Purpose of review
Treatment outcome of relapsed or refractory AML patients remains dismal and new treatment options are needed. Adoptive cell therapy using CAR-T cells is a potentially interesting approach in this.

Recent findings
Several potentially interesting AML targets are being investigated with CAR-T therapy with over 60 clinical trials listed on clinicaltrials.gov. The first clinical data are only just emerging with mixed results, once more proving that further research is needed.

Summary
Adoptive cell therapy using chimeric antigen receptor T cells is being investigated in AML through many clinical trials. So far, no AML-specific antigen has been identified, requiring additional strategies to mitigate on-target off-tumor toxicity and to increase efficacy. Focus point is to acquire control over the CAR T cells once administered. Strategies to do so include biodegradable CARs, inducible CARs, suicide-switch containing CARs and two-component modular CARs. Limited and mixed results are available, confirming the risk of lasting toxicity for nonswitchable CARs. Initial results of modular CARs suggest toxicity can be mitigated whilst maintaining CAR activity by the use of modular CAR concepts that allows for ‘ON’ and ‘OFF’ switching.

Keywords
adaptor chimeric antigen receptor, adoptive immunotherapy, acute myeloid leukemia, chimeric antigen receptor T cell

INTRODUCTION
Acute myeloid leukemia (AML) is an aggressive hematologic malignancy mainly affecting elderly patients, with a poor prognosis despite the introduction of new treatment options [1,2]. A promising immunotherapeutic approach is the genetic modification of the patient’s own (autologous) or a donor’s (allogeneic) T cells with chimeric antigen receptors (CAR-T). In this way, leukemia-associated antigens can be specifically targeted. In B-cell-derived malignancies, CAR-T has been clinically very successful, whereas in AML, a convincing proof of the applicability of CAR-T is still missing. In this review, we highlight the pros and cons of targeting different antigens, including clinical results published to date, as well as strategies to increase safety and efficacy of CAR-T therapies in AML. Optimization of chimeric constructs as well as improvements in production processes are not within the focus of this review. The authors refer to the numerous review articles on these topics published in recent years [3–7].

CHALLENGES IN IDENTIFYING POTENTIAL TARGETS FOR CHIMERIC ANTIGEN RECEPTOR T-CELL THERAPY IN ACUTE MYELOID LEUKEMIA
The ideal target antigen for CAR-T-cell therapy is characterized by four key characteristics:
Expression in a substantial part of AML patients ensuring broad applicability.

Expression on immature leukemia-initiating cells to allow for complete eradication of the leukemic clone.

Stable expression without downregulation or shedding to avoid antigen-loss escape variants.

Negligible expression on normal hematopoietic stem cells and other normal tissue reducing the risk for prolonged pancytopenia or other on-target/off-tumor effects.

So far, no antigen on AML cells fulfills all mentioned criteria. Although the biology and expression patterns of potential CAR-T-cell therapy targets in AML have been discussed elsewhere [6,8], this review will focus on published clinical data and novel strategies to handle the issue of imperfect target antigens. Table 1 shows clinical studies with results reported. An extended tabulated summary of all identified clinical trials in AML with CAR-T is provided as supplementary information, http://links.lww.com/COH/A24.

**POTENTIAL TARGET ANTIGENS FOR CHIMERIC ANTIGEN RECEPTOR T CELLS IN ACUTE MYELOID LEUKEMIA**

Below, several potentially interesting targets will be discussed shortly.

**NKG2D ligands**

NKG2D ligands are widely expressed on many malignancies and preclinical experiments with respective CAR constructs showed promising results [9]. In a single-center phase I study with a first-generation CAR targeting NKG2D ligands, the treatment was found to be well tolerated and 3 of 12 patients, 7 with AML and 5 with multiple myeloma, achieved a Complete Response (CR) [10–12]. In contrast, another study did not report objective
| Study short title                                                                 | Study identifier   | Status       | Phase | Condition | Interventional targets | Responsible party                                                                 | Reference       |
|----------------------------------------------------------------------------------|--------------------|--------------|-------|-----------|------------------------|-----------------------------------------------------------------------------------|-----------------|
| Genetically Modified T-cell Immunotherapy in Treating Patients With Relapsed/     | NCT02159495       | Recruiting   | I     | prBPDCN   | rrAML CD123            | City of Hope Medical Center, Duarte, California, United States                    | Budde et al., 2018 |
| Refractory Acute Myeloid leukemia and Persistent/Recurrent Blastic Plasmacytoid   |                    |              |       |           |                        |                                                                                   |                 |
| Dendritic Cell Neoplasm                                                          |                    |              |       |           |                        |                                                                                   |                 |
| Phase I Study of UCART123 in Patient With Adverse Genetic Risk Acute Myeloid      | NCT04106076       | Withdrawn    | I     | AML       | CD123                  | Cellectis S.A., Paris, France                                                    | Cummins and Gill, 2019 |
| Leukemia                                                                         |                    |              |       |           |                        |                                                                                   |                 |
| Study Evaluating Safety and Efficacy of UCART123 in Patients With Relapsed/       | NCT03190278       | Recruiting   | I     | rrAML     | CD123                  | Cellectis S.A., Paris, France                                                    | Roboz et al., 2020 |
| Refractory Acute Myeloid leukemia                                                 |                    |              |       |           |                        |                                                                                   |                 |
| Safety Study of Anti Lewis Y Chimeric Antibigen Receptor in Myeloma, Acute        | NCT01716364       | Unknowna     | I     | [r]AML,   | Lewis Y                | Peter MacCallum Cancer Centre, Melbourne, Australia                               | Ritchie et al., 2013 |
| Myeloid Leukemia or Myelodysplastic Syndrome                                      |                    |              |       | MDS, MM   |                        |                                                                                   |                 |
| Safety Study of Chimeric Antibigen Receptor Modified T-cells Targeting NKG2D,     | NCT02203825       | Completed    | I     | AML, MDS-| NKG2D Ligands          | Celyad Oncology SA, New York, New York, United States                            | Baumeister et al., 2019 |
| Ligands                                                                          |                    |              |       | RAEB, MM  |                        |                                                                                   |                 |
| A Dose Escalation Phase I Study to Assess the Safety and Clinical Activity of     | NCT03018405       | Unknowna     | I/II  | AML, CRC, | NKG2D Ligands          | Celyad Oncology SA, New York, New York, United States                            | Sallman et al., 2018, 2019 |
| Multiple Cancer Indications (THINK)                                               |                    |              |       | EOC, NKG2D|                        |                                                                                   |                 |
| DEPLETHINK - LymphoDEPLEtion and T-erapeutic Immunotherapy With NKR-2             | NCT03466320       | Completed    | I/II  | AML, MDS  | NKG2D Ligands          | Celyad Oncology SA, New York, New York, United States                            | Sallman et al., 2020 |
| (DEPLETHINK)                                                                      |                    |              |       |           |                        |                                                                                   |                 |
| EPTHINK: Epigenetic Drug Treatment and T-erapeutic Immunotherapy With NKR-2      | NCT03612739       | Withdrawn    | I     | AML       | NKG2D ligands          | Celyad Oncology SA, New York, New York, United States                            | Sallman et al., 2020 |
| Study in Relapsed/Refractory Acute Myeloid Leukemia or Myelodysplastic Syndrome | NCT04167696       | Recruiting   | I     | MDS, rrAML| NKG2D ligands          | Celyad Oncology SA, New York, New York, United States                            | Deeren et al., 2020 |
| Patients to Determine the Recommended Dose of CYAD-02                             |                    |              |       |           |                        |                                                                                   |                 |
| Dose-escalating Trial With UniCAR02-T Cells NCT04230265                          | Recruiting        |             | I     | AML, B-ALL, BPDCN | CD123                  | CPT Cellex Patient Treatment GmbH, Dresden, Germany                              | Wermke et al., 2021 |
| and CD123 Target Module (TM123) in Patients With Hematologic and Lymphatic       |                    |              |       |           |                        |                                                                                   |                 |
| Malignancies                                                                      |                    |              |       |           |                        |                                                                                   |                 |
| *As referenced on clinicaltrials.gov                                               |                    |              |       |           |                        |                                                                                   |                 |

AML, acute myeloid leukemia; B-ALL, B-Cell acute lymphoblastic leukemia; BPDCN, Blastic Plasmacytoid Dendritic-Cell Neoplasm; CRC, Colorectal Cancer; EOC, Epithelial Ovarian Cancer; FTC, Fallopial Tube Carcinoma; MDS, Myelodysplastic Syndrome; MM, Multiple Myeloma; PC, Prostate Cancer; RAEB, Refractory Anaemia with Excessive Blasts; rrAML, relapsed refractory AML; TCC, Transitional Cell Carcinoma; TNBC, Triple-Negative Breast Cancer.
responses in 17 AML patients [12]. Intermittent surface expression of NKG2D ligands by activated T cells possibly inducing fratricide might explain limited clinical efficacy [13,14] and CAR-T production failures. The addition of blocking antibodies to the production process or deletion of the antigen of interest by gene editing are possible solutions to these problems [15]. A study has been initiated using NKG2D CAR-T-cells with intentional down-regulation of MICA/B by co-expression of short hairpin RNA. Promising early results show bone marrow blast reduction from baseline in seven patients treated [16]. Further studies will have to prove whether NKG2D is a valid target for AML CAR-T therapy.

**C-type lectin-like molecule-1**

C-type lectin-like molecule-1 (CLL-1) is expressed in about 92% of AML patients including the leukemia-initiating cell compartment whereas normal hematopoiesis lacks significant CLL-1 expression [17–19]. Early clinical results in pediatric AML patients showed promising results with three out of four patients achieving a complete remission and measurable residual disease (MRD) negativity [20]. The same group reported results from 11 patients treated either with CLL-1 or CLL-1-CD33 dual targeting CAR-T and reported five CRs being MRD-negative, and additionally three CRs with MRD detectable, one Progressive Disease (PD) and one Stable Disease (SD) [21]. Toxicity was manageable with grade 1–3 cytokine release syndrome (CRS) and myelosuppression. Another group reported seven MRD-negative CRs out of nine patients with dual targeting CD33 and CLL-1-specific CAR-T [22,23]. Several clinical trials are ongoing using CLL-1 as a target, alone or in combination with other targets (Supplementary Information, http://links.lww.com/COH/A24), but to our knowledge, no further clinical data are available to determine whether CLL-1 is a safe antigen to be targeted with conventional CAR-T without damaging hematopoiesis.

**FMS-like Tyrosine Kinase 3**

The FMS-like Tyrosine Kinase 3 (FLT3) is an established AML target and expressed in 80% of patients, making it an interesting target for CAR-T treatment. CAR constructs currently under development are not specifically targeting the mutated FLT3 but FLT3, in general [24–27]. FLT3 expression on normal tissue seems to be restricted to a subpopulation of hematopoietic stem and precursor cells [25,28]. Nevertheless, significant hematopoietic toxicities have been observed in mouse models using murine or human FLT3-specific CAR-T [26,27]. Therefore, CAR constructs with a safety switch allowing for complete elimination of CAR-T cells have been developed to mitigate this risk for prolonged suppression of normal hematopoiesis [26]. At least three CAR-T trials targeting FLT3 are listed in the registries but no clinical data have been reported yet.

**CD33**

CD33 is a transmembrane receptor of the SIGLEC family and a proven target for AML treatment [29]. CD33 targeting CAR-T were evaluated preclinically demonstrating the potential of this approach [30,31]. As CD33 is also expressed on myeloid progenitor cells, there is a substantial risk for lasting aplasia, implying the need for a subsequent hematopoietic cell transplantation (HCT). This was indeed reported in a trial combining CLL-1 and CD33 as a target on a CAR-T cell [22]. In a single case report, a patient infused with CD33-specific CAR-T experienced CRS and pancytopenia whilst achieving a reduction in bone marrow blasts [32]. To mitigate CD33-associated toxicities, deleting CD33 expression from hematopoietic stem cells is investigated to enable CD33 CAR-T therapy along with transplantation of the gene-edited stem cells [33]. Alternatively, a safety suicide gene is implemented [34].

**CD123**

IL-3Ra/CD123 is overexpressed in AML and other hematological malignancies including the leukemia-initiating cell compartment [29,35–38]. Various agents have shown the potential of CD123 as a target for AML including Tagraxofusp (Elzonris) and Flotetuzumab inducing CRs in relapsed/refractory patients [39,40]. However, data from mouse models suggests that continuous anti-CD123 activity of CAR-T may be harmful for the hematopoietic progenitor cell compartment [41,42]. Outcomes of the first cohorts of a FIH clinical trial with a conventional CD123 targeting CAR-T showed responses not only including CRi but also lasting aplasias and as such an HCT was planned after CAR-T therapy [43]. The potential of allogeneic CD123 CAR-T is also under exploration. After an initial trial has been put on hold because of a patient’s death, the study was re-initiated with a modified product and a strong dose reduction [44]. The initial dose level was cleared without any DLT but no efficacy data have been reported, thus far [45].

The apparent lack of clinical data amidst a multitude of clinical trials investigating CAR-T treatments in AML clearly indicates the complexity to safely target antigens that are not solely expressed on tumor cells.
STRATEGIES TO ALLOW SAFE TARGETING OF IMPERFECT ACUTE MYELOID LEUKEMIA ANTIGENS

Being able to switch CAR-T cells off is key to allow for targeting of antigens, which are shared between AML and normal hematopoiesis or other nontarget tissue. Moreover, switchable constructs may increase safety in situations of serious CAR-T-cell-associated toxicity, for example, CRS or immune effector cell-associated neurotoxicity syndrome (ICANS).

Potential strategies to turn CAR-T-cells into a tightly controllable therapeutic tool will be discussed below and are illustrated in Fig. 1.

**FIGURE 1.** Schematic representation of various approaches to improve safety of Chimeric antigen receptor T-cell constructs. Panel a: Biodegradable CAR-T cell. mRNA encoding disease-specific receptor genes is inserted into the T-cell by means of nanoparticles, electroporation, or photoporation. Panel b: suicide switch CAR-T cell. Activity is abrogated by the introduction of a small molecule or antibody that triggers the degradation of the T cell. Panel c: logic gated CAR-T. The construct has two binding domains for two separate targets, CAR-T activation is only initiated upon simultaneous binding of the CAR with both domains. Panel d: inducible CAR-T. Administration of specific molecules trigger the expression or functional assembly of the CAR. Panel e: MODULAR, switchable CAR-T on the left side the UniCAR having the epitope on the target module and on the right side Rev(ersible) CAR with the epitope expressed on the CAR-T cell. In both cases, the CAR can only be activated upon connection via the targeting component connecting the tumor cell with the Universal CAR. CAR-T, Chimeric antigen receptor T-cell.
Biodegradable chimeric antigen receptor T cells

‘Biodegradable’ CAR-T cells refers to T cells that express the CAR only transiently, mostly achieved by transient transfection of mRNA encoding the CAR via, for instance, nanoparticles, electroporation, or photoporation [46–49].

Transient expression of a CD123-specific CAR-T for AML treatment was explored in a clinical trial initiated at UPenn [50]. Seven patients were enrolled, with two patients receiving all planned doses. Successful manufacturing was only achieved for approximately 60% of doses and median time from enrolment to infusion was 50 days, highlighting the technical hurdles still to be overcome. All but one patient experienced CRS upon cell product administration but the effects were only transient and CD123 CAR-T cells were neither detectable nor a reduction of CD123-positive cells observed in the bone marrow. Thus, the trial was terminated early due to lack of efficacy.

Chimeric antigen receptor T cells containing a suicide off-switch

The severe and sometimes fatal side effects observed in the CD19 CAR-T studies prompted researchers to incorporate safety mechanisms allowing for elimination of the genetically modified cells in an emergency. In addition to the well known Herpes simplex virus thymidine kinase, which has been studied as an eliminator for CAR-T [51,52], an inducible caspase system has also been developed and preclinically tested in the context of CD33-specific CAR-T [53]. However, the authors observed that only about three quarters of CAR-T were eliminated by caspase induction and concluded that a combination with other T-cell inhibitory agents, such as BCL-2 inhibitors is required. Another way to eliminate CAR-T is antibody-dependent cellular toxicity (ADCC). In addition to the CAR, the T cells are modified with epitopes that are recognized by clinically approved monoclonal antibodies (mabs). Examples are the co-expression of a truncated epidermal growth factor receptor (tEGFR) or a portion of CD20, which are recognized by cetuximab and rituximab, respectively. Another approach would be pan T-cell ablation by T-cell targeting mabs like alemtuzumab. When the two strategies were compared in a murine xenograft model, CAR-T-cells could be ablated by using either alemtuzumab or rituximab, facilitating hematopoietic engraftment after elimination of leukemic cells [54]. The disadvantage of these strategies is that the CAR-T cells are finally eliminated and cannot combat any relapse. The elimination of an expensive autologous product does not seem very reasonable from an economic point of view either.

Logic-gated chimeric antigen receptor T cells

Another approach is to logically link signals via two synthetic receptors that recognize different antigens. The signals can act either cooperatively (‘AND’ gating) or inhibitory (‘NOT’ gating). Preclinical proof-of-concept for these approaches has been provided with non-AML-specific antigens [55,56]. At the Memorial Sloan Kettering center, combinatorial CAR-T targeting ADGRE2 and CLL-1 is currently under development [57].

Inducible chimeric antigen receptor T cells

Conditional rather than constitutive CAR expression is another way to avoid or mitigate toxicities. Inducible promoter systems like the tetracycline-inducible promoter can be used for this purpose [58]. Another elegant approach is using synthetic Notch receptors, via which the expression of a CAR can be induced [59]. Small molecules like methotrexate (MTX) can be used to modify the affinity of CAR binders. CD33-specific single-chain antibodies were generated as CAR binders, which led to a reversible attenuation in the presence of MTX [60].

Modular chimeric antigen receptor T cells allowing for off-switching and on-switching

An alternative to address the limitations of aforementioned systems is to use soluble adaptors conferring tumor antigen-binding capacities to otherwise inert CARs also referred to as adaptor CARs. Various platforms exist, all based on the principle of the CAR-T cell recognizing and binding to a tag that is connected to a molecule that recognizes the tumor-associated antigen. CAR-T-cell activation only occurs upon binding of the second component. A well designed system allows for rapid up-titration and down-titration of the nonimmunogenic module as well as the ability to rapidly eliminate the binding component in case of acute life-threatening toxicity. Several reviews detail on the design of various adaptor CARs and the most prominent ones are depicted in Fig. 2 and discussed below [61**,62–66].

Early adapter CAR systems used a biotin-binding structure or a FITC-based connection system, mostly combined with labeled full-length antibodies [67–69] or CD16 extracellular domain combined with naked antibodies [70]. The use of molecules that have a long half-life does, however, not seem to be a very suitable approach, given their slow elimination resulting in long latency switches. Fluorescein

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isothiocyanate (FITC) is a synthetic molecule absent in the human body and broadly used as a flow cytometry dye. The feasibility of the FITC-based system was demonstrated but no clinical data are available yet [71,72]. Both biotin-based as well as FITC-based systems face the risk of immunogenicity, both being haptons [73–76].

Another modular CAR-T switch system uses a leucine-zipper-based CAR and a fusion molecule of a leucine zipper and a tumor-targeting scFv. CAR activity can only commence once the two leucine zippers have connected. Competitive zipFv’s with leucine zippers binding to the original zipFvs but not to the zipCAR can prevent activation of the CAR. Clinical trials are needed to confirm the concept’s activity and controllability [77]. Whether the used leucine zippers will interact with naturally occurring leucine zippers and hence create off-target toxicity is still unclear.

Yet another strategy is the use of a modified NKG2D extracellular domain, also referred to as convertible CAR [78]. The system uses a mutated form of the NKG2D extracellular domain on the CAR (iNKG2D CAR) and ligands that engage with this CAR but not wild-type NKG2D. The ligands are fused with monoclonal antibodies against a potential tumor target, creating the connection between tumor cell and CAR-T cell. Clinical trials will start in 2021 with rituximab and trastuzumab as initial antibodies to be used, raising concern for the rapid switchability because of the long half-lives of Abs [79].

An alternative approach is the use of a unique peptide-based connection between the CAR-T cell and the target-binding component like in the UniCAR system [80,81], which has been developed by our group and will be reviewed in the following paragraph.

**THE UniCAR SYSTEM, A MODULAR CHIMERIC ANTIGEN RECEPTOR-T PLATFORM WITH CLINICAL RESULTS IN ACUTE MYELOID LEUKEMIA**

UniCAR is a rapidly switchable, two component CAR-T platform using a CD28 co-stimulatory domain. The first component is a universal CAR-T cell that is inert by itself. The second component is a targeting module, which confers specificity towards the cancer antigen as it contains a tumor-binding moiety linked to the epitope recognized by the UniCAR-T cell. The UniCAR-T cell can be rapidly switched on and off to abrogate and prevent toxicities simply by administering or withholding the continuous infusion of TM, which has a very short half-life. The system allows for sequential or
simultaneous targeting of multiple antigens using different TMs.

UniCAR-T in combination with a CD123-specific TM (TM123) is currently explored in a phase 1 trial in relapsed/refractory AML. Clinical results of this ongoing study were presented at the 2021 AACR Annual Meeting, and preliminary initial results were published [82,83**]. All reported patients showed a robust expansion of UniCAR-T cells in peripheral blood and bone marrow, leading to an overall response rate of 80% including two patients with Cri. Both granulocytes and thrombocytes recovered quickly after stop of TM administration in all patients, eliminating the need for HCT. Two patients received a second cycle of TM123 leading to the re-activation of UniCAR-T cells and renewed clinical responses, indicating the opportunity to further deepen responses and optimize response duration. Low grade (1–2) CRS was experienced in 80% of patients, rapidly abating after cessation of TM123 infusion. No ICU admissions were needed. To avoid the potential exhaustion of T cells, alternative schedules are considered, limiting the duration of TM administration and allowing T cells to recuperate.

OFF-THE-SHELF CHIMERIC ANTIGEN RECEPTOR T-CELL PRODUCTS

The rapid progressive nature of AML complicates CAR-T implementation because of lengthy manufacturing time for autologous CAR-T products. In addition, T cell quality from patients is often hampered because of the underlying disease and long history of previous treatments. Off-the-shelf allogeneic CAR-T products generated from healthy donors may provide a solution. There are, however, still many hurdles to take., Genetic edits are necessary to avoid instant rejection by the host on the one hand and to prevent development of Graft versus Host Disease (GvHD) on the other hand, making the production technically more complex and complex. The novel gene editing techniques allow targeted knockout of, for example, the T-cell receptor and further modifications to enhance efficacy and reduce risk for GvHD, and numerous approaches are under development (e.g. [84–86]). Allogeneic CARs are also expected to significantly reduce costs, which is a major concern in CAR-T treatment [87–89]. However, it is currently unclear how many viable products can be generated from one healthy donor, impacting cost reduction and practical implementation significantly. Numerous allogeneic CAR-T-cells against various targets are currently in development [90,91]. Early clinical results from allogeneic CAR-T in B-cell malignancies not only show signs of efficacy (e.g. [92]) but also suggest that limited persistence has a negative effect on effectiveness. Apart from the study with UCART123 mentioned above, the authors are currently not aware of any other published results on allogeneic CAR-T in AML but the use of ‘off-the-shelf’ CAR-T for the treatment of AML promises great potential.

CONCLUSION

CAR-T-cell therapy in AML is not as straightforward as in B-NHL because of the lack of truly AML-specific antigens, and the rapidly progressive nature of the disease further complicates treatment. Early results indicate, however, CAR-T may have a place in AML. The optimal construct is preferably modular and allowing for rapid on-switching and off-switching and targeting multiple targets. Additionally, allogeneic CARs may increase applicability to a broader population and decrease costs.

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Conflicts of interest

J.K. and M.C. are employees of AvenCell Europe GmbH, M.W. has received honoraria from AvenCell Europe GmbH for consultancy services for the development and conduct of the UniCAR study, A.E. is employee and co-owner of AvenCell Europe GmbH, G.E. is founder and co-owner of AvenCell Europe GmbH.

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