cell inhibiting receptors can also suppress the alloreactive response. CRISPR-Cas9 and shRNA techniques are the most common instruments applied to manipulate the expression of these genes.

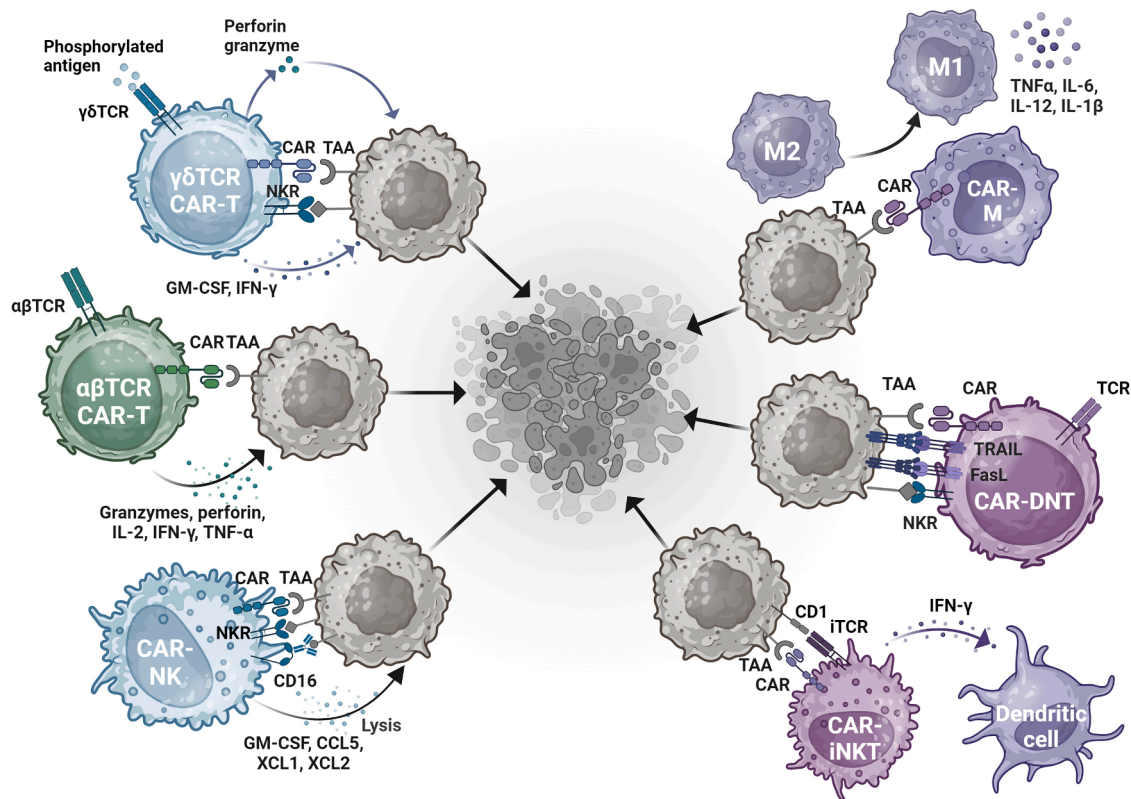


Fig. 3. A summary of tumor cell killing mechanisms by CAR cells originating from different cell types. In conventional CAR T therapy, tumor cell apoptosis is primarily triggered by CAR recognition of tumor-associated antigens (TAA) and the release of granzymes, perforins, and other cytokines. Since this therapy is prone to side effects like GVHD, other cell types such as NK cells and certain T cell subtypes are being investigated. These cell types exert tumor-killing effects independent of the $\alpha\beta$ TCR, mainly through receptors like Fas, NKR, and CD16. Moreover, macrophages compensate for the ineffectiveness of other cells in targeting solid tumors. In addition to CAR-based recognition, CAR-Macrophages can reeducate the M2 subtype of macrophage into the M1 subtype, releasing proinflammatory cytokines to trigger further immune response against the tumor.

can further inhibit host innate immune attacks (e.g., NK cells) while suppressing adaptive immunity responses (i.e., T cells) [23].

Immunotoxicity

As the effector capacity of CAR-T cells increases, the side effect rate also rises. CAR-T therapy can trigger the overactivation of T cells, releasing high levels of cytokines such as IL-6, IL-10, and interferon- γ (IFN- γ) [10]. These pro-inflammatory cytokines may elevate blood vessel permeability, causing life-threatening diseases like vascular leakage syndrome and disseminated intravascular coagulation [24]. Approximately 77–93 % of patients treated with FDA-approved autologous CAR cell therapies result in cytokine release syndrome (CRS), with grade 3 or greater occurring in 7–46 % of patients [25]. Some patients may further develop immune effector cell-associated neurotoxicity syndrome (ICANS) [1]. To address the immunotoxicity issue, several solutions employing the concept of switchable CAR have been developed to control T cell activity.

The ON-switch CAR aims to reduce immunotoxicity by regulating T-cell activation. The signaling domain of the ON-Switch CAR is usually separate from the co-stimulation domain. Only upon the addition of heterodimerized small molecules can the two CAR domains assemble. As a consequence, the activation state of the T cells can be controlled by the dosage of the heterodimerizing small molecules [26]. On the other hand, the OFF-switch CAR primarily mitigates immunotoxicity by deactivating the CAR cells. Several strategies to degrade CARs, including suicide genes, are now under investigation. Suicide genes such as herpes-simplex-thymidine-kinase (HSV-TK) and inducible-caspase-9 (iCasp9) are intended to selectively destroy transferred cells [26].

These genes activate upon the administration of corresponding molecules, resulting in irreversible T-cell termination. Likewise, a suicide receptor is introduced, which functions by inducing the expression of a common antigen CD20 on the CAR-T cells [26]. Infusion of anti-CD20 antibodies further triggers the elimination of CAR-T cells.

Other challenges

Another challenge faced in UCAR cell therapy is the low persistence [7]. The determinants of CAR-T persistence in vivo are unknown, possibly related to intrinsic T-cell mass, the ratio of CD4⁺ to CD8⁺ T-cells, or specific co-stimulatory domains in individual CAR constructs [27]. Currently, researchers have focused on increasing the persistence by adding proinflammatory cytokines or altering co-stimulatory structural domains of the CAR structure. For example, IL-7 and IL-15 secreting domains are effective in inducing the memory phenotype of T cells, which increases the durability of UCAR cells [28,29].

Moreover, while there is now plenty of research into the gene-edited UCAR cells to reduce side effects, this might lead to another potential safety issue, the genotoxicity of gene-edited cells, which will be discussed later.

Recent advances in universal CAR-T therapy

In the last few years, with the development of gene editing techniques and the understanding of basic cell biology, several new solutions to the above challenges are constantly being investigated. Some of these novel discoveries have already progressed to the clinical stage, as outlined in Table 2.

Table 2

List of clinical trials of UCAR-T therapy with available results. Updated till 07/04/2024.

Title	Safety assessment population (N)	CRSN (%)	ICANSN (%)	InfectionsN (%)	GVHDN (%)	Efficacy assessment population (N)	ORRN (%)	CRN (%)	Sponsor	Strategies	Target	Indication	Phase of study	Location
UCAR-T														
ALLO-501[30]	46	Gr I-II: 10 (21.7); ≥Gr III: 1 (2.2)	≥Gr II: absent	≥Gr III: 11 (23.9)	absent	32	24(75)	16 (50)	Allogene Therapeutics	Disruption of TRAC and CD52 genes by TALENs	CD19	relapsed/refractory (r/r) B-Lymphoma	Phase 1	US
TRUUCAR™ GC502[31]	4	Gr I-II: 1 (33.3); ≥Gr III: 2(66.7)	≥Gr II: absent	absent	absent	4	4(100)	3(75)	Gracell Biotechnologies	TRAC and CD7 loci were disrupted	CD19/CD7	relapsed/refractory B-cell acute lymphoblastic leukemia (r/r B-ALL)	Phase 1	China
ALLO-715[32]	52	Total: 27 (52); ≥Gr III: 1 (1.9)	Gr I-II: 5 (1)]	Total: 29 (56); ≥Gr III: (29)	absent	36	27(75)	18 (50)	Allogene Therapeutics	Lymphodepletion with an anti-CD52 antibody (ALLO-647)-containing regimen	BCMA	r/r multiple myeloma	Phase 1	US
UCART22[33]	3	Gr I-II: 2 (67)	absent	-	-	3	2 (66.7)	1 (33.3)	Collectis	Disruption of TRAC and CD52 genes by TALENs	CD22	B-ALL	Phase 1 process 2	US
CYAD-211[34]	9	Gr I: 1 (11)	absent	Gr I-II: 2(22)	absent	9	2 (12.6)	-	Celyad Oncology	Use of shRNA to silence mRNA coding for the CD3ζ component of the TCR	BCMA	r/r Multiple Myeloma	Phase 1	US
CTX110[35]	32	Total: 18 (56.3); ≥Gr III: 2 absent	Total: 3 (9.4); ≥Gr III: 2 (6.2)	≥Gr III: 4 (12.5)	absent	32	18 (56.3)	11 (34.4)	CRISPR Therapeutics	Disruption of TRAC gene by CRISPR/Cas9	CD19	B cell malignancies	Phase 1	US
FT819[36]	12	≤Gr II: 3 (25)	absent	-	absent	-	-	-	Fate Therapeutics	iPSc derived T cells, disruption of TRAC gene by CRISPR/Cas9	CD19	B cell malignancies	Phase 1	US
ThisCART19A [37]	8	Gr2: 6 (75) ≥Gr III: 2(25)	Total: 3 (37.5)	Total: 1 (12.5)	absent	7	-	7 (100)	Fundamenta Therapeutics	Intracellular retention of TCR/HLA-I	CD19	r/r B-ALL and relapsed non-Hodgkin Lymphoma (NHL)	Phase 1	China
UCART19[38]	21	19(91); ≥Gr III: 3(14)	Gr I-II: 8 (38); ≥Gr III: absent	13(62)	Gr I: 2 (10); ≥Gr II: absent	21	-	14 (67)	Servier	Disruption of TRAC and CD52 genes by TALENs technology	CD19	r/r B-ALL	Phase 1	US
P-BCMA-ALLO1[39]	24	Gr I: 3 (14)	Gr I: 1(4)	-	absent	-	-	-	Poseida Therapeutics	Disruption of TRBC and B2 M genes by the Cas-CLOVER™ Gene Editing tool	BCMA	Multiple Myeloma	Phase 1	US
PBCAR0191 [40]	15	Total: 9 (60); ≥Gr III: absent	Total: 4 (26.6); ≥Gr III: 1 (6.6)	Total: 6(40)	absent	15	-	9(60)	Precision BioSciences	Disruption of TRAC gene by the versatile genome-editing platform ARCUS	CD19	B-ALL	Phase 1 - 2	US
TT52CAR19 [41]	6	Gr II: 2 (33.3)	Gr IV: 1 (16.6)	-	Gr I: 1 (16.6)	6	-	-	University College, London	Disruption of TRAC and CD52 genes by CRISPR/Cas9	CD19	B-ALL	Phase 1	UK

(continued on next page)

Table 2 (continued)

Title	Safety assessment population (N)	CRSN (%)	ICANSN (%)	InfectionsN (%)	GVHDN (%)	Efficacy assessment population (N)	ORRN (%)	CRN (%)	Sponsor	Strategies	Target	Indication	Phase of study	Location
WU-CART-007 [42]	18	Gr I-II: 13(72) Gr III: 1 (5.56)	Gr I: 1 (5.56)	Total: 2 (11.1)	absent	12	-	7 (58.3)	Washington University School of Medicine	Disruption of CD7 & TRAC genes by CRISPR/Cas9	CD7	r/r T-Cell Acute Lymphoblastic Leukemia/ Lymphoblastic Lymphoma (T-ALL/LBL)	Phase 1	US
P. MUC1C-ALLO1[43]	7	absent	absent	-	absent	-	-	-	Poseida Therapeutics	Disruption of TRBC and B2 M genes by the Cas-CLOVER™ Gene Editing tool; iCasp9-based safety switch gene	Mucin1 cell surface associated C-Terminal (MUC1-C) antigen	Advanced or Metastatic Solid Tumors	Phase 1	US
CTA101[44]	6	Total: 6 (100), Gr I:3(50), GrII:2 (33.3), GrIII:1 (16.6)	absent	Total: 6 (100), GrI:1 (16.6), GrII:2 (33.3), GrIII:3(50)	absent	6	6(100)	2 (33.3)	Zhejiang University	TRAC and CD52 knockout by Cas9	CD19/CD22	r/r B-ALL	Phase 1	China
RD13-01[45]	12	Gr I-II: 10 (83.3), ≥Gr III: absent	absent	12(100)	absent	11	9 (88.8)	7 (63.6)	Zhejiang University	CD7, TCR/CD3 knockout by CRISPR/Cas9	CD7	r/r T-ALL/LBL	Phase 1	China
CARCIK-CD19 [46]	13	GrI-II: 3 (23), ≥Gr III: absent	absent	4(30)	absent	13	-	8 (61.5)	Fondazione Matilde Tettamanti Menotti De Marchi Onlus	Sleeping beauty transposon induction of CIK cells	CD19	r/r B-ALL	Phase 1	Italy
SC291[47]	1	absent	absent	-	-	-	-	-	MD Anderson Cancer Center	CD3 and HLA class I/ II gene knoout&CD47 overexpression	CD19	NHL and Chronic Lymphocytic Leukemia (CLL)	Phase 1	US
AVC-101 (UniCAR-T-CD123)[48]	19	Gr I-II: 12 (63.2); Gr III: 3 (15.8)	Gr II: 1 (5.3)	-	-	15	8(53)	-	-	Adapter CAR-T consists of a universal CAR-T cell (UniCAR-T) and a CD123 targeting module™	CD123	r/r AML	Phase 1	Germany
CD33CART [49]	19	Total: 13 (68); ≥Gr III: 4(21)	Total: 1 (5)	-	-	19	-	2(11)	Center for International Blood and Marrow Transplant Research	-	CD33	r/r AML	Phase 1	US
Nathali-01[50]	3	3(100)	absent	-	absent	3	3(100)	2 (66.7)	Collectis	Inactivation of TRAC and CD52 by TALENs	CD20, CD22	r/r NHL	Phase 1	US
BE-CAR7[51]	3	3(100)	2 (66.7)	1 (33.3)	1 (33.3)	-	-	-	Great Ormond Street Hospital for Children NHS Foundation Trust	CRISPR/nCas9 base editing to inactivate three genes encoding CD52 and CD7 receptors and the β chain of the αβ T-cell receptor	CD7	relapsed T-ALL	Phase 1	UK

Table 3

List of clinical trial results for UCAR therapies based on other cell types. Updated till 07/04/2024.

Title	Safety assessment population (N)	CRSN (%)	ICANSN (%)	InfectionsN (%)	GVHDN (%)	Efficacy assessment population (N)	ORRN (%)	CRN (%)	Sponsor	Strategies	Target	Indication	phase of study	Location
Other T cell subtypes														
ADI-001[63]	9	Gr I- II: 2 (22)	Gr I: 1 (11)	≥Gr III: 1(11)	absent	9	7(78)	7(78)	Adicet Bio	γδ T	CD20	r/r B-cell NHL	Phase 1	US
KUR-502[64]	NHL (n = 5) and ALL (n = 2)	Gr I: 2/2 (100) in ALL group	absent	–	absent	NHL (n = 5) and ALL (n = 2)	4(57)	3(43)	Athenex	iNKT cell, IL15, and short hairpin RNA expression (downregulates HLA I, II)	CD19	r/r B-Cell Malignancies	Phase 1	US
CAR EBV-CTL [65]	16	absent	absent	–	3	–	–	–	Memorial Sloan Kettering Cancer Center	EBV virus specific T cell	CD19	r/r B-Cell Malignancies	Phase 1	US
CD30. CAR-EBVST cells [66]	14	Gr I: 4 (28.5)	–	–	absent	14	9 (69.2)	5 (38.5)	Baylor College of Medicine	EBV virus specific T cell	CD30	CD30-Positive Lymphoma	Phase I/II trial	US
CD19CAR/virus specific T cells [67]	6	absent	absent	–	absent	6	2 (33.3)	2 (33.3)	Baylor College of Medicine	Virus specific T cells	CD19	CD19 ⁺ B cell malignancies	Phase 1	US
RJMty19[68]	12	Gr I /II: 4 (33.3)	absent	1 (8.3)	absent	5	3(25)	1 (8.3)	Zhejiang University	DNT cells	CD19	r/r B-cell NHL	Phase 1	China
UCAR-NK														
iC9/CAR.19/IL15-Transduced CB-NK Cells [69]	11	absent	absent	GrI: 2 (18.1) GrIII:1 (9.1)	absent	11	8(73)	7(64)	M.D. Anderson Cancer Center	NK cells; iCasp9-based safety switch gene; IL-15	CD19	r/r B-cell lymphoma or leukemia	Phase 1	US
NKX101[70]	6	absent	absent	3(50)	absent	6	–	3(50)	Nkarta	NK cells	NKG2D	Acute Myeloid Leukemia (AML)	Phase 1	US
AFM13-NK [71]	42	absent	absent	–	absent	42	39 (92.8)	28 (66.7)	M.D. Anderson Cancer Center	NK cells; combined with AFM13 (a CD30/CD16A bispecific antibody)	CD30	Refractory CD30-Positive Lymphomas	Phase 1	US
Cnty-101[28]	1	absent	absent	–	–	1	–	1 (100)	Century Therapeutics	iPSC-derived NK cells, IL-15, EGFR safety switch, and Alloevasion™ edits (B2 M and CIITA knockouts, and HLA-E knock-in)	CD19	r/r NHL	Phase 1	US
CAR19/IL-15 [72]	37	Gr I: 1 (2.7)	absent	Gr I-II: 7 (18.9); Gr III:8 (21.6)	absent	37	18 (48.6)	14 (37.8)	M.D. Anderson Cancer Center	Cord blood-derived NK cells, IL-15, iCasp9-based safety switch gene	CD19	B Lymphoid Malignancies	Phase 1/2	US

Advances in gene editing tools

Gene editing techniques offer a powerful tool for modifying allogeneic CAR T cells, addressing concerns related to GvHD and HVGR while enhancing the therapeutic potential. In recent years, CRISPR/Cas9 has been one of the most widely adopted gene editing tools [52]. For example, disrupting the immune checkpoint PD1 via CRISPR techniques has shown promise in increasing the potency of CAR-T therapy, surpassing traditional allogeneic CAR-T approaches [53]. CRISPR techniques have also been employed in genome-wide screening, identifying genes such as IL-23 and KLF4 that can facilitate the differentiation of T cell subtypes with superior proliferative potential and memory-like phenotypes [54]. They can also be adopted to screen novel antigen targets for unknown diseases via single-guide RNA (sgRNA) libraries, reducing the likelihood of immune escape as well as improving therapeutic efficacy [55].

However, gene editing is not devoid of challenges. Conventional gene editing techniques (including CRISPR/Cas9 and TALENs) perform editing by inducing double-strand breaks (DSB) in DNA, with risks of occurring at off-target sites [56]. Besides, universal CAR cell therapy often requires simultaneous multiplexed editing, which may trigger chromosomal translocations and other aberrations [57]. Fortunately, new solutions to these challenges are now emerging. Several Cas9 enzyme variants have been developed to overcome the disadvantages of DSB. For example, the Nickase Cas9 (nCas9) contains mutations in the RuvC or HNH domains that allow Cas9 to cleave only targeted or non-targeted single-strand DNA. The combination usage of two pairs of nCas9/gRNA complexes can reduce the generation of off-target DSBs [58]. Moreover, nCas9 can be conjugated to cytosine and adenosine deaminases for single-base pair editing. Recently, nCas9-mediated base editing inducing premature stop codons for gene knockout has progressed into the clinical stage, exhibiting molecular remission in patients [56]. On the other hand, enzymatically dead Cas9 (dCas9) can be ligated to other functional epigenetic enzymes or transcriptional regulators to induce gene expression alterations independent of direct DNA editing [58].

Furthermore, the delivery system for conventional CAR-T therapies is often lentiviral, posing risks on random genomic integration, which increases the chance for insertional mutagenesis and functional gene disruption [2]. As a result, recent advancements in gene-edited CAR-T cells have been focused on employing virus-free transfer systems. For example, one group reported adopting the CRISPR-Cas9 system and virus-free gene-transfer strategies with Sleeping Beauty transposons to deplete HLA-1 and TCR in CAR-T cells [59]. These edited cells demonstrate comparable *in vivo* and *in vitro* anti-tumor abilities compared to conventional CAR-T cells, with highly specific editing of HLA-I and TCR and no significant off-target effects [59].

In addition to CRISPR-like techniques that directly alter DNA material, several other innovative and safer options have been proposed—for example, epigenetic editing. Unlike conventional gene editing techniques that rely on DNA breakage, epigenetic editing can durably regulate the expression of multiple genes at once without introducing genomic changes [60]. Utilizing a dCas9-based epigenetic editor, one group successfully silenced the expression of TCR and major histocompatibility complex (MHC) class I and II without causing translocations or truncations, as observed with Cas9-based multiple DNA editing [60]. Epigenetic drugs have also been proven potent in enhancing the anti-tumor function of CAR-T cells, maintaining the memory phenotype, and reducing T cell exhaustion [61]. Another approach involves an RNA-based gene writer system that catalyzes various editing reactions employing target-stimulated reverse transcription (TPRT) biochemistry [62]. This technique allows simultaneous multiplex editing without inducing translocations, which is crucial since UCAR cells often require the deletion of multiple genes to avoid side effects.

Alternative T cell subtypes

To date, the majority of the CAR-T cells are derived from T cells in peripheral blood mononuclear cells (PBMC), resulting in a highly heterogeneous pool of cells. Nevertheless, research has indicated that certain T cell subsets may exhibit greater effectiveness than a mixture of different T cell subtypes [2]. Another focus of innovation in CAR-T therapy has therefore been the selection of preferred T cell types (Table 3).

$\gamma\delta$ T cell

According to the peptide chain structure of the TCR, T cells can be classified into $\alpha\beta$ T cells, which consist of α and β chains, and $\gamma\delta$ T cells, characterized by γ and δ chains [2]. $\gamma\delta$ T cells typically represent 1–5 % of the total circulating T cells [4]. These $\gamma\delta$ T cells play a crucial role in the innate immune response, whose killing is independent of HLA and antigen-presenting cells (Fig. 3). This intrinsic feature provides $\gamma\delta$ T cells an inherent advantage in targeting solid tumors that lack specific tumor-associated antigens [2]. Since its killing mechanism is independent of TCR and HLA, concerns related to GVHD are alleviated [73]. One group has reported that over 98 % of CAR $\gamma\delta$ T cells express IFN- γ under basal conditions, indicating high efficacy killing [73]. Furthermore, a pioneering allogeneic CD20-targeted CAR $\gamma\delta$ T cell therapy has already entered clinical trials and demonstrated promising results in patients [74].

invariant Natural Killer T (iNKT) cell

Invariant Natural Killer T cells (iNKT) are rare, CD1d-restricted glycolipid-reactive T cells that link adaptive and innate immunity [75]. iNKT cells possess the ability to kill CD1d⁺ tumor cells through direct cytotoxicity (Fig. 3). Additionally, they can modulate the immune responses of NK cells and dendritic cells through cytokine secretion, thereby enhancing the anti-tumor response in collaboration with endogenous T cells [4]. Notably, allogeneic iNKT cells do not cause GVHD, making them an ideal candidate for “off-the-shelf” immunotherapy [75].

Previous studies have demonstrated that iNKT cells engineered with CAR exhibit comparable or superior cytotoxicity and a better safety profile than conventional CAR-T cells, particularly in the context of solid tumors [2]. Currently, there is an ongoing investigation into bispecific CAR-iNKT cell therapy. Experiments in mice have indicated that CD19/CD133 CAR-iNKT cell therapy, in an NKG2D-dependent manner, surpasses CAR-T cell immunotherapy and holds potential in preventing immune escape, leptomeningeal disease, and lineage switching without significant hematological toxicity [75].

Moreover, novel bispecific engagers have been developed to enhance the anti-cancer activity of iNKT cells. Researchers have designed a bispecific iNKT cell engager (biNTE) wherein one arm engages the invariant TCR (iTTCR), and the other engages the tumor antigen of interest [76]. Data indicates that biNTE is highly effective in killing and virtually eliminating patient-derived bone marrow myeloma plasma cells [76]. If this biomaterial can be used in conjunction with CAR to enhance the efficacy of iNKT cells, it could serve as an “off-the-shelf” platform for treating myeloma and other hematological or solid tumors.

Double-negative T (DNT) cell

Double-negative T cells (DNT) represent another rare subtype of mature T cells, expressing CD3 but lacking CD4 and CD8[77]. DNTs possess both anti-tumor and immunomodulatory properties. Research has demonstrated the strong inhibitory capacity of DNTs against CD8 T cells, CD4 T cells, B cells, and NK cells, leading to tolerance of allografts and potent prevention of GVHD [78]. Additionally, DNTs exhibit robust anti-tumor properties through HLA-independent killing, primarily mediated by FasL, NKG2D, DNAM-1, TRAIL, and NKp 30[77,78] (Fig. 3).

Currently, “Off-the-shelf” CAR-DNT cell therapy targeting C-type

lectin-like molecule-1 (CLL1) has been developed for the treatment of Relapsed/Refractory Acute Myeloid Leukemia and has demonstrated promising results [79]. A first-in-class, open-label, single-dose, phase 1 study of CD19 CAR-DNT cell therapy RJMty19 has also been initiated [80]. Remarkably, there were no cases of GvHD or ICANS observed after a single infusion of RJMty19, highlighting the safety profile of CAR-DNT cell therapy.

Virus-specific T cells

In addition to gene editing approaches, another strategy for mitigating GvHD involves the usage of CAR-T cells based on virus-specific T cells, such as Epstein-Barr virus (EBV) T cells. EBV T cells can maintain the expression of endogenous TCR with inherently low allosteric reactivity due to the fixed recognition of defined viral antigens [81,82]. Consequently, the risk of GVHD is significantly diminished. Recent studies have illustrated that CD20/CD19 bispecific CAR EBV T cells generated from healthy donors exhibit stable CAR expression, a high proportion of T cells with memory phenotypes, and efficient HLA-independent killing [81]. Furthermore, animals treated with these bispecific CAR EBV T cells showed superior tumor growth inhibition without treatment-related toxicity compared to those treated with autologous CD20/CD19 bispecific CAR T cells.

T memory stem cell (TSCM)

T memory stem cells (TSCM) are a rare subpopulation of memory lymphocytes with self-renewal and pluripotent capacity. As previously mentioned, one of the challenges in universal CAR T cell therapy is the low persistence in vivo. Results from the clinic have demonstrated that CAR T cells originating from less differentiated TSCMs exhibit higher self-renewal ability, increased homing to tumor sites, and higher in vivo persistence compared to the effector T cells [83]. Common approaches in favoring TSCM over effector T cells include the application of cytokines such as IL-7 and IL-15. In addition, some pharmacological methods have been discovered recently to increase T cell stemness. For example, metabolic reprogramming inhibitors targeting the PI3K/AKT/mTOR pathway can restrict the differentiation of T cells [84]. Agonists targeting the Wnt/ β -catenin pathway could also restrict T cell differentiation by upregulating transcription factors such as Tcf1 and Lef1 or epigenetic regulator PRMT1 [85–87]. Furthermore, regulating genes in CAR T cells, such as the transcription factor FOXO1, might provide a more prolonged increase in T-cell stemness and CAR T cell persistence compared to the treatment of inhibitors [88,89].

Recent advances in universal CAR-NK therapy

In addition to T cells, NK cells offer a promising alternative for allogeneic cellular immunotherapy. NK cells, which fall within the innate immune system, are cytotoxic lymphocytes that differentiate between healthy and abnormal cells primarily through the balance between inhibitory and activating receptors [90]. If the activating signal (e.g., NKG2D, NKG2C, CD16) delivered is stronger than the inhibitory signal (e.g., KIR, NKG2A), it will result in the activation of NK cells [91]. This ability allows NK cells to recognize tumors with down-regulated MHC-I expression, making them effective against cancer cells [92]. Upon activation, NK cells may perform killing mechanisms such as cytolysis via granzymes and perforins, and antibody-dependent cytotoxicity (ADCC) which function through the binding of CD16 receptors to the Fc region of IgG (Fig. 3). All of these mechanisms are independent of the $\alpha\beta$ TCR, addressing the susceptibility of GVHD.

Compared to CAR-T therapy, CAR-NK therapy offers several advantages. First, NK cells have various modalities of tumor killing. CAR NK cells can eliminate cancer cells expressing the target through CAR-mediated killing and those lacking the target through CAR-independent innate NK cytotoxicity [91]. This HLA-independent property prevents NK cells from causing GVHD. Second, compared to CAR-T therapy, the incidence of CRS in patients treated with CAR NK cells is

very low [91]. CAR T cell activation releases proinflammatory cytokines, while NK cells primarily secrete chemokines and granulocyte-macrophage colony-stimulating factor (GM-CSF) [1,91]. Third, CAR-NK cells can be generated from various allogeneic cell sources since NK cell activation does not necessitate an MHC route. Current clinical trials on CAR-NK cells are primarily based on the NK92 cell line and PBMC, with umbilical cord blood and embryonic stem cells as alternative sources [2,93,94]. Finally, CAR-NK therapy is safer for treating T-cell-derived malignancies, as the risk of fratricide attack is significantly reduced due to the absence of shared antigens between malignant T cells and CAR-NK cells [95,96].

Recently, there has been a trend in the immunotherapy field to adopt induced pluripotent stem cells (iPSCs) as a cell source for CAR-NK cell therapy [97]. iPSC-derived NK cells are homogeneous, display long-term expansion potential, and allow large-scale production of standardized products that can be administered in multiple doses. In preclinical studies, one cryopreserved human iPSC-derived CAR-NK cell therapy has been found to maintain considerable antigen-specific cytotoxicity in vitro [98]. Moreover, a case study has shown promising results with multiple doses of Cnty-101, which is an iPSC-derived allogeneic CD19 targeting CAR-NK product [28].

Furthermore, previously mentioned CAR-NK cell therapies have been focused on tilting the balance of activating and inhibiting receptors towards the side of activating receptors. Now, CAR-NK cell therapy is also being developed for ADCC-mediated killing. Engineering an Fc-receptor that combines a high-affinity Fc-binding domain with an SLNK12 CAR signaling domain can lead to ADCC-mediated killing at a lower antibody concentration compared to conventional NK cells [99].

At present, a recognized drawback regarding CAR-NK cell therapy is the short survival cycle of NK cells, with a half-life of <10 days [1]. As a result, various strategies are now being explored to enhance their durability. IL-7 and IL-15 have proven effective in increasing the longevity of CAR-NK cells [28,29]. Reprogramming CAR NK cells for memory phenotypes and prolonged in vivo survival is another area of active exploration. Recently, one study showed that DAP10 co-stimulation-induced epigenetic reprogramming of CD5 CAR-NK cells can lead to enhanced cellular adaptation and memory formation, resulting in better anti-tumor potential [100]. It is noteworthy, however, that the durability requirements for CAR-cell therapy may vary between diseases, requiring a tailored approach [101].

Recent advances in universal CAR-Macrophage therapy

CAR-T cell therapy has achieved remarkable success in hematological malignancies, but it has yet to yield outcomes in solid tumors. Its limited effect on solid tumors may be due to impediments in the trafficking, infiltration, recognition, and killing of cancer cells by T cells [1]. Solid tumors often have barriers such as the extracellular matrix (ECM) and an immunosuppressive tumor microenvironment (TME), hindering the efficacy of CAR-T and CAR-NK cells. To address these challenges, researchers are exploring new avenues. CAR-macrophages have recently emerged as a potential solution for targeted therapies in solid tumors.

CAR-macrophages offer several advantages in targeting solid tumors. Macrophages, with the highest infiltration rate among innate immune cells in the TME, play a crucial role in modulating immune responses [102]. In addition to direct tumor eradication through phagocytic mechanisms, they also act as antigen presenters, immune stimulators, and modulators of the TME. Tumor-associated macrophages (TAMs) are usually activated as M1 (classically activated) or M2 (selectively activated) phenotypes [103]. M1 macrophages demonstrate anti-tumor properties through phagocytosis and the release of reactive oxygen and nitrogen species (ROS/iNOS) [102]. They can also initiate immune responses by stimulating NK cells and cytotoxic T cells via pro-inflammatory cytokines [104]. On the other hand, M2 macrophages have pro-tumor effects and contribute to an immunosuppressive TME. CAR-macrophages can counteract the immunosuppressive

microenvironment by re-educating M2 macrophages to the M1 phenotype (Fig. 3). Furthermore, CAR-macrophages can enhance the expression of matrix metalloproteinases (MMP), contributing to the remodeling of the ECM and potentially aiding the infiltration of T cells and NK cells [103].

To date, CAR-Macrophage therapy is largely in the preclinical phase, with a Phase I trial targeting HER2 overexpressing solid tumors. However, CAR macrophage therapies face multiple challenges in the manufacture and treatment process. Unlike other immune cells, macrophage has a low desire to circulate in the bloodstream and has limited proliferation capacity [103]. As a result, patients can only receive a limited number of macrophages, highly reducing the effectiveness of the treatment. Fortunately, similar to CAR-NK cells, CAR-macrophages can be derived from a variety of sources, including iPSCs, which have shown potent phagocytosis and immune activity [102]. In terms of CAR design, a second generation of CAR-macrophages has been reported, featuring a CD3 ζ -TIR dual-signaling CAR [105]. This design demonstrates targeted phagocytosis, antigen-dependent M1 polarization, and resistance to M2 polarization in a nuclear factor kappa B (NF- κ B)-dependent manner. While CAR-macrophage is a promising immunotherapy for solid tumors, further studies are needed to improve their efficacy and safety, especially in the clinical setting.

In situ CAR cell therapy

Despite the considerable success achieved by adoptive cell therapy over the last decade, their manufacture still relies on manual or semi-manual ex vivo production, which is of high complexity and bench-to-bench product variability [106]. Moreover, although allogeneic CAR cell therapy has many advantages over autologous therapies, the additional gene editing and cell purification steps complicate the manufacturing protocol, which not only delays production and increases costs but also reduces lymphocyte viability [107].

At present, a novel type of “Off-the-shelf” CAR therapy has emerged

Table 4
List of recent findings on in situ CAR cell therapy. Updated till 07/04/2024.

Reference	State	Target Antigen	Vehicle	Target Cell
Magee et al. [62].	Preclinical	–	Lipid nanoparticle	T cell
Parker et al. [108].	Preclinical	CD20	Lentiviral vector	T cell
Mei et al. [109].	Preclinical	CD3	Lentiviral vector	T cell
Krotova et al. [110].	Preclinical	CD19	Lentiviral vector	T cell
Andorko et al. [111].	Preclinical	CD20	Lentiviral vector	T cell and NK cell
Beltran-Garcia et al. [112].	Preclinical	–	Lipid nanoparticles and virus-like particles	T cell, NK cell and macrophage
Green et al. [113].	Preclinical	CD19	Fusosomes (viral vectors pseudotyped with modified paramyxovirus envelopes targeting specific cell types)	T cell
Rurik et al. [114].	Preclinical	CD5	Lipid nanoparticle	T cell
Smith et al. [115].	Preclinical	CD3	PBAE polymers with PGA coating	T cell
Parayath et al. [116].	Preclinical	CD8	PBAE polymers with PGA coating	T cell

in the field, focusing on the possibility of generating CAR cells in vivo (Table 4). For instance, the VivoVec™ platform is a CAR engineering platform comprising lentiviral particles and CAR transgene payloads. The surface of the lentiviral particles includes a multidomain fusion protein with T-cell activating ligands and agonistic ligands. With a single injection of VivoVec™ particles, CAR T cells are efficiently generated in vivo and expanded against specific antigens without the need for lymphodepletion chemotherapy or exogenous supportive cytokines, while removing cells expressing target antigens and forming a memory population of CAR T cells [108]. Besides, dual-targeting lentiviral vectors utilizing bio-orthogonal chemistry and click chemistry have demonstrated effective anti-tumor effects in humanized mouse models [109].

In recent years, biomaterials have gained attention in tumor immunotherapy due to their biocompatibility, targeting capability, and controllable release. Biomaterials also offer cost-effectiveness and versatility, enabling industrial production and commercialization. Therefore, the concept of generating CAR-modified cells via biomaterials has been proposed [10,117]. Previously described RNA-writer system is delivered through lipid nanoparticles, which achieved significant reporter gene expression in humanized mouse and non-human primate models [62]. Feeding the RNA writer system with LNP into primary human T cells resulted in a substantial population of CAR⁺ T cells without comprising cell viability and proliferative capacity. Studies have also explored mRNA LNPs and cell-targeted mRNA virus-like particles (VLPs), both of which enhanced the killing function of transduced T cells, NK cells, and macrophages [112]. While the use of biomaterials is promising, concerns exist regarding the interaction of nanomaterials with the immune system [5]. Overall, further efforts are needed to improve the safety and utility of biomaterials in CAR-T therapy.

Discussion

Universal CAR cell therapy, often referred to as “Off-the-shelf” CAR cell therapy, holds significant promise for cancer treatment and market viability. “Off-the-shelf” CAR cell therapies have four main advantages, 1) Sufficient cell stock. Current UCAR cell therapies can be derived from PBMCs, cell lines, and iPSCs. 2) Ready-to-use. UCAR cells are pre-treated and cryopreserved for immediate use, eliminating the time delay in manufacturing autologous CAR cell therapy. 3) Multiple doses are permitted. UCAR immune cell therapy allows for the redosing and switching of target antigens to counteract patient resistance. 4) Inexpensive. Through large-scale production, universal CAR cell therapies are expected to reduce production costs.

Nevertheless, UCAR cell therapies face challenges such as GVHD, HVGR, and CRS. For GVHD and HVGR, current research has focused on eliminating the TCR and HLA on CAR-T cells via gene editing techniques. To address immunotoxicity side effects, the latest research has been focused on developing suicide genes and safety switches to artificially control the CAR-engineered cells. In addition, alternative cell types such as NK cells, macrophages, and certain T cell subtypes can perform killing in an HLA-independent manner, offering a potential solution. In the future, with advances in the field of gene therapy such as more specific delivery to target cell types and tissues, in situ CAR-T might present unlimited potential.

Both CAR-T and CAR-NK therapies exhibit limited efficacy in solid tumors, prompting the exploration of combination therapies. Combining CAR therapy with chemotherapy, immune checkpoint inhibitors, oncolytic viruses, and radiotherapy may enhance CAR cell infiltration and remodel the tumor microenvironment. Low-dose chemotherapy has been found to promote antigen presentation to CAR-T cells, increasing persistence [104]. Additionally, oncolytic viruses can convert “cold” tumors into “hot” tumors, improving the recruitment and effector function of T cells and NK cells [118].

Looking forward, the concept of CAR-EVERYTHING is proposed. The application of CAR cell therapy may extend beyond cancer, but to treat other diseases, such as cardiac fibrosis [114,119]. In addition, the use of

CAR T-cell therapy in autoimmune diseases is of great interest. Pre-clinical studies have strongly supported the use of B-cell-targeted CAR T cells in autoimmune diseases, with CD19 CAR T cells abrogating disease-specific B-cell autoimmunity and organ inflammation in a mouse model of systemic lupus erythematosus [120]. Recently, the first application of allogeneic CAR T cell therapy has been reported in patients with systemic autoimmune diseases [121]. Still, target antigen selection, modification of CAR structures, and adjuvant therapeutic regimens for these cells require further optimization. In conclusion, continued research and optimization are expected to lead the development of advanced CAR-based cell therapies, benefiting a broader range of patients in the future.

CRedit authorship contribution statement

Ziyu Wu: Writing – review & editing, Writing – original draft, Investigation, Data curation. **Yifan Wang:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Data curation, Conceptualization. **Xin Jin:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Data curation, Conceptualization. **Luqiao Wang:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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