

Chimeric antigen receptor T-cell therapy for T-cell acute lymphoblastic leukemia

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Abstract

Chimeric antigen receptor (CAR) T-cell therapy is a new and effective treatment for patients with hematologic malignancies. Clinical responses to CAR T cells in leukemia, lymphoma, and multiple myeloma have provided strong evidence of the antitumor activity of these cells. In patients with refractory or relapsed B-cell acute lymphoblastic leukemia (ALL), the infusion of autologous anti-CD19 CAR T cells is rapidly gaining standard-of-care status and might eventually be incorporated into frontline treatment. In T-ALL, however, leukemic cells generally lack surface molecules recognized by established CAR, such as CD19 and CD22. Such deficiency is particularly important, as outcome is dismal for patients with T-ALL that is refractory to standard chemotherapy and/or hematopoietic stem cell transplant. Recently, CAR T-cell technologies directed against T-cell malignancies have been developed and are beginning to be tested clinically. The main technical obstacles stem from the fact that malignant and normal T cells share most surface antigens. Therefore, CAR T cells directed against T-ALL targets might be susceptible to self-elimination during manufacturing and/or have suboptimal activity after infusion. Moreover, removing leukemic cells that might be present in the cell source used for CAR T-cell manufacturing might be problematic. Finally, reconstitution of T cells and natural killer cells after CAR T-cell infusion might be impaired. In this article, we discuss potential targets for CAR T-cell therapy of T-ALL with an emphasis on CD7, and review CAR configurations as well as early clinical results.

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) accounts for about 10-15% of childhood ALL and 20-25% of adult ALL.^{1,2} Historically, T-ALL had a poorer overall outcome when compared with B-lineage ALL, and patients with T-ALL were often regarded as having high-risk ALL.^{1,3} Among patients with T-ALL, those with early T-cell progenitor (ETP)-ALL have a particularly poor response to remission induction chemotherapy and often require intensive chemotherapy and/or hematopoietic stem cell transplant (HSCT).⁴⁻⁹ For patients with T-ALL who are refractory to chemotherapy or relapse, new and effective therapies are urgently needed.¹⁰⁻¹² In this context, the success of chimeric antigen receptor (CAR) T-cell therapy in B-lineage ALL provides a strong impetus to develop similar technologies applicable to T-ALL. CAR can direct T lymphocytes against antigens expressed by tumor cells. Antigen binding transduces signals which

induce T-cell activation, resulting in both tumor cell killing and T-cell proliferation.¹³ Consequently, CAR T cells can expand and persist after infusion, reducing tumor load and eliminating minimal residual disease (MRD). The antitumor potential of this approach has been amply demonstrated through the clinical use of CAR T cells in B-cell malignancies with CAR targeting CD19 or CD22.¹⁴⁻²⁶ Both target antigens are detectable by flow cytometry on the surface membrane of the vast majority of leukemic cells in most cases of B-lineage ALL and other B-cell malignancies.²⁷⁻³¹ CD19 and CD22 are normally expressed only by B lymphoid cells.³² Although the infusion of the corresponding CAR T cells induces B-cell lymphopenia and agammaglobulinemia, these are clinically manageable.³³ Moreover, CD19 and CD22 are not expressed by T cells and their targeting does not affect CAR T-cell manufacturing. The surface marker profile of T-ALL and of its lymphomatous counterpart, T-cell lymphoblastic lymphoma (T-LL),³⁴

is considerably different from that of B-cell leukemias and lymphomas. Proven CAR T-cell targets, such as CD19 and CD22, are generally absent and new targets must be identified. Moreover, T-ALL and T-LL typically share surface antigens with peripheral blood T lymphocytes.^{4,27,34} This poses three major potential problems. First, the manufacturing of viable CAR T cells might be complicated by CAR-mediated killing within the CAR T-cell population, the so-called “fratricide” effect.^{35,36} Secondly, T lymphocytes are difficult to separate from T-ALL cells should the latter contaminate the cell source for CAR T-cell manufacturing. Finally, the degree of T-cell ablation resulting from the infusion of CAR T cells directed against T-cell antigens and its clinical consequences are unclear. One of the important factors for the success of autologous anti-CD19 CAR T-cell therapy is the expansion and prolonged persistence of CAR T cells,^{14,17,18} but infusion of autologous CAR T cells directed against T-cell antigens might require HSCT soon after to correct T-cell aplasia.

We here review potential targets for CAR T-cell therapy of T-ALL with an emphasis on CD7, which has been the focus of most clinical studies reported to date. We discuss CAR design and testing, approaches to avert fratricide and lessons learned from the early clinical experience.

Targeting CD7 for chimeric antigen receptor T-cell therapy of T-cell acute lymphoblastic leukemia

CD7 expression

CD7 is a 40 kDa type I transmembrane glycoprotein belonging to the immunoglobulin superfamily and is one of the earliest surface markers in the developmental pathway of human T cells.³⁷⁻³⁹ CD7 is widely used as a key marker for the diagnosis of T-ALL because of its high and often uniform expression in leukemic blasts.⁴⁰⁻⁴³ Importantly, CD7 is also expressed in ETP-ALL; by definition, this T-ALL subtype lacks some markers often expressed in non-ETP T-ALL, such as CD5 and CD1a.⁴ We studied leukemic cells from diagnostic bone marrow samples of 49 T-ALL patients (14 with ETP-ALL) and found that the median percent CD7 expression exceeded >99% in 46 cases.³⁶

In addition to high levels of expression, an effective CAR T-cell target must be present in chemoresistant leukemic clones which drive relapse. We studied 14 samples collected at relapse and found that CD7 expression in these samples was as high as that in samples studied at diagnosis.³⁶ We also determined whether chemotherapy would alter CD7 expression by measuring it in leukemic cells from bone marrow samples examined for MRD during chemotherapy. In 54 samples (from 21 patients), >99% of leukemic cells expressed CD7.³⁶ Sequential samples were available from 18 patients and the results clearly showed high stability of

CD7 expression.³⁶ Taken together, these results provided a strong rationale for targeting CD7 with CAR T cells in T-ALL. CD7 is also expressed by most normal T cells in peripheral blood, and CD7 mRNA expression in T cells rapidly increases after activation.^{44,45} CD7 function in human T cells is unclear, and CD7-deficient mice showed normal lymphocyte populations in primary and secondary lymphoid tissues without significant immunodeficiency.^{46,47} Nevertheless, it was noted that mice had reduced *in vitro* antigen-specific interferon- γ production and CD8 cytotoxic capacity, and *in vivo* resistance to lipopolysaccharide-induced shock syndromes.⁴⁸ Human peripheral blood T cells contain a small CD7-negative fraction typically accounting for <10% of T cells,⁴⁹ which progressively increases with age.⁵⁰ This CD7-negative T-cell population contains a higher proportion of CD4-positive cells than that in the CD7-positive cell population and a relatively higher proportion of cells with a memory phenotype.^{50,51}

Among normal tissues, CD7 expression is generally limited to the T-cell lineage.^{36,40,42-44} An anti-CD7-ricin A chain immunotoxin did not seem to affect other cells when administered to patients with T-cell lymphoma; the vascular leak syndrome observed in some patients appeared to be unrelated to the CD7 specificity, as there was no anti-CD7 reactivity with endothelial cells.⁵²

Anti-CD7 chimeric antigen receptor design and expression

CD7 holds potential as a CAR T-cell target because of its high expression in T-ALL/T-LL cells and its lack of expression in non-hematopoietic cells. We designed an anti-CD7 CAR composed of the single-chain variable fragment (scFv) of the anti-CD7 antibody TH-69 joined to the signaling domains of 4-1BB (CD137) and CD3 ζ via the hinge and transmembrane domain of CD8 α . In addition to the scFv derived from the antibody TH-69,⁵³ other scFv derived from the 3A1 antibody and other 3A1 hybridomas⁴⁴ have also been studied (Table 1). TH-69 has been reported to have the highest antigen binding affinity.^{54,55}

Because an scFv construct does not always recapitulate the binding profile of the original antibody, it is important to test its specificity and binding capacity. To this end, we generated a TH-69 scFv in soluble form and ensured that it could only label cell lines known to express CD7.³⁶ Further assurance was provided by experiments in which preincubation with the scFv inhibited staining of CD7-positive cells with an anti-CD7 monoclonal antibody.³⁶ Finally, we used two differently labeled CD7-positive cell lines and found that the TH-69 scFv induced significant and specific cell aggregation.³⁶ This extensive set of experiments provided solid data regarding the capacity of the TH-69-derived scFv to bind CD7 strongly and specifically.

After incorporating the TH-69 scFv into the CAR construct, we used a retroviral vector to express the anti-CD7-41BB-CD3 ζ CAR in the Jurkat T-cell line. CAR expression was high

Table 1. Targets and receptor design for chimeric antigen receptor T-cell therapy in T-cell acute lymphoblastic leukemia/T-cell lymphoblastic lymphoma.

Target antigen	% positive cases	Components of the chimeric antigen receptor					Reference
		Antigen-binding domain	Hinge region	Transmembrane domain	Co-stimulatory domain	Signaling domain	
CD7	>99	TH-69	CD8 α	CD8 α	4-1BB	CD3 ζ	Png <i>et al.</i> ³⁶
		VHH6 ^a	IgG1	CD8 α	ICOS and 4-1BB	CD3 ζ	Zhang <i>et al.</i> ⁶⁵
		3A1e	IgG1	CD28	CD28	CD3 ζ	Gomes-Silva <i>et al.</i> ⁵⁹
		TH-69	CD8 α	CD28	CD28 and 4-1BB	CD3 ζ	Cooper <i>et al.</i> ⁶⁰
		3A1e	CD8 α	CD8 α	4-1BB	CD3 ζ	Georgiadis <i>et al.</i> ⁶¹
		3A1e	CD8 α	CD8 α	CD28	CD3 ζ	Diorio <i>et al.</i> ⁷⁰
		TH-69	CD8 α	CD28	4-1BB	CD3 ζ	Lu <i>et al.</i> ⁸⁰
		3A1e	CD8 α	CD8 α	CD28	CD3 ζ	Watanabe <i>et al.</i> ⁷⁷
CD5	~80	H65	IgG1	CD28	CD28	CD3 ζ	Mamonkin <i>et al.</i> ³⁵
		H65, FHV _H 1 ^b , FHV _H 3 ^b	CD8 α	CD8 α	4-1BB	CD3 ζ	Dai <i>et al.</i> ⁸⁵
		H65	CD28	CD28	CD28	CD3 ζ	Raikar <i>et al.</i> ⁸⁴
		H65	IgG1	CD28	4-1BB	CD3 ζ	Mamonkin <i>et al.</i> ⁸⁶
CD1a	~40	NA1, 34.HLK	CD8 α	CD8 α	4-1BB	CD3 ζ	Sanchez-Martinez <i>et al.</i> ⁹³
TRBC1	~30	JOVI-1	CD28	CD28	CD28 and OX40	CD3 ζ	Maciocia <i>et al.</i> ⁹⁵
CD38	>80	THB-7	CD8 α	CD8 α	4-1BB	CD3 ζ	Mihara <i>et al.</i> ¹⁰⁴
		028, 056, 026, daratumumab	CD8 α	CD8 α	4-1BB	CD3 ζ	Drent <i>et al.</i> ¹⁰⁷
		Nb-1G3 ^c	CD8 α	CD8 α	4-1BB	CD3 ζ	An <i>et al.</i> ¹²⁹
		MOR202, AB79, MOR3080	CD8 α	CD8 α	4-1BB	CD3 ζ	Glisovic-Aplenc <i>et al.</i> ¹⁰⁸

^aDerived from llama immunized with CD7-positive Jurkat cells. ^bDerived from fully human heavy-chain-only phage display antibody library.

^cDerived from camel immunized with recombinant CD38.

and its ligation induced signal transduction, as revealed by increases in the expression of T-cell activation markers, such as CD69 and CD25.³⁶

We selected 4-1BB (CD137) as a co-stimulatory molecule in our second-generation CAR.³⁶ 4-1BB is the co-stimulatory molecule in the CAR for B-lineage ALL originally developed in our laboratory,⁵⁶ and is the one incorporated in the first CAR T-cell product approved for the treatment of B-lineage ALL (tisagenlecleucel).²⁶ The addition of 4-1BB to CAR markedly increases their capacity to induce and sustain T-cell expansion and antitumor activity.⁵⁶⁻⁵⁸ As summarized in Table 1, most reported CAR directed against CD7 or other T-cell antigens have either 4-1BB or CD28 as co-stimulatory molecules, while CD3 ζ is universally used to deliver the primary activation stimulus.

The problem of chimeric antigen receptor T-cell fratricide and its solutions

Given the high expression of CD7 in peripheral blood T cells, it was not surprising that expression of anti-CD7-41BB-CD3 ζ CAR dramatically reduced cell viability, regardless of whether the CAR was permanently expressed by retroviral transduction or only transiently expressed by mRNA electroporation; in 17 experiments, CAR expression

reduced viable cell recovery to about one-third 24 hours after CAR introduction and cell viability decreased with time.³⁶ The occurrence of CAR T cells that killed other CAR T cells (i.e., fratricide) was corroborated by the appearance of exocytosis of lytic granules in the CAR T cells. Similar findings were also reported by other investigators studying different anti-CD7 CAR constructs, all of whom noted poor viability and limited *in vitro* expansion of CAR T cells.⁵⁹⁻⁶² These results collectively indicate that downregulation of CD7 in CAR T cells is a critical step for robust manufacturing of anti-CD7 CAR T cells.

Several approaches have been developed to mitigate the issue of fratricide by generating CD7-negative anti-CD7 CAR T cells, including post-translational interference of CD7 expression, gene editing, and the use of naturally occurring CD7-negative T cells.

To interfere with the expression of CD7 on the surface membrane of T cells we used the protein expression blocker (PEBL) technology previously developed in our laboratory.^{63,64} These blockers consist of a scFv (anti-CD7 in this case) linked to amino acid sequences containing endoplasmic reticulum/Golgi retention domains. We observed that transduction of anti-CD7 PEBL resulted in almost immediate suppression of CD7 expression while CD7 mRNA expression

was retained.³⁶ A similar approach using a construct that contains a heavy-chain-only CD7 binding domain instead of a scFv was subsequently reported.⁶⁵ Expression of anti-CD7 PEBL and downregulation of CD7 do not appear to affect the expression of other surface antigens.³⁶ Moreover, T-cell survival in culture was not altered; when the T cells rendered CD7-negative by PEBL were transduced with an anti-CD19 CAR, they were functionally indistinguishable from CD7-positive anti-CD19 CAR T cells.³⁶ In sum, PEBL expression and the formation of PEBL-CD7 complexes in the endoplasmic reticulum did not have discernible effects on T-cell function. Lack of CD7 did not produce any detectable functional impairment.

When the PEBL-transduced CD7-negative T cells were transduced with the anti-CD7 CAR, post-transduction viability improved markedly, demonstrating that downregulation of CD7 prevented fratricide.³⁶ In several experiments *in vitro* and in xenograft models of leukemia, anti-CD7 PEBL CAR T cells were remarkably effective anti-leukemic agents.³⁶ During the testing of anti-CD7 PEBL CAR T cells we compared their potency to that of T cells expressing the anti-CD19-41BB-CD3 ζ CAR, using T-ALL cell lines transduced with CD19. Cytotoxicity and long-term proliferative capacity were similar, indicating that the anti-CD7 CAR T cells would be expected to produce an antitumor effect similar to that of the established anti-CD19 CAR T-cell product.³⁶ We also compared their antitumor capacity to that of residual T cells recovered after CAR expression without prior CD7 downregulation. In 45 experiments, the former were consistently more potent, and their superiority extended to exocytosis of lytic granules, cytokine production, and proliferative capacity in co-cultures with CD7-positive target cells.³⁶ These results indicate that CD7 downregulation is not only essential to avoid fratricide but also benefits CAR T-cell function. We speculate that apparently CD7-negative cells may express CD7 during CAR signaling and activation resulting in engagement of the CAR by self-CD7, which would create a decoy that diverts the CAR from antigen expressed by target cells.

A unique aspect of the PEBL technology as applied to CAR T-cell therapy manufacturing is that it fits well with current Good Manufacturing Practice (GMP) protocols. For example, PEBL and CAR genes can be included in a single bicistronic vector allowing CAR T-cell production with minimal fratricide using a work schedule essentially identical to that used for anti-CD19 CAR T-cell manufacturing.³⁶ Therefore, it can be easily implemented by any GMP facility familiar with CAR T-cell manufacturing. Because both CAR and PEBL genes are within a single viral vector, there should not be an increased risk of integration in potentially oncogenic sites as compared to expression of a CAR gene alone. The CAR-PEBL technology has been translated into a process that meets current GMP regulations and is, at present, being studied in an ongoing clinical trial (ClinicalTrials.gov Identifier: NCT05043571).

Gene editing technologies have also been used to eliminate CD7 expression for anti-CD7 CAR T-cell manufacturing. Cytidine deamination guided by clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 has been successfully utilized to delete CD7 and prevent CAR-mediated fratricide.^{59,60,62} While CRISPR-Cas9 is effective in knocking out targets, the formation of double-stranded DNA breaks might result in genetic aberrations, especially in multiplexed editing scenarios.^{61,66,67} Base-editors rely on CRISPR-Cas9 technology, wherein a defective Cas9 that cannot cause double-stranded breaks is linked to a cytosine or adenine deaminase, and uracil glycosylase inhibitor.^{61,68,69} When base-editors are guided to the target DNA sequence by guide RNA, the deaminase converts cytosine to uracil or adenine to inosine. The resulting mismatch is then repaired by cellular machinery, resulting in CG to AT, or AT to CG mutations.⁶⁸ Base-editors have been used for multiplexed editing of anti-CD7 CAR T cells, primarily to prevent fratricide and, in the setting of allogeneic CAR T cells, to knockout other surface proteins such as T-cell receptor (TCR) (to prevent graft-versus-host disease [GvHD]) and CD52 (to protect CAR T cells from alemtuzumab administered to suppress rejection).^{70,71}

While gene editing completely eliminates the expression of a specific target protein, off-target mutations may potentially lead to carcinogenicity or cytotoxicity in normal cells.^{66,67} Additionally, genome-wide, non-specific editing may also arise from multiplexed editing.^{68,69} Although unbiased genome-wide screening can be used to detect potential off-target mutations, such technologies are limited by the 0.1% detection limit of next-generation sequencing.^{72,73} Next-generation base-editors might have a reduced non-specificity but this may be accompanied by a lower on-target efficiency and higher sequence-specific requirements.⁷⁴⁻⁷⁶

Other investigators have proposed alternative approaches to anti-CD7 CAR T-cell manufacturing that do not include downregulation of CD7. Thus, Watanabe *et al.*⁷⁷ used ibrutinib and dasatinib which inhibit tyrosine kinases, such as ITK and LCK, involved in CAR signaling,^{78,79} to allow survival and expansion of anti-CD7 CAR T cells during manufacturing. The cytotoxic, proliferative, and cytokine secreting functions of the resulting CAR T cells were restored following removal of dasatinib. While this method allows production of sufficient functional CAR T cells *ex vivo*, it is unclear whether fratricide after dasatinib wash-out will result in lower persistence of these cells *in vivo*, possibly reducing longevity of the therapy.

The selective transduction of naturally occurring CD7-negative T cells through magnetic bead separation with CD7 depletion followed by CD3 enrichment has been described as an approach to bypass fratricide.^{80,81} Through this approach, CD7-negative T cells were engineered to express a CD7-targeting CAR that was shown to trigger cytotoxic activity *in vitro* with good cell viability and expansion. How-

ever, the major foreseeable challenge to clinical application of this approach is the potentially low cell numbers, particularly in heavily pre-treated patients from whom it is often difficult to obtain sufficient numbers of T cells regardless of their phenotype. Moreover, as discussed above, anti-CD7 CAR T cells with downregulated CD7 were more potent than those derived from T cells surviving fratricide during manufacturing.³⁶

Chimeric antigen receptor T-cell targets beyond CD7

CD5

CD5 is a 67 kDa cell surface glycoprotein expressed in approximately 80% of T-ALL.^{4,27,82} In comparison to CD7, its expression is generally lower, and dim or absent in ETP-ALL.^{4,9,83} CD5 is also expressed by peripheral blood T cells but Mamonkin *et al.* was able to develop anti-CD5 CAR T cells without prior CD5 downregulation,³⁵ perhaps because of the relatively weak expression of CD5 in some T-cell subsets. Subsequent studies relied on CD5 knockout by CRISPR-Cas9 prior to transduction of the CD5 CAR, which led to higher CAR expression and reduced tonic activation, as compared to unedited CAR T cells.^{84,85} In a separate approach using a system wherein CAR expression is controlled by doxycycline, anti-CD5 CAR T cells were cultured with doxycycline and T-cell expansion improved due to reduced fratricide. CAR expression was then restored after wash-out and allowed the elimination of CD5-positive targets in a mouse model.⁸⁶

CD1a

CD1a is related to the major histocompatibility complex and is expressed during T-cell development in the thymus.^{87,88} It is also expressed in approximately 40% of T-ALL cases and is a defining feature of the cortical T-ALL subtype.^{27,89-92} Absence of CD1a expression is one of the defining features of ETP-ALL.⁴ CD1a is not expressed by peripheral blood T cells and Sanchez-Martinez *et al.*⁹³ showed that fratricide was not an issue when manufacturing anti-CD1a CAR T cells, which had specific cytotoxicity against CD1a-positive T-ALL cell lines and primary leukemic cells. Obviously, the main restriction of using CD1a as a target is the limited number of T-ALL cases that express CD1a.

TRBC1

TCR β -chains can be encoded by either the *TRBC1* or the *TRBC2* gene, and their expression is mutually exclusive.^{94,95} Thus, targeting TRBC1 would eliminate TRBC1-expressing normal T cells but spare a substantial number of normal T cells expressing TRBC2. Maciocia *et al.*⁹⁵ generated anti-TRBC1 CAR T cells and showed that these cells had the capacity for specific killing of TRBC1-positive cell lines and

primary cells *in vitro* and in mouse models of leukemia. In the context of T-ALL, the application of this elegant approach is limited by the fact that CD3/TCR $\alpha\beta$ expression is found in a minority of cases and expression is often dim.^{27,90,96}

CD38

CD38 is a 45 kDa type II, transmembrane glycoprotein expressed on activated T cells, terminally differentiated B cells, monocytes and natural killer (NK) cells,⁹⁷ and is reportedly not expressed by multipotent hematopoietic stem cells.⁹⁸⁻¹⁰⁰ CD38 is also expressed in hematologic malignancies, including ALL, acute myeloid leukemia, lymphoma and multiple myeloma. CD38 is expressed in most cases of T-ALL,¹⁰¹ suggesting that it could be possible to target T-ALL with the anti-CD38 antibody daratumumab.^{102,103} Anti-CD38 CAR T cells were originally developed to target B-cell non-Hodgkin lymphoma and multiple myeloma.¹⁰⁴⁻¹⁰⁷ CD38 is also expressed in normal activated T cells; cell recovery after CAR expression was reported to be low due to cell fratricide and was improved by the addition of a blocking anti-CD38 antibody during T-cell manufacturing.¹⁰⁴ More recently, the anti-CD38 CAR approach was extended to preclinical models of T-ALL.¹⁰⁸ In these studies, fratricide did not appear to be an issue.

Clinical results with chimeric antigen receptor T cells targeting T-cell antigens

Table 2 summarizes data from clinical studies of CAR T cells in T-ALL/T-LL which have used either autologous or allogeneic cells as a source for CAR T-cell manufacturing. The studies reported so far have targeted either CD7 or CD5.

Autologous cell products

Zhang *et al.*⁶⁵ used autologous anti-CD7 CAR T cells with nanobody-derived fratricide resistance to treat seven patients with T-ALL. Despite significant disease burden prior to treatment, six of these patients achieved complete responses with tolerable toxicities, although four relapsed 3 months later.⁶⁵ Information regarding the quality of the manufactured products, in terms of transduction efficiency and leukemic cell contamination, was not extensive in the report, and it is not clear whether these factors could have contributed to the relapses. Lu *et al.*⁸⁰ used autologous anti-CD7 CAR T cells without any additional modifications to reduce fratricide to treat 20 patients with T-ALL/T-LL; 16 had bone marrow involvement prior to CAR T-cell infusion and 15 of these achieved a complete response 1 month later. Twelve of these 15 patients received a HSCT within 100 days of infusion.⁸⁰ Autologous cells have also been tested in the context of CD5-specific CAR. Of four reported

Table 2. Data from clinical studies of chimeric antigen receptor T cells in T-cell acute lymphoblastic leukemia/T-cell lymphoblastic lymphoma.

Cell source	Target	Fratricide prevention	Additional modifications	N of pts enrolled	N of pts in CR ~4 weeks after infusion ^a	Lymphodepletion	Reference
Autologous	CD7	Post-translational interference of CD7 expression	None	8	6	Flu 90 mg/m ² Cy 1,000 mg/m ²	Zhang <i>et al.</i> ⁶⁵
	CD7	None	None	18	17	Flu 90 mg/m ² Cy 900 mg/m ²	Lu <i>et al.</i> ⁸⁰
	CD5	None	None	4	1	Flu 90 mg/m ² Cy 1,500 mg/m ²	Hill <i>et al.</i> ¹³⁰
Allogeneic from HLA-matched or haploidentical donors	CD7	Post-translational interference of CD7 expression	None	20	17	Flu 90 mg/m ² Cy 750 mg/m ² or 90 mg/kg (^b ~3,150 mg/m ²) for new donor-derived CAR T cells	Pan <i>et al.</i> ¹¹⁷
		None	None	2	2	Flu 90 mg/m ² Cy 900 mg/m ²	Lu <i>et al.</i> ⁸⁰
Allogeneic not from HSCT donors	CD7	Deletion of CD7 by base-editing	Deletion of TRBC, CD52	3	2	Flu 150 mg/m ² Cy 120 mg/kg (^b ~4,200 mg/m ²) Alemtuzumab: 1 mg/kg	Chiesa <i>et al.</i> ⁷¹
		Deletion of CD7 by CRISPR-Cas9	Deletion of TRAC	2	2	Flu 180-222 mg/m ² Cy 1,200-3,600 mg/m ² Prednisone 240 mg/m ² + melphalan 74 mg/m ² or methylprednisolone 360 mg/m ²	Li <i>et al.</i> ¹²⁰
			Deletion of TRAC, RFX5; expression of E-cadherin-CD28, IL2RG	9	5	Flu 150 mg/m ² Cy 1,500 mg/m ² Etoposide 500 mg	Hu <i>et al.</i> ¹²¹
			Deletion of TRAC	7	2	Flu 90 mg/m ² Cy 1,500 mg/m ²	Ghobadi <i>et al.</i> ¹³¹

^aIn some but not all patients with T-cell acute lymphoblastic leukemia, complete remission was confirmed by flow cytometry and/or polymerase chain reaction. In some patients with T-cell lymphoblastic lymphoma, the definition of complete remission was based on imaging only. ^bBased on a 70 kg adult with an estimated body surface area of 2 m². Pts: patients; CR: complete remission; Flu: fludarabine; Cy: cyclophosphamide; CAR: chimeric antigen receptor; HSCT: hematopoietic stem cell transplant; TRBC: T cell receptor beta constant gene; TRAC: T-cell receptor alpha constant gene; RFX5: regulatory factor X 5 gene; IL2RG: interleukin 2 receptor subunit gamma gene.

patients with T-ALL, only one had an initial response but could not proceed with the planned HSCT and relapsed with CD5-positive disease.¹⁰⁹

None of the clinical studies of autologous CAR T cells in T-ALL/T-LL reported to date has indicated the occurrence of relapse driven by reinfusion of leukemic cells that survived CAR T-cell manufacturing. This is, however, a potential risk of autologous CAR T-cell infusions. If circulating leukemic cells survive the culture period required for CAR T-cell manufacturing, they may be genetically modified and express the CAR, which may mask the target antigen and cause escape from CAR T-cell cytotoxicity, as has been reported with anti-CD19

CAR T cells.¹¹⁰ This occurrence is likely to be rare because ALL cells require mesenchymal cell support to survive *in vitro*;^{111,112} they are unlikely to persist and even less likely to be amenable to viral vector transduction under the culture conditions used to manufacture CAR T cells. Nevertheless, levels of MRD in peripheral blood in T-ALL are generally higher than in B-lineage ALL,^{113,114} increasing the risk of leukemic cell contamination in the starting material. Moreover, enrichment of normal T cells during manufacturing using antibodies is often impossible in T-ALL, with the exception of a minority of cases in which leukemic cells lack surface expression of CD3 or CD4 and CD8.⁸⁰ It is therefore important to reduce

levels of peripheral blood MRD as much as possible prior to leukapheresis, and test for the presence of residual T-ALL cells at the end of the cultures, including their expression of CAR and targeted antigen.

Allogeneic chimeric antigen receptor T cells derived from HLA-matched hematopoietic stem cell donors

In patients with refractory T-ALL, it may be challenging to obtain an adequate number of peripheral blood mononuclear cells for CAR T-cell manufacturing. Such patients often have high numbers of circulating leukemic cells and chemotherapy to reduce the tumor burden also reduces lymphocyte counts. Thus, in patients who relapse after HSCT, the use of HLA-matched donors as a source of peripheral blood is an attractive option. As a caveat, this approach has the potential to trigger GvHD as CAR T cells are strongly stimulated during manufacturing and the numbers of T cells infused (including those not expressing the CAR) might exceed the threshold regarded as safe during HSCT and for donor lymphocyte infusions.^{115,116} Pharmacological interventions to control GvHD are generally targeted at T cells and would most likely nullify any benefits derived from CAR T-cell therapy. Ultimately, decisions to use matched-donor cells should be weighed against individual risks that are both specific to the type of product infused and the patient's clinical status and previous treatment. Pan *et al.*¹¹⁷ obtained peripheral blood from HLA-matched or haploidentical HSCT donors to manufacture anti-CD7 CAR T cells. CD7 expression was downregulated using an approach seemingly identical to the PEBL strategy described earlier.³⁶ The study included patients who had already undergone HSCT who received cells from their previous donors as well as patients who had not undergone HSCT who received cells from prospective matched donors. The latter group of patients were given higher doses of lymphodepleting cyclophosphamide (30 mg/kg/day vs. 250 mg/m²/day). Among 20 patients treated, 17 achieved MRD-negative complete remission; all seven patients who had received cells from new donors went on to receive HSCT after CAR T-cell infusion. Fifteen of the 17 initial responders remained in remission, with a median follow-up of 6.3 months. Of note, all patients who had received cells manufactured from previous donors had complete chimerism. Although early-onset GvHD was reported in 60% of patients, most cases were grade 1 involving the skin. The follow-up of this study extended to 2 years was reported more recently.¹¹⁸ Six patients relapsed within 4 to 11 months after CAR T-cell infusion, and four of these lacked CD7 expression. A follow-up paper further reported on the initial cohort of patients who received HSCT consolidation with the addition of another six patients.¹¹⁹

Matched-donor cells were also tested in the clinical trial by Lu *et al.*⁸⁰ who used anti-CD7 CAR transduced cells that had survived fratricide during manufacturing despite the lack of CD7 downregulation. The two patients treated

both had complete responses (1 with mild skin GvHD) and subsequently received HSCT.

Allogeneic chimeric antigen receptor T cells not from hematopoietic stem cell donors

Some inroads have been made in the generation and clinical testing of allogeneic CAR T cells not derived from HSCT donors. A recent paper reported interim results of a phase I study in which base-editing was used to remove CD7 (to avoid fratricide after anti-CD7 CAR expression), the β chain of the $\alpha\beta$ TCR (to avoid GvHD) as well as CD52 (to allow the possible use of alemtuzumab to delay rejection).⁷¹ Of the three patients who received cells derived from various healthy donors, one achieved a MRD-negative status within 1 month. She went on to receive HSCT on day 49, ahead of reduced toxicity conditioning and antithymocyte globulin to remove allogeneic CAR T cells and prevent GvHD. The patient was in remission at the time of the report, 9 months later. Another patient remained MRD-positive, as determined by polymerase chain reaction analysis, and died of a fungal infection. The third patient achieved MRD negativity and had HSCT with no further follow-up at the time of the report.

Li *et al.*¹²⁰ reported the use of “off-the-shelf” anti-CD7 CAR T cells. They used CRISPR-editing of CD7 to avoid fratricide and of TRAC to abrogate TCR expression. Of the two treated patients, one remained in remission for a year after the transplant and one relapsed 48 days later.

Hu *et al.*¹²¹ introduced a multitude of other gene modifications, including deletion of CD7, TCR, and HLA class II, expression of a NK inhibiting receptor (e-cadherin + CD28), and expression of the interleukin 2-receptor γ chain, an approach that seems likely to considerably increase the risk of non-specific genetic alterations. Remission was achieved in five of eight evaluable patients with T-ALL/T-LL but only two remained in remission, one after a transplant.

Overall, the three studies provide evidence that allogeneic anti-CD7 CAR T cells can produce tumor responses and that GvHD risk can be markedly reduced by removing TCR expression. In the studies by Li *et al.*¹²⁰ and Hu *et al.*¹²¹ the number of cells infused was 1×10^7 cells/kg or greater (a dose 10 times higher than that in the study by Chiesa *et al.*⁷¹) but no GvHD was reported. The usefulness of targeting other genes beyond CD7 and TCR remains unclear in the context of using CAR T-cell therapy as a bridge to transplant.

Lymphodepletion, toxicities and immune cell reconstitution

Regardless of the autologous or allogeneic origin of CAR T cells, their infusion is commonly preceded by lymphodepleting chemotherapy which creates an environment that favors engraftment of CAR T cells.¹²² The intensity of lymphodepletion can affect the degree of expansion of autologous CAR T cells after infusion.¹⁷ In the case of allogeneic CAR T cells,

lymphodepletion also temporarily suppresses rejection. Table 2 summarizes the lymphodepleting regimens that have been used prior to CAR T-cell infusion in patients with T-ALL/T-LL. Given the small numbers of patients, it is unclear whether the differences in lymphodepletion regimens have had any impact on clinical responses.

Pancytopenia was one of the most common toxicities reported after CD7-directed CAR T-cell therapy, and it was associated with viral reactivation and fungal infections.^{71,117}

It is unclear whether specific targeting of CD7 affects the severity and duration of pancytopenia. However, patients with T-ALL who received CAR T-cell therapy in the reported studies had usually been given multiple lines of intensive chemotherapy and many had undergone HSCT, as well as further bridging chemotherapy and lymphodepletion prior to receiving the CAR T cells. Pancytopenia was not, therefore, unexpected. The types of infections reported following CAR T-cell therapy were not distinctly different from those observed in patients with prolonged pancytopenia. Reported infections after CAR T-cell infusion in patients with T-ALL appeared to be primarily associated with pancytopenia and not due to CAR T-cell-driven immune cell deficiencies.

The toxicity profile typically associated with CAR T cells, including cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS),³³ does not appear different from that when CAR T cells target CD7. No dose-limiting toxicities were reported in the reviewed clinical studies; manageable grade 1-2 CRS was the most common adverse effect reported, while grade 3-4 CRS occurred rarely.^{80,117} The severity of reported cases of ICANS was also mild; the cases resolved spontaneously in most studies and were associated with a high burden of disease.^{71,80,117}

CD7 expression in T-ALL cells is typically higher than in normal T cells³⁶ and some normal T cells may have very dim or undetectable CD7 expression.⁴⁹⁻⁵¹ Whether such T cells can repopulate the T-cell compartment after anti-CD7 CAR T-cell therapy is unclear. As mentioned earlier, CD7-deficient mice did not have significant lymphopenia or immunodeficiency,^{46,47} but the specific deficiency of CD7 has not been described in humans. In the study by Pan *et al.*¹¹⁷ with donor-derived CAR T cells, there was recovery of T and NK cells that lacked CD7 expression after initial depletion of CD7-positive immune cells by the CAR T cells. The T cells were also reactive to stimulation with fungal and viral peptides. Cell profiling by single-cell RNA-sequencing-based profiling indicated a higher expression of T-cell functional pathways as compared to T cells prior to CAR T-cell therapy.¹²³ Further studies are required to determine the long-term impact of the loss of CD7 expression on T cells and NK cells.

Conclusions and future perspectives

Effective CAR T-cell technologies targeting antigens expressed by T-ALL and T-LL have been developed and are

being translated into clinical-grade products. The collective data reported so far show that their infusion produces marked antitumor responses with an acceptable safety profile. Although some investigators rely on a minority of T cells that escape killing by the CAR T cells during manufacturing, others prefer to downregulate the antigen targeted by the CAR in the CAR T cells to avert fratricide, as well as maximize the potency and T-cell repertoire of the infused T cells.

Because of its high expression in T-ALL, including ETP-ALL, CD7 is an excellent target for CAR T-cell therapy of T-ALL and T-LL. Other targets, however, could be important in cases in which not all leukemic cells are CD7-positive; such cells may drive CD7-negative relapse after anti-CD7 CAR T-cell infusion.^{65,80,117} Should CD5, CD1a, TRBC1, CD38 and other targets be expressed in the CD7-negative cells, they could provide additional opportunities for treatment, and might also be useful in treating patients with more mature T-cell malignancies, which often lack CD7.¹²⁴ Conceivably, levels of target antigen expression in leukemic cells influence the cells' susceptibility to CAR T-cell killing, and leukemic cells with undetectable target antigen by flow cytometry are likely to escape cytotoxicity. If these cells have clonogenic potential, they can be the source of relapse after CAR T-cell infusion. Nevertheless, data are still lacking to determine specific thresholds to use as inclusion criteria.

The use of autologous cells remains the most widely used form of CAR T-cell therapy for ALL. The development of methods that allow manufacturing of autologous CAR T cells on site, i.e., at the point-of care and not in a remote facility, should further widen their accessibility and timely infusion.^{125,126} Cells derived from healthy donors should have more predictable expansion and better capacity for persistence than those derived from patients who received multiple courses of chemotherapy; culture conditions that promote expansion of cells with superior longevity and antitumor capacity could further enhance the quality of allogeneic products.^{13,127,128} The immediate availability of allogeneic off-the-shelf products would make them clinically valuable, particularly for patients with rapidly progressing disease, when it might be difficult to collect a sufficient number of effective T cells and the risk of transducing and/or genetically modifying contaminating leukemic cells is high. While allogeneic cells have the potential to cause GvHD, this can be effectively prevented by abrogating TCR expression in CAR T cells.^{13,127,128} Without any further modification, however, allogeneic CAR T cells are likely to be quickly rejected and have insufficient time to eradicate leukemia. Therefore, at present, their use is limited to being a last-ditch effort to reduce MRD before HSCT. By contrast, autologous CAR T cells (or those derived from HSCT donors) have the potential to persist for years after infusion and, hence, exert a profound and durable anti-leukemic effect. It remains to be seen whether allogeneic CAR T cells can

be used only as a bridge to HSCT (or to autologous CAR T cells) or can represent a stand-alone treatment.

In sum, CAR T-cell therapy is an emerging new treatment option for patients with T-ALL/T-LL. Much work still needs to be done but the tremendous knowledge already gained with CAR T-cell therapy in B-cell malignancies will serve as a guide and hopefully accelerate progress in this area, which holds promise for cure in patients with an otherwise lethal disease.

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BLZO has no conflicts of interest to disclose. NV, DMHW, and DC receive royalties for patents related to the development of CAR

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Contributions

BLZO led the writing of the article with input from the other authors.

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