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► **To cite this version:**

Marius Maucher, Micha Srour, S. Danhof, H. Einsele, M. Hudecek, et al.. Current Limitations and Perspectives of Chimeric Antigen Receptor-T-Cells in Acute Myeloid Leukemia.. *Cancers*, 2021, *Cancers*, 13, 10.3390/cancers13246157 . hal-04481460

HAL Id: hal-04481460

<https://hal.univ-lille.fr/hal-04481460v1>

Submitted on 28 Feb 2024

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Review

Current Limitations and Perspectives of Chimeric Antigen Receptor-T-Cells in Acute Myeloid Leukemia

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Simple Summary: Acute myeloid leukemia (AML) is the most frequent type of acute leukemia in adults. Allogeneic hematopoietic cell transplantation (allo-HCT) has been the only potentially curative treatment for the majority of patients. The ability of chimeric antigen receptor (CAR)-modified T-cell therapy directed against the CD19 antigen to induce durable remissions in patients with acute lymphoblastic leukemia (ALL) has provided optimism that this novel treatment paradigm can be extrapolated to AML. In this review, we provide an overview of candidate target antigens for CAR-T-cells in AML, an update on recent progress in preclinical and clinical development of investigational CAR-T-cell products, and discuss challenges for the clinical implementation of CAR-T-cell therapy in AML.



Citation: Maucher, M.; Srour, M.; Danhof, S.; Einsele, H.; Hudecek, M.; Yakoub-Agha, I. Current Limitations and Perspectives of Chimeric Antigen Receptor-T-Cells in Acute Myeloid Leukemia. *Cancers* **2021**, *13*, 6157. <https://doi.org/10.3390/cancers13246157>

Academic Editor: Christian Récher

Received: 7 October 2021

Accepted: 25 November 2021

Published: 7 December 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Abstract: Adoptive transfer of gene-engineered chimeric antigen receptor (CAR)-T-cells has emerged as a powerful immunotherapy for combating hematologic cancers. Several target antigens that are prevalently expressed on AML cells have undergone evaluation in preclinical CAR-T-cell testing. Attributes of an 'ideal' target antigen for CAR-T-cell therapy in AML include high-level expression on leukemic blasts and leukemic stem cells (LSCs), and absence on healthy tissues, normal hematopoietic stem and progenitor cells (HSPCs). In contrast to other blood cancer types, where CAR-T therapies are being similarly studied, only a rather small number of AML patients has received CAR-T-cell treatment in clinical trials, resulting in limited clinical experience for this therapeutic approach in AML. For curative AML treatment, abrogation of bulk blasts and LSCs is mandatory with the need for hematopoietic recovery after CAR-T administration. Herein, we provide a critical review of the current pipeline of candidate target antigens and corresponding CAR-T-cell products in AML, assess challenges for clinical translation and implementation in routine clinical practice, as well as perspectives for overcoming them.

Keywords: AML; CAR-T-cell; hematology; gene therapy; adoptive cell therapy



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1. Introduction

Acute myeloid leukemia (AML) is a clonal proliferative neoplasm usually characterized by bone marrow, blood, and other tissue infiltration. Current AML cure rates range from 35% to 40% and 5% to 15% in patients over 60 years and up to and including 60 years of age, respectively [1]. The prognosis depends on the molecular and cytogenetic features approved by the 2016 revision of the WHO classification of myeloid neoplasm and acute leukemia [2] and the ELN recommendation [3]. Treatment is determined according to the risk stratification based on the WHO classification. Intensive induction therapy remains the backbone of therapy in younger patients, while hypomethylating agents are

recommended in older patients [4]. For the last three decades, allogeneic hematopoietic cell transplantation (allo-HCT) has been the first immune-based therapy and only potentially curative approach widely used in intermediate and high-risk AML. Despite considerable improvement in the conditioning regimen, HLA donor selection, and stem cell sourcing, non-relapse mortality remains a major concern. Because allo-HCT is restricted to a subset of relatively young patients with a low comorbidity index and good performance status [5–7], new tolerable and effective therapeutic approaches are needed.

Over the past two decades, AML and other hematological malignancies have become targets for the developing field of immune therapy, such as antibody-based therapeutics, dendritic cell vaccines, TCR-T-cell therapy, and gene-engineered chimeric antigen receptor (CAR)-T-cells, coupled with conventional chemotherapy and molecular targeted therapy [8–11]. Following the discovery of immune inhibitory mechanisms and cancer-related antigens on leukemic cells, immune approaches are evolving constantly.

2. Emerging CAR-T-Cell Therapy in AML

Adoptive transfer of gene-engineered CAR-T-cells has emerged as a powerful arm of immunotherapy to combat hematologic cancers [12–14]. These artificial receptors are genetic hybrids of antibody and T-cell receptor (TCR) domains that are composed of an extracellular binding unit, typically a single-chain variable fragment (scFv) at the N-terminus connected to spacer or hinge domains, a transmembrane domain, and an intracellular signal module at the C-terminus [12,15]. The principle behind CARs is to elicit a cellular anti-tumor response through an artificial receptor that acquires specificity for distinct tumor-associated antigens (TAAs). We currently distinguish between at least four generations of CARs. First-generation CARs only have the CD3 ζ domain as intracellular compound, whereas second-generation CARs are built from the CD3 ζ and a co-stimulatory domain such as 4-1BB or CD28 [16,17]. Adding a co-stimulatory domain improved efficacy, expansion, and persistence of CAR-T-cells [18,19]. Third-generation CARs carry two co-stimulatory domains in the same receptor, e.g., 4-1BB and CD28, while fourth-generation CAR-T-cells consist of one antigen-specific receptor with the addition of other transgenes that enhance anti-tumor function [20–22]. All CAR-engineered cell products are designed to eliminate tumor cells upon cell surface target antigen recognition [12]. In the case of CAR-T-cells, an anti-tumor memory that will prevent relapse of antigen-positive cancer cells may be established [23].

The growing evidence of T-cell anti-leukemic responses derived from healthy donors in the context of allo-HCT has resulted in leukemic and lymphoid malignancies becoming a focus of advanced research into T-cell based immunotherapy [24]. This research has rapidly established CD19 as a key target antigen for B-cell lymphomas and leukemias. This has led to the drug approval of blinatumomab and other bispecific biologicals engaging CD19 and T-cells, as well as the approval of autologous CD19-directed CAR-T-cell products [25,26]. Four CAR-T-cell products targeting CD19 and one targeting B-cell maturation antigen (BCMA) have been approved by the FDA: tisagenlecleucel (Kymriah[®], Novartis, Basel, Switzerland), axicabtagene ciloleucel (Yescarta[®], Gilead, Foster City, CA, USA), brexucabtagene autoleucel (Tecartus[®], Gilead), lisocabtagene maraleucel (Breyanzi[®], Bristol-Myers Squibb, New York, NY, USA), and idecabtagene vicleucel (Abecma[®], Bristol-Myers Squibb) [27–29].

A customized ‘living drug’, autologous CAR-T-cell production is a sophisticated process demanding careful and precise manufacturing and logistics. As a first step, leukocytes of cancer patients are obtained by leukapheresis. Subsequent isolation of T-cells is performed depending on the specific T-cell product and the manufacturer [30,31]. Enriched T-cells are activated, typically via anti-CD3/CD28 magnetic beads or antibody mixtures, and transduced with retroviral or lentiviral vectors to stably express the CAR [12,15,32] and expanded with cytokine supplementation. All commercial CAR-T-cells are delivered to the treatment center as frozen products, thawed and infused into the patients as a single

dose. Prior lymphodepletion is generally foreseen in treatment protocols as CAR-T-cell expansion after injection is markedly promoted [33].

Ongoing research is seeking to extend CAR-T treatment to other cancer entities, such as solid tumors, while rendering CAR-T-cell manufacture safer and more time- and cost-efficient. Because lenti- and retroviral vectors are expensive to produce and demand careful preparation and application, virus-free gene transfer methods have clear advantages [34,35]. Transposon-transposase gene delivery platforms, e.g., the Sleeping Beauty transposon system, are increasingly popular in preclinical CAR-T manufacturing and have been successfully tested in clinical trials (NCT04499339 [33,34,36]). Targeted genome-editing tools, first and foremost CRISPR-Cas9, offer additional possibilities for advanced CAR-T-cell engineering [37–39].

One of the main challenges in clinical CAR-T-cell usage is the appearance of cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) [12,23,40]. Recent therapeutic advances have been made by interfering with the CRS ‘lead’ cytokines IL-1, IL-6 and GM-CSF [41–43], in developing so-called STOP-CARs [44], and by successful administration of the tyrosine kinase inhibitor Dasatinib as a pharmacological CAR-T-cell on/off-switch [45].

3. AML Target Antigens under Investigation

The development of CAR-T-cell therapy in AML depends on the identification of suitable target antigens. Though several candidate antigens have been evaluated in preclinical experiments, few of them have been found to satisfy all attributes of a ‘perfect’ AML antigen, i.e., high-level expression on leukemic blasts and leukemic stem cells (LSCs) and absence on healthy normal hematopoietic stem and progenitor cells. In addition, the use of different cut-off and threshold values for flow cytometric analyses results in a controversy over when to define AML cells as target antigen positive [46,47]. In contrast to other blood cancer types where CAR-T-cell therapies are being studied, only a small number of AML patients has received CAR-T treatment in clinical trials, resulting in limited clinical experience for this therapeutic approach in AML (Table 1). In the following sections, we give an overview of AML target antigens under current investigation (Figure 1).

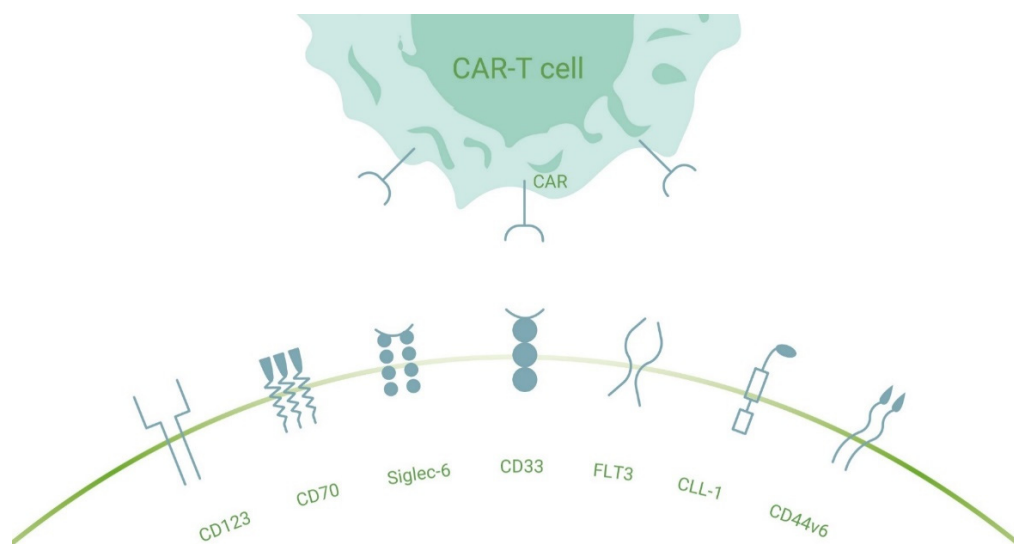


Figure 1. Selection of investigated surface antigen molecules for CAR-T-cell therapy in AML. Depicted membrane proteins on AML blasts or LSCs are recognized by their matching chimeric antigen receptors on gene-engineered T-cells. Abbreviations: CD123: the alpha subunit of the interleukin IL-3 receptor; CD70: the physiological ligand of CD27; CD33: a member of sialic acid-binding immunoglobulin-like lectins (Siglecs); Siglec-6: a member of CD33 (Siglec-3)-related protein superfamily; FLT3: FMS-like tyrosine kinase 3; CLL-1: C-type lectin like molecule-1; CD44v6: a ubiquitous glycoprotein that enables cell adhesion and cell-cell contacts.

Table 1. Main current active or recruiting clinical trials with CAR-T-cell therapies for AML.

Status	Interventions	Phase	NCT Number	Locations
Recruiting	FLT3 CAR-T cells	Early Phase 1	NCT04803929	Zhejiang Provincial People's Hospital, China
Recruiting	CLL-1 CAR-T cells	Phase 1	NCT04219163	Texas Children's Hospital, Texas, United States
Recruiting	CD123/CLL1 CAR-T cells	Phase 2 Phase 3	NCT03631576	Fujian Medical University Union Hospital, China
Recruiting	CD19 CAR-T cells	Phase 2 Phase 3	NCT04257175	Chaim Sheba Medical Center, Israel
Recruiting	CLL-1, CD33 and/or CD123 CAR-T cells	Phase 1 Phase 2	NCT04010877	Shenzhen Geno-immune Medical Institute, China
Recruiting	CLL1 CAR-T cells	Phase 1 Phase 2	NCT04884984	The First Affiliated Hospital of Soochow University, China
Recruiting	CD38 CAR-T cells	Phase 1 Phase 2	NCT04351022	The First Affiliated Hospital of Soochow University, China
Recruiting	CD33, CD38, CD56, CD123, CD117, CD133, CD34 and/or MUC-1 CAR-T cells	n/a	NCT03473457	Southern Medical University Zhujiang Hospital, China
Recruiting	CD33 CAR-T cells	Phase 1	NCT04835519	Beijing Boren Hospital, China
Recruiting	CD33 CAR-T cells	Phase 1 Phase 2	NCT03971799	Children's Hospital of Los Angeles, California, United States [and others]
Recruiting	CD123 CAR-T cells	Phase 1	NCT04014881	Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, China
Recruiting	CD19 CAR-T cells	Phase 1 Phase 2	NCT03896854	The First Affiliated Hospital of Soochow University, China
Recruiting	B7-H3 CAR-T cells	n/a	NCT04692948	Anhui Provincial Hospital, China
Recruiting	CD44v6 CAR-T cells	Phase 1 Phase 2	NCT04097301	IRCCS San Raffaele, Italy
Recruiting	CD123 CAR-T cells	Phase 1	NCT04318678	IRCCS Ospedale Pediatrico Bambino Gesù, Italy
Recruiting	Allogeneic CD123 CAR-T cells	Phase 1	NCT03190278	St. Jude Children's Hospital, Tennessee, United States
Recruiting	CD123 CAR-T cells	Phase 1 Phase 2	NCT04272125	University of California, San Francisco (UCSF)-Helen Diller Family Comprehensive Cancer Center, California, United States [and others]
Recruiting	Allogeneic CD19 CAR- $\gamma\delta$ T cells	n/a	NCT04796441	Chongqing University Cancer Hospital, China
Recruiting	CLL1 CAR-T cells	Early Phase 1	NCT04923919	Hebei yanda Ludaopei Hospital, China No.212 Daguang Road, Xishan District, China
Recruiting	CD123 CAR-T cells	Phase 1 Phase 2	NCT04265963	920th Hospital of Joint Logistics Support Force, China
Recruiting	CLL-1 CAR-T cells	Phase 1	NCT04789408	Washington University School of Medicine, Missouri, United States [and other]
Recruiting	CD19, CD20, CD22, CD10, CD33, CD38, CD56, CD117, CD123, CD34, or MUC-1 CAR-T cells +/- peptide-specific DCs	Phase 1	NCT03291444	Zhujiang Hospital, Southern Medical University, China
Recruiting	CD123 CAR-T cells (autologous vs allogeneic)	Phase 1	NCT02159495	City of Hope Medical Center, California, United States
Recruiting	CD7 CAR-T cells	Phase 1 Phase 2	NCT04762485	The First Affiliated Hospital of Soochow University, China
Active, not recruiting	CD123 CAR-T cells	Phase 1	NCT03766126	University of Pennsylvania, Pennsylvania, United States
Recruiting	CD123 CAR-T cells	Phase 1	NCT04678336	Children's Hospital of Philadelphia, Pennsylvania, United States
Recruiting	CD123 UniCAR-T cells	Phase 1	NCT04230265	Universitätsklinikum Ulm, Germany [and others]
Recruiting	CD7 CAR-T cells	Phase 1 Phase 2	NCT04033302	Shenzhen Geno-immune Medical Institute, China
Recruiting	NKG2D Ligands CAR-T cells	Phase 1	NCT04167696	Mayo Clinic Cancer Center, Florida, United States [and others]

3.1. Lewis Y Antigen

The first antigen that was targeted with CAR-T-cells in AML was the Lewis Y antigen, as reported by Ritchie et al. in 2013 [48]. Though the patient with the deepest partial remission relapsed, this study provided proof-of-concept and spurred the quest for additional targets and improved CAR-T-cell products in AML.

3.2. CD123

CD123 is the alpha subunit of the interleukin IL-3 receptor, enabling proliferation and survival of AML cells by high-affinity stem cell factor (SCF) binding. Because it is a receptor of normal hematopoietic lineage formation and renewal, complete abrogation of CD123 positive cells is undesirable [49]. Almost every AML patient counts positive for this cell surface molecule with sometimes even higher expression in LSCs than in blasts [46,50]. Several groups developed CD123-specific CAR-T-cells and showed efficacy in *in vivo* models, including against AML blasts with relatively low CD123 expression [51–54]. These promising outcomes are countered by reports of *in vitro* toxicity screens utilizing colony forming unit assays that demonstrate significantly hindered differentiation of healthy hematopoietic stem cells when co-cultured with CD123-specific CAR-T-cells [51,55]. Similarly, CD123-specific scFvs inhibited hematopoiesis in preclinical *in vivo* models [56].

CD123 CAR-T-cells have been administered in completed and ongoing clinical trials (Table 1). Despite severe toxicity observed in most study participants, complete remission was achieved in at least three patients in two studies [57–59]. Though CD123 may be an efficient target for CAR-T-cell therapy in AML, enhanced technologies such as logic gating and dual-antigen targeting are desirable to reduce toxicity and increase tolerability.

3.3. CD33

CD33 is a member of the sialic acid-binding immunoglobulin-like lectin (Siglec) superfamily. It is primarily expressed on myeloid cell surfaces [60]. Although monocytes and mature granulocytes show the highest expression, CD33 is detectable on normal hematopoietic stem and progenitor cells (HSPCs) [46,61,62], rendering CD33-directed CAR-T-cells, similar to CD123, toxic to healthy bone marrow cells. It is important to note that CD33 is prevalent on the surface of AML cells in greater than 96% of adult patients, with higher expression levels on blasts compared to LSCs [46,63]. While complete tumor eradication remained difficult to achieve, including in animal models [61,64], preclinical results with CD33-specific CAR-T-cells targeting AML cell lines and primary patient samples raised hope for successful clinical translation [65–68]. Though the first in-human study proved transient reduction of CD33-positive blasts in one patient and a subsequent clinical trial demonstrated complete remission of one study participant, severe but reversible adverse events were reported in one patient, including induced rigorous chills and fevers, drastic fluctuations of preexisting pancytopenia, elevated serum cytokine levels, *i.e.*, interleukin (IL)-6, IL-8, tumor necrosis factor- α , and interferon- γ ; slight transient hyperbilirubinemia within two weeks, a subsequent intermittent moderate fever, and reversed fluctuation of the pancytopenia [69]. In contrast, CD33-CAR NK-92 cells have been safely infused in three AML patients eliciting short response in two patients [70]. Considering the ablation of healthy HSPCs, CD33- and CD123- directed CAR-T therapy is therefore most likely best implemented as a bridging therapy between primary chemotherapy and allogeneic hematopoietic cell transplantation (allo-HCT).

3.4. CD44v6

CD44 is a ubiquitous glycoprotein that enables cell adhesion and cell-cell contacts through which it seems to play a role in cancer initiation [71]. Because the isoform variant 6 (CD44v6) is expressed in 64% of AML cells, but absent in HSPCs [72], HSPCs have been spared from CAR-T-cells that target CD44v6 in preclinical models [73]. The first clinical experience with low doses of CD44v6 CAR-T-cells demonstrated a favorable toxicity profile (personal communication C. Traversari, Table 1).

3.5. FLT3

FMS-like tyrosine kinase 3 (FLT3) is a key player in normal hematopoiesis and is therefore expressed on HSCs, myeloid, and lymphoid immune cells [74–76]. Through tumor growth promotion, FLT3 is uniformly present in AML blasts [74,76,77]. In many AML cases, FLT3 is mutated such that its intracellular kinase domain is constitutively active. Most prominent aberrations are internal tandem duplications (ITDs) in juxta membrane domain as well as tyrosine kinase domain mutations [74,78,79]. Specifically targeting FLT3-mutated AML patient cells with FLT3-specific CAR-T-cells should carry a low risk for FLT3 antigen loss as the mutated FLT3 represents an AML ‘driver mutation’ [80]. FLT3 CAR-T-cell efficacy could be enhanced synergistically both in vitro and in vivo by co-administration of crenolanib, a specific type-I-inhibitor that targets the active FLT3 kinase conformation [80,81]. Because normal hematopoiesis is hampered by FLT3 CAR-T therapy, an adoptive therapy with FLT3 CAR-T-cells will most likely require incorporation into a treatment algorithm with subsequent CAR-T-cell depletion and allo-HCT. Under these circumstances, carefully conducted clinical trials are warranted, especially for high-risk FLT-ITD+ AML patients. Three clinical trials have been initiated with CAR-T-cells against FLT3 in AML (NCT05023707, NCT03904069, NCT05017883).

3.6. CD70

CD70 is the physiological ligand of CD27. It belongs to the tumor necrosis factor superfamily and occurs as type II transmembrane glycoprotein that can be shed off to soluble ligand form [82,83]. Monocytes and regulatory T-cells show the highest expression of CD70, facilitating expansion of cytolytic, mostly virus-specific CD8+ T-cells. In addition to overexpression in multiple solid tumors, i.e., renal cell carcinoma, CD70 overexpression has been described for various lymphoid and myeloid cancers, including AML. More than 85% of AML patient samples were CD70-positive with both expression on blasts and LSCs [84,85]. A recent pre-clinical study reported that CD70-directed CAR-T-cells are efficient against AML cells, thereby sparing normal HSCs. Virus-specific T-cells were also recognized and eliminated, constituting an on-target off-tumor toxicity to be considered [86]. Because some healthy tissues, e.g., endothelial cells, also express CD70, careful monitoring for on-target off-tumor reactivity is warranted. Human ProteinAtlas: <https://www.proteinatlas.org/ENSG00000125726-CD70> (accessed on 24 November 2021).

3.7. Siglec-6

Siglec-6 is a member of the CD33 (Siglec-3)-related protein superfamily, resembling CD33 molecule composition and structure [87–89]. Because Siglec-6 contains intracellular immunoreceptor tyrosine-based inhibition motifs (ITIMs), it is considered a negative activation regulator in immune cells [87–89]. Its expression pattern is highly restricted to a few healthy cell types and tissues, comprising B-cells, mast cells, basophilic granulocytes, and placenta [88,90–92]. In malignant cells, Siglec-6 expression has been described for mucosa-associated lymphoid tissue lymphoma, thyroid cancer, CLL, and clonal mast cell diseases [88,93–95]. A recent study reported absence of Siglec-6 expression on normal HSPCs and other healthy blood and solid tissue cells, and demonstrated anti-leukemic efficacy of engineered T-cells with a Siglec-6-specific CAR against AML cells in vitro and in vivo [55]. Although Siglec-6 expression varies among AML patients, high expressors are expected to benefit from Siglec-6-directed CAR-T therapy. Because this treatment strategy does not cause HSPC ablation, it may not require subsequent allo-HCT. Clinical studies of this novel gene therapy are highly recommended [55].

3.8. CLL-1

C-type lectin like molecule-1 (CLL-1/CLEC12A) is another target candidate for anti-AML CAR-T therapies. As orphan type II transmembrane glycoprotein receptor, its expression has been reported for AML blasts of nearly every pediatric patient and approx. 80% of adult patients [46,63]. In addition, higher expression levels on LSCs than on normal HSCs

have been described, with a distinct incidence in childhood AML patients [46,63]. More than 60% of CD33+ AML patient samples were found to be CLL-1 positive [96]. CLL-1 is not exclusively expressed on a subset of early hematopoietic cells, AML blasts, and LSCs, but it is also present on healthy monocytes and other immune cells [97,98]. The first clinical applications of CLL-1-targeting CAR-T-cells led to complete remission in three pediatric patients who underwent allo-HCT afterwards [99]. Further investigations are needed in order to determine if these encouraging outcomes can be achieved in adult AML patients (Table 1).

3.9. Other Target Antigens

Multiple additional antigens have been tested preclinically or in a clinical setting in the quest for better CAR-T cell target proteins. Apart from those described above, the following antigens have either been examined for AML or are being investigated: CD7, CD13, CD19, CD34, CD38, CD56, CD117, B7-H3, mesothelin, NKG2D ligands, IL-10 receptor, GM-CSF receptor, ILT3, TIM-3, MUC-1, Lewis Y, and folate receptor β [46,100–109] (Table 1).

4. Challenges and Perspectives for Successful Implementation of CAR-T-Cell Therapy in AML

4.1. Considerations for the Choice of Target Antigen

4.1.1. Antigen Selection

In general, stable and homogenous expression of the target antigen on the tumor cell surface is a prerequisite for potent CAR-T-cell reactivity, whereas absence from relevant healthy tissues is required to avoid on-target/off-tumor toxicities [110,111]. The latter is particularly challenging in AML due to lack of specific tumor-restricted antigens that are not simultaneously co-expressed on normal hematopoietic stem cells or myeloid progenitors.

Different tumor-specific neoantigens that arise from AML-specific mutations have been identified, even though AML is a disease with very low mutational burden [112]. Neoantigens are potentially of high tumor specific immunogenicity, e.g., NPM1 [113], IDH1/2 [114], or pml/RAR alpha [115], but are ‘intracellular antigens’ and therefore inaccessible for conventional CAR binding. Based on the four-nucleotide duplication in the oncogene nucleophosmin (NPM1c), a recent study presented a CAR that binds to the NPM1c-HLA-A2 complex, but not HLA-A2 alone or in complex with other peptides [116]. Similarly, prototypic CARs with TCR-like binding to WT1-HLA-A2 complexes have been generated [117]. Though both approaches demonstrated potent preclinical anti-leukemic activity and while it is attractive to include intracellular target antigens for CAR-T-cell therapy, the downregulation or loss of HLA expression, as previously demonstrated in AML [118], is a pitfall of this strategy.

Alternative splicing is yet another mechanism resulting in relatively tumor-restricted isoform variants. Mis-splicing has been demonstrated for various genes in AML, including FLT3, NOTCH2 [119], and CD44 [73].

4.1.2. Modulation of CAR-T-Cell Persistence

Controlling the persistence of CAR-T-cells is another approach for improving CAR-T-cell safety in the absence of an ideal target antigen. The incorporation of safety switches that eliminate CAR-T-cells in case of major toxicities is one such strategy, exemplified by the herpes simplex virus-thymidine kinase (HSV-TK) suicide gene system developed over 25 years ago [120,121]. A more recently developed suicide strategy relies on the inducible caspase 9 (iCasp9) that dimerizes after administration of the small-molecule AP1903, resulting in downstream activation of pro-apoptotic molecules [122]. Multiple clinical trials with CAR-T-cells have incorporated this safety strategy, e.g., NCT03594162, NCT03696784, NCT03721068, yet other one for haploidentical stem cell transplantation [123], clinical use of the iCas9 suicide switch has not been reported in the context of CAR-T-cell therapy to date. A further strategy for targeted CAR-T cell depletion is the co-expression of a truncated EGFR on the T-cell surface. This makes the engineered T-cells targetable with the anti-

EGFR monoclonal antibody cetuximab [124]. Though this strategy has been incorporated into clinical protocols, i.e., NCT02028455, NCT04483778, and NCT04499339, a systematic clinical evaluation of the kinetic and extent of CAR-T cell depletion by this strategy is yet to be performed.

In order to prevent long-term persistence of CAR-T-cells without incorporating a suicide mechanism, mRNA electroporation was used to generate CAR-T cells used in a clinical study evaluating CD123-specific CAR-T-cells for the treatment of AML [57]. Though seven patients were to receive repetitive doses of CAR-T-cells, less than 60% of planned doses were successfully manufactured. No anti-leukemic activity was noted, and the study was terminated prematurely. The mRNA-based approach to CAR-T-cell engineering is still an attractive option and ongoing preclinical refinements are justified.

Another strategy to interrupt CAR-T-cell function is the use of soluble adaptor molecules that bind to CAR-T-cells that recognize a peptide motif of the adaptor molecule. Given their typically short half-life, the interruption of adaptor molecule infusion facilitates terminating or mitigating CAR-T-cell induced toxicities [125]. Promising data from an ongoing phase I clinical trial using a targeting module directed against CD123 was recently reported [58]. Further updates are eagerly awaited.

The approach of STOP-CARs is intriguing because it can decrease the activity of CAR T-cells temporarily, as opposed to shutting it down completely. The CAR construct consists of a recognition chain (antigen binding) and a signaling chain (T cell activation). A computationally designed protein in the endodomain of these two chains can dimerize into a functional heterodimer without the need of a dimerizing agent. This chemically disruptable heterodimer (CDH) can be disrupted exclusively when small molecule agents, such as the Bcl-XL inhibitors A1331852 and A1155463, are administered. Once dissociated in their monomeric forms, CAR-T-cell activation upon antigen recognition is blocked. The main challenge is that the development of designer fusion proteins bears the risk of creating immunogenic epitopes [44].

4.1.3. Combination Strategies with Allo-HCT

One on-target/off-tumor toxicity of considerable concern for antigens expressed on AML and hematopoietic stem cells is the eradication of the latter, potentially resulting in severe and persistent myelosuppression. In order to broaden the therapeutic window of CD33-directed immunotherapies, several groups have preclinically evaluated the effects of genetically modifying the allograft by deleting CD33 in HSCs [62,126,127]. In murine and non-human primate models, deletion of CD33 from hematopoietic stem cells did not compromise their hematopoietic capacities [62]. A clinical trial to evaluate this approach is ongoing [128].

4.1.4. Multiantigen Targeting

Targeting more than one antigen with CAR-T-cells in order to reduce the risk of antigen escape can be achieved by infusing either more than one CAR-T-cell product of different specificities, or by infusing one CAR-T-cell product with either bicistronic CARs (two independent CARs co-expressed in the same T cell), or bispecific CARs (two scFv binding domains on the same CAR backbone) [129]. Ma and colleagues clinically evaluated the potential of a bicistronic CAR-T-cell product targeting CLL1 on leukemic stem cells and CD33 on bulk AML cells [130] and observed MRD-negativity in seven out of nine patients prior to six patients receiving subsequent allo-HCT. The toxicity profile was considerable with grade 3 CRS in two patients and neurotoxicity in three patients [131].

Increasing the specificity of CAR-T-cells to the malignant cells while sparing healthy tissue can be achieved by engineering T-cells with suboptimal activation upon binding of one antigen and full activation requiring co-stimulation through binding of a second antigen [132,133]. This strategy was pursued by He et al. with a combined bispecific and split CAR (BissCAR) T-cell system [106] directed against the myeloid antigens CD13 and TIM-3. While CD13 binding in the absence of TIM-3 on hematopoietic stem cells resulted

in low T-cell activation, simultaneous binding of CD13 and TIM-3 as co-expressed on leukemic stem cells enabled CD28-mediated co-stimulation and led to full T-cell activation. As a result, leukemic stem cells were effectively eliminated while large parts of normal hematopoiesis were spared. Clinical evaluation of this and other strategies, such as synthetic notch receptors [134,135], will be required to fully elucidate the degree of tumor specificity and T-cell functionality for these next-generation CAR-T-cells in AML.

4.2. Considerations Regarding the Immunosuppressive Character of AML and the Tumor Microenvironment

4.2.1. Modulation of Soluble Factors in the AML Microenvironment

AML is a disease with an immunosuppressive nature and AML cells have properties comparable to myeloid derived suppressor cells (MDSCs) [136]. STAT3 overexpression or induction commonly found in AML is related to downstream immunosuppressive mechanisms [137], such as the release of indoleamine 2–3 dioxygenase (IDO) and arginase (ARG), both soluble factors that impair T-cell function and drive T-cell apoptosis [138].

In addition, IDO promotes Th2 responses that are thought to be counterproductive for CAR-T-cell efficacy [139]. A preclinical strategy that modulates the IDO levels in the tumor microenvironment is the overexpression of miR-153, a tumor-suppressive miRNA, that downregulates the IDO1 expression and enhances CAR-T-cell cytotoxicity in murine models for colonic cancer [140]. Fludarabine and cyclophosphamide, the two lymphodepleting components of the most commonly used conditioning regimen prior to CAR-T-cell transfer, downregulate IDO expression in lymphoma cells and augments CAR-T-cell efficacy [141]. The relevance of either strategy in AML has not been investigated to date. IDO catalyzes the conversion of tryptophan into immunosuppressive metabolites [142] that inhibit the expansion of CAR-T-cells in response to interleukins such as IL-7 or IL-15. Approaches to attenuate the deleterious effects of IDO include engineering strategies to equip CAR-T-cells with IL-secreting properties [143,144] or combining CAR-T-cell therapy with the tyrosine kinase inhibitor sorafenib that increases IL-15 production in FLT3-ITD+ AML [145].

4.2.2. Modulation of Cellular Components in the AML Microenvironment

Accumulation of bone marrow and peripheral blood MDSCs has been reported in patients with MDS and AML [146]. MDSCs directly suppress T-cell activity [147,148] through different mechanisms [149]. Given that CD33 is also expressed on MDSCs, CAR-T-cells targeting CD33 represent an option for depleting MDSCs [61,67]. Various other strategies to inhibit or eliminate MDSCs have been proposed, including the use of phosphodiesterase-5 inhibitors [150], class I histone deacetylase inhibitors [151], and all-trans retinoic acid [152]. Tregs, another relevant immunosuppressive population, are also increased in blood and bone marrow of patients with AML [153,154]. Given that IDO belongs to the factors that drive differentiation into Tregs [155,156], strategies that reduce IDO levels are attractive also in this regard. Another approach to inhibit Tregs by depriving them of IL-2, stemming from CAR-T-cells, is the generation of CARs deficient in Lck signaling [157]; however, the effects on pro-inflammatory bystander cells remain to be determined. Unlike for other types of cancer, the role of immune checkpoints for pathogenesis and treatment of AML remains unclear [158,159]. The fact that single agent therapy checkpoint inhibitors have not yet shown a clear benefit in published clinical trials [160] could be explained by the generally low mutational burden of AML. Combination strategies with hypomethylating agents are under ongoing clinical investigation, and CAR-T-cell therapy may benefit from combinations with both groups of drugs.

4.2.3. Clonal Heterogeneity and Clonal Evolution of AML

AML is clonally and genetically more heterogenous in comparison to ALL, which may contribute to the low response rates with CAR-T-cells in AML [161–163]. Consolidation with allo-HCT should therefore be considered in patients undergoing CAR-T-cell therapy.

4.2.4. Combination Therapies with Approved Anti-Leukemic Drugs

Hypomethylating agents belong to the therapeutic backbone of AML treatment. In vitro treatment of AML blasts with decitabine and 5-azacytidine has been shown to result in a significant increase in NKG2D ligand expression and makes AML cells more susceptible to CD33-targeted antibody dependent cellular cytotoxicity [164]. Decitabine has been shown to increase the anti-leukemic effects of CD123 CAR-T-cells by differential methylation leading to the enrichment of naïve and early memory T-cells [165]. Further, 5-azacytidine sensitizes tumor cells to CAR-T-cell therapy by upregulation of the co-stimulatory OX40 ligand [166]. These and other observations [167,168] prompted clinical testing of decitabine-primed CAR-T-cells in B-cell malignancies (NCT04697940, NCT04553393). Even though further relevant mechanisms have yet to be understood, combination strategies of hypomethylating agents with CAR-T-cells appear attractive in the context of AML and should be evaluated clinically.

Current data demonstrates that venetoclax, a bcl-2 inhibitor recently approved for combination AML therapy in the elderly, increased the anti-leukemic effects of T-cells through enhanced secretion of reactive oxygen species [169]. Anthracyclines, a component of AML treatment for decades, are known to induce immunogenic cell death mediated by dendritic cells that elicit T-cell immunity [170,171]. Indeed, first data combining CAR-T-cells with sub-therapeutic doses of doxorubicin showed improved tumor elimination in a murine model of osteosarcoma [172].

The effect of other anti-leukemic drugs on CAR-T-cell performance is less favorable. Especially cytarabine reduces T-cell viability and cytokine secretion even at low doses [173] and depletes early lineage T-cells that are essential for T-cell expansion [174]. Immunosuppressive drugs commonly used in allo-HCT, such as calcineurin inhibitors [175] or mycophenolic acid [176], also limit T-cell activity and represent a potential obstacle to effective CAR-T-cell therapy in the post-allo-HCT setting. Strategies therefore include the implementation of CAR-T-cell therapy into earlier treatment lines in high-risk patients to ensure minimal exposure of T-cells to deleterious anti-leukemic drugs and the use of allogeneic T-cell sources. The latter comes with the risk of inducing or exacerbating graft-versus-host disease (GvHD) in recipients. Recent advances in gene engineering strategies, such as CRISPR–Cas9 technology [177] or TALEN-mediated approaches [178], have opened new possibilities for off-the-shelf CAR-T-cell products. While allogeneic CAR-T-cells are still in preclinical testing for AML [179], a favorable clinical safety profile has been documented in B-cell malignancies [180]. The use of allogeneic $\alpha\beta$ T-cells as a source for CAR-T-cell production may require additional genetic engineering such as the deletion of the endogenous T-cell receptor to prevent GvHD [181] and the deletion of HLA class I/II to prevent rejection by the recipient's immune system. Alternative T-cell sources without inherent alloreactivity, such as $\gamma\delta$ T-cells [182] or NK cells derived from healthy donors, cord blood [183], induced pluripotent stem cells [184], and immortalized cell lines, are therefore under investigation. Given the logistical and financial challenges of autologous CAR-T-cell therapy, the development of universal CAR-T-cell products remains highly relevant in AML.

5. Conclusions

For curative AML treatment, abrogation of bulk blasts and LSCs is mandatory with the necessity of hematopoietic recovery after CAR-T-cell administration. Efforts in development of new CAR-T-cell therapies for AML have resulted in a rich pipeline of candidate target antigens and corresponding CAR-T-cell products. Considering that AML is a heterogeneous disease, it is conceivable that single antigens or combinations of antigens will have to be selected for patients to receive customized CAR-T-cell products and increase the likelihood for inducing durable responses.

Author Contributions: Conceptualization, S.D., M.H., H.E. and I.Y.-A.; writing—original draft preparation, M.M., M.S. and S.D. All authors have read and agreed to the published version of the manuscript.

Funding: The authors are supported by Deutsche Forschungsgemeinschaft (project number 324392634, TRR 221 to M.M., H.E. and M.H.), the European Union’s Horizon 2020 research and innovation programme under grant agreements No 733297 (EURE-CART to M.H. and H.E.) & No 754658 (CARAMBA to M.H., S.D., H.E. and I.Y.-A.). Further, the authors are supported by the patient advocacy group ‘Hilfe im Kampf gegen den Krebs e.V.’, Würzburg, Germany; ‘Forschung hilft’ Stiftung zur Förderung der Krebsforschung an der Universität Würzburg (M.M., S.D., H.E., M.H.); and BPI program from the CHU of LILLE University Hospital.

Acknowledgments: The authors thank R. Tipton and N.C. Øien for revising and editing the manuscript.

Conflicts of Interest: M.M., M.S. and S.D. declare no conflict of interest. M.H. is listed as a coinventor on patent application related to CAR target antigens and CAR technologies that have been filed by the University of Würzburg, Germany and that have been licensed, in part, to industry. M.H. is a co-founder and equity owner of T-CURX GmbH, Würzburg, Germany; I.Y.-A. has received honoraria from Kite/Gilead, Novartis, Celgene/BMS and Janssen, the companies that are involved in commercial CAR-T-cells. H.E. declares research support/funding: Janssen, BMS/Celgene, Amgen, Sanofi, GSK; consultant work for Janssen, BMS/Celgene, Amgen, Novartis, Takeda; work on scientific advisory boards for Janssen, BMS/Celgene, Amgen, Novartis, Takeda, Sanofi, GSK; honoraria from Janssen, BMS/Celgene, Amgen, Novartis, Takeda, Sanofi, GSK.

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