Genome-Edited T Cell Therapies

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Key Points

- Genome editing can help address allogeneic barriers for 'off-the-shelf' T cell therapies against cancer
- First clinical applications of genome edited T cells have provided preliminary safety and efficacy data
- Further applications are addressing 'fratricide' effects and manipulating checkpoint pathways for enhanced cellular immunotherapy and wider applications

Synopsis

Chimeric antigen receptor (CAR) T-cells are widely being investigated against malignancies, and allogeneic 'universal donor' CAR-T cells offer the possibility of widened access to pre-manufactured, off-the-shelf therapies. Different genome-editing platforms have been used to address human leukocyte antigen (HLA) barriers to generate universal CAR-T cell therapy and early applications have been reported in children and adults against B cell malignancies. Recently developed Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-based systems and related technologies offer the prospect of enhanced cellular immunotherapies for a wider range of hematological malignancies.

Introduction

Genome editing offers the prospect of enhanced gene therapy and new therapeutic avenues beyond those envisaged by conventional 'gene-addition' strategies. The breakthrough innovation of genome editing is the ability to alter cellular DNA at very specific sites for precise and advantageous therapeutic effects.

Early emerging tools to target specific genome sequences shared a common mechanism of action: a variety of engineered DNA nuclease platforms allow recognition of specific DNA loci and can

generate double strand brakes (DSBs) that, in combination with the endogenous DNA repair pathways, result in permanent disruption of targeted genes (**Figure 1**). For over a decade, genome editing platforms including Zinc Finger Nucleases (ZNF) (1), Transcription Activator-Like Effector Nucleases (TALENs) (2) Meganucleases (MNs) (3) and mega-TALENs (4) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-based systems, have been investigated for their potential in cellular therapies. Structural differences among these tools include the modality of target site recognition, either by customized DNA-binding proteins (for ZFNs, MNs and TALENs) or single guide RNA (sgRNA for CRISPR-based systems), and the cleavage modules that generate precise DNA DSBs (Fok-1 for ZFNs/TALENs or bacterial-derived Caspase9 for CRISPR). (**Table 1**). Early designs of modular zinc-fingers proteins that could recognize multiple DNA triplets provided initial proof-of-concept that specific DNA sites could be edited in mammalian cells. Increased specificity of DNA sequence recognition was obtained with TALE–DNA binding repeats delivering more flexibility than triplet-limited zinc-finger domains. Limitations of these strategies included difficulties in targeting multiple sites and laborious development and validation pathways. More recently RNA-guided engineered nucleases based on CRISPR/Cas9 or derivatives comprising alternative Cas or guide systems (such as Cas12 and Clover-Cas) have provided more cost-effective and efficient genome-editing avenues and promise simpler bench to bedside development and clinical phase testing (5-7). Similar to previous iterations of genome editing platforms, initial applications aimed to disrupt the expression of one or more cell surface proteins through non-homologous end joining (NHEJ) at sites of targeted DNA breakage, but recently more complex strategies have included delivery of therapeutic transgene DNA templates through site specific integration by homologous recombination (HR)(8). Further improvements, including precise nucleotide conversion using base-editing is also being advanced for multiplexed editing (9) and investigations into modifications using prime-editing are also well underway (10).

T cells are attractive targets for such emerging technologies, with fewer hurdles and risks than following ex-vivo manipulation of hematopoietic stem cells (11), or the immunological and biodistribution challenges of direct in vivo approaches (12-14). T cells are easily accessible from peripheral blood, resist transformation and can be readily manipulated and cryopreserved. They are receptive to viral and non-viral engineering, and the efficiency of the latter has been notably refined with the availability of improved electroporation devices and stabilized RNA.

Proof of concept studies that collected T cells for ex vivo culture ahead of adoptive cell therapy for cancer have been underway since the early 1980's (15) and included trials of tumor-infiltrating lymphocytes (TILs), anticipating that the triggered cytotoxic activity could be exploited to tackle certain cancers (16). Following initial experiences and emergence of gene transfer technologies, development of adoptive therapies employing recombinant TCR (rTCR)(17) or chimeric antigen receptor (CAR) (18) emerged as targeted immunotherapy for cancer. Autologous CAR-T cell therapies have led the way and have been amongst the first to be authorized as marketable advanced therapies and are now being developed worldwide (19). As genome editing has become available, applications for "next generation" adoptive T cell therapies based on TILs, rTCR and CAR-T cells have aimed to improve anti-tumor activity (8), persistence (20), safety and accessibility (21).

In particular, genome-edited allogeneic "universal" donor CAR T cells represent an attractive strategy to guarantee rapid availability of pre-manufactured cells for use in multiple recipients, thereby reducing costs and widening accessibility (22). T cells from healthy donors may exhibit superior fitness compared to patient derived T cells, especially after multiple cycles of chemotherapy or recent stem cell transplantation (23). Premanufactured CAR-T cells can be stored in cell banks, with minimal discrepancies in product specification across different batches, and be readily available, avoiding treatment delays in patients with aggressive disease (**Figure 2**). Furthermore, healthy donor cells address the risk of product contamination with unwanted leukemic blasts that could be inadvertently transduced and refractory to CAR effects (24). Genome editing of T cells has also been applied to reduce exhaustion and promote persistence by targeting checkpoint pathways such as PD1, and strategies are in development to allow targeting of other hematological lineage malignancies (9) and to tackle hurdles for cellular immunotherapy against solid tumors.

Addressing HLA Barriers for 'universal' CAR T cell therapy against B cell malignancies

HLA mismatched T cells operating in a hostile environment are prone to rejection following host immune system recognition of non-self HLA molecules and may themselves be triggered through their antigen specific TCRαβ to cause graft versus host disease (GVHD). To overcome these obstacles, T cell manipulation through genome editing has been implemented to prevent TCRαβ

expression, and strategies to remove HLA molecules or render cells insensitive to lymphodepletion drugs are being investigated (**Figure 3**).

Autologous CAR-T cell therapies for CD19 expressing malignancies became rapidly accessible over the past 5 years, and as "real-life" data are being collected, the optimal strategies for a higher chance of success are being established. For instance, it is now clear that CAR-T cell therapies are more effective when used after preparative lymphodepletion regimens, most commonly comprising fludarabine and cyclophosphamide (25). This strategy appears to favor homeostatic expansion of infused cells, perhaps by reducing competition of cytokines and growth factors, and possibly by disrupting constraints in immunological niches and regulatory pathways (25, 26). In the allogeneic HLA-mismatched setting, lymphodepletion scheme must also sufficiently inhibit host immunity to prevent rapid rejection of allogeneic T cells by host immune system. Patients can be screened for pre-existing anti-HLA antibodies against incoming cells, but host cellular immunity mediated by T and NK cells requires additional intervention. In addition to chemotherapy, anti-CD52 monoclonal antibody (alemtuzumab) has been used in the allogeneic setting to deplete host cellular immunity and allow CAR T cells edited to remove CD52 an in vivo survival advantage after infusion (27). Suppression of host NK and T cells for 2-3 weeks could be sufficient to allow incoming engineered T cells to mediate potent anti-leukemic effects, and alemtuzumab may also dampen macrophage activity linked to cytokine release phenomena during CAR activity. In 2015, two infants with relapsed B-cell acute lymphoblastic leukemia (B-ALL) received single dose infusions of UCART19 cells knocked out using TALENs for TRAC and CD52 and achieved molecular remissions allowing them to proceed to allo-SCT (27). Subsequent multi-center trials in children and adults encountered only mild GvHD and manageable CAR related side effects such as cytokine release syndrome (CRS) and neurotoxicity, though the consequences of deeper lymphodepletion in the allogeneic setting in terms of cytopenia and viral infections was noted (21). UCART19 cells are under further investigation in adults with non-Hodgkin lymphoma (NHL) (28), and next-generation universal cells disrupted for TCR/CD52 using CRISPR/Cas9 were generated using a lentiviral system that couples TCR knockout and CAR expression and are currently used in study in children with refractory/relapsed B-ALL who failed or are ineligible to an autologous approach (29, 30). Additional data from CRISPR-edited CAR with disruption of TRAC and CD52 have been reported in 6 adults with B-ALL that received an "universal" bi-specific CD19/CD22 (CTA101)(31). CD22 has also been targeted as a single

antigen in r/r B-ALL in adults as a single target by Cellectis using TALENs to disrupt TRAC and CD52 (32).

Another strategy to address host mediated immune rejection involves disruption of HLA molecules: targeting of HLA class I by editing β2-microglobulin chain (B_2M), a conserved domain across all class I molecules, has been readily achievable. The strategy aims to prevent recognition of mismatched donor HLA class I by host $CD8^+$ T cells and thereby avoid direct cytotoxic effects. Additional manipulations have been proposed to address the issue of host NK cell triggering by 'missing self' mechanisms (33). Addressing interactions between host $CD4^+$ T cells and HLA class II molecules on activated donor CAR T cells has been investigated through disruption of CIITA, a critical transcriptional regulator of HLA class II expression in preclinical studies (34). In the clinic, CRISPR Therapeutics have reported preliminary data of a trial where universal donor CAR19 T cells, incorporating a CAR19 cassette integrated into the TRAC locus using adeno-associated virus (AAV), were also modified at B_2M locus using CRISPR/Cas9 for class I immunological stealth. Previously, animal studies have suggested that endogenous control of CAR gene mediated by TCR transcriptional machinery at the TRAC locus may provide improved cytotoxic activity and reduce exhaustion (8). The study treated adults with relapsed refractory diffuse large B-cell lymphoma and preliminary data from 26 patients were communicated in 2021, reporting 38% remission rate, no GvHD, mild CRS occurring in 50% of patients, and one case of severe neurotoxicity attributed to viral reactivation.

Others are also testing site specific transgene integration. Precision Biosciences have used a similar AAV delivery system combined with a proprietary endonuclease platform again to direct a CAR transgene expression cassette integration into the TRAC locus, although with an internal promoter rather than using TRAC transcriptional machinery (3). Interim reports of 13 NHL patients treated showed an encouraging safety profile and overall complete response rate of 54%, with indicators favoring more intense lymphodepletion (35, 36).

Finally, as an alternative to viral delivery, Poseida biotech is developing non-viral PiggyBac transposon platform for CAR expression and are targeting BCMA in multiple myeloma, to be combined with genome editing of TRAC and B_2M with Cas-Clover. The latter comprises inactivated Cas9 fused with dimerization dependent Clo51 endonuclease and requires two RNA guides and potentially offers enhanced editing specificity (37). Whether disruption of HLA class

I is sufficient to evade immune rejection, or if additional removal of class II antigens may also be required is an important issue still to be addressed in human studies.

Although crucial questions remain unanswered, data from the above-mentioned clinical trials investigating genome edited CAR-T cells in B cell malignancies (summarized in **Table 2**) will contribute to optimize strategies for universal allogeneic approaches.

Engineered T cells for non-B lineage hematological malignancies

Two major reasons why CD19 is an ideal target for engineered T cells against B-cell malignancies are the consistently high expression across a variety of B cell malignancies and the limited risk of "on-target/off-tumor" toxicity other than protracted B cell aplasia. Long term hypogammaglobulinemia might persist for years after treatment (38) and can be managed with immunoglobulin replacement therapy if required. However, a similar T-cell therapy strategy against T cell malignancies has to accommodate fratricide effects between T cells during manufacturing, and immunodeficiency caused by protracted T-cell aplasia mediated by engineered effector T cells in vivo. Suitable T-cell antigens include the TCR $\alpha\beta$/CD3 complex, CD5, and CD7 and the issue of fratricide during manufacture has been addressed by protein inhibition strategies as well as genome editing. For example, anti-CD7 CAR T cells have been generated following expression of inhibitory proteins to restrict CD7 surface expression (39). Trials are underway in Singapore (NCT05043571) and in China (NCT04689659), where a cohort of patients infused with CAR7 T cells derived from their hematopoietic stem cell or alternate donors achieved remission in 15/20 patients, albeit with relatively high incidence of GVHD and lymphopenia (40). Genome editing with TALENs against TRAC has been shown to inhibit expression of the multimeric TCRαβ/CD3 complex on T cell surface ahead of lentiviral transduction of an anti-CD3ε CAR transgene. CAR3 expression was followed by 'self-enrichment' of engineered T cells during culture to yield an anti-T cell product devoid of TCR (and less able to mediate GVHD) but with potent anti-T cell immunity, both in vitro and in humanized mice in vivo (41). Similarly, CRISPR/Cas9 editing of CD7 in combination with TRAC knockout, has produced 'universal' donor CAR7 T cells (42), and an allogeneic donor strategy may offer a "bridge to transplant", for rapid donor-derived T cell recovery once remission is secured. Similar to B-cell malignancies, an allogenic approach also avoids the risk of unintended transduction of leukemic blasts during

manufacture (24). Preclinical development and comparison of CRISPR/Cas9 and base edited CAR7 T cells (9) found that efficient multiplexed disruption was achieved with both platforms, although low level of translocation events associated with Cas9 nuclease activity were virtually undetectable in base edited CAR7 T cells, suggesting advantages in terms of transformation risk. First-in-human application of anti-CD7 CAR T cell therapies are emerging, with encouraging remissions in the small number of individuals with refractory T-cell malignancies treated to date. Multiplexed CD7 and TRAC edited T cell trials using CRISPR/Cas9 in China have already reported remissions with manageable toxicities (43), and a trial of multiplexed base edited CAR7 T cells is planned in the UK (**Table 3**).

CAR-T cells redirected against myeloid targets in acute myeloid leukemia (AML), have also been constrained by the risk of "on-target/off-tumor" consequences against the normal hematopoietic compartment given that common myeloid markers such as CD33 and CD123 are shared between myeloid derived blasts and healthy progenitors. Approaches to remove target antigen expression from healthy hematopoietic progenitors using CRISPR/Cas9 have been developed (44) although have not yet been tested in clinic. Alternatively, genome editing is being applied to generate 'universal' allogeneic CAR-T cells against such antigens, as a prelude to allo-SCT and donor derived reconstitution once remission is achieved. For example, UCART123 are TALEN edited donor T cells with anti-CD123 CAR that have been investigated against adult acute myeloid leukemia (NCT03190278) and blastic plasmacytoid dendritic cell leukemia (NCT03203369), with toxicities reported early in Phase 1 testing (45). A clinical study of base edited anti-CD33 and anti-CD123 universal CAR T cells against paediatric AML plans to use combinations of CAR T cells to address the issue of disease escape when single antigens are targeted (**Table 3**).

Addressing T cell exhaustion and persistence

Promoting persistence and addressing exhaustion is widely being explored through manipulation of checkpoint pathways. PD-1-PDL-1 pathway represents an attractive target due to its role in inhibition of T cell activation, proliferation, and survival within inhibitory tumor microenvironments (46). Anti- PD-1 and PD-L1 antibodies (e.g., nivolumab, pembrolizumab) have been approved for a variety of cancers (47) and there has been extensive interest in using genome editing to disrupt expression in T cells (48-50). The first human application of CRISPR/Cas9 involved editing autologous TILs at the PDCD1 locus (encoding PD-1) in adults

with refractory lung cancer, and there were no immediate or short-term toxicities uncovered (51). A similar engineered T cell therapy, manufactured with multiplex genome editing of TRAC, TRBC, and PDCD1 loci and lentiviral transduction of a synthetic cancer-specific rTCR against NY-ESO-1 has been used in adults with advanced cancer (49). In both these reports, extended persistence of engineered T cells was attributed to PD-1 disruption, although no definitive conclusions could be drawn for efficacy. A similar approach has been applied to allogeneic CAR19 cells for treatment of B-cell NHL by Caribou Biosciences: the ANTLER trial is investigating the safety of CB-010, PD-1 disrupted allogeneic CAR19 T cells, manufactured using proprietary CRISPR hybrid RNA-DNA guides (chRDNA) in combination with Cas12a for high efficiency gene insertions of template delivered using AAV (52). As discussed above, as PD-1 has been reported to act as haplo-insufficient immune suppressor of T-cell lymphomagenesis (53), one concern of using PD-1 genome edited T cells is the potential risk of malignant T cell transformation. Although the above-mentioned reports appear reassuring to date, further monitoring will be required to better evaluate such risks and benefits.

Safety and long-term monitoring

Experience with gamma-retroviral and lentiviral transduction for gene-addition to hematopoietic stem cells for the correction of inherited monogenic disorders has uncovered transformation risks following vector mediated manipulation (54-57). Description of transactivation effects from enhancer elements in retroviral long terminal repeats almost twenty years ago led to the development of self-inactivating configurations, advancement of lentiviral systems and long-term monitoring plans for all patients receiving gene therapies. Genetic manipulation of human T cells using retroviral vectors has not been linked to transformation (58), although recent concerns emerged from new-onset CAR-T lymphoma in two patients following modification of T cells using piggyBac transposons for the expression of CAR19 with mechanisms yet to be defined (59). Clonal dominance after lentiviral transduction of T cells has been described in two subjects after CAR19 (60) and CAR22 (61) therapy and attributed to specific integration sites. In the context of genome editing, a trial of TALEN edited CAR19 T cells in r/r B cell lymphoma was temporarily placed on hold while regulators in the US considered possible translocation related adverse effects (62). Pre-clinical studies and early clinical applications of genome-edited T cells quantified

frequencies of translocation between chromosomes using both TALEN and CRISPR/Cas9 technology, with up to 5% abnormal karyotypes observed for the former ahead of trial applications (27, 49). In other circumstances studies with CRISPR/Cas9 in T cells have previously indicated T cell transformation could arise through disruption of PD1 checkpoint pathways (53). Alternative genome editing strategies using base editing for precise genetic modification and single base conversion may address some concerns. Cytidine deamination mediated based editing has demonstrated that translocations can be virtually eliminated in T cells in comparison to CRISPR/Cas9 (9, 63). Nevertheless, there remains the possibility of unpredictable genome-wide off-target activity (64, 65) warranting careful monitoring of patients as the technology reaches clinical application.

Summary

The first licensed gene modified T cell products represent a beginning for new opportunities to exploit the immune system to fight cancer. Emerging genome-editing applications represent efforts to extend novel therapies including pre-manufactured 'off the shelf' CAR-T cell banks that might contribute to reducing costs while widening accessibility. While the potential of the technology is rapidly evolving, watchful and continuous monitoring for longer terms effects will be part of the investigational landscape. Clinical trial observations will contribute to defining optimal strategies, including the degree of immunosuppression required, duration and persistence for anti-leukemic effects, and the role of hematopoietic stem cell transplantation to consolidate responses.

Clinical Care Points

- Genome edited T cells are being investigated for cancer immunotherapy and could help extend the application of CAR-T cell therapy.
- Hurdles of allogeneic CAR-T cells (rejection and allo-reactivity) are being addressed using different preparative strategies and cell engineering platforms
- Long-term monitoring of safety and outcomes will be essential as treatments roll out

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Figure Legends

Figure 1. Platforms for precise genome editing have already moved into clinic and include ZFN, TALEN and CRISPR/Cas. A common strategy to deliver on-target alteration of DNA sequences relies on the generation of DNA double strands breaks. Endogenous repair systems in the cell, such as non-homologous end-joining repairing generate insertions or deletions within the target sequence and disrupt expression. Alternatively, homologous repair can be activated, and homology flanked DNA sequences might be recruited by DNA repair machinery to incorporate a transgene into a specific locus.

Abbreviation: gRNA= guide RNA; HDR= homologous direct repair; NHEJ= non-homologous end-joining.

Figure 2. Manufacturing outline of autologous and allogeneic CAR-T cells. After collection of autologous peripheral blood lymphocytes from a patient at a clinic or harvest center, the cells are usually transported to a centralized manufacturing site. T cells are engineered in a clean room facility, using compliant reagents and validated processes. CAR T cells are subsequently cryopreserved before release for shipping to the healthcare provider. The cells are infused back to the patient after they have received lymphodepletion. On the other hand, healthy donor PBLs are collected from a volunteer to generate allogeneic CAR-T cells and one donation can generate dozens of doses or CAR T cells for multiple recipients. Additional genome editing strategies are used to address HLA barriers and "universal" CAR-T cells are stored frozen in multi-dose cell banks.

Figure 3. Strategies for generation of CAR-T cells using genome-editing platforms. CAR: chimeric antigen receptor; CD52: cluster domain-52; MHC: major histocompatibility complex; PD-1: programmed cell death-1; TCR: T cell receptor.

Tables

Table 1. Characteristics of widely investigated strategies for genome editing and examples of investigated clinical application.

	ZFN	TALEN	CRISPR/Cas9	Base-editor
Advantages	Specific editing Limited off-target effect	Highly specific and more versatile editing	Adaptable design and efficient editing of multiple targets Low costs	Precise base conversion without double strand breaks Low risk of translocations Highly efficient
Limitations	Difficult to design for multiple editing Risk of translocations	High costs Large size Risk of translocations	DNA off-target effects Risk of translocations Possible immunogenicity from non-human components	By-stander conversions and off-target effects on DNA or RNA Possible immunogenicity from non-human components
Example of human application for T cell engineering	CCR5 ko in T cells to prevent HIV entry (66)	TCR and CD52 ko For universal CAR T (27)	PD1 ko for lung cancer TILs TCR and PD1 ko in rTCR engineered cells for cancer (49, 51)	Anti CD7 universal CAR T cells (pending)

Table 2. Selected active and/or enrolling clinical trials of "universal" genome edited T cell against B cell malignancies with early clinical data available.
Abbreviations: CRS= cytokine release syndrome; ICANS= immune-effector cell-associated neurotoxicity syndrome; LV= lentivirus; AAV= adeno-associated virus; N/A= not available

Sponsor/Study	Product	Indication	Target edits	Platforms	Patients (n)	Safety	Reference
Servier/Allogene NCT02808442 NCT02746952	UCART19	r/r CD19+ B - ALL	TRAC CD52	TALEN LV	Children (7) and adults (14)	Grade 3+ CRS 15%; Grade 3+ infections 39%	(21)
Allogene NCT04416984 NCT03939026	ALLO-501A ALLO-501	r/r large B cell CD19+ lymphoma	TRAC CD52	TALEN LV	Adults (47)	Grade 3+ CRS: 2%; Grade 3+ infections 24%	(28, 67, 68)
Cellectis NCT04150497	UCART22	r/r CD22+ B-ALL	TRAC CD52	TALEN LV	Adults (9)	No Grade 3+ infections/CRS/ICANS	(32, 69)
Precision Bio NCT03666000	PBCAR0191	r/r CD19+ B cell malignancy	TRAC	Homing endonuclease AAV	Adults (21)	Grade 3+ CRS 6%; Grade 3+ infections 31%	(36)
CRISPR Therapeutic NCT04035434	CTX110	r/r CD19+ B cell malignancy	TRAC B2M	CRISPR/Cas9 AAV	Adults (26)	No Grade 3+ CRS; Grade 3+ infections 9%; Grade 3+ ICANS 4%	(8, 70); sponsor communications
Great Ormond Street Hospital NCT04557436	TT52CAR19	r/r CD19+ B - ALL	TRAC CD52	CRISPR/Cas9 LV	Children (2)	No Grade 3+ CRS/ICANS; Grade 3+ infections 2/2	(29)
Nanjing Bioheng Biotech Co. NCT04227015	CTA101	CD19+CD22+ B cell malignancy	TRAC CD52	CRISPR/Cas9 LV	Adults (6)	Grade 3+ CRS 17%; Grade 3+ infections 50%	(31)

Poseida NCT04960579	P-BCMAAllo1	r/r Multiple myeloma	TRAC B2m	Cas-Clover PiggyBac transposon	Adults	N/A	(37)
Caribou Biosciences NCT04637763	CB-010	CD19+ r/r B cell NHL	TRAC PDCD1	chRDNA/Cas12a AAV	Adults	N/A	(52)

Table 3. Selected clinical trials investigating allogeneic CAR-T cells for T-ALL and AML.

Sponsor/Country/Trial ref.	Indication	Target	Platform	Reference
Wugen, USA NCT04984356	$CD7^+$ T-ALL	CD7 TRAC	CRISPR/Cas9	(71, 72)
Gracell Bio, China NCT04264078	$CD7^+$ T-ALL	CD7 TRAC	CRISPR/Cas9	(43)
Nanjing Bioheng Biotech Co., China	$CD7^+$ T-ALL	CD7 TRAC HLA-II	CRISPR/Cas9	(73)
Great Ormond Street Hospital, UK (Opening in 2022)	$CD7^+$, $CD33^+$, $CD123^+$ AML/T-ALL	CD7 TRBC CD52	Base editor	(9)
Yake Bio, China NCT04599556	$CD7^+$ T-ALL	CD7	Protein expression blockers (PEBLs)	(40)
Cellectis, USA NCT03190278	$CD123^+$ AML	TRAC CD52	TALEN	(45)