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Novel approaches to CAR T cell target identification in acute myeloid leukemia

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Identifying safe and effective CAR T cell targets in acute myeloid leukemia (AML) is challenging due to the disease's complexity and overlap with normal hematopoiesis. This review highlights advances in target discovery for AML, emphasizing innovative approaches. Structural surfaceomics identifies tumor-specific protein conformations, while AI-driven single-cell RNA sequencing integrates multi-source data to pinpoint optimal targets. Refined cell surface capture technology maps the AML surfaceome without relying on predefined antibodies. These strategies enhance CAR T cell specificity and minimize off-tumor effects, offering promising pathways for safer and more effective AML treatments and broader cancer therapies.

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Introduction

Chimeric antigen receptor (CAR) engineered T cells are a revolutionary form of immunotherapy. They are genetically engineered T cells that utilize a modified B cell receptor-like structure (mostly VH-VL domain) to recognize and bind specific tumor-associated antigens (TAAs) [1,2]. Upon binding its target antigen, the CAR transmits an activation signal via the cluster of differentiation 3 zeta (CD3ζ) intracellular domain, leading to an MHC-independent activation of the T cell and, ultimately, a powerful and significant anti-tumor effect [3]. The development of CAR T cell therapy has shown great advancement in several hematologic malignancies, mostly B cell-derived cancers, where antigens such as CD19 and B cell maturation antigen (BCMA) were proven as well-defined, safe, and effective targets [4–7].

Despite the success of CAR T cell therapy in B cell malignancies, the application of this therapy in acute myeloid leukemia (AML) has encountered significant challenges [8]. One of the most prominent pitfalls is the lack of AML-specific antigens that can be safely targeted without harming healthy tissue and particularly healthy hematopoiesis [9]. This review will explore the evolving strategies and technological advancements for discovering novel and more suitable CAR T cell targets in AML, focusing on their potential to improve the safety and efficacy of this therapy. Moreover, these advancements could serve as a blueprint for expanding CAR T cell therapy to other types of cancer, paving the way for broader oncological applications.

CAR T cell therapy in B cell malignancies: establishing a successful model

The development of CAR T cells for B cell malignancies has transformed treatment outcomes for patients with B cell acute lymphoblastic leukemia (B-ALL) and B cell non-Hodgkin lymphoma. By targeting CD19, which is highly expressed on malignant B cells but also present on healthy B cells, CAR T cells have demonstrated high response rates and significant survival benefits.

Maude et al. reported that tisagenlecleucel, a CD19-directed CAR T cell, showed unprecedented remission rates in pediatric and young adult patients with B-ALL [4]. A key aspect of CD19-targeting therapies is the on-target off-tumor effect, where healthy B cells are also depleted due to their expression of CD19 [10]. This off-tumor effect, while notable, is manageable because

patients can a) live without B cells and b) when needed, receive intravenous immunoglobulin (IVIG) to compensate for B cell loss [11].

Similarly, in multiple myeloma (MM), CAR T cell therapy targeting BCMA has demonstrated impressive, long-lasting efficacy. In KarMMa-3, as well as CARTITUDE-1 study patients with relapsed/refractory MM treated with idecabtagene vicleucel (BCMA CAR T cell therapy) or ciltacabtagene autoleucel respectively, experienced a longer progression-free survival compared to patients receiving standard therapies [5,12].

These examples set a strong foundation for CAR T cell therapy in hematologic malignancies, but they also underscore the importance of identifying a safe target antigen, a challenge that remains to be solved until today for many if not most disease entities.

Current immunotherapeutic strategies in AML

Historically, antigen discovery for cancer therapies has relied on the development of monoclonal antibodies (mAbs). This approach began with the pioneering work of Köhler and Milstein, who developed a technique to generate mAbs by immunizing animals (such as mice) with specific antigens and fusing isolated B cells from the animal's spleen with an immortal myeloma cell line to create hybridomas [13,14]. These hybridomas produce large amounts of antibodies against specific antigens, which could then be screened and isolated.

This technique enabled the identification of CD19 and CD33 as target antigens for B cell and myeloid malignancies, respectively [15,16]. In AML, CD33 became an attractive target because of its high expression on AML cells [17,18]. Thus far, CD33 remains the only targetable antigen validated in AML. Gemtuzumab-Ozogamicin (GO), a CD33-targeting antibody drug conjugate (ADC) is the only approved immunotherapy in AML and has shown to have an acceptable safety profile [19,20]. Pivekimab sunirine (IMGN623), a CD123 targeting antibody-drug conjugate has been studied in a phase 1/2 trial, combined with azacitidine and venetoclax in patients presenting with CD123-positive R/R AML disease. The trial results were encouraging as they showed complete response rate (CRR) of 25 % and overall response rate (ORR) of up to 45 %. However, this high intensity treatment might lead to significant treatment associated side effects such as myelosuppression [21,22].

T cell bispecific antibody therapy in B cell malignancies targeting antigens such as CD19 or CD20 have shown very promising results and impressive response rates in B cell tumors [23,24]. Clinical trials evaluating bispecific antibody therapies in AML, targeting CD3 and CD123 or CD33 have however shown relatively low response rates [25–27]. Flotetuzumab, a bispecific dual-affinity re-targeting antibody directed against CD123, achieved a CRR of 20 % and an ORR of 24 % in R/R AML. Treatment was also associated with a high frequency of cytokine release syndrome (CRS) and other infusion-related side effects, which raised significant safety concerns and resulted in the decision to halt further clinical development [25]. The expression of CD33 and CD123 on hematopoietic stem progenitor cells poses a significant challenge, as targeting said antigen leads to significant myelotoxicity due to depletion of healthy progenitor cells in the bone marrow [28,29].

Challenges of targeting antigens in AML

One of the main challenges in developing CAR T cell therapy for AML is the lack of AML-specific target antigens that are uniquely or preferentially expressed on AML cells without being expressed on healthy cells, particularly HSPCs.

Several factors make AML a difficult target for CAR T cell therapy.

1. Shared antigen expression: Many of the antigens expressed on AML cells are also found on healthy myeloid cells, making it difficult to target AML without causing significant toxicity [30]. As AML originates from early stem or progenitor cells, expression of any targets is at high risk of being conserved in the downstream progeny, creating a large pool of on-target-off-tumor events.
2. Heterogeneity of target expression: AML is known for its molecular and genetic heterogeneity, with diverse subtypes and mutational profiles. Antigen target expression is known to be heterogenous both intra-as well as interindividually [31]. This makes it difficult to find a solitary antigen that is uniformly and broadly expressed [32,33]. Combinatorial approaches may be necessary to overcome the heterogeneity of the disease [34]. Interestingly, a study by Krupka et al. showed that >99 % of patient samples processed showed positivity for CD33 [35].
3. Clonal evolution: AML is a disease notoriously evolving throughout its history and particularly under therapeutic pressure, with both clonal shifts as well as de novo clonal occurrence arising simultaneously or sequentially [36]. Furthermore, the antigen expression within the myeloid compartment can contribute to toxicity, specifically CRS [37,38]. It is unclear whether this clonal evolution and potential antigen loss may have an impact on antigen targetability in AML for immunotherapies, however phenotypic changes in AML patients undergoing chemotherapy have been reported in up to 90 % of cases [39].
4. Immunosuppressive tumor microenvironment (TME): The bone marrow and vascular microenvironments in AML create an immunosuppressive

TME through the action of T regulatory cells (Tregs), myeloid-derived suppressor cells (MDSCs), mesenchymal stromal cells (MSCs), and macrophages. These cells contribute to the downregulation of MHC expression on AML blasts and natural killer (NK) or T cell ligands, impairing immune recognition [40,41]. To address this, strategies such as engineering CAR T cells to synthesize arginine, using Clustered Regularly Interspaced Short Palindromic Repeats/Cas9 (CRISPR/Cas9) to delete Regnase-1, and knocking out tumor growth factor β (TGF-β) receptor 2 have been explored to enhance CAR T cell efficacy and overcome the TME's immunosuppressive effects [42–44].

Defining an adequate CAR T cell target in AML

To be considered an ideal target for CAR T cell therapy in AML, an antigen should meet the following criteria (Table 1) [45].

1. Extracellular accessibility: The antigen needs to be present on the surface of the target cell, enabling the CAR T cell to bind and trigger an immune response.
2. Tumor-selective expression: The antigen should be highly expressed on AML and leukemic stem or leukemia initiating cells and minimally or not at all expressed on healthy tissues, particularly HSPCs, to minimize off-tumor toxicities.
3. Ubiquitous expression in AML: Ideally, the antigen should be expressed across different genetic subtypes of AML. This would ensure that CAR T cells could target disease across various AML-subtypes, making patient selection more feasible.
4. Absence of expression on T cells: The antigen epitope should not be expressed on T cells to avoid fratricide. T cell fratricide occurs, when T cells recognize themselves, preventing adequate expansion and activation, ultimately preventing strong antitumor efficacy and T-cell persistence.
5. Minimal expression on vital tissues: The antigen should not be expressed on any essential tissues, particularly those that could not be compensated for, to avoid severe side effects.

Approaches for discovery of novel CAR targets in AML

Identification of safe and effective targets for CAR T cell therapy in AML remains challenging. Due to the heterogeneity of AML and the lack of distinct tumor antigens that are absent from healthy tissues, researchers have been exploring novel technologies for target identification. These include mass spectrometric approaches to analyze the surface proteome of AML cells, structural surfaceomics to detect tumor-specific conformations of proteins, and transcriptomic studies combined with artificial intelligence (AI) to pinpoint AML-specific markers. Below, we discuss these cutting-edge techniques and their implications for the discovery of novel CAR T cell targets in AML (graphically described in Figure 1 and summarized in Table 2). Importantly, while many of these methods have been developed and primarily tested in AML, they can be readily translated to other diseases.

Mass-spectrometric determination of AML cell surface proteome

Köhnke et al. integrated cell surface proteomic data with gene expression and mutational analyses to identify potential CAR T cell targets in AML [46]. This was done by optimizing previously established cell surface capture (CSC) protocols [47] and implementing these specifically for primary AML patient samples. Their approach used biotinylation to label the surface proteins of these cells, followed by mass spectrometric analysis of peptides. This allowed mapping the AML surfaceome without the need for antibody-based detection, which might miss novel targets, due to the lack of suitable or specific antibodies.

In addition to this proteomic data, they incorporated gene expression data from databases such as BloodSpot, HemaExplorer, and Genotype-Tissue Expression

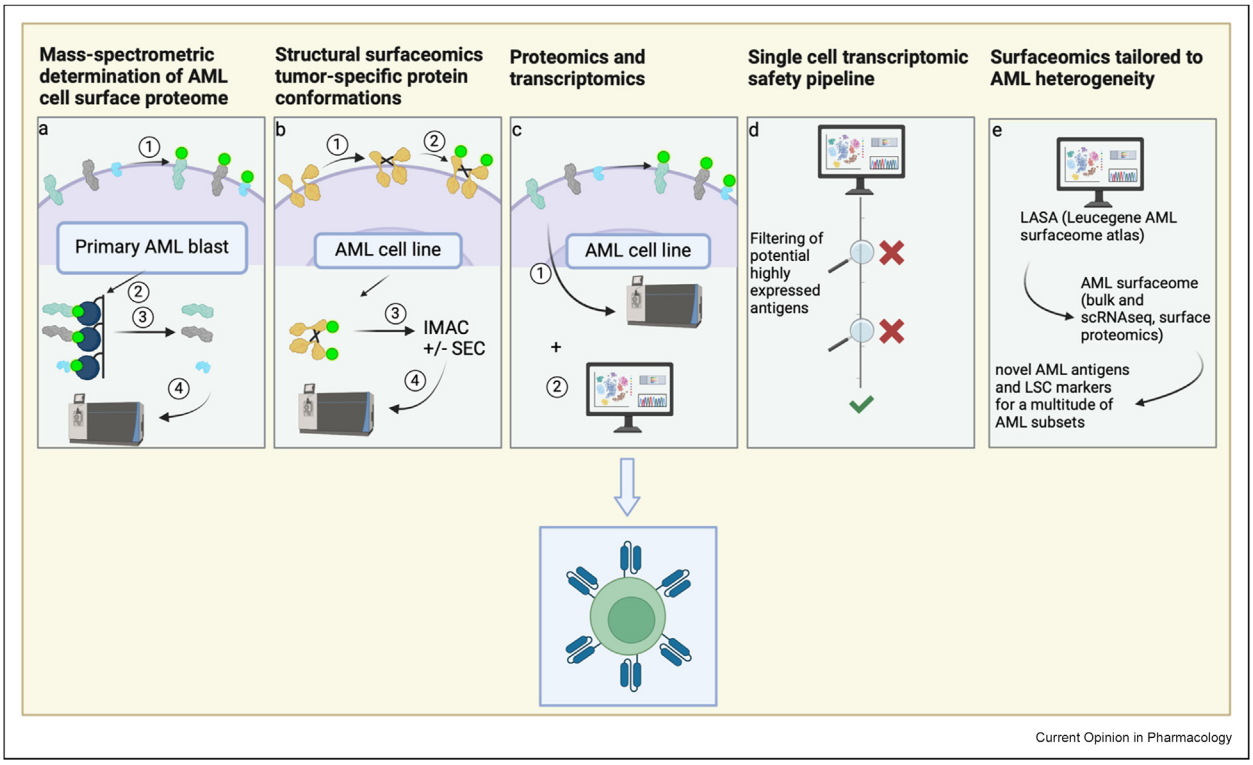
Table 1

Characteristics of an ideal CAR T cell target.

Characteristics	Description
Extracellular accessibility	Must be present on the surface of AML cells to allow CAR T cells to bind and trigger an immune response
Tumor-selective expression	Should be highly expressed on AML cells
Ubiquitous expression in AML	Should ideally be expressed across all AML subtypes or at least in most patients to ensure broad applicability
Absence of expression on T cells	Should not be present on T cells to prevent CAR T cell fratricide
Minimal expression on vital tissues	Should not be expressed on essential tissues, especially those vital for survival, to avoid severe adverse effects

Table 1 explains all the characteristics a novel CAR T cell target must fulfill to qualify as a reliable target.

Figure 1



New strategies for target identification in AML. **a,** Cell surface proteins on primary AML blasts are biotinylated (1) and subsequently bound by streptavidin (2). The cells are lysed and digested with trypsin (3) before being analyzed using mass-spectrometry which allows the determination of the cell surface proteome. **b,** Cell surface proteins on AML cell lines are cross-linked (1), biotinylated (2) before being digested with trypsin and enriching cross-linked peptides using size-exclusion chromatography (SEC) only or supplemented with immobilized metal affinity chromatography (IMAC). This process allows the analysis of tumor-specific protein conformations. **c,** Analog to (a), after biotinylation of the cell surface of AML cell lines and MS-analysis of the cell surface proteome (1), this data is correlated to RNA sequencing data to filter out antigens which, while being highly expressed on AML cells, are equally present on healthy tissue. **d,** An AI-generated safety pipeline was developed to filter RNA sequencing data to distinguish highly expressed antigens on AML cells. These putative antigens were subsequently exemplary filtered based on expression on healthy tissue. Following these steps novel antigens were found to generate anti-AML CAR T cells. **e,** The comprehensive surfaceome profiling of 100 primary AML samples using the LAS atlas identified pan-AML antigens, leukemia stem cell markers, and subgroup-specific targets, revealing distinct surface expression patterns.

(GTEx), allowing them to discard targets highly expressed on healthy tissues such as HSPC [48–50]. Mutational analysis was performed using COSMIC, which enabled them to filter targets that were not expressed in at least 50 % of patient samples. Identified candidate target antigens, were then validated by flow cytometry. This study identified three promising targets: CD148, integrin subunit alpha 4 (ITGA4; CD49d), and Integrin beta 7 (ITGB7), which were considered suitable for further development of targeted immunotherapies such as CAR T cell therapy.

Structural surfaceomics for AML-specific conformations

Mandal et al. utilized a novel approach termed "structural surfaceomics," which combines cross-linking mass spectrometry (XL-MS) with cell surface capture technology to detect unique conformational states of proteins on AML cells [51]. The cross-linking approach allowed the identification of specific inter- and intra-protein links, capturing the structure of proteins as they natively occur on the cell surface. Two different cross-linking agents—disuccinimidyl sulfoxide (DSSO) and disuccinimidyl phenyl phosphonic acid (PhoX)—were used to analyze both inter- and intra-peptide linkages, providing a detailed map of protein structures.

This study focused on integrin beta 2, a protein that exhibited a tumor-specific "open-active" conformation on AML cells compared to healthy cells. Anti-active integrin beta 2 CAR T cells were then engineered and demonstrated potent anti-tumor activity in both AML cell line xenograft models (CDX) and patient-derived xenografts (PDX). Importantly, safety of these novel CAR T cells was validated in a humanized non-tumor-bearing mouse model. In these patient-relevant disease models, anti-active integrin beta 2 CAR T cells showed lower signs of hematopoietic toxicity than state-

Table 2

Novel approaches to target identification in AML.

Article information	Novel approach to AML target identification	Novel target found
Köhnke et al., Biomarker Research, 2022	Cell surface capture, mass spectometry technique in primary AML samples	CD148, ITGA4, Integrin beta 7
Mandal et al., Nature Cancer, 2023	Structural surfaceomics (Combination of cross-linking MS and CSC) to determine cancer-specific conformations of antigens	Activated conformation of Integrin Beta 2
Perna et al., Cancer Cell, 2017	Correlation of proteomic data with transcriptomic data, combinatorial targeting strategy	ADGRE2, CCR1, CD70, LILRB2
Gottschlich et al., Nature Biotechnology, 2023	Development of a single-cell RNA sequencing algorithm pipeline to determine safe but highly expressed antigens	CSF1R, CD86
Bordeleau et al.	Surfaceome profiling using the LAS atlas (sc and bulk RNAseq data, surface proteomics), with a focus on AML heterogeneity and primitive markers	Including but not limited to: Pan AML: - MILR1, CTSG, PTPRC, CD47, CD37, ITGA4, CD74 Primitive: - CD34, NPR3, SLC38A1 CK-AML: - ENG, PTPRC, CD47, PROM1

Table 2 summarizes the different novel approaches as well as specific antigen findings for AML-specific CAR T cell antigen targets.

of-the-art anti-CD33 CAR T cells. However, a limitation of this approach was that the active conformation of integrin beta 2 was also detected on activated monocytes and granulocytes, highlighting the potential risk of on-target, off-tumor toxicity.

Proteomics and transcriptomics for surfaceome analysis

Perna et al. leveraged a combined proteomic and transcriptomic strategy to study the surface proteins of AML cells, focusing on how mutations reshape the surfaceome [52]. They used a multi-step procedure, starting with the surface biotinylation of AML cell lines and subsequent mass spectrometric analysis. This proteomic data was then compared to mRNA expression databases like the Human Protein Atlas and the Human Proteome Map to exclude targets that are highly expressed in healthy tissues. The results were validated by flow cytometry using patient samples, and a subset of top targets was further analyzed.

This study revealed four key AML-specific targets—adhesion G protein-coupled receptor E2 (ADGRE2), C–C chemokine receptor type 1 (CCR1), CD70, and leukocyte immunoglobulin-like receptor subfamily B member 2 (LILRB2)—that were validated as potential candidates for CAR T cell therapy. By comparing these targets to established markers such as CD33 and CD123, the researchers demonstrated their superior specificity. They also explored the potential of combinatorial targeting approaches to enhance the effector function of CAR T cells while maintaining safety, a strategy that could be crucial for improving CAR T cell therapy outcomes in AML.

AI-powered single-cell RNA sequencing for target discovery

Recently, we developed an innovative single-cell RNA-sequencing (scRNA-seq)-based approach to identify novel AML-specific target antigens [53]. By leveraging scRNA-seq data, we could compare gene expression profiles of malignant AML blasts and HSPC at an unprecedented resolution, identified several so far unrecognized target antigens, highly overexpressed on AML blasts. Importantly, by integrating 11 different scRNA-seq datasets of 9 healthy organs into a "cross-organ off-target transcriptomic atlas" (COOTA), we tried to identify target antigens with minimal expression on healthy tissues.

Two promising targets—CSF1R and CD86—were discovered through this method. These antigens were subjected to extensive preclinical testing, including *in vitro* functional assays using human and murine models, as well as *in vivo* validation in CDX and PDX models. Anti-CSF1R and anti-CD86 CAR T cells demonstrated strong anti-tumor effects both *in vitro* and *in vivo*. Importantly, safety profile was confirmed in syngeneic mouse models and using advanced *in vitro* models of HSPC and induced-pluripotent stem cell (iPSC)-derived microglia.

Comprehensive surfaceome profiling and subgroup analysis

A recent study conducted an extensive surfaceome analysis of 100 primary AML patient samples to explore immunotherapeutic targeting of surfaceome heterogeneity in AML [54]. Given the highly heterogeneous nature of AML, the study aimed to identify differentially expressed antigens on leukemia stem cells (LSCs), as well as novel antigens specific to AML subgroups and pan-AML markers. These findings have the potential to facilitate more personalized and precise treatment strategies by targeting both LSCs and AML-specific antigens.

Deploying a multi-step approach, with proteomics-based surfaceome analysis using the Leucegene collection, 100 primary AML samples were investigated. Of these, 20 samples underwent further subanalysis through single-cell RNA sequencing to identify primitive AML blasts. Transcriptomic data from this cohort were used as a follow-up quality check to validate acquired proteomic data. To determine if the analyzed proteins were cell surface-bound, the Surface Protein Annotation Tool (SPAT) was employed. Researchers then performed differential surfaceome analysis across AML subgroups, revealing distinct surfaceome expression patterns among various AML subunits.

The study identified several antigens of interest, including pan-AML antigens such as mast cell immunoglobulin-like receptor 1 (MILR1), cathepsin G (CTSG), protein tyrosine phosphatase receptor type C (PTPRC), CD47, CD37, ITGA4, and CD74. Additionally, AML antigens related to LSCs were identified, notably pan-AML hematopoietic stem cell markers like CD34, natriuretic peptide receptor 3 (NPR3) and solute carrier family 38 member 1 (SLC38A1). Further subgroup-specific analyses revealed unique antigen targets, including Semaphorin 4D (SEMA4D) (with low correlation between surface proteomic and RNA sequencing data), solute carrier family 4 member 1 (SLC4A1), and ficolin 1 (FCN1) in RUNX1-mutated AML. In CK-AML, targets such as endoglin (ENG) (also overexpressed in MECOM-rearranged AML), protein tyrosine phosphatase receptor type C (PTPRC), CD47, and prominin-1 (PROM1) were highlighted.

This comprehensive atlas of AML surfaceome profiles, encompassing subgroup analyses and LSC marker identification, serves as a valuable resource for future immunotherapeutic targeting strategies.

Conclusion and discussion: overcoming limitations and moving forward

The integration of novel technologies such as mass spectrometry, structural surfaceomics, and scRNA-seq has opened new avenues for identifying AML-specific targets for CAR T cell therapy. Each of these approaches addresses a different aspect of the challenge—whether it's refining specificity of target antigens, reducing off-tumor effects, or improving anti-tumor efficacy. However, limitations remain, such as low sample input requirements, partial expression of some targets on healthy cells, and the need for further *in vivo* validation. Despite these challenges, the combined efforts of researchers in these areas bring the field closer to developing safer and more effective CAR T cell therapies for AML. Future directions will likely involve refining these technologies, integrating them with gene editing techniques like CRISPR/Cas9 [55,56], or epitope editing [57] and exploring combinatorial or multi-target approaches [29,58] to maximize therapeutic benefit while minimizing risks.

Targeting a single antigen in AML poses significant challenges for CAR T cell therapy due to disease's high heterogeneity and expression of candidate antigens on healthy, vital cells. Consequently, alternative approaches such as dual targeting or combinatorial strategies are emerging as promising solutions to enhance therapeutic efficacy and safety.

One such approach was recently introduced by Volta et al., who developed a combinatorial adaptor-mediated targeting platform for AML known as AdCAR T cells [59]. This innovative strategy utilizes adaptor CAR T cells that bind to fluorochrome-bound antigen-binding diabody adaptors. In their study, Volta et al. targeted CD33 and CD117, demonstrating potent anti-tumor effects both *in vitro* and *in vivo*. The flexibility of this system lies in the half-life of the adaptor molecules and the ability to combine multiple adaptors, allowing for a more personalized treatment strategy. This approach not only improves efficacy but also mitigates toxicity by enabling precise antigen targeting.

Another promising strategy, involved co-targeting CAR T cells with an attenuated ADGRE2 and CLEC12A costimulatory receptor using the IF-BETTER gate CAR T cell design [60]. This method selectively targets cancer cells with high ADGRE2 and C-type lectin domain family 12 member A (CLEC12A) expression, sparing HSPCs that exhibit low or no expression of these antigens. The IF-BETTER approach effectively minimizes off-tumor toxicity while maintaining potent anti-tumor activity.

Building on the concept of combinatorial targeting, Silva et al. introduced a dual-strategy approach combining two targets [61]. They engineered a CD70 CAR T cell to secrete an anti-CD33, anti-CD3 dual-targeting antibody. This local secretion mechanism reduces systemic on-target off-tumor effects, thereby minimizing toxicity. Additionally, the secreted dual-targeting antibody enhances the activation and efficacy of non-CAR T cells

within the tumor microenvironment, further potentiating the anti-tumor response.

These advanced targeting strategies underscore the importance of combinatorial approaches in overcoming the challenges associated with antigen heterogeneity and minimizing adverse effects in AML treatment. By leveraging dual targeting and adaptor-mediated systems, these innovative methods pave the way for safer, more effective, and personalized CAR T cell therapies in AML.

Declaration of the use of generative AI

This review was edited for clarity and improved legibility using ChatGPT, an AI language model developed by OpenAI. The authors critically reviewed, edited and adapted the content generated by the generative AI model.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: S.K. has received honoraria from Cymab, Plectonic, TCR2 Inc, Novartis, BMS, Miltenyi and GSK. S.K. is an inventor of several patents in the field of immunooncology. S.K. received license fees from TCR2, Inc., and Carina Biotech. A.G. received research support from Tabby Therapeutics and Plectonic for work unrelated to the paper. S.K. received research support from TCR2 Inc., Plectonic GmBH, Catalym GmBH and Arcus Bioscience for work unrelated to the manuscript. M.S. received research support from Amgen, BMS/Celgene, Gilead/Kite, Janssen, Miltenyi Biotec, Molecular Partners, Novartis, Roche, Seagen, Takeda; participated in speakers' bureau for AstraZeneca, BMS, Gilead/Kite, GSK, Janssen, LAWG, Novartis, Pfizer, Roche, Springer Healthcare; performed a consultancy or advisory board role for AbbVie, Amgen, Autolus, AvenCell, BMS, CanCell Therapeutics, Genmab US, Gilead, Ichnos Sciences, Incyte Biosciences, Interius BioTherapeutics, Janssen, Miltenyi Biomedicine, Molecular Partners, Nektar Therapeutics, Novartis, Orbital Therapeutics, Pfizer, Roche, Sanofi, Scare, Takeda; and received travel support from Gilead, Pfizer, and Roche.

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