REVIEW SERIES

Chimeric antigen receptor T-cell therapy in childhood acute myeloid leukemia: how far are we from a clinical application?

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Abstract

Recurrent and/or refractory (R/R) pediatric acute myeloid leukemia (AML) remains a recalcitrant disease with poor outcomes. Cell therapy with genetically modified immune effector cells holds the promise to improve outcomes for R/R AML since it relies on cytotoxic mechanisms that are distinct from chemotherapeutic agents. While T cells expressing chimeric antigen receptors (CAR T cells) showed significant anti-AML activity in preclinical models, early phase clinical studies have demonstrated limited activity, irrespective of the targeted AML antigen. Lack of efficacy is most likely multifactorial, including: (i) a limited array of AML-specific targets and target antigen heterogeneity; (ii) the aggressive nature of R/R AML and heavy pretreatment of patients; (iii) T-cell product manufacturing, and (iv) limited expansion and persistence of the CAR T cells, which is in part driven by the immunosuppressive AML microenvironment. Here we review the results of early phase clinical studies with AML-specific CAR T cells, and avenues investigators are exploring to improve their effector function.

Introduction

The overall survival of pediatric patients with newly diagnosed acute myeloid leukemia. (AML) has improved over the last two decades with rates reported to be close to 70%.[1-4] However, the event-free survival of these patients has plateaued at about 55% despite the use of intensive therapies.[1-3] Furthermore, the outcome of children with relapsed and/or refractory (R/R) disease remains poor. Recent data from the Berlin Frankfurt Münster (BFM) group and Children's Oncology Group (COG) showed that the overall survival rates of pediatric patients with first relapse of AML were 42% and 35%, respectively.[4] For patients with second relapse of AML, the overall survival at 5 years remains dismal (15 ± 4%) based on BFM and COG data.[5] Similar outcomes were reported in a recent analysis by a Nordic-Dutch-Belgian-Spain-Hong-Kong-Israel-Portugal (NOPHO-DB-SHIP) consortium.[6]

This high rate of treatment failure has been attributed in part to the existence of chemotherapy-resistant leukemic stem cells, a minor fraction of leukemic cells that are capable of maintaining and re-initiating the disease.[7,8] While allogeneic hematopoietic stem cell transplant (HSCT) at present offers the highest probability of long-term sustained remission,[3] its success relies on several factors with remission status prior to HSCT being one of the most important prognostic factors.[9] However, it is challenging to re-induce remission in these patients with salvage chemotherapy due to toxicities and limited efficacy of standard or investigational therapies. Many patients are therefore unable to proceed to allogeneic HSCT.

Adoptive transfer of chimeric antigen receptor (CAR) T cells has emerged as a promising treatment option for pediatric patients with CD19-positive B-cell acute lymphoblastic leukemia.[10,11] CD19-CAR T cells have shown potent anti-leukemia activity in the R/R setting, leading to their approval by the Food and Drug Administration in 2017.[10-14] In contrast, the clinical experience with AML-specific CAR T cells is

limited with information on several studies only reported in abstract form. Overall, the clinical activity of AML-specific CAR T cells, irrespective of the targeted antigen, has been underwhelming and has shown variable complete response (CR) rates.[15-33] Importantly, early phase clinical and preclinical studies have highlighted several roadblocks (Figure 1), including: (i) a limited array of AML-specific targets and target antigen heterogeneity; (ii) the aggressive nature of R/R AML and heavy pretreatment of patients; (iii) T-cell product manufacturing; and (iv) limited expansion and persistence of the CAR T cells, which is in part driven by the immunosuppressive AML microenvironment.[18,34-39]
In this review we summarize published results of early phase clinical studies with AML-specific CAR T cells from centers around the globe, including the USA, Europe, Australia and China, presenting pediatric as well as adult studies. In the second part of the review, we then outline potential ways to overcome current roadblocks to effective CAR T-cell therapies for AML. This review is not meant to be comprehensive, and we refer the reader to other excellent reviews on CAR immune cell therapies for AML that have been published recently.[36,40-42] Likewise, due to space limitations, we will not review combinatorial approaches in which CAR T cells are combined with other treatment modalities.

Immunotherapy targets in acute myeloid leukemia

An ideal antigen for CAR T-cell therapy is a cell surface antigen that is highly expressed in all malignant cells but not expressed in healthy tissues. As for many other malignancies, such an ideal target remains elusive for AML. To date, CD33, CD123, and CLL1 (CLEC12A) have been the most frequently targeted AML antigens.[43-49] While these are overexpressed on the cell surface of AML blasts, they are also expressed on normal hematopoietic cells, raising

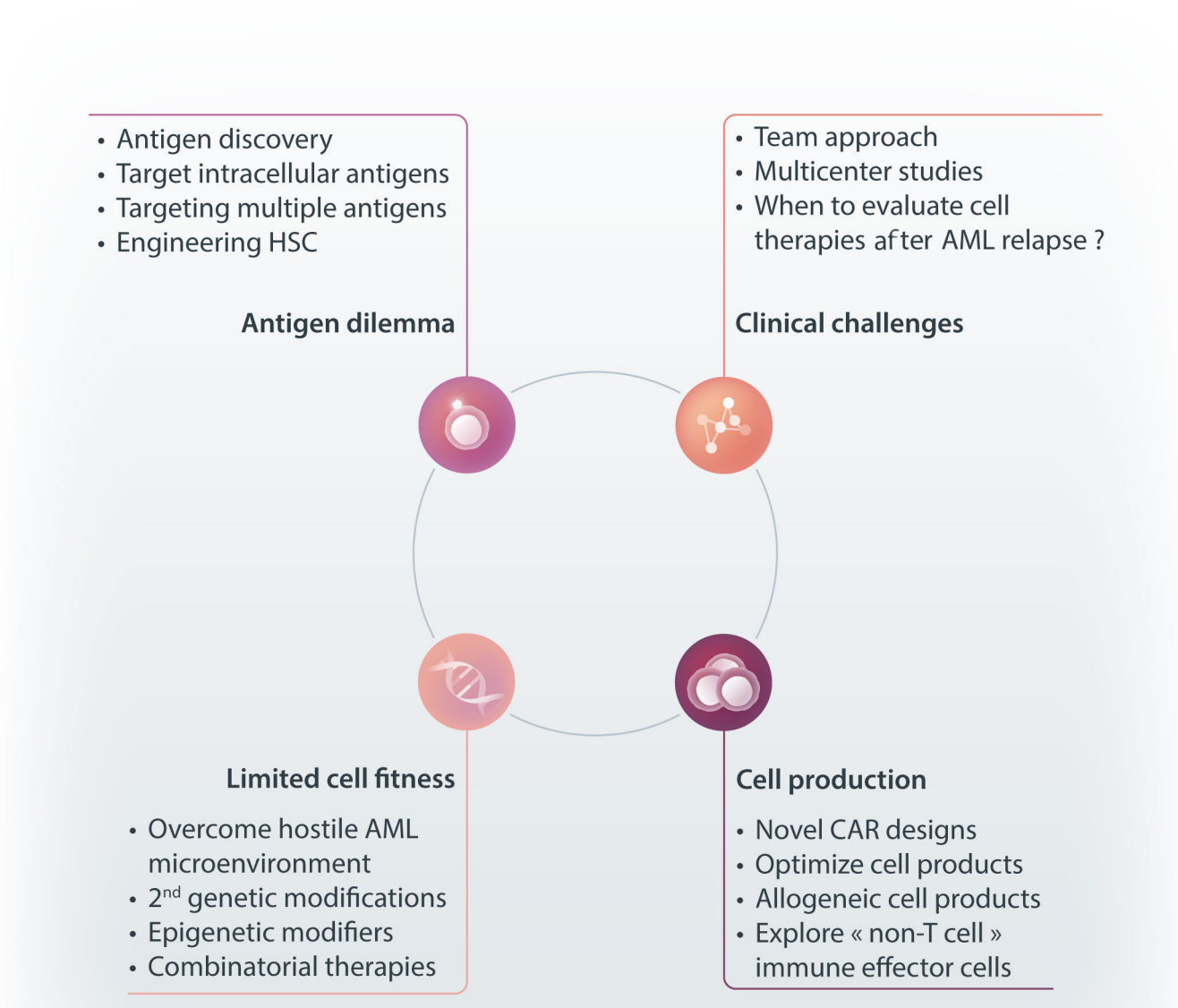

Figure 1. Strategies to overcome acute myeloid leukemia-specific chimeric antigen receptor T-cell therapy roadblocks. Roadblocks to acute myeloid leukemia (AML)-specific chimeric antigen receptor (CAR) T-cell therapy include: (i) a limited array of AML-specific targets and target antigen heterogeneity (the antigen dilemma); (ii) the aggressive nature of relapsed/refractory AML and the fact that patients are heavily pretreated (fragile) (clinical challenges); (iii) T-cell product manufacturing (cell production); and (iv) limited expansion and persistence (limited fitness) of the CAR T cells. Potential strategies to overcome each roadblock are highlighted. For additional details see the text. HSC: hematopoietic stem cells.

concerns about myelotoxicity, with CD33 and CD123 being pan-myeloid antigens that are also expressed on hematopoietic stem cells and CLL1 being expressed on mature myeloid cells, including mature granulocytes. Nevertheless, the safety of targeting CD33 and CD123 has been shown in the pediatric clinical setting with the use of gemtuzumab, a CD33-specific monoclonal antibody drug conjugate,[50,51] leading to its approval by the Food and Drug Administration, and flotetuzumab, a bispecific CD123xCD3 antibody.[52] Early phase clinical trials of CAR T cells targeting CD123, CD33 and CLL1, as described in the next section, have used either safety switches or adopted a bridge-to-transplant strategy to overcome this issue. Targets that are expressed on the cell surface of AML blasts and not on normal myeloid cells are also being explored, including NKG2D, CD70, and GRP78.[29,53-55] However, these are also associated with potential on-target/off-cancer toxicity, including being upregulated in normal cells under conditions of cellular stress (NKG2D, GRP78) or in subsets of activated immune cells (CD70). Other AML targets that have been or are being actively explored include CD38, CD44v6, CD117 (c-KIT) CD276 (B7-H3), CD327 (Siglec-6), FLT3, FRβ, GRP78, LILRB3, LILRB4, and TIM3.[56-65] While these promise to have limited myelotoxicity, this aspect needs to be carefully evaluated in preclinical studies. For example, CD38-CAR T cells had potent anti-AML activity and prolonged survival in xenograft models, but limited hematopoietic stem cell toxicity was observed, consistent with the pattern of CD38 expression.[56] Finally, a recent single-cell transcriptomic study of AML blasts discovered and validated CD86 and CSF-1R as AML-CAR T-cell therapy targets.[35]

Clinical experience with chimeric antigen receptor T cells for acute myeloid leukemia

In the following section we summarize results of clinical studies (Table 1) which have been either published or presented in abstract form. Data from the USA, UK, Europe, and China are included.

Lewis-Y-chimeric antigen receptor T cells

Lewis-Y (LeY) is an aberrantly glycosylated carbohydrate antigen that is overexpressed on AML blasts but has limited expression on normal tissues. While to our knowledge, LeY is no longer being pursued as an AML target, LeY-CAR T cells were the first CAR T-cell product that was evaluated in a phase I clinical study for AML (NCT03851146).[15] Four adult patients with AML received LeY.CD28.ζ-CAR T cells. No significant toxicities were reported, but only transient responses or stable disease were observed. However, this study provided valuable information since CAR T cells were labeled with indium enabling tracking by single-photon emission computed tomography, which demonstrated CAR T-cell trafficking to bone marrow and at extramedullary disease sites, highlighting the potential benefit of using CAR T-cell therapy for AML.

CD123-chimeric antigen receptor T cells

Autologous CD123-chimeric antigen receptor T cells

Investigators evaluated T cells that expressed a CD123.CD28.ζ-CAR and a truncated EGFR safety switch which were infused after lymphodepletion induced by fludarabine/cyclophosphamide (NCT02159495). From this study, the outcome of six patients with AML, who were infused with CD123-CAR T cells at either dose level 1 (DL1: $5x10^7$ CAR T cells) or dose level 2 (DL2: $2x10^8$ CAR T cells), have been reported.[16] Two patients achieved a CR and proceeded to allogeneic HSCT. Blood counts recovered in all patients following initial pancytopenia. Four patients showed either grade 1 or 2 cytokine release syndrome (CRS) but no dose-limiting toxicities were observed.

T cells that express CD123.CD28.ζ-CAR and a CD20 safety switch are actively being evaluated in a phase I clinical trial (NCT04318678) and preliminary results have been presented.[17] Following lymphodepletion with fludarabine/cyclophosphamide, two patients were infused at DL1 ($3x10^5$ CAR T cells/kg), and four patients at DL2 ($3x10^5$ CAR T cells/kg), with one patient being infused off protocol. Isolated fevers occurred after infusion but resolved within 24 hours, and no dose-limiting toxicities were observed. At DL2, one patient with extramedullary disease achieved a CR that lasted for 2 months; she then received a second infusion and again achieved a short-lived CR. One additional patient was infused at DL2 off protocol and achieved a morphological CR but with detectable low-level minimal residual disease (MRD).

To overcome the potential concern regarding prolonged myelosuppression and vascular toxicity, investigators have evaluated T cells that were transfected with messenger RNA encoding a CD123.41BB.ζ-CAR, which results in transient CAR expression in adoptively transferred T cells (NCT02623582). Five patients received one to three serial infusions of CD123-CAR T cells following lymphodepleting chemotherapy. All five patients had grade ≥2 CRS, but the study was terminated as no patient had a clinical response. However, the lack of significant vascular toxicity prompted the development of a follow-up study utilizing a lentiviral vector to generate CD123-CAR T cells (NCT03766126).[18,19]

Another unique approach to overcome the risk of myelosuppression has been the use of switchable CAR. The universal CAR platform (UniCAR) is a two-component, second-generation CD28.ζ-based CAR T-cell platform in which an adapter molecule, targeting module (TM), confers specificity against the cancer antigen of choice.[66] The targeting module has a short half-life of less than 30 minutes, enabling a rapid switch-off. Ten patients have been treated on a phase I trial with UniCAR-T cells directed at

Table 1. Selected published acute myeloid leukemia-specific chimeric antigen receptor T-cell therapy clinical studies.

Target	Trial ID	Product	LD	Peds/Adult	N of patients	Comments	Ref
LeY	NCT03851146	Auto CD28.ζ CAR T cells	Flu	Adult	4	Transient responses, SPECT was used to track infused cells	15
CD123	NCT02159495	Auto CD28.ζ CAR T cells	Flu/Cy	N/A	32*	6 patients reported 2 CR CRS grade 1 to 2 (N=4)	16
	NCT04318678	Auto CD28.ζ CAR T cells	Flu/Cy	Peds	7	1 CR (EMD only), 1 CR (MRD^+), isolated fevers after infusion	17
	NCT02623582	Auto 41BB.ζ CAR T cells**	Flu/Cy	Adult	5	≥ Grade 2 CRS in all patients	18,19
	NCT03766126	Auto 41BB.ζ CAR T cells	Flu/Cy	Adult	12*	Outcome not reported	
	NCT04230265	Auto CD28.ζ CAR T cells with adapter molecule	Flu/Cy	Adult	10	1 CR, 2 CRi, 4 PR	20, 21
	NCT04106076	Allo 41BB. ζ CAR T cells (KO TRAC, CD52)	Flu/Cy +/- anti-CD52	Adult	16	2 SD, 1 MLFS, 1 MRD^- CR, grade 1 to 2 CRS (N=12), grade 4 CRS (N=2), grade 5 CRS (N=1), grade 3 ICANS (N=1)	22
CD33	NCT03126864	Auto 41BB.ζ CAR T cells	Flu/Cy	Adult	10	Only 3 infused, NR, grade 2 CRS (N=1), grade 3 CRS (N=1), grade 2 ICANS (N=1)	23
	NCT01864902	Auto 41BB.ζ CAR T cells	None	Adult	1	CRS (not graded), transient response	24
	NCT03971799	Auto CD28.ζ CAR T cells	Flu/Cy	Peds/ Adult	19	CRS (N=13), CR (N=2)	68
CLL1	NCT03222674	Auto 41BB.ζ CAR T cells	Flu/Cy***	Peds	8	5 MLFS (4 MRD^-, 1 MRD^+), 1 CRi (MRD^+), 1 PR, 1 SD grade 1-2 (all patients)	25
		CD28.CD27.ζ CAR T cells (iC9I	Flu/Cy	Peds	4	3 CR (MRD^-) ≤ grade 2 CRS (N=3), ≤ grade 2 ICANS (N=1)	26
		Auto 41BB.ζ CAR T cells or CD28.CD27.ζ CAR T cells (iC9)	Flu/Cy	Peds	7	5 CR (3 MRD^-, 2 MRD^+) ≤ grade 2 CRS, grade 2 ICANS (n=1)	27
	ChiCTR2000041054	Auto 41BB.ζ CAR T cells	Flu/Cy	Adult	10	7 CR/CRi, grade 2 (N=4) and grade 3 (N=3) CRS, CRES (N=1)	28
NKG2D	NCT02203825	Auto ζ CAR T cells	None	Adult	7	NR, safe	29
	NCT03018405	Auto ζ CAR T cells	None	Adult	16	3 infusions at 2-week intervals 3 of 12 evaluable patients: CR/ CRi (2 AML, 1 MDS) grade 3 or 4 CRS (N=5)	30, 31
CLL1 and CD33	NCT03795779	Auto# costim?.ζ CAR T cells	Flu/Cy	Peds/ Adult	9	7 CR/CRi (MRD^-), grade 1-3 CRS (N=8), grade 1-3 neurotoxicity (N=4)	32, 33

*Actual enrollment according to the clinicaltrials.gov webpage; unclear how many patients were infused. **The only product that was generated by mRNA transfection; all other utilized viral vectors. ***Seven out of eight patients received fludarabine/cyclophosphamide; #One patient received a chimeric antigen receptor T-cell product from a matched sibling donor. ID: identity; LD: lymphodepletion; Peds: pediatric population; N: number; Ref: reference; auto: autologous; CAR: chimeric antigen receptor; Flu: fludarabine; SPECT: single photon emission computed tomography scanning; Cy: cyclophosphamide; N/A: data not available; CR: complete response; CRS: cytokine release syndrome; EMD: extramedullary disease; MRD: measurable residual disease; CRi: complete response with incomplete hematologic recovery; allo: allogeneic; PR: partial response; KO: knock out; TRAC: T-cell receptor α constant; SD: stable disease; MLFS: morphological leukemia-free state; ICANS: immune effector cell-associated neurotoxicity syndrome; CRES: T cell-related encephalopathy syndrome; NR: no response; iC9: inducible caspase 9; AML: acute myeloid leukemia; MDS: myelodysplastic syndrome; costim?: co-stimulatory domain not disclosed.

the CD123 antigen (NCT04230265).[20,21] Following lymphodepletion, patients received continuous intravenous infusion of a CD123 targeting module (TM123; 0.5 to 1 mg/day) from days 0 to 24. CD123-directed UniCAR-T-cell therapy was safe and tolerable with limited toxicities. Encouraging anti-AML activity was observed, including one CR, two CR with incomplete hematologic recovery (CRi), and four partial responses (PR).

Allogeneic CD123-chimeric antigen receptor T cells

To overcome the concern about T-cell fitness and in an effort to enhance persistence, allogeneic CD123-CAR T cells have been evaluated, including an off-the-shelf, 'universal' CD123-CAR T-cell product, which is generated by transduction with a lentiviral vector encoding a CD123.41BB.ζ-CAR and gene edited with transcription activator-like effector nuclease to disrupt the T-cell receptor α constant gene locus to minimize risk of graft-*versus*-host disease and the CD52 locus to enable the use of alemtuzumab (anti-CD52) as part of the lymphodepletion regimen (NCT04106076).[22] Sixteen patients received off-the-shelf CD123-CAR T-cell infusions with half undergoing lymphodepletion with fludarabine/cyclophosphamide and the other half receiving fludarabine/cyclophosphamide plus alemtuzumab. Four patients experienced dose-limiting toxicities including grade 4 CRS (n=2), grade 5 CRS (n=1), and grade 3 immune effector cell-associated neurotoxicity syndrome (ICANS) (n=1) irrespective of the lymphodepletion used. Low-grade (<3) CRS occurred in 12 patients. Adding alemtuzumab to fludarabine/cyclophosphamide was associated with improved lymphodepletion and significantly higher CAR T-cell expansion and persistence. Four patients had a response, including two cases of stable disease, one morphological leukemia-free state, and one durable MRD-negative CR. In addition to this study, a case report of a single patient given donor-derived CD123-CAR T cells as part of a reduced intensity conditioning regimen prior to HSCT has been published.[67]

CD33-chimeric antigen receptor T cells

Investigators evaluated the feasibility and safety of autologous T cells modified to express a CD33.41BB.ζ-CAR and truncated human epidermal growth factor receptor (HER1t) (NCT03126864).[23] Ten adults with R/R AML were enrolled; T cells were collected by apheresis from eight, although only four T-cell products were successfully manufactured, and three patients were infused at the lowest dose level of $0.3x10^6$ cells/kg. There were no dose-limiting toxicities; two patients had CRS (grades 2 and 3) and one had ICANS (grade 2). Although CD33-CAR T cells were detected in blood samples from patients following infusion, with associated symptoms and increased cytokine levels, no anti-leukemic responses were seen. One case report on the use of CD33.41BB.ζ-CAR T cells for R/R AML described transient clinical activity but also pancytopenia.[24] Finally, there is an ongoing multicenter study, being conducted through the Pediatric Transplantation and Cellular Therapy Consortium, evaluating the safety and efficacy of autologous CD33.CD28.ζ-CAR T cells in pediatric patients with R/R AML (NCT03971799). Interim results from this study were presented at the American Society of Hematology annual meeting in 2023. Nineteen of the 24 patients enrolled were infused with CD33 CAR T cells. CRS was early in onset and was reported in 13 (68%) patients, being high grade (≥3) in four (21%) of these patients. Complete remission was seen at DL4, with two patients achieving MRD-negative CR. Dose escalation is currently underway in the phase II portion of the study.[68]

CLL1-chimeric antigen receptor T cells

CLL1.41BB.ζ-CAR T-cell products have been evaluated in a phase I clinical trial in eight pediatric patients with AML.[25] The children were given $0.35\text{-}1x10^6$ CAR T cells/kg as a single dose after fludarabine/cyclophosphamide lymphodepleting chemotherapy. All developed grade 1-2 CRS and pancytopenia following the infusion. Five patients achieved a morphological leukemia-free state, being MRD-negative in four cases and MRD-positive in one case, one patient had an MRD-positive CRi, one patient had a PR, and one patient has stable disease. In a second report, four pediatric patients received T cells expressing CLL1.CD28.CD27.ζ-CAR and an inducible caspase 9 suicide gene after lymphodepleting chemotherapy. Infusions were well tolerated with only low-grade CRS (≤2) and/or ICANS (≤2). Three patients achieved an MRD-negative CR.[26] The investigators subsequently compared autologous T cells expressing CLL1.CD28.CD27.ζ-CAR and the inducible caspase 9 suicide gene (n=4) or CLL1.41BB.ζ-CAR (n=3) in seven children with R/R AML following lymphodepleting chemotherapy.[27] The CAR T-cell infusions were well tolerated and only grade ≤2 CRS and one grade 2 ICANS were observed. Five patients achieved CR (3 negative and 2 positive for MRD) with a 1-year overall survival rate of 57.1%. Due to the small number of patients, no significant differences were noted between the two CAR T-cell products.

Finally, CLL1.41BB.ζ-CAR T cells have been evaluated in ten adults with R/R AML.[28] Patients received fludarabine/cyclophosphamide lymphodepleting chemotherapy followed by an infusion of a single dose of $1\text{-}2x10^6$ CAR T cells/kg. All patients developed CRS, including grade 2 (n=4) and grade 3 (n=3) cases, and one patient developed T cell-related encephalopathy syndrome. Severe pancytopenia occurred in all patients, and two patients died of severe infection due to chronic agranulocytosis. The CR/CRi rate was 70% and, at a median follow-up time of 173 days, six patients were alive.

NKG2D ligand-chimeric antigen receptor T cells

NKG2D ligand (NKG2D-L)-positive tumor cells can be targeted with human leukocyte antigen (HLA) killer immu-

noglobulin-like receptor mismatched natural killer (NK) cells.[69] Another approach relies on expressing a NKG2D-L-CAR which consists of NKG2D directly linked to the CD3ζ chain.[70] NKG2D-L-CAR T cells were infused without prior lymphodepletion to alleviate concerns that chemotherapy might induce NKG2D-L expression on healthy tissues.[29] Seven patients with NKG2D-L-positive R/R AML received NKG2D-L-CAR T cells in four escalating dose levels ($1x10^6$ to $3x10^7$ total viable T cells). No dose-limiting toxicities were reported, but no objective responses were noted either. It is likely that the low cell dose without antecedent chemotherapy played a role in limited expansion and persistence of CAR T cells, resulting in limited anti-AML activity.

Building on these initial results and preclinical data supporting the use of multiple NKG2D-L-CAR T-cell infusions, a follow-up study was conducted (NCT03018405).[30,31] This multicenter study enrolled 25 patients of whom 16 were infused at three dose levels, DL1: $3x10^8$, DL2: $1x10^9$, and DL3: $3x10^9$ T cells per infusion. Patients received three infusions at 2-week intervals followed by potential consolidation treatment consisting of three additional infusions. Of the infused patients, one had myelodysplastic syndrome, three had multiple myeloma, and 12 had R/R AML. Five patients across all dose levels had grade 3 or 4 CRS, and one dose-limiting toxicity was observed in a patient on DL3. Three of 12 evaluable patients with R/R AML (n=11) or myelodysplastic syndrome (n=1) had CR/CRi. Among responders, two patients with R/R AML proceeded to allogeneic HSCT with durable ongoing remissions (at 5 and 61 months).

Multi-antigen-specific chimeric antigen receptor T cells

In addition to monospecific CAR T-cell products, multi-antigen-specific CAR T cells are starting to be evaluated in early phase clinical studies.[32,33] Nine patients received escalating doses ($1\text{-}3x10^6$/kg) of CAR T cells expressing CLL-1- and CD33-CAR as single or split doses following lymphodepletion with fludarabine/cyclophosphamide. The T-cell products were autologous in eight cases and from an HLA-matched sibling donor in one case. Toxicities included grade 4 pancytopenia (n=9), grade 1-3 CRS (n=8), and grade 1-3 neurotoxicity (n=4). Seven of the nine patients achieved a CR/CRi (MRD negative by flow cytometry), while two had no response (the AML blasts of one of the non-responders expressed only CD33). Six of the seven patients who achieved MRD-negative CR proceeded to a subsequent HSCT.

The prospect of generating effective chimeric antigen receptor T-cell products for acute myeloid leukemia

Based on the available clinical experience with CAR T cells for AML, there is a long road ahead before these cells can be applied in the routine clinical setting akin to CD19-CAR T cells. The timeline until CAR T-cell therapy for AML achieves similar effectiveness as CD19-CAR T cells for B-cell acute lymphoblastic leukemia remains undefined. The current status of the field of AML-CAR immune cell therapy is reminiscent of the early days of HSCT in the 1960s, when outcomes were poor and many experts believed that this approach would never become part of our treatment armamentarium for patients with hematologic malignancies.[71] Current preclinical and clinical studies have identified formidable roadblocks that center around: (i) which antigen(s) to target and heterogenous antigen expression (the antigen dilemma); (ii) limited T-cell effector function through intrinsic and extrinsic mechanisms; and (iii) the ideal starting cell source for generating AML-specific T-cell products. In the following we summarize current efforts to overcome these roadblocks,, which are also illustrated in Figure 1.

Roadblock #1: the antigen dilemma

A major obstacle to AML-specific CAR T-cell therapies is the identification of target antigens that are expressed on leukemic blasts but are absent or expressed at low levels on normal myeloid cells and/or other healthy tissues.[34,35,72,73] Moreover, AML blasts and leukemic stem cells are highly heterogeneous cell populations and more than one antigen may need to be targeted to increase antitumor activity.[7] While target antigen discovery is ongoing, we believe that targeting a single antigen is highly unlikely to achieve outstanding cure rates, i.e., we will not suddenly discover a CD19-equivalent pan-AML target that is consistently expressed with an acceptable on-target/off-cancer toxicity profile. We will likely have to develop CAR T-cell products directed at certain AML subsets. The majority of CAR-targeting approaches have relied on targeting cell surface molecules. However several studies have highlighted that CAR with an antigen recognition domain which recognize a peptide in the context of an HLA molecule can target intracellular proteins in cancer cells, including AML.[74-76] While this approach increases the repertoire of targetable antigens it is HLA-restricted and therefore raises feasibility concerns for small populations unless we revolutionize cell production to enable the generation of patient-specific genetically modified T-cell products in a cost-effective and timely manner.

In addition to antigen discovery, investigators have focused on developing dual targeting strategies for AML.[34,72,77,78] This approach has the potential not only to mitigate the risk of antigen loss variants, but also specificity since CAR immune cells will only be fully activated in the presence of both antigens. For example, a multiomic approach systematically analyzed potential AML target antigen combinations,[34] and based on these data an AML-specific T-cell product was developed that expresses an ADGRE2-CAR and a CLEC12A-chimeric co-stimulatory receptor (CCR).[78] Other CAR/CCR combinations that are being explored include

CD123-CAR/CD33-CCR and CLEC12A-CAR/TIM3-CCR.[79,80] Other studies have suggested that targeting either CD33 and TIM3 or CLL1 and TIM3 has the potential to overcome heterogenous antigen expression of AML blasts.[72] Finally, dual targeting of CD123 and GRP78 is being explored as an approach to prevent immune escape.[81] In this regard, utilizing NK cells as immune cells to express CAR may be beneficial due to their inherent ability to target NKG2D-L-positive tumor cells (see the section on NKG2D-L CAR T cells). Other efforts are focused on limiting on-target/off-cancer toxicities, including myelotoxicity, by deleting AML target antigens, including CD33, from hematopoietic stem cells or by using base editing to delete a specific epitope of an AML target in the hematopoietic stem cells (e.g. CD45).[82,83] Finally, investigators have focused on fine-tuning antigen recognition and logical gating approaches to drive the specificity of CAR T cells.[84-88]

Roadblock #2: limited effector function through T-cell intrinsic and extrinsic mechanisms

Intrinsic and extrinsic mechanisms contribute to the limited effector function of CAR T cells for cancers including AML. The immunosuppressive AML microenvironment presents a key extrinsic mechanism, which has been recently reviewed, and we defer the interested reader to these publications.[37-39] Clearly, the microenvironment needs to be studied in pediatric as well as adult AML since these are distinct disease entities.[89] Several strategies have been evaluated to enhance the effector function of CAR T cells in preclinical models focusing on genetic engineering.[90,91] While many of these have not been directly evaluated in AML models, they could potentially also enhance the anti-AML activity of CAR T cells. Conceptually, additional genetic modifications of CAR T cells can be divided into two approaches. One approach is focused on identifying and deleting negative regulators of immune cell function, and the other approach relies on transgenic expression of molecules to enhance their function. The former strategy includes targeting molecules that play a role in (i) limiting T cell activation,[92,93] and (ii) regulating T-cell fate through epigenetic mechanisms.[94-98] The latter approach includes expressing (i) transcription factors,[99] (ii) secretory or membrane-bound cytokines,[100-104] (iii) chimeric cytokine receptors that are either constitutively active or ligand-activated,[105-107] (iv) chimeric switch receptors that convert a T-cell inhibitory signal into an activating signal,[108,109] or (v) co-stimulatory receptors.[110,111] Specific examples for each approach are listed in Table 2. Some of these approaches are actively being explored for CAR immune cell therapies for solid tumors, and it is unclear which approach will have the desired outcome of achieving long-term CR. It is likely that several strategies will have to be combined to generate effective AML-specific CAR T cells, for example improving T-cell activation and manipulating epigenetic pathways. In addition, other factors need to be considered. For example, pro-inflammatory cytokines have been reported to promote leukemogenesis, which would make the transgenic expression of cytokines in AML-specific immune cells less desirable.[112]

Roadblock #3: acute myeloid leukemia-specific immune cell production

The optimal CAR design, CAR immune cell production, as well as the donor source of AML-specific CAR immune cells remain unknown. The original CAR design celebrates its 30th anniversary this year,[113,114] and is still being used by the majority of synthetic T-cell receptors that are currently being evaluated in clinical studies, including those on AML. It has a cytoplasmic signaling domain that consists of a signaling domain derived from one or two co-stimulatory molecules and an activation domain derived from the CD3ζ chain.[115] Several preclinical studies have highlighted that adding a signaling domain from cytokine receptors or replacing CD3ζ with ZAP70 improves CAR functionality.[85,116] Likewise, adding a domain to enhance recruitment of intracellular signaling molecules has been shown to improve the effector function of CAR T and NK cells.[117] Novel synthetic T-cell receptors have also been developed, including T-cell antigen couplers (TAC),[118] T-cell receptor fusion constructs (TRuC),[119] HLA-independent T-cell receptors (HIT),[120] and synthetic T-cell receptor and antigen receptors (STAR),[121] which endow T cells with enhanced effector function in comparison to CAR T cells. Finally, modifying T cells with a bispecific T-cell engager is another approach to generate

Table 2. Selected approaches to enhance chimeric antigen receptor T-cell function.

Approach	Biological pathway	Example	Ref
Deleting negative regulators	Molecules that limit immune cell activation	RASA2, Regnase-1	92, 93
	Molecules that regulate immune cell fate through epigenetic mechanisms	DNMT3A, TET2, Suv39h1	94-98
Transgenic expression	Transcription factors	cJun	99
	Secretory or membrane-bound cytokines	IL12, IL15, mIL15, IL18	100-104
	Chimeric cytokine receptors (constitutively active, ligand-activated)	C7R, orthogonal IL-2Rβ	105-107
	Chimeric switch receptors	IL4/2R, IL4/7R	108, 109
	Co-stimulatory receptors	Chimeric antigen receptor without ζ activation domain	110, 111

T cells that recognize AML blasts.[122] While it will be impossible to compare all these different approaches in clinical studies, revisiting which genetic engineering approach is optimal to generate tumor-specific immune cells for AML in preclinical models seems advisable, given their limited clinical activity thus far.

Currently, most investigators use the same CAR T-cell-product manufacturing approach irrespective of the targeted disease. At least for autologous products it is likely that cytokine requirements might differ to generate CAR T cells with optimal antitumor activity, since for example T cells from patients with AML or acute lymphoblastic leukemia have been exposed to very different classes of chemotherapeutic agents, and the leukemia microenvironments differ. To our knowledge, there are no published reports of studies investigating the effects of different cytokine cocktails or other culture media supplements on the anti-AML activity of autologous CAR T cells. Integrative bulk- and single-cell sequencing approaches have been used to characterize CD19-CAR T-cell products in detail and track infused T cells at a clonal level.[123,124] We are hopeful that insights gained from these types of studies conducted on autologous AML-specific CAR T-cell products will direct the development of a manufacturing process to optimize their effector function in the future.

What is the ideal donor source of immune cells to develop AML-specific immune cell therapies? This of course depends on the type of immune cells. For example, while autologous CAR T cells can be generated from AML patients, this might be less than ideal since the effector function of autologous T cells is compromised by previous exposure to chemotherapeutic agents.[125,126] Likewise, it presents a logistical challenge since patients must stop their AML-targeted therapies for 1 to 2 weeks (or even longer) before T cells can be collected for manufacturing.

These challenges could be overcome by using allogeneic donors. These include the previous HSCT donor for patients who relapse after a transplant or off-the-shelf CAR T-cell products from healthy donors. While generating CAR T-cell products from the previous HSCT donor requires no additional genetic modifications, off-the-shelf CAR T-cell products require additional modifications to prevent graft-*versus*-host disease and/or allorejection.[127,128] In addition, allogeneic NK cells, including cytokine-induced memory NK cells and CAR NK cells, are actively being explored as a source of immune effector cells for the immunotherapy of AML.[76,129-131]

Conclusions

We believe that developing safe and effective CAR T-cell therapy for AML is not a pipe dream. However, there are formidable challenges that have to be addressed, including targeting multiple antigens to prevent immune escape and on-target/off-cancer toxicities, and to overcome the immunosuppressive AML microenvironment. Likewise, T-cell production as well as evaluating these therapies initially in heavily pretreated patients poses significant challenges. Clearly a concerted team effort will be required in which basic/translational science and clinical investigators from multiple centers work together to achieve this goal. We hope that this review will stimulate discussions on how to reach the summit of effective cell therapies for AML.

Disclosures

SG and MPV are co-inventors of products with patents or patent applications in the fields of T-cell and gene therapy for cancer. SG is a member of the Scientific Advisory Board of Be Biopharma and CARGO, and the Data and Safety Monitoring Board (DSMB) of Immatics and has received honoraria from TESSA Therapeutics within the last year.

Contributions

SN, MPV, and SG co-wrote the manuscript.

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