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# Bispecific CD33-TIM3 CAR T cells enhance specificity while maintaining efficacy against AML

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# 1. Table of content

# 2. Abstract

Background: CD33, a specific antigen prevalent on myeloid cells, is found in 88% of samples from patients with acute myeloid leukemia (AML), highlighting its potential as an immunotherapeutic target. Despite numerous ongoing clinical trials targeting AML, challenges related to CAR T-cell specificity—termed "on-target off-leukemia"—continue to present significant hurdles. In response, this research focuses on the development of dual CAR T cells that concurrently target CD33 and TIM3. TIM3 has been identified on leukemic stem cells (LSCs) and is absent on normal hematopoietic cells. Additionally, the role of TIM3 in impeding immune regulation underscores its suitability as a secondary target in AML immunotherapy strategies. The objective of this investigation is to construct and evaluate dual CAR T cells targeting CD33 and TIM3 to enhance the specificity and maintain the anti-leukemic efficacy of these cells.

Methods: This study initiated by producing anti-TIM3 antibodies using hybridoma technology. These antibodies were then validated for their specificity through enzyme-linked immunosorbent assay (ELISA) and fluorescence-activated cell sorting (FACS). DNA sequencing was performed of anti-TIM3 single-chain variable fragments (scFv) derived from these hybridomas. The structural interactions of CD33 and TIM3 scFvs with each antigen were modeled using AlphaFold2, referencing the TIM3 structure (PDB ID: 5F71) and CD33 structure (PDB ID: 6D48). The CD33 scFv was derived from gemtuzumab ozogamicin (clone hP67.6) and both incorporated along with costimulatory domains (CD28 or 4-1BB) into a pMP71 vector. A retroviral production system utilizing 293Vec-GALV and RD114 cells facilitated the production of the retroviruses. *In vitro*, the cytotoxicity of the CAR-T cells was tested against both wild-type and TIM3-transduced AML cell lines (THP-1 and OCI-AML3) through co-culture assays by multiparametric flow cytometry (MPFC). Cytokine release assays (CBA) were used to measure IFN-γ and IL-2 secretion, and cell-target avidity was analyzed using a Z-Movie analyzer. Off-target effect was monitored through colony-forming unit (CFU) assay on $CD34^+$ cells from healthy donors after 14 days.

For long-term efficacy assessments, CAR-T cells were periodically re-stimulated every four days by co-culturing with irradiated TIM3 expressing SKM-1 cells at a 1:1 effector-to-target (E:T) ratio over 24 days. During these intervals, analyses of CAR-T cell proliferation, checkpoint marker expression, and T cell subset differentiation were conducted via MPFC.

Conclusion: This study successfully generated dual-target CAR T cells utilizing both "AND" and "OR" gating strategies to target CD33 and TIM3, enhancing their efficacy to AML cells *in vitro*. The findings demonstrated that these CAR T cells exhibit superior binding avidity and cytotoxic capabilities towards cells expressing both CD33 and TIM3 antigens, as opposed to targeting a single antigen. Notably, the split CAR T cell approach effectively eradicated $CD33^+TIM3^+$ cell lines and primary AML cells, while minimizing impact on healthy hematopoietic cells. The implications of these results suggest that dual CAR T cell configurations might serve as potential bridging therapies before hematopoietic stem cell transplantation. The split CAR T cell particularly offered a potential safer alternative, possibly obviating the need for allogeneic stem cell transplantation due to on -target-off-leukemia toxicity. These advancements have the potential to markedly alter the therapeutic landscape for AML, offering more precise, effective, and safer options for treatment.

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Acute lymphoblastic leukemia (ALL)
Acute myeloid leukemia (AML)
Acute myeloid leukemia with myelodysplasia-related changes (AML-MRC)
Acute promyelocytic leukemia (APL)
Allelic ratio (AR)
Allogeneic stem cell transplantation (AlloSCT)
All trans retinoic acid (ATRA) and arsenic trioxide (ATO)
Anti-2,4,6-trinitrophenyl (TNP)
B cell acute lymphoblastic leukemia (B-ALL)
Bone marrow (BM)
CCAAT/enhancer binding protein α (CEBPA)
Universal CAR T cell (UCART)
Central nervous system (CNS)
Chimeric antigen receptor T cells (CAR T)
Chimeric T cell receptor (TCR)
Chronic lymphocytic leukemia (CLL)
Chronic myeloid leukemia (CML)
Cluster of differentiation (CD)
Complete remission (CR)
Complex karyotype (CK)
Core-binding factor (CBF)
Donor lymphocyte infusions (DLI)
Effector-to-target (E: T)
Enzyme-Linked Immunosorbent Assay (ELISA)
First complete remission (CR1)
Fluorescence in situ hybridization (FISH)
Fludarabine, cytarabine, idarubicin, and G-CSF (FLAG-IDA)
FMS-like tyrosine kinase 3 (FLT3)
FLT3 internal tandem duplication (FLT3-ITD)
FLT3 tyrosine kinase domain (FLT3-TKD)
French-American-British (FAB)
Gemtuzumab Ozogamicin (GO)
Graft versus host disease (GvHD)
Graft versus leukemia (GvL)
Hematopoietic stem and progenitor cells (HSPCs)
Hypomethylating agents (HMA)
Immune effector cell-associated neurotoxicity syndrome (ICANS)
Inhibitory CAR (iCAR)
Interferon-gamma (IFN-γ)
Interleukin-10 (IL-10)
Interleukin-2 (IL-2)
Interleukin-6 (IL-6)
International Consensus Classification (ICC)
Lewis Y (LeY)
Medical Research Council (MRC)
Measurable residual disease (MRD)
Monosomal karyotype (MK)
Multiple myeloma (MM)
Multiparameter flow cytometry (MPFC)
Myelodysplasia-related changes (AML-MRC)
Myelodysplastic syndromes (MDS)
National Comprehensive Cancer Network (NCCN)
Next-generation sequencing (NGS)

Nuclophosmine 1 (NPM1)
Optical density (OD)
Overall response rate (ORR)
Overall survival (OS)
Peptide-loaded major histocompatibility complex (pMHC)
Peripheral blood (PB)
Polymerase chain reaction (PCR)
Referencing European LeukemiaNet (ELN)
Second complete remission (CR2)
Single-chain variable fragment (scFv)
Sinusoidal obstruction syndrome (SOS)
Stem cell transplantation (SCT)
Synthetic Notch receptor (synNotch)
T cell receptor (TCR)
T-cell immunoglobulin and mucin domain 3 (TIM3)
The National Cancer Institute (NCI)
Therapy-related AML (t-AML)
Tumor lysis syndrome (TLS)
Tumor necrosis factor (TNF)
Tyrosine kinase inhibitor (TKI)
World Health Organization (WHO)

# 6. Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease characterized by the excessive proliferation of myeloid cells [1]. This condition is marked by the rapid expansion of abnormal myeloid cells in bone marrow (BM) and peripheral blood (PB), disrupting normal hematopoiesis. AML accounts for approximately 80% of all leukemia cases in adults [1]. In the United States, it is estimated that 20,800 people of all ages will be diagnosed with AML in 2024 [2]. In adult patients as well as children, it's the second most common type of leukemia [2]. According to a 2022 statistical survey, 81,900 patients were diagnosed with leukemia in China [3]. Although AML can be diagnosed at any age, it is rare before the age of 45, with the average age at diagnosis being 68 [2]. The 5-year overall survival (OS) rate is estimated at 30%, with significant variations across age groups [2]. It reaches 50% among younger patients but falls below 10% for those aged over 60 [4]. Therefore, scientists and physicians are still working towards more precise diagnostics and treatment of AML.

## 6.1 AML classification

AML is classified by three primary systems: the French-American-British (FAB) classification (Table 1) [5], which defines distinct immunotypes based on morphology, the World Health Organization (WHO) classification (Tables 2 and 3) [6], and the International Consensus Classification (ICC) [7]. The latter two systems incorporate chromosome translocations and dysplasia evidence as foundational criteria.

The FAB classification system was validated by several studies in 1976 for its ability to predict AML prognosis and evolution [8], [9], [10], [11]. It remains the standard for morphological classification of AML with no significant changes. However, advancements in genetic testing technologies have challenged its effectiveness, demanding a more nuanced classification approach.

**Table 1: FAB subtype [5].**

| Subtype | Description |
|---|---|
| M0 | Minimally differentiated AML |
| M1 | AML without maturation |
| M2 | AML with maturation |
| M3 | Acute promyelocytic leukemia (APL) |
| M4 | Acute myelomonocytic leukemia |
| M4E0 | AML with eosinophils |
| M5a | Acute monoblastic leukemia |
| M5b | Acute monocytic leukemia |
| M6 | Acute erythroleukemia |
| M7 | Acute megakaryoblastic leukemia |
| Acute leukemia of ambiguouse lineage | Acute leukemia of ambiguous lineage |

**Table 2: 5th edition of the WHO classification of myeloid neoplasms [6].**

| Type | Gene mutation |
|---|---|
| AML defined by genetic abnormalities | APL with PML::RARA fusion |
| | RUNX1::RUNX1T1 |
| | CBFB::MYH11 |
| | DEK::NUP214 |
| | RBM15::MRTFA |
| | BCR::ABL1 |
| | K MT2A rearrangement |
| | MECOM rearrangement |
| | NUP98 rearrangement |

| | |
|---|---|
| | NPM1 mutation |
| | CEBPA mutation |
| | myelodysplasia-related AML with other defined genetic alterations |
| AML defined by differentiation | AML with minimal differentiation |
| | AML without maturation |
| | AML with maturation |
| | Acute basophilic leukemia |
| | Acute myelomonocytic leukemia |
| | Acute monocytic leukemia |
| | Acute erythroid leukemia |
| | Acute megakaryoblastic leukemia |

**Table 3: WHO differentiation markers and criteria for AML defined by differentiation [6].**

| Type | Diagnostic criteria |
|---|---|
| AML with minimal differentiation | • blasts are negative (< 3%) for MPO and SBB by cytochemistry<br>• two or more myeloid-associated antigens expression, e.g. CD13, CD33, and CD117<br>• Criteria for mixed-phenotype acute leukemia are not met |
| AML without maturation | • blasts positive for MPO (≥3%) and NSE by cytochemistry negative<br>• granulocytic lineages constitute mature cells < 10%<br>• two or more myeloid associated antigens expression, e.g.CD13, CD33, MPO, and CD117 etc. |
| AML with maturation | • blasts positive for MPO (≥3%) or SBB by cytochemistry<br>• granulocytic lineages constitute mature cells ≥10%<br>• monocyte lineage cells constitute < 20% of BM cells<br>• two or more myeloid associated antigens expression, e.g.CD13, CD33, MPO, and CD117 etc. |
| Acute basophilic leukemia | • blasts or immature/mature basophils with metachromasia on toluidine blue staining<br>• blasts are negative for cytochemical MPO, SBB, and NSE<br>• strong CD117 equivalent negative (exclude mast cell leukemia) |
| Acute myelomonocytic leukemia | • monocytes and their precursors (≥20%)<br>• maturing granulocytic cells (≥20%)<br>• MPO blasts positive (≥3%) |
| Acute monocytic leukemia | • monocytes and/or their precursors (≥80%)<br>• maturing granulocytic cells (< 20%)<br>• two more monocytic markers e.g. CD11c, CD14, CD36 and CD64 (or NSE positivity on cytochemistry) expression on blasts and promonocytes |
| Acute erythroid leukemia | • immature erythroid cells (≥30%)<br>• BM with erythroid predominance (≥80%) |
| Acute megakaryoblastic leukemia | • one or more platelet glycoproteins e.g.CD41, CD61, or CD42b expression on blast cells |

The WHO 2022 classification describes AML diagnosis through various methods, emphasizing not only the quantifiable measurement of more than 20% myeloblasts in BM or PB but also recognizing the clinical significance of extramedullary manifestations [6]. Integral to this framework is the identification of pathognomonic genetic aberrations, including translocations e.g. PML::RARA, RUNX1::RUNX1T1, and CBFB::MYH11 [6]. It is important to note that APL, which accounts for 5-10% of AML cases, represents a distinct subgroup characterized primarily by the PML::RARA gene

translocation [12], [13].

Both, the WHO2022 and the ICC2022 use different blast thresholds to define AML [6-7]. The WHO2022 doesn't set a minimum threshold for AML with chromosomal abnormalities, whereas the ICC2022 requires a minimum of 10% blast cells in BM and PB. For other AML subgroups, the WHO still mandates a 20% blast threshold, while the ICC2022 has introduced a new category for Myelodysplastic Syndrome/Acute Myeloid Leukemia (MDS/AML) characterized by 10-19% blasts [6], [7], [14].

Both WHO2022 and ICC2022 use different criteria to definite myelodysplasia-related changes (AML-MRC) AML subtype. Neither of them includes a specific AML-MRC category within their frameworks. Instead, they introduce classifications based on molecular and cytogenetic abnormalities which indicate secondary ontogeny. These abnormalities are correlated with adverse prognostic outcomes [15]. The WHO2022 taxonomy classifies these cases as AML myelodysplasia-related (AML-MR), requiring a documented history of MDS or MDS/MPN [6]. A history of MDS/MPN in contrast to AML is used only as an adjunct to diagnosis by the ICC2022 [7]. The criteria for defining myelodysplasia lesions also differ. For example, the ICC2022 considers a RUNX1 mutation as indicative of myelodysplasia which is not reflected in the WHO's 2022 criteria. Other difference between WHO2022 and ICC2022 are listed below (Table 4) [16].

**Table 4: Differences between WHO2022 and ICC2022 [16].**

| WHO2022 | ICC2022 |
|---|---|
| Structure | |
| Two subgroups:<br>• AML defined by genetic abnormalities.<br>• AML defined by differentiation requires exclusion of AML with defined genetic alterations, MPAL, myeloid neoplasm pCT and history of proven MPN. | • Hierarchical diagnosis of AML with recurrent genetic abnormalities<br>• mutated TP53 (VAF > 10%); AML with myelodysplasia related gene mutations<br>• AML with myelodysplasia-related cytogenetic abnormalities<br>• AML NOS |
| Blast threshold | |
| • AML defined by genetic abnormalities does not require any blast threshold (except for AML with BCR::ABL1 and AML with biallelic/single bZIP mutations in CEPBA mutation which require 20%).<br>• AML defined by differentiation requires 20% blasts. | • AML with recurrent genetic abnormalities requires 10% blasts (except AML with BCR::ABL1 requires 20%).<br>• Other subtypes are defined as MDS/AML (blasts 10%–19%) or AML (blasts ≥20%). |
| AML-MR vs AML with myelodysplasia related gene mutations/cytogenetic abnormalities | |
| Molecular: ASXL1, BCOR, EZH2, SF3B1, SRSF2, STAG2, UAKF2, and ZRSR2 | Molecular: ASXL1, BCOR, EZH2, SF3B1, SRSF2, STAG2, U2AF1, ZRSR2, and RUNX |
| Prior history of MDS or MDS/MPN accounts for AML-MR | Prior history of MDS or MDS/MPN accounts as a qualifier and not a separate group |
| Prior History of MDS or MDS/MPN, prior cytotoxic therapy, germline disposition | |
| • New Definition of secondary myeloid neoplasm (separate from AML defined groups above)<br>• Myeloid neoplasms post cytotoxic therapy (pCT)<br>• Myeloid neoplasms associated with germline predisposition | Diagnostic qualifiers in addition to AML group<br>• Therapy related.<br>• Progression from MDS or MDS/MPN<br>• Germline predisposition |

The WHO2022 and ICC2022 have distinct methodologies for accounting for AML that arises post-cytotoxic treatment and those with inheritable genetic predispositions. The WHO categorizes these entities as secondary myeloid neoplasms, which includes myeloid neoplasms that are secondary to cytotoxic interventions or characterized by genetic predispositions. Contrary to this, the ICC2022 includes these considerations in the AML diagnostic criteria, without the need for a separate categorization. This highlights a paradigm shift driven by advancements in molecular diagnostics, which improve the identification of germline predispositions in cases of myeloid malignancy, even those diagnosed in later life stages [17]. The need for genetic screening in cases of MDS and AML, which are characterized by unique molecular/cytogenetic profiles, syndromic manifestations, or familial histories, is becoming more widely acknowledged [18]. These findings of inherited predispositions are crucial as they affect treatment options, such as the choice of donor for allogeneic stem cell transplantation (AlloSCT), conditioning regimens selection and require family monitoring and evaluation [19].

Recent advancements in the AML diagnostic landscape offer the potential for improved therapeutic customization across distinct AML subsets. However, the introduction of novel classification frameworks, notably WHO2022 and ICC2022, presents communication barriers among physicians, pathologists, treating clinicians, and patients. This divergence highlights the need for clinicians to comprehensively synthesize diagnostic and clinical data to formulate optimal treatment strategies [20]. The ICC2022 approach proposes broader criteria to expand the range of clinical trials available to patients and healthcare providers.

## 6.2 AML risk stratification

### 6.2.1 Current risk stratification rule

Estimating the prognosis of AML patients remains challenging due to various factors, including patient-related factors, clinical symptoms at diagnosis, and genetic analysis. Additionally, the use of measurable residual disease (MRD) levels to assess prognosis has been validated as an effective approach for certain groups of AML patients. The assessment of emerging AML treatments, including immunotherapies, using conventional methods such as standard chemotherapy, still presents difficulties. As a result, the latest European Leukemia Net (ELN) 2022 guidelines have been revised to place greater emphasis on the significance of new genetic mutations. The current standards for risk assessment are primarily based on ELN2022, as shown in Table 5 [21].

**Table 5: ELN2022 risk category [21].**

| Risk Category | Genetic Abnormality |
|---|---|
| Favorable | t(8;21)(q22;q22.1); RUNX1-RUNX1T1 |
| | inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFβ-MYH11 |
| | Mutated NPM1 without FLT3-ITD |
| | bZIP in-frame mutated CEBPA |
| Intermediate | Mutated NPM1 with FLT3-ITD |
| | Wild-type NPM1 with FLT3-ITD |
| | t(9;11)(p21.3;q23.3); MLLT3-KMT2A |
| | Cytogenetic abnormalities not classified as favorable or adverse |
| Adverse | t(6;9)(p23;q34.1); DEK-NUP214 |
| | t(v;11q23.3); KMT2A rearranged |
| | t(9;22)(q34.1;q11.2); BCR-ABL1 |
| | inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM(EVI1) |
| | t(3q26.2;v); MECOM (EVI1)-rearranged |
| | -5 or del (5q); -7; -17/abn (17p) |
| | Complex karyotype, monosomal karyotype |

| | |
|---|---|
| | Mutated ASXL1, BCOR, EZH2, RUNX1, SF3B1, SRSF2, STAG2, U2AF1, or ZRSR2 |
| | Mutated TP53 |

### 6.2.2 AML clinical risk profile

Current research consistently identifies age as a critical factor influencing outcomes in AML. In patients over 60, the five-year survival rate for AML is between 10-15%, and in those over 70, the rate is even lower [22]. This phenomenon can be attributed to the close association between advanced age, the adverse risk cytogenetic abnormalities, and genetic alterations, including complex karyotypes, mutations related to myelodysplastic syndromes, and secondary disease [14], [23], [24], [25].

Furthermore, the outcomes of AML patients are also influenced by their performance status and comorbidities. Performance status provides a simple and immediate assessment of overall health, significantly affecting early mortality, the likelihood of achieving CR, and long-term survival [24]. The performance status of an individual depends on their age, their medical history, and their comorbidities, as well as the manifestations of their diseases. Addressing initial complications and initiating treatment can markedly enhance outcomes for AML patients [26].

Multicomorbidity is common in AML patients, increasing the risk of early mortality and treatment-related complications. This makes effective salvage therapy less likely in cases of relapse or failure to respond to initial treatment [14], [22], [23].

Between 5-20% of AML patients exhibit leukocytosis (white blood cell counts more than 50,000 or 100,000/mL), which is related with an increased complications and premature mortality risk. Severe initial complications (leukocytosis, tumor lysis syndrome, and disseminated intravascular coagulation) can compromise to satisfactory treatment chance [14], [27], [28], [29]. Extramedullary disease is also associated with leukocytosis, but its prognostic significance remains undetermined [30].

### 6.2.3 Cytogenetics risk profile

With the continuous progress in the research on the biological mechanisms of AML, the correlation between cytogenetics and mutation abnormalities has been further clarified. Both are integrated for an accurate prognostication and are integrated in the ELN 2022 classification [21], [31], [32], [33]. An example for a recognized favorable cytogenetic risk group is core binding factor (CBF) leukemia, which includes AML characterized by t(8;21)(q22;q22) and either inv(16)(p13.1q22) or t(16;16)(p13.1;q22). These genetic alterations result in the fusion genes RUNX1;RUNX1T1 and CBFB;MYH11 [34], [35]. In addition, c-KIT has been the subject of extensive research, with some studies reporting its adverse impact on AML characterized by the RUNX1;RUNX1T1 fusion [23], [36], [37], [38].

In AML with KMT2A rearrangements, the t(9;11)(p21.3;q23.3)/KMT2A::MLLT3 subtype shows a higher response rate to intensified chemotherapy and is therefore classified as intermediate risk according to the ELN2022 classification, in contrast to other KMT2A rearranged subtypes that are considered high risk [21]. In adverse cytogenetic abnormalities, the t(6;9)(p23;q34)/DEK;NUP214 lesion is often associated with BM dysplasia and additional cytogenetic abnormalities. Furthermore, FLT3-ITD aberrations, which occur in 70-80% of these cases, have not yet been definitively associated with prognostic significance in this high-risk group [39], [40].

In addition, AML with inv(3)(q21.3;q26.2) or t(3;3)(q21.3;q26.2) represents a subgroup with particularly poor outcomes, where conventional treatment is unlikely to result in long-term survival [31], [41], [42]. Other rearrangements involving 3q26, including the MECOM (EVI1) gene, are also associated with a poor prognosis and have been classified in the adverse risk group by the ELN2022 guidelines [21], [43]. Despite the challenges in distinguishing it from chronic myeloid leukemia (CML), AML with the t(9;22)(q34.1;q11.2)/BCR;ABL1 translocation is classified in the adverse risk category by WHO2022 guidelines and ICC2022 [6], [7], [21]. Researchers have confirmed that the rare t(8;16)(p11.2;p13.3)/KAT6A; CREBBP rearrangement has poor prognostic value [34], [43]. Poor

prognosis is associated with partial deletions and monosomies of chromosomes 5, 7 and 17 [21], [31], [32]. Isolated del(7q-) is typically considered intermediate risk and is frequently observed in older leukemia patients [7], [33].

Complex karyotype (CK) is characterized as more than three unrelated cytogenetic chromosome abnormalities, occurring without other recurrent genomic lesions that categorize specific classes. CK is consistently associated with a poor prognosis [31], [32], [33]. However, there is considerable heterogeneity in this patient cohort, with prognosis generally worsening with each additional chromosomal aberration[31], [44]. CK can be further subdivided into typical and untypical CK based on the involved chromosomes. Typical CK is characterized by deletions or monosomies of chromosomes 5, 7, and 17, exhibiting a higher degree of cytogenetic complexity. In contrast, atypical CK is characterized by frequent TP53 mutations, which generally correlate with slightly better prognostic outcomes.[16], [45], [46].

The established significance of monosomies is evident both in CK and especially in monsoonal karyotype (MK) cases. The prognosis of monosomies is typically poor, with mortality rates below 5% over the long-term [47], [48]. In addition, coexistence of CK and MK leads to significantly worse OS than CK alone.

### 6.2.4 Gene mutation risk profile

Genetic mutations have been shown to significantly influence leukemia prognosis. Currently, research into AML-pathogenic gene mutations has focused on several key genes, although the role of some is still under debate: mutations in FMS-like tyrosine kinase 3 (FLT3), nucleo-phosmin 1 (NPM1), CCAAT/enhancer binding protein α (CEBPA), TP53, RUNX1 and ASXL1.

Approximately 30% of AML patients have FLT3 mutations [49]. These mutations often occur with a normal karyotype, are associated with NPM1 mutations in 40% of cases, and with DEK;NUP214-AML mutations in 70% of cases [50]. The FLT3-ITD mutation is consistently linked to poor prognosis and a high relapse risk, which varies depending on its allelic ratio, length, and insertion site within the TKD1 domain [42], [51], [52]. The genetic classification criteria proposed by Tazi group also indicate an increased risk of escalating risk in intermediate-risk patients with FLT3-ITD [53].

In addition, the NPM1 mutation often occurs in patients with a normal karyotype and is associated with other gene mutations in about 70% of cases [6], [7], [53]. NPM1 and FLT3 mutations often occur together, with the favorable prognosis associated with NPM1 primarily restricted to cases without the FLT3-ITD mutation [50]. However, a favorable prognosis associated with NPM1 mutations is primarily observed in cases without FLT3-ITD mutation [54]. NPM1 mutations are also rarely observed in therapy-related AML (t-AML), but their genetic characteristics and prognostic implications closely resemble those of de novo NPM1-mutated AML [55]. The 2022 ICC2022 and WHO2022 classification both categorize NPM1-mutated AML as independent of prior clinical history [6], [7].

CEBPA mutations are present in approximately 10% of AML patients [56], with a favorable prognosis observed in those with biallelic mutations [57], [58]. Meanwhile, recent studies suggest that in-frame mutations within the bZIP region of the CEBPA locus correlate with a favorable prognosis [59], [60].

TP53 is a common mutation in cancer, present in approximately 10% of AML patients [44]. It's often found in cases with chromosomal abnormalities such as CK and MK, as well as in the therapeutic context [61]. Patients with TP53 mutations often show resistance to intensive chemotherapy and have a poorer prognosis [62].

RUNX1 and ASXL1 mutations have been shown to have adverse prognostic implications in several studies [63], [64], [65], [66], [67]. However, in most cases these mutations co-occur with other mutations [68].

### 6.2.5 MRD risk profile

Current research suggests that MRD plays an important role in leukemia relapse, particularly in ALL [69]. However, further progress has also been made in AML, albeit the assessment and clinical application of MRD in AML face challenges due to the genetic and immunophenotypic heterogeneity among patients [70]. Many detection methods have been used to overcome these challenges, including multiparameter flow cytometry (MPFC), polymerase chain reaction (PCR), and next-generation sequencing (NGS).

The ELN2017 guidelines classified MRD remission patients as an independently subcategory of complete remission (CR), recognizing that MRD negative CR patients after intensified chemotherapy have a favorable prognosis than those with MRD positive status [21]. One year later, ELN2018 provided a comprehensive overview of the critical role of MRD in AML treatment and prognosis [71]. Furthermore, a study clearly demonstrated the correlation between MRD and patient prognosis, with a 5-year OS of 68% in MRD negative patients, significantly higher than the 34% in the MRD positive group. In various clinical treatment contexts, MRD negativity is strongly correlated with better long-term survival outcomes [72]. In the latest ELN2022 guidelines, MRD has been fully integrated into the diagnostic and management framework [21].

### 6.2.6 Leukemia stem cell and other risk factors

It has been proposed to evaluate the gene expression profile of AML leukemic stem cells (LSCs) to improve prognosis when AML relapses [73], [74], [75]. Currently, the LSC17 score, a 17-gene stemness score, is used to assess the prognosis associated with LSC. In several AML cohorts, the presence of LSC has been correlated with poor clinical outcomes [76], [77].

Beyond the major risks previously discussed, AML encompasses additional risk factors awaiting exploration, such as RNA dynamics, DNA hypermethylation, and proteomics [78], [79], [80], [81].

## 6.3 AML treatment

### 6.3.1 AML chemotherapy and its combination therapy

Young patients without comorbidities may be considered suitable for intensive chemotherapy. Currently, advanced age is relative contraindication to intensive chemotherapy. Additionally, the FDA has established criteria for ineligibility for intensive chemotherapy, including poor liver, kidney, heart, or lung function. The Ferrara group's criteria for predicting short-term mortality following intensive chemotherapy for AML have been validated in a large patient population [82]. Even those with good health and no adverse biological risks may not be suitable for intensive chemotherapy [83]. Patients with intensive induction chemotherapy typically have a higher rate of CR, partly as a result of a lower treatment-related mortality rate (TRM) [83].

**Standard and intensive chemotherapy**

Standard AML treatment is to achieve CR by minimizing the leukemia burden, then follow up with chemotherapy and/or AlloSCT after remission [84]. Furthermore, the selection of induction and post-remission therapies have to consider various factors: patient comorbidities, medical history (especially cytotoxic chemotherapy or previous myelodysplastic syndrome), cytogenetic and molecular risk of AML, potential MRD status, and the availability of donors [21].

Intensive chemotherapy for AML is mainly based on an anthracycline- and cytarabine-based regimen (e.g. 7+3) [85], [86], in which cytarabine (100-200 mg/m$^2$ for 7 days) and daunorubicin (60-90 mg/m$^2$ for 3 days) are administered [87], [88]. Thereafter, there is a spectrum of alternative induction regimens, including CLAG-M, G-CLAM, IA, FLAG-IDA, and lomustine-IA [89], [90], [91], [92], [93]. The comparative efficacy of these regimens versus conventional 7+3 treatment remains a subject of ongoing investigation [94]. In particular, enhancements of the 7+3 regimen by the addition of agents such as lomustine, nucleoside analogues or FLAG-IDA have been postulated to improve patient

outcomes in certain contexts, as evidenced by prospective randomized trials [95], [96]. However, the use of FLAG-IDA has been limited by its associated toxicity, highlighting the need for a balanced consideration of efficacy versus side effects in regimen selection [97]. Consequently, current treatment strategies now include chemotherapy combined with monoclonal antibodies and small molecule inhibitors, including Gemtuzumab Ozogamicin (GO), FLT3/IDH, CPX351 and Venetoclax combination therapies.

**Chemotherapy + GO**

GO is a type of anti-CD33 antibody conjugated to calicheamicin, has shown efficacy in AML treatment. A study reported that the addition of GO to standard chemotherapy regimens (7+3 or FLAG-IDA) significantly improved survival compared to regimens without GO [98]. The study also showed the benefit of GO was limited to AML patients with favorable and intermediate cytogenetics [98]. Furthermore, no GO-treated CBF-AML patients showed a particularly low OS (five-year OS rate only 55%) [98]. Given these findings, coupled with concerns about the myelosuppressive and hepatic toxicity of GO, there has been considerable debate regarding the routine inclusion of GO in induction therapy. However, one study has also shown that GO administered in fractionated doses, as opposed to a single dose, deepened responses across a broad spectrum of AML molecular subtypes without increased toxicity [99]. Furthermore, elderly AML patients receiving fractionated doses showed improved survival rates when their induction therapy was followed by consolidation with AlloSCT. Some favorable or newly diagnosed intermediate risk AML patients appear to benefit most from the addition of GO to standard AML therapy [100]. Given the modest GO adding benefit to induction chemotherapy for intermediate-risk patients and the increased risk of sinusoidal obstruction syndrome (SOS) following stem cell transplantation (SCT) in first complete remission (CR1), many physicians prefer to reserve GO for induction treatment exclusively with favorable cytogenetic risk profiles patients [101].

**Chemotherapy + Midostaurin**

Midostaurin is a pioneering first-generation type I FLT3 inhibitor that has demonstrated activity to FLT3-ITD and FLT3-TKD mutations in AML. Midostaurin was approved FLT3 mutated AML patient after the RATIFY trial, which showed a significant survival benefit in individuals aged 18-59 [102]. This trial compared outcomes between patients receiving the standard 7+3 chemotherapy regimen with/without the addition of midostaurin, and demonstrated the enhanced therapeutic benefit of including midostaurin in the regimen [102]. The FDA approved the combination of cytarabine and daunorubicin (7+3 regimen) with midostaurin for patients with FLT3-mutated AML who are eligible for intensive chemotherapy. Among patients aged 60-70, this regimen showed improved outcomes compared to historical cohorts [103], [104]. Other FLT3 inhibitors are under evaluation, with quizartinib emerging as a particularly active candidate in early phase trials for the treatment of AML. The QUANTUM first phase III trial marks a significant advance in this area. It showed that in newly diagnosed, under 75 years old FLT3-ITD mutated AML patient, treatment by standard 7+3 chemotherapy regimen in combination with quizartinib significantly improved outcomes [105]. In addition, a retrospective analysis of GO in combination with midostaurin shows a high response rate and good tolerability with no evidence of an increase when added to standard induction chemotherapy in newly diagnosed FLT3-mutated/CD33+ AML patients [106]. Such studies underscore the critical role of FLT3 inhibitors in optimizing AML treatment strategies, marking a significant advance in targeted cancer therapy.

**Chemotherapy + CPX-351**

CPX-351 was approved in 2017 for the treatment of t-AML and AML-MRC. It is a liposomal formulation of daunorubicin and cytarabine. The approval of CPX-351 for clinical application was based on a pivotal phase 3 clinical trial which involved 309 patients aged 60-75 years [107]. These patients were diagnosed with t-AML, myelodysplastic syndromes (MDS), chronic myelomonocytic leukemia (CMML) or AML with myelodysplasia-related changes (AML-MRC) and were randomized to receive either CPX-351 or the conventional 7+3 chemotherapy regimen [107]. Some study results also showed higher CR rates and median OS in patients treated with CPX-351 [108], [109]. And real-world evidence has further confirmed the efficacy of CPX-351 in a cohort of 188 patients, 24.5% of whom were under the age of 60 [110]. Given the WHO2022 and ICC2022 definitions of myelodysplastic neoplasms and AML-MR, the applicability of CPX-351 to patients who may not have qualified for the CPX versus 7+3 trial

is still uncertain[6], [7], [16]. Certain subgroups may benefit more from either venetoclax in combination with HMAs or CPX-351. While prospective randomized trials are pending, some evidence suggests that monocytic subtypes of AML patients, who typically have poor outcomes with aza-citidine plus venetoclax, may potentially benefit more from CPX-351. On the other hand, AML subtypes that are refractory to chemotherapy, particularly those with TP53 mutations, may be better treated with azacitidine/venetoclax or other protocol regimens [111]. However, in patients with unfavorable cytogenetics and mutated TP53, the combination of venetoclax and azacitidine has shown high response rates but has not significantly prolonged OS [112], [113].

**Chemotherapy + Venetoclax**

The combination therapy of venetoclax has been an important part of AML treatment. Venetoclax is a kind of BH3 mimetic which inhibits BCL-2 protein, also a key regulator that prevents apoptosis in cells. This inhibition facilitates the induction of apoptosis specifically in AML, targeting the pathology of the disease at the molecular level [114]. Venetoclax has been approved for use in combination with hypomethylating agents (HMA) or low-dose cytarabine in newly diagnosed over 75 aged AML patients, or in those deemed ineligible for induction with intensive chemotherapy [16], [115], [116]. There are several phase I-II studies evaluating venetoclax with fludara-bine, cytarabine, idarubicin, and G-CSF (FLAG-IDA) as well as daunorubicin and cytarabine for the treatment of newly diagnosed AML [117], [118], [119] . Preliminary results from these studies show that the FLAG-IDA regimen in combination with venetoclax achieved a composite 89% CR, with 93% of CR patients reaching negative MRD [16], [120]. In addition, an estimated 2-year OS rate of 76% highlights the potential of this combination therapy to improve outcomes for AML patients [117]. In a phase I study of venetoclax in combination with a 7+3 chemotherapy regimen, all 11 patients evaluated achieved CR, and most of them were MRD negative [118]. In a 47 patients AML study, a regimen combining venetoclax with cladribine, idarubicin and cytarabine achieved a composite CR rate of 94%[119]. In addition, 85% of these patients achieved MRD-negative status, with an observed one-year OS of 85%, demonstrating the regimen's high efficacy and potential impact on patient outcomes [119]. Although initial results are promising, these regimens are associated with significant myelosuppression and the risk of prolonged cytopenia and infections. Therefore, the use of intensive chemotherapy in combination with venetoclax in the upfront treatment of AML should be limited to clinical trials.

### 6.3.2 Post remission therapy in AML

AML post-induction therapy is essential for enhancing the likelihood of favorable long-term outcomes, including potential cure. The strategy post-remission treatment is informed by the ELN with high-dose chemotherapy recommended for those classified under the favorable risk category [7]. In contrast, intermediate or adverse risk scores AML patients are advised to consider AlloSCT, due to risks of mortality from disease relapse or resistance, which may surpass the TRM risks associated with AlloSCT. Notably, AlloSCT is not routinely pursued for patients with CBF-AML in CR, barring the detection of MRD positivity. A pivotal prospective trial demonstrated that MRD-positive patients get benefit from AlloSCT, exhibiting significantly lower relapse rates and superior disease-free and OS outcomes compared to those who continued with chemotherapy. Thus revealing a nuanced approach to managing AML after remission [121].

Although consensus exists that high-risk AML patients achieve long-term survival with AlloSCT during CR1, an UK MRC trials recommend delaying transplantation to CR2 for intermediate-risk patients [122]. This strategy could avoid unnecessary SCT-related morbidity and mortality, especially in patients with MRD negativity and a low relapse risk. Conversely, a comprehensive meta-analysis comparing AlloSCT in CR1 with other treatments showed an OS benefit for both intermediate (HR 0.83; 95% CI 0.74–0.93) and high-risk (HR 0.73; 95% CI 0.59–0.90) groups [123]. The current approach primarily involves transplanting all adverse and intermediate-risk patients in their CR1 stage provided they have available donors and no contraindications [124].

Maintenance therapy following AlloSCT is scrutinized for its potential to extend patient survival. An analysis encompassing five trials highlighted a significant survival benefit with maintenance therapy compared to absence of such intervention [125]. Notably, the benefit was particularly pronounced

with the administration of sorafenib, a pioneering second-generation tyrosine kinase inhibitor (TKI), efficacious in patients harboring FLT3-ITD mutations [126]. This observation underscores the pivotal role of targeted therapy in enhancing post-AlloSCT outcomes [126], [127], [128], [129]. Furthermore, innovative maintenance strategies, including the combination of hypomethylating agents (HMAs) with venetoclax and the application of second-generation TKI Gilteritinib for FLT3 mutant cases are currently under rigorous evaluation in phase III clinical trials [130], [131]. These endeavors aim to refine post-transplant care by integrating novel therapeutic agents, potentially setting new standards for AML management post-AlloSCT.

### 6.3.3 Non-intensive therapy for newly diagnosed AML patients

Most of AML patients who are ineligible for intensive therapy are typically elderly. This population often faces significant treatment challenges due to an increased risk of toxicity and the presence of comorbidities that can complicate the use of aggressive chemotherapy regimens. Until a few years ago, the management of elderly AML patients did not lend itself to intensive therapy and consisted primarily of supportive care, resulting in poor prognosis. Although decitabine and azacytidine were commonly used in this population, their efficacy compared to other therapies remained uncertain. Recently, the approval of various drugs and combination regimens, particularly the incorporation of venetoclax with low-dose chemotherapy, has dramatically changed the therapeutic landscape for AML patients ineligible for intensive chemotherapy induction [132], [133]. This advance has significantly improved the prognosis for these patients, marking a pivotal shift in AML treatment strategies and contributing to prolonged survival rates. In addition to the previously mentioned treatments, combinations including Gilteritinib, Glasdegib, IDH inhibitor Ivosidenib and Venetoclax combination therapy have been identified as effective in prolonging long-term patient survival.

**Venetoclax**

Venetoclax combinations with HMA or LDAC have shown significant efficacy and have been approved for use in elderly AML patients. The phase III VIALE-A trial demonstrated that the addition of azacitidine to venetoclax significantly improved response rates and OS compared to azacitidine monotherapy in patients older than 75 years or with significant comorbidities [134]. Venetoclax also shows synergy with various therapies, including low-dose chemotherapy, targeted therapy and immunotherapy [135]. One study showed that venetoclax combined with low-dose chemotherapy benefited those patients who considered ineligible for intensive chemotherapy and older patients [16], [136].

**Ivosidenib**

Ivosidenib is an IDH1 inhibitor which was granted FDA approval as monotherapy in IDH1-mutated AML patients, specifically in newly diagnosed individuals ineligible for intensive therapy [137]. The phase III AGILE trial evaluated the efficacy of adding ivosidenib to azacitidine versus azacitidine alone in patients with IDH1-mutated AML who were older than 75 years or had significant comorbidities [138]. Median OS was significantly longer in patients treated with ivosidenib compared to controls (24 months vs. 7.9 months) [138]. Howerer, there is no data to compare HMA combined with venetoclax with the current standard of care for IDH1-mutant older AML patient [16], [139]. The decision between the use of azacitidine + venetoclax or ivosidenib remains unresolved, particularly given the high relapse rates and the role of ivosidenib as salvage therapy. The reduced hematologic toxicity of ivosidenib plus venetoclax, as evidenced by faster neutrophil recovery, suggests its suitability for frailer patients compared to HMA/venetoclax therapy.

**Glasdegib**

The FDA has approved glasdegib as an inhibitor of the Hedgehog pathway. A study evaluated glasdegib + LDAC versus LDAC alone in ND AML patient ineligible for intensive chemotherapy because they are over 75 years of age or have significant comorbidities [140]. The study found significant improvements in CR rates (17% vs. 2%) and median OS (8.8 vs. 4.9 months) with glasdegib + LDAC [140]. A retrospective study evaluated the glasdegib + LDAC efficacy in relapsed and refractory (R/R) setting and showed a composite CR rate of 21% and a 3.9 months median OS [141]. Due to limited reports on glasdegib in treatment regimens for newly diagnosed elderly patients, its specific role is still unclear.

In addition, a type of tyrosine kinase inhibitor named gilteritnib get FDA approved for R/R FLT3-mutated AML [141], [142]. It is reported to be more potent and specific than midostaruin [145]. The Phase III LACEWING trial reported gilteritinib in combination with additional therapies as an initial treatment strategy [143]. gilteritinib and azacitidine were randomized in patients with ND FLT3-mutated AML who were not eligible for intensive chemotherapy [143]. The median OS did not show a significant difference (9.8 vs. 8.9 months), the composite CR rate was significantly higher in the azacitidine + gilteritinib group (58% vs. 26.5%) [143]. Notably, 44% of patients in the azacitidine group received FLT3 inhibitor subsequent therapy, compared to 20% in the gilteritinib and azacitidine group [143]. This discrepancy could have influenced the observed efficacy of the combination therapy in the treatment of FLT3-mutant AML in newly diagnosed elderly patients.

### 6.3.4 AML therapy for R/R patients

R/R AML remains a significant therapeutic challenge with a only 10% 5-year survival rate [144], [145]. Prognostic factors in the R/R setting include age, duration of CR1, initial cytogenetics, and prior AlloSCT [72]. The prevailing strategy for R/R AML, particularly for patients who are inclined to receive additional treatment for a modest chance of a favorable long-term outcome, focuses on achieving CR2 and consolidation with AlloSCT [146], [147], [148]. For individuals with R/R AML who receive AlloSCT during their CR1, therapeutic strategies extend beyond conventional treatments to include innovative approaches aimed at harnessing the immune system to fight leukemia. These methods involve modulating the immune response to enhance the graft versus leukemia (GvL) effect. Strategies include the gradual reduction of graft versus host disease (GvHD) prophylaxis to stimulate the immune response, the administration of donor lymphocyte infusions (DLI), immune checkpoint inhibitors incorporation, or the consideration of a second AlloSCT [149], [150], [151], [152]. In addition, in recent years, several targeted therapies have been approved specifically for R/R AML patients with mutations, marking a significant advance in personalized medicine for this challenging disease. These therapies include gilteritinib, IDH inhibitors, GO and venetoclax + salvage chemotherapy.

**Gilteritinib**

Gilteritinib, known for its potent FLT3 inhibition, includes ITD and TKD mutations[153]. The approval of gilteritinib as a monotherapy for this subset of patients was based on the results of the ADMIRAL trial [143]. This pivotal study underscored the therapeutic efficacy of gilteritinib and marked a significant advancement in R/R FLT3-mutated AML treatment [143]. It demonstrated a significant improvement in median OS for gilteritinib cohort (9.3 vs. 5.6 months) for those receiving chemotherapy [143]. In addition, the incidence of adverse events was lower in the gilteritinib group than in the chemotherapy group, demonstrating not only the therapeutic benefit of gilteritinib but also its improved tolerability profile [143]. For FLT3 mutant relapsed AML, a novel regimen combining HMAs, ve-netoclax and gilteritinib shows promise, achieving an ORR of 67% (CR, CRi and MLFS) with a 10.5 months median OS [154]. While there is a notable risk of myelosuppression with these combinations, they are being explored as potential bridging strategies to transplantation, although evaluation of their long-term application is ongoing.

**IDH inhibitors**

IDH inhibitors (ivosidenib, olutasidenib and enasidenib) continue to play an important role. A recent phase Ib-II study evaluated the efficacy of venetoclax + ivosidenib, with or without azacitidine, in patients with IDH1-mutated MDS, ND AML or R/R AML [16], [155]. The study reported high response rates, with a composite CR of 87% and 63% of patients achieving MRD-negative [155]. And two-year OS rates were notably 67% in the ND AML and 50% in the R/R AML groups [155]. However, these results are preliminary, underscoring the need for validation in a more cases and comparison with care standards.

Olutasidenib is a novel selective IDH1 inhibitor that has recently been approved for the treatment of R/R IDH1-mutated AML following encouraging results from a phase I-II clinical trial [156], [157]. In AML patients receiving monotherapy, the overall response rate (ORR) was observed to be 25% in ND and 32% in R/R cases [156]. Notably, median OS reached 8.7 months for R/R AML patients on

monotherapy and improved to 12.1 months with combination therapy [152]. The incidence of differentiation syndrome was reported to be 13%, in line with rates observed with other IDH inhibitors [152]. The FDA summary highlighted a CR plus CR with partial hematologic recovery (CRh) rate of 35% in 147 R/R IDH1 mutated AML patients[158]. In addition, 34% of transfusion-dependent patients achieved independence from red blood cell and platelet transfusions [158]. Despite these advances, the use of IDH1 inhibitors as single agents in newly diagnosed patients remains rare, with combinations such as HMA+venetoclax or HMA+ivosidenib. The optimal approach for the treatment of R/R IDH1 mutant AML remains to be clarified, particularly regarding the most effective combination partners for ivosidenib or olutasidenib. This uncertainty is compounded by a lack of comparative data between these agents and is further complicated by the widespread prior use of first-line combination therapies.

Enasidenib is an IDH2 inhibitor that has received FDA approval for IDH2-mutated R/R AML [159]. The Phase III IDHENTIFY trial comparing enasidenib to standard of care in this patient population did not demonstrate an improvement in OS [56]. A post-hoc analysis of IDHENTIFY showed that enasidenib improved CR rates and OS compared to lower intensity regimens (1-year OS 41% vs. 26%) [160]. In addition, combination regimens with enasidenib, tested in a small R/R case series as a doublet with azacitidine or as a triplet with venetoclax, showed a 58% composite (CR + CRi) rate and suggested a venetoclax addition survival benefit (1-year OS: 67% vs. 20%) [161].

**Gemtuzumab Ozogamicin**
In addition to IDH inhibitors, several studies have evaluated GO in R/R AML. GO was also approved for R/R AML patients largely due to MYELOFRANCE-1 clinical trial outcomes, which showed a 26% CR rate and a 11 month median RFS [162]. However, its use is limited due to modest efficacy and a higher risk of SOS in patients who ideally progress to AlloSCT [101].

**Combination therapy**
Intensive chemotherapy regimens for R/R AML include combinations such as mitoxantron/etoposide/cytarabine and fludarabine/idarubicin/cytarabine/G-CSF, with no clear superiority among them. Venetoclax in combination with intensive chemotherapy, specifically the FLAG-IDA + venetoclax regimen, demonstrated composite 61-75% CR rates and a 68% 1-year OS estimate in a phase Ib/II study [117], [163]. AlloSCT proved to be critical for long-term survival, significantly improving survival compared to chemotherapy alone in a landmark analysis (median OS not reached vs. 7 months). TP53-mutated R/R AML patients had poor outcomes (OS 7 months). Due to the high risk of myelosuppression, prophylactic antibacterial, anti-fungal and antiviral therapies were administered and the venetoclax duration was adjusted from 14 to 7 days during the study [159]. FLAG-IDA and venetoclax real-world data reported significant infection risks [164]. Although Venetoclax plus HMA therapy is not approved for R/R AML, it has been evaluated with 31-60% response rate, which suggest potential efficacy over HMA alone despite similar OS rates [165], [166], [167].

### 6.3.5 AML molecular targeted therapy

Despite significant advances in the treatment of AML, outcomes for high-risk patients remain suboptimal. A plethora of trials are evaluating targeted- and immune-therapies, both as monotherapy and in combination. While most of these therapies are in the investigational phase with undefined long-term outcomes, some have shown encouraging initial responses in difficult-to-treat populations, including those with TP53 mutations or MLL rearrangements. These types of molecularly targeted therapies mainly include menin, uproleselan.

**Menin**
MLL translocations at chromosome 11q23 involving KMT2A affect 5-10% of adult AML patients and generally indicate a poor prognosis according to ELN2022 guidelines, exclude the intermediate-risk t(9;11)(p21.3;q23.3)/MLLT3::KMT2A translocation [168], [169]. These genetic alterations lead to upregulated HOX gene expression, similar to that seen in NPM1-mutated AML, which promotes self-renewal of hematopoietic stem cells. Menin, a type of scaffolding protein encoded by MEN1, is

essential for the role of KMT2A in leukemogenesis. Early phase trials are exploring KMT2A-menin interaction inhibitors, notably SNDX 5613, which achieved a 44% CR rate in patients with NPM1 mutations or MLL rearrangements, showing promising preliminary results [170], [171].

**Uproleselan**

Uproleselan (GM-1271) is a kind of E-selectin inhibitor which disrupts vascular niche-mediated chemoresistance[16], [172]. In a phase I/II study, R/R AML patients treated with the MEC regimen + uproleselan showed a 41% CR rate and a 8.8 months median OS [173]. Notably, uproleselan significantly reduced the rate of oral mucositis associated with MEC.

**CD47**

CD47 is known as the "don't eat me" signal on AML cells, which helps AML cells evade macrophage-mediated phagocytosis. Magrolimab is a type of anti-CD47 antibody that has shown promise in AML treatment. A phase Ib study combining magrolimab + azacitidine in ND-AML patients, predominantly with TP53 mutations, reported a response rate of 65% and a CR rate of 45% [174]. In TP53 mutation carriers, the response was even more pronounced with a 71% response rate and 45% CR rate [174]. CD47 expression on red blood cells requires a dose escalation strategy to mitigate hemolysis and monitor anemia. A phase Ib trial of azacitidine, venetoclax, and magrolimab triplet therapy yielded impressive results, including high response (91%) and CR rates (81%) in ND-AML and variable response rates in R/R AML depending on prior venetoclax exposure [175]. These findings have spurred phase III trials to further evaluate magrolimab in combination with aza-citidine and venetoclax in ND-AML and TP53-mutated AML populations.

**Sabatolimab**

Sabatolimab (MBG453), a type of T cell immunoglobulin and mucin domain 3 (TIM3) targeting on immune and leukemic cells, showed a 41.2% response rate in ND-AML patients in a phase Ib trial [176]. It's clearly not expressed on normal hematopoietic stem cells. Currently, a phase II study is evaluating sabatolimab in combination with azacitidine and venetoclax in AML patients ineligible for intensive chemotherapy [177]. In this study, the combined rate of CR, mCR and PR was 31.8% in 22 patients.

In summary, the AML treatment paradigm is characterized by a rigorous search for the most effective therapeutic strategies. In this context, alloSCT is emerging as a cornerstone therapy characterized by its unique mechanism of mobilizing the donor immune system, particularly T cells, to induce a GvL effect. This effect is critical for targeting and eradicating leukemic cells, highlighting the critical role of T cells in mediating anti-leukemic responses. The success of alloSCT therapy is attributed to the immunological interactions between the donor immune cells and the patient's residual leukemia cells, providing clear evidence of the essential function of T cells against AML. However, the application of alloSCT is only feasible for a limited segment of the AML patient population due to challenges such as donor availability, patient health status and compatibility issues. In addition, allo-HCT is recognized as a resource-intensive method and the only potentially curative treatment for AML, which is a significant barrier to access, even as health insurers seek to alleviate financial constraints [178]. Despite these efforts, there is still a substantial unmet need that is exacerbated by sociodemographic disparities that limit access to alloHCT [1]. This landscape highlights the urgent need for the development of alternative T cell-based therapies that can either mimic or enhance the GvL effect without the limitations of alloHCT. Advances in this area have the potential to significantly diversify the treatment options available to AML patients, particularly those who are ineligible for transplantation. Ongoing research and innovation in T cell-based modalities offer promising prospects in the fight against AML, with the goal of improving patient outcomes through advanced therapeutic avenues.

## 6.4 CAR T therapy in AML

### 6.4.1 CAR T therapy history

The exploration of immunotherapy to combat cancer began in the 1980s, with significant advances in the understanding and development of vaccines. In the field of cancer treatment, a pioneering approach has been adoptive cell therapy using T cells engineered to recognize tumor antigens via tumor-specific receptors [179], [180], [181], [182]. Since the 1980s, the advent of chimeric antigen receptor T cells (CAR T) has significantly changed the treatment landscape for hematologic malignancies [183], [184], [185], [186].

The beginnings of chimeric antigen receptor (CAR) adoptive cell therapy approach which integrates antibody-derived variable regions with T cell receptor constant segments, were first published in Japan in 1987 [187]. Their seminal research demonstrated that murine T cell lymphoma EL4 cells modified to express chimeric anti-phosphorylcholine receptors-initiated calcium influx upon exposure to phosphorylcholine-positive bacteria, indicating antigen-driven T cell activation. Subsequently, Dr. Zelig Eshhar's group advanced this concept by developing cTCR in 1989 that combined the variable regions of the anti-2,4,6-trinitrophenyl (TNP) antibody with TCR constant regions [183]. This design facilitated MHC-independent T cell engagement, as demonstrated by the activation and IL-2 secretion of transduced murine T cells in response to TNP, highlighting the potential for targeted cellular immune responses. The initial dual-chain cTCR constructs faced challenges with co-transduction efficiency. In response, Eshhart's group innovated a single-chain format that fused a single-chain variable fragment (scFv) with a T cell signaling domain, pioneering the first generation of CAR T cells [184]. This scFv-based receptor retained the original antibody specificity while simplifying the genetic engineering of T cells, enhancing transduction efficacy, and ensuring robust antigen-specific activation [185], [188], [189]. These early iterations represent the foundational structures of today's CAR technology and represent a significant leap forward in the field of adoptive immunotherapy.

The first generation of CAR T cells, characterized by scFv linked to CD3ζ or FcR signaling domains, showed promising anticancer effects in preclinical models [190]. For example, targeting ERBB2 and HER2 with CD3ζ signaling domains significantly inhibited tumor progression in mouse models. Similarly, T cells engineered with MOv-γ targeting the α-folate receptor and fused to the Fc receptor γ chain demonstrated effective antitumor responses in various murine setups. In these studies, the modified T cells were typically treated with high doses of interleukin-2 (IL-2), which enhanced the therapeutic results.

Despite the preclinical successes, the transition to human trials revealed limitations [191], [192]. Initial clinical applications in ovarian cancer and metastatic renal cell carcinoma using MOv-γ and scFv(G250) CAR T cells, respectively, failed to achieve tumor reduction and the modified T cells exhibited poor *in vivo* persistence [186], [191] Subsequent studies also highlighted challenges, with only transient efficacy observed for treatments targeting CD20 and neuroblastoma-specific antigens, despite some *in vivo* persistence [193], [194]. The modest clinical efficacy of first-generation CARs, characterized by their transient presence and limited antitumor activity, underscored the need for further innovation. This led to the development of next-generation CAR T cells with the goal of improving their persistence, efficacy, and overall therapeutic potential in human patients.

Recognizing the critical role of these signals, Sadelain group developed a chimeric receptor that fuses CD3ζ and CD28 signaling domains [195]. This innovative design facilitates both activation and costimulation, significantly enhancing T cell functions such as proliferation, interleukin-2 secretion, and the ability to effectively target cancer cells *in vitro* [196]. T cell activation requires two signals: the initial signal from TCR interaction with peptide-loaded major histocompatibility complex (pMHC) molecules and a secondary signal from costimulatory receptors such as CD28 or 4-1BB [197], [198]. Moreover, T cells equipped with this dual-signaling chimeric receptor demonstrated superior expansion and longevity in human subjects compared to those with a receptor containing only the CD3ζ domain, increasing the efficacy of incorporating costimulatory domains.

Further progress was made with the integration of the 4-1BB costimulatory domain signal into CAR structures [199]. This modification significantly increased both the durability and anti-tumor capacity of engineered T cells in animal models. In addition, the use of the EF-1α promoter within the lentiviral vector framework ensured more consistent and prolonged expression of the chimeric receptors in T cells compared to alternatives such as CMV, PGK, and ubiquitin promoters.

This strategic incorporation of costimulatory signals alongside the CD3ζ domain culminated in the second-generation CAR, which has shown significant clinical successes [200]. This generation marks a pivotal evolution in CAR T cell therapy, with the accompanying illustration highlighting the differences between traditional TCR engagement with pMHC and the advanced recognition of tumor-associated antigens by second-generation CARs, including the derivation of the scFv domain from monoclonal antibodies.

The advancement of second-generation CAR T cell therapy has demonstrated remarkable success in the treatment of hematologic malignancies, with significant results in individual cases and clinical trials [201]. Notably, the National Cancer Institute (NCI) reported a case of advanced follicular lymphoma in which the patient experienced partial remission and selective elimination of B-lineage cells following treatment with CD19-specific CAR T cells [202]. This therapy used the MSGV retroviral vector to express a CD19-targeting CAR derived from the murine monoclonal antibody FMC63, which incorporates both the CD28 costimulatory and CD3ζ signaling end domains. The treatment regimen included lymphodepletion, CAR T cell infusions and IL-2 administration.

Similarly, a phase 1 study evaluated the efficacy of autologous CD19 targeted CAR T cells (19-28z) in patients with relapsed or refractory chronic lymphocytic leukemia (CLL) and B-cell acute lymphoblastic leukemia (B-ALL) in the United State [202]. This study highlighted the importance of prior conditioning, such as cyclophosphamide, in achieving partial responses, in contrast to the lack of objective responses in patients without such conditioning. A pivotal moment in CAR T cell therapy research occurred with the documented complete or partial remissions in three adult patients with advanced CLL following treatment with CD19-specific CAR T cells in the US [203], [204]. The CAR structure of this study featured an anti-CD19 scFv from FMC63, a 4-1BB costimulatory domain, and a CD3ζ signaling domain, driven by the EF1-α promoter in a lentiviral vector. The remarkable expansion of CAR T cells after infusion, up to a thousandfold increase, underscored the potential of the therapy to effectively treat advanced CLL and other B-cell malignancies.

These findings underscore the critical role of preparative lymphodepletion in enhancing the success of CAR T cell therapy, as demonstrated by the benefits of cyclophosphamide and fludarabine in promoting proliferation and tumor targeting of infused T cells [179]. Lymphodepletion, by reducing competitive endogenous lymphocytes and increasing levels of circulating T cell growth factors such as IL-7 and IL-15, promotes an environment conducive to the expansion and efficacy of infused CAR T cells [205], [206], [207]. Collectively, these studies are advancing our understanding of the mechanisms of CAR T cell therapy and laying the groundwork for its application in the treatment of various hematologic cancers.

The clinical success of CAR T cell therapies has been profound, as evidenced by dramatic improvements in survival and quality of life for patients who were previously at the end of treatment with conventional therapies. To date, FDA has approved six CAR T cell products, with additional approvals pending around the world (Table 6).

**Table 6: FDA approved CAR T therapies.**

| Target antigen | Costimulatory domain | Vector | Targeted cancers | Pivotal trial | Reference |
|---|---|---|---|---|---|
| CD19(FMC63) | 4-1BB | Lentiviral | R/R B-ALL | ELIANA | [208] |

| | | | | | |
|---|---|---|---|---|---|
| CD19(FMC63) | CD28 | Retroviral | R/R LBCL | ZUMA-1 | [209] |
| CD19(FMC63) | CD28 | Retroviral | R/R MCL | ZUMA-2 | [210] |
| CD19(FMC63) | 4-1BB | Lentiviral | R/R LBCL | Transvend NHL001 | [211] |
| BCMA(BB2121) | 4-1BB | Lentiviral | R/R MM | KarMMa | [212] |
| BCMA | 4-1BB | Lentiviral | R/R MM | CARTITUDE-1 | [213] |

CD19 CAR T cell therapy has achieved high response rates in pediatric and young adult B-ALL patients, but many patients relapse with CD19 negative disease [204], [214]. Follow-up AlloSCT improves long-term disease control. CD22 CAR T therapy shows promise in B-ALL, effective against both CD19 CAR T naive and resistant cases. The combination of bispecific CD19/CD22 CAR T with AlloSCT yields favorable results in R/R B-ALL in younger patients [215]. In adults, high disease burden predicts poorer outcomes after CD19 CAR T therapy, with limited success from salvage therapies including a second CAR T infusion or chemotherapy, despite AlloSCT consolidation [216]. In contrast, CLL is an early target for CAR T therapy, which remains FDA-unapproved for this treatment, showing only a 30% response rate with CD19 CAR T, possibly due to T cell exhaustion. CAR T therapy in T cell malignancies faces hurdles such as fratricide and contamination, but targeting CD5, TRBC, or TRBV shows potential to circumvent these issues while sparing normal T cells [217]. Some studies demonstrate the durable efficacy of CAR T cells, highlighting a shift from $CD8^+$ to $CD4^+$ CAR T cells maintaining long-term remission in CLL [218]. In B-cell malignancies, durable remissions over three years after anti-CD19 CAR T therapy were observed in a significant subset of patients, underscoring the long-term therapeutic promise of CAR T [219], [220]. A meta-analysis in R/R B-ALL associates 4-1BB costimulatory domains, low-dose lymphodepletion, and pretreatment remission with better survival [221]. A retrospective study shows higher efficacy but increased toxicity in R/R DLBCL, highlighting the impact of costimulatory domains and vector types on outcomes [222].

### 6.4.2 Current status of AML CAR T therapy

Although CAR T immunotherapy has achieved tremendous success in the treatment of ALL, it still faces significant challenges in AML. *In vitro* and *in vivo* studies have demonstrated the potential of CAR T cells targeting surface proteins such as CD33, CD123, CLL1, CD13, CD7, NKG2D ligand, CD38, CD70, and TIM3 to effectively eliminate AML cells [223], [224], [225], [226], [227], [228]. Despite these promising findings, clinical trial outcomes for AML have been somewhat disappointing, and modest response rates and significant “on-target, off-tumor” toxicity (Table 7). This adverse effect results from the expression of targeted antigens on healthy hematopoietic stem or progenitor cells and other tissues, resulting in unintended damage.

**Table 7: AML CAR T ongoing clinical trials and case reports.**

| Antigen | CAR structure | Study | Patient number | Safety | Reference |
|---|---|---|---|---|---|
| CLL1 | - | Phase1/2 | 8 | 5 patients grade1. 3 patients gradeCll2 | [229] |
| | CLL1-CD28-CD3ζ | Phase1 | - | - | NCT04219163 |
| | - | Phase1 | - | - | NCT04789408 |
| | - | Phase1/2 | - | - | NCT04884984 |
| | - | Phase1 | - | - | NCT04923919 |
| CD33 | - | Phase1 | - | - | NCT03126864 |

| | | | | | |
|---|---|---|---|---|---|
| | - | Phase1 | - | - | NCT02799680 |
| | - | Phase1 | - | - | NCT04835519 |
| | - | Phase1 | - | - | NCT03927261 |
| | CD33-41BB-CD3ζ | Phase1/2 | - | - | NCT01864902 [230] |
| | CD33-CD28-41BB-CD3ζ | Phase1/2 | - | - | NCT02944162 |
| | - | Phase1/2 | - | - | NCT03971799 [231] |
| | - | Phase1/2 | - | - | NCT02958397 |
| CD38 | - | Phase1 | 6 | 5 patients presented mild CRS (Grade 1-2) | NCT04351022 [232] |
| NKG2D | - | Phase1 | - | 1 | NCT02203825 |
| | - | Phase1 | - | - | NCT04167696 |
| | - | Phase1 | - | - | NCT04623944 |
| | - | Phase1/2 | - | - | NCT03018405 |
| CD123 | - | Phase1 | - | - | NCT03585517 |
| | CD123-41BB-CD3ζ-EGFRt | Phase1 | - | - | NCT03114670 |
| | CD123-CD28-41BB-3ζ | Phase1 | - | - | NCT04014881 |
| | - | Phase1 | - | - | NCT04318678 |
| | - | Phase1/2 | - | - | NCT04272125 |
| | - | Phase1/2 | - | - | NCT04265963 |
| | UniCAR + CD123 recombinant antibody | Phase1 | 19 | 12 patients grade 1-2; 3 grade 3 CRS 1; 1 grade 2 ICANS | NCT04230265 [233] |
| | - | Phase1/2 | - | - | NCT03556982 |
| | CD123-TCR-41BB-CD3ζ | Phase1 | - | - | NCT02623582 |
| | CD123-CD28-CD3ζ-EGFRt | Phase1 | 6 | CRS: 4 grade 1, 1 grade 2 | NCT02159495 [234] |
| | - | Phase1 | - | - | NCT03672851 |
| | CD123-TCR-4-1BB | - | - | - | NCT03766126 |
| | - | - | - | - | NCT03796390 |
| | CD123 Universal CAR T cell (UCART) | - | - | - | NCT03190278 |
| Ley | - | Phase1 | 4 | grade 2 neutropenia | NCT01716364 [235] |
| CD7 | - | Phase1 | - | - | NCT04762485 |
| | - | Phase1/2 | - | - | NCT04033302 |
| FLT3 | - | Phase1/2 | - | - | NCT05023707 |
| | - | - | - | - | NCT05017883 |
| CD44v6 | - | Phase1/2 | - | - | NCT04097301 |
| Dual CAR or | Muti-CAR T cell combined Eps8 or | Phase1 | - | - | NCT03291444 |

| | | | | | |
|---|---|---|---|---|---|
| Multiple CAR | WT1 CD33, CD38 CD56, CD117, CD123, CD34 and Muc1 | | | | |
| | Single CAR T or double CAR T cells with CD33, CD38, CD56, CD123, CD117, CD133, CD34 or Mucl | Phase1 | - | - | NCT03473457 |
| | Multi-CAR T cell CD33, CD38, CD123, CD56, Mucl, and CLL1 | Phase1/2 | - | - | NCT03222674 |
| | CD123-CLL1 CAR T | Phase2/3 | - | - | NCT03631576 |
| | Dual CD33-CLL1 CAR T | Early Phase1 | - | - | NCT05016063 |
| | Dual CD33-CLL1 CAR T | Phase1 | - | - | NCT05248685 |

CAR T cells can bind directly to cell surface molecules, bypassing antigen processing or HLA expression [236]. The key aspect of their development is the selection of the target antigen. An ideal target should have clear, high expression on tumor cells sufficient for CAR T cell recognition and activation, while having minimal or no expression on healthy tissues to minimize toxicity. CD19 is an example of a successful target that is expressed on B-cell malignancies but not on normal HSC and non-lymphoid tissues [237].Despite advances in understanding AML immunopathology, identifying a specific AML-exclusive target remains challenging. This complexity is evidenced by the significant toxicities observed in early phase clinical trials, underscoring the need for cautious target selection and the potential risks of CAR T therapy in AML [230].

### 6.4.3 Potential targets of AML CAR T therapy

**CD123**

CD123, also known as interleukin-3 receptor alpha chain, is expressed on AML blasts and LSCs, while its expression on normal hematopoietic stem cells is minimal [238], [239]. Some studies show it to be a promising target for CAR T cell therapy in AML [240], [241]. Mardiro group developed a CD123 CAR T cell exhibited potent effector functions against CD123 postive cell lines and primary AML samples, effectively lysing autologous AML blasts without affecting normal myeloid or erythroid colony formation *in vitro* [242]. These CD123 CAR T cells also demonstrated anti-leukemic efficacy *in vivo* in a diverse AML model. Gill et al. highlighted that CD123 targeted CAR T cells could eradicate primary AML cells and disrupt normal human myelopoiesis in immunodeficient mice, suggesting a novel BM conditioning approach for hematopoietic cell transplantation [243]. Modifications in CAR design using variable heavy and light chains of different CD123 specific antibodies optimized the balance between minimizing HSC toxicity and maintaining anti-tumor activity. The potential toxicity to normal hematopoietic stem cells due to low levels of CD123 expression necessitates further research into innovative CAR T cell designs, including universal, bispecific CARs and those equipped with safety switches to mitigate risk.

**CD33**

In addition, CD33 has received significant attention due to its high expression on myeloid cells. CD33, a member of the sialic acid-binding immunoglobulin-like lectin family, plays a critical role in modulating leukocyte behavior during immune responses. This surface glycoprotein is primarily found on cells of the myeloid lineage, including myeloid precursors and mature myeloid cells. Notably, it is present in over 90% of cells implicated in AML, establishing its importance in both diagnostics and treatment

strategies [244]. The therapeutic GO represents the only FDA-approved therapy targeting AML, underscoring the therapeutic importance of CD33 as a target antigen in the fight against this malignancy [245], [246].Empirical evidence from both *in vivo* and *in vitro* analyses has illuminated the sustained antitumor efficacy of CD33 CAR T cells, demonstrating robust tumor elimination and sustained T cell functionality during the cytotoxic onslaught against AML cells [247], [248]. Notably, clinical trials have shown that treatment with CD33 CAR T cells can significantly reduce BM blasts within two weeks, although cases of relapse have been documented, indicating the need for ongoing research to optimize therapeutic outcomes [230].Current studies are evaluating the safety and feasibility of administering various doses of CD33 CAR T cells to patients with resistant or relapsed AML [230]. Despite the therapeutic promise, the administration of autologous CD33 CAR T cells in R/R AML has been associated with serious adverse effects, including CRS, ICANS, tumor lysis syndrome (TLS), severe respiratory distress, and septic shock, due to excessive secretion of inflammatory cytokines. This underscores the imperative need for refined safety protocols in the use of CD33 CAR T cell therapies.

**NKG2D**

NKG2D is a type II membrane receptor that plays a key role in immune surveillance and is expressed predominantly on natural killer cells, $CD8^+$ T cells and γδ T cells. This receptor is instrumental in identifying and responding to cells under stress, such as those infected or undergoing transformation, by recognizing specific stress-induced ligands. The ligands of NKG2D are particularly present in a variety of hematological malignancies, including not only AML and MM, but also in solid tumors, distinguishing them from healthy cells. Despite the relatively low expression levels of NKG2D ligands in AML, NKG2D CAR T cells have significant activity against these cancer cells. This activity can be further enhanced by selectively enhancing NKG2D ligand expression through histone deacetylase inhibition, thereby improving the efficacy of NKG2D CAR T cell therapy [249], [250], [251]. A clinical trial focused on autologous CAR T cells targeting NKG2D ligands in patients with AML/MDS and MM showed encouraging results. Of seven AML patients treated with NKG2D CAR T cells, six cases showed strong responses against tumor cells without dose-limiting toxicities, CRS or CAR T cell-related neurotoxicity [251].

**CLL1**

CLL1 (or known as CLEC12A) is a type II transmembrane glycoprotein expressed on myeloid cells and a significant fraction of AML blasts. Its pronounced overexpression in LSCs, in contrast to its scarce presence during normal hematopoiesis, delineates CLL1 as a promising target for AML-targeted CAR T cell therapy, suggesting a reduced likelihood of collateral damage to healthy tissues. Since 2017, CLL1-directed CAR T cells have demonstrated targeted cytotoxicity *in vitro* and *in vivo* [252]. The introduction of a controllable caspase-9 suicide gene system into these cells facilitates targeted ablation of CAR T cells, mitigating potential adverse effects on normal myeloid cells. One research group developed a CLL1-specific CAR using scFv derived from antibodies to CLL1 isolated from C57BL/6 mice, which achieved targeted destruction of CLL1-positive cell lines and primary AML specimens *in vitro* [253]. At the same time, other investigators developed a composite CAR by coupling anti-CLL1 CAR with anti-CD33 CAR via a self-cleaving P2A peptide, allowing simultaneous expression of both CAR structures on T cells[253]. This innovative approach led to a landmark clinical trial in which this dual CAR T cell therapy showed substantial efficacy in participants. In particular, a 6-year-old patient with a complex FLT3-ITD mutation achieved CR within 19 days after two administrations of the combined CAR T therapy [254]. In addition, another group introduced a PD1-suppressed CLL1 CAR, addressing the issue of increased PD-1 expression following CAR T cell activation, which represents a significant step forward in overcoming immune evasion mechanisms in AML [255]. Another group has also developed a CLL1 CAR incorporating the apoptosis inducing gene FKBP-caspase9. In their study, 75% of R/R AML patients achieved CR with no detectable minimal residual disease. The treatment was associated with low-grade, manageable adverse events [256].

**CD7**

CD7 is a member of the immunoglobulin superfamily expressed on the surface of T cells and NK cells. This glycoprotein is also present in over 90% of T cell lymphoblastic leukemias and lymphomas and in about 30% of AML cases, while it is absent in healthy myeloid cells [257]. The presence of CD7 on

T cells necessitates their modification in the structureion of CD7-targeted CAR T cells. One group has successfully engineered CD7 CAR T cells by using T cells with edited CD7 genes [258]. These modified cells demonstrated efficacy in eradicating CD7-expressing AML cell lines, primary AML cells, and colony-forming units, while sparing myeloid and erythroid progenitor cells from any adverse effects.

**FLT3**

FLT3 is a class III receptor tyrosine kinase that localizes to hematopoietic stem and progenitor cells but not to BM. In particular, FLT3 internal tandem duplication (FLT3-ITD) mutations occur in approximately 20% of AML patients, while 7% have FLT3 tyrosine kinase domain (FLT3-TKD) mutations [259]. The FDA has approved several FLT3 inhibitors, such as gilteritinib, midostaurin, and quizartinib, for the treatment of FLT3-mutant AML [104], [143], [260]. One research group has demonstrated the potential of FLT3-directed CAR T cells in targeting $FLT3^+$ AML, showing substantial anti-leukemic efficacy both *in vitro* and in murine models [261]. In addition, another group has developed FLT3 ligand-specific CAR T cells that selectively eliminate FLT3-expressing leukemic cells and AML patient-derived BM mononuclear cells *in vitro* and significantly increase survival in FLT3+ AML xenograft mice without affecting normal human cord blood hematopoietic stem cells [262].

**CD70**

CD70 is a ligand of the tumor necrosis factor (TNF) family that is expressed on AML blasts and stem and progenitor cells, but is minimally or not detectable in normal tissues and hematopoietic cells. The interaction between CD70 and its receptor CD27 on T cells reveals a promising therapeutic pathway. The monoclonal antibody cusatuzumab inhibits the CD70/CD27 signaling axis and targets CD70-expressing LSCs, highlighting the potential of CD70 as a valuable antigen for AML treatment [263], [264], [265]. Sauer's group conducted a study comparing the efficacy of CAR T cells engineered with human CD27 (the ligand for CD70) fused to the CD3ζ chain (termed CD27z) against those with CD70 CAR [266]. Their results showed that CD27z CAR T cells had superior proliferation and anti-leukemic efficacy both *in vitro* and *in vivo* [266].

**LeY**

The Lewis Y (LeY) antigen is part of the Lewis blood group system, also a carbohydrate antigen, which is prominently overexpressed in numerous epithelial cancers and hematologic malignancies, but is rarely found in normal tissues [252], [267]. One research group synthesized LeY CAR T cells and evaluated their efficacy against AML and MM cells in preclinical studies, demonstrating that these CAR T cells specifically target LeY+ cells and secrete IFN-γ in response [267]. In addition, another group conducted a phase I clinical trial of LeY-targeted CAR T cells in relapsed AML patients. In this study, four participants were treated with anti-LeY CAR T therapy, resulting in one patient achieving transient cytogenetic remission and another showing temporary blast reduction, while two others maintained stable disease. However, all participants eventually experienced disease relapse within 23-28 months after treatment [268].

**TIM3**

TIM3 is known as the T cell immunoglobulin and mucin domain 3 immune checkpoint molecule. It has been found to be predominantly expressed on LSCs and leukemic progenitor cells in the majority of AML patients, while absent on normal hematopoietic stem cells [269], [270]. Studies analyzing protein expression in AML patients revealed high levels of TIM3 on LSCs at diagnosis (78.5%) and during relapse (64.7%), with its overexpression correlating with adverse clinical outcomes [271]. Therefore, targeting TIM3 represents a strategic therapeutic approach aimed at selectively eliminating AML LSCs, thereby preserving healthy hematopoietic stem cells. In preclinical studies, the anti-TIM3 monoclonal antibody ATIK2 demonstrated efficacy in inducing complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity, effectively preventing AML progression in xenograft models and leading to *in vivo* eradication of LSCs without affecting normal hematopoietic processes [272], [273]. Several clinical trials are currently evaluating the efficacy of TIM3 inhibitors, either as monotherapy or in combination with other therapies, in various cancer types. In particular, sabatolimab (MBG453) has highlighted the efficacy of its combination with an HMA, showing promising survival results for individuals with MDS or AML. In a pivotal Phase 1 study, patients with

relapsed/refractory AML or previously untreated de novo AML who were ineligible for standard chemotherapy received sabatolimab in combination with an HMA. Sabatolimab in combination with intravenous HMA was safe and well tolerated in patients with high-risk MDS, with overall response rates of 61.1% and 57.9% with decitabine and azacitidine, respectively [177]. The ongoing investigation of the combination of sabatolimab with azacitidine, notably in a phase 1/2 trial in AML patients in CR after AHCT with detectable residual disease, and the phase 2 STIMULUS-AML1 trial adding venetoclax to the regimen of newly diagnosed AML patients ineligible for intensive chemotherapy, underscore the continued exploration and potential of this therapeutic approach [274]. Several targeted agents against TIM-3 are in various stages of development (Table 8).

**Table 8: Ongoing TIM3 CAR T and antibody studies.**

| Name | Product | Clinical trial | Disease | Reference |
|---|---|---|---|---|
| TIM3 antibody | Sabatolimab | phase III | IR/HR/-MDS | [177] |
| | BGB-A425 | phase II | solid tumors | NCT03744468 |
| | INCAGN02390 | phase II | advanced malignancies | NCT03652077, NCT04370704 |
| | LY3321367 | phase I | solid tumors | NCT03752177 |
| | RO7121661 | phase II | solid tumors | NCT03708328, NCT03869190, NCT04785820 |
| | Sym023 | phase I | advanced solid tumor malignancies or lymphomas | NCT03489343 NCT03311412 NCT04641871 |
| | TSR-022 | phase II | advanced solid tumors | NCT02817633 NCT03680508 |
| TIM3-CD28 fusion protein | TIM3-CD28/ TIM3-CD28-6 | preclinical | K562 cell line | [275] |
| TIM3 CAR T cells | TIM3-CD28 CD19 CAR T cell | preclinical | B cell lymphoma | [276] |
| | TIM3 CAR T | preclinical | AML | [273] |
| | CD13-TIM3 split CAR T cell | preclinical | AML | [277] |
| | CLL1-TIM3 split CAR T cell | preclinical | AML | [278] |

In addition to the targeted antigens mentioned, it is also noteworthy to identify dual or multiple targets. Our previous studies have shown that the combination of CD33, CD123 and CLL1 can effectively and specifically identify AML [271]. Specific combinations of CD123/CD33, CLL1/TIM3, CD312/CLL1, etc. have been investigated for AML [277], [279], [280]. These provide a preliminary basis for exploring dual-targeted CAR T immunotherapy.

### 6.4.4 Challenges and solution for CAR T cell therapy in AML

**Antigen loss**

Despite rapid advances in CAR T cell therapy and the identification of numerous targets, including CD33, CD123, and CLL1, the intrinsic variability of AML has rendered single-target CAR T cell therapies only moderately effective [228], [281], [282]. To date, AML treatment still faces significant challenges, one of these possibility is due to the loss of target antigens [283], [284]. Tumor cells can reduce or eliminate antigen expression through regulatory gene alterations, impairing the ability of CAR T cells to recognize and destroy them [285], [286]. Resistance to CAR T therapy can also result from genetic changes in AML cells, such as antigen targeting mutations or deletions, or from increased expression of immunosuppressive factors, such as PD-L1, which deactivate CAR T cells. This issue highlights the

limitations of focusing on a single antigen in CAR T therapy. For example, one study of CAR T cells showed an impressive initial complete response rate of 93%, but this dropped to about 55% after one year, indicating significant development of resistance [287], [288]. The strategy of integrating multiple CARs targeting different AML antigens is emerging as a viable solution, reflecting successes observed in B-cell disorders and solid tumor models in a preclinical context [289], [290], [291], [292]. Other Innovative engineering methods comprise multi-specific T cells, such as using bicistronic vectors for dual CAR expression, incorporating tandem CAR designs with dual binding domains, or employing co-transduction with different CAR-encoding vectors [281], [293], [294]. The latter strategy generates diverse T cell populations, each expressing different combinations of CARs, thereby facilitating *in vivo* identification of the most effective cellular configuration against target cells.

Clinical evidence supporting this approach includes a study in which CAR T cells co-transduced with CD19 and CD22 CARs induced CRs in the majority of patients with B-cell acute lymphoblastic leukemia without evidence of antigen escape [295]. In addition, dual CAR co-transduction targeting BCMA and GPRC5D demonstrated superior tumor control in a multiple myeloma model, underscoring the potential of this strategy [296]. Hazelton group pioneered a tri-specific CAR T cell structure targeting CD33, CD123 and CLL1, and showed that these Tandem CAR T cells operate on an "OR gate" mechanism [297]. This allows activation by any single tumor-associated antigen, potentially resulting in cytolytic activity comparable or superior to monospecific counterparts. In addition, Ghamari group also developed a bispecific CAR targeting CD123 and folate receptor β to enhance tumor control and signaling upon costimulation by both receptors [298]. Another group also innovated a bispecific CAR T cell therapy targeting CD13 and TIM3 overexpressed on AML cells, which demonstrated significant leukemia eradication *in vivo* with minimal off-target effects due to the selective absence of TIM3 on healthy cells [277]. Thus, Multi-antigen CAR T cell therapy offers significant advantages: it reduces the risk of antigen escape leading to disease persistence and relapse, allows targeting of tumor cells with low antigen density, and enhances AML-specific cytotoxicity, thereby improving therapeutic outcomes.

**Hematotoxicity**

In addition to the lack of antigenic targets, another challenge for CAR T cell therapy is hematotoxicity. This is a major challenge because CAR T cells can attack normal, healthy cells due to shared antigens, leading to off-target effects that can impair hematopoiesis and cause myelosuppression. Reported hematotoxicities include pancytopenia, coagulopathies, neutropenia, thrombocytopenia, and anemia [299]. This is important because prolonged cytotoxicity can put healthy tissues at risk and increase the risk of infection due to prolonged blood cell deficiency [300], [301]. Strategies to reduce the risks associated with the long-term presence of CAR T cells include incorporating safety switches into CAR T cells or using mRNA electroporation to create transiently active CAR T cells [302], [303], [304]. One research group has developed an innovative strategy called epitope editing in hematopoietic stem and progenitor cells (HSPCs) [305]. This involves introducing single amino acid changes into antigens such as FLT3, CD123 and KIT. The goal is to make these antigens invisible to CAR T cells, thereby increasing the selectivity and safety of the therapy. Another groundbreaking work by the Cancer Genome Atlas Research Network has elucidated the de novo mutational landscape of AML and identified key recurrent mutations that drive leukemogenesis[306]. Approximately 30% AML cases have a somatic mutation in exon 12 of NPM1 gene which provide a clear therapeutic target [307]. Xie group also highlighted the potential of CAR T cells engineered to target the NPM1c epitope in complex with HLA-A2, demonstrating specific cytotoxicity against NPM1c+HLA-A2+ AML cells *in vitro* and *in vivo* [308]. In addition, dysregulated splicing produces more neoantigens, particularly Notch2 and FLT3 variant isoforms, identified on AML blasts [309]. These prevalent variants represent an untapped frontier in CAR T cell therapy for AML and signal potential avenues for innovative treatment strategies. However, none of these variants have been used in preclinical CAR T products.

Logic-gated CAR T cells offer another advanced strategy to reduce the risk of “on-target-off-tumor” toxicity. This approach features configurations such as AND, OR, and NOT gates. In this system, one CAR molecule triggers the activation signal, similar as first-generation CAR (eg. CAR-CD3ζ), while the second provides co-stimulatory signals, similar as second-generation CARs, but without an activation

domain (eg.CAR-CD28-41BB). Thus, the simultaneous recognition of two distinct antigens on tumor cells is critical for complete T cell activation [310]. This approach has shown promise in preclinical studies, with prolonged survival observed in AML-affected mice treated with AND-gated CARs targeting both CLL1 and CD33 or CLL1 and CD123 [311]. However, the variability in antigen expression among AML cases increases tumor evasion risk. In addition, the AND configuration could erroneously activate a signal upon detection of an antigen on normal cells. To address this issue, advanced AND-gated CARs with sequential signaling have been developed. These use a second-generation CAR regulated by an inducible promoter is activated only by signal from the synthetic Notch receptor (synNotch) receptor [312], [313].

Furthermore, the NOT-gated CAR framework with an inhibitory CAR (iCAR) allows differentiation between malignant and non-malignant cells. This system ensures that cell destruction only occurs when the target cells exclusively bind to the specific receptors on the CAR T cells. The key challenge with NOT-gated CARs is to identify a target antigen which present in normal tissues but not in cancer cells. In a landmark study, Richards group demonstrated the efficacy of NOT-gated CAR T cells in targeting AML using NOT-gated $CD93^+$ CAR T cells that selectively target $CD93^+$ AML cells [314]. They used single-cell RNA sequencing of endothelial and AML cell lines to identify 232 potential targets for a NOT-gated CAR targeting CD93, including key endothelial markers such as PECAM1 and TIE1.

**CRS and ICANS**

CRS caused by CAR T cells should not be overlooked in addition to the structural design of CAR T cell. CRS is an adverse effect associated with CAR T cell therapy. It is characterized by an intense immunological response triggered by the activation of CAR T cells. This activation leads to a significant release of cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-10 (IL-10), and interferon-gamma (IFN-γ). This increase in cytokine levels in the PB can lead to CAR T cell proliferation and a systemic inflammatory response, which can result in significant organ damage [315]. The spectrum of CRS manifestations ranges from mild, with symptoms such as fever and fatigue, to severe, which can include life-threatening conditions such as hypotension and respiratory distress. The incidence of CRS is often indicative of CAR T cell proliferation and efficacy, with a higher prevalence observed in patients with significant tumor burden. At the same time, the infiltration or activation of CAR T cells and peripheral monocytes in the central nervous system (CNS) can lead to immune effector cell-associated neurotoxicity syndrome (ICANS). This syndrome presents with a variety of neurological disorders ranging from cognitive impairment and headache to coma and seizures. ICANS is often associated with the migration of CAR T cells across the blood-brain barrier or the activation of peripheral monocytes. The occurrence of CRS and ICANS is attributed to the reactivity of CAR T cells against malignant cells and is not exclusive to myeloid malignancies [315], [316]. This occurs across different cancer types due to the universal mechanism of CAR T cell activation and action. Therefore, it's important to monitor patients closely and intervene promptly to optimize patient safety and therapeutic outcomes. In this context, tocilizumab is often used for prevention or early treatment of CRS, while corticosteroids and interleukin-1 receptor antagonists are used to treat both CRS and ICANS [317], [318].

### 6.4.5 The impact of CAR T therapy on AlloSCT

The refinement of CAR T cell approaches for AML during the interim "bridge period" warrants further investigation. The interval between T cell collection via apheresis and CAR T cell administration normally is 2-6 weeks. This duration has been problematic in many clinical trials, as a considerable proportion of participants experience disease progression or serious complications related to their disease. An analysis of 62 adult B-ALL patients showed that 12 individuals who received bridging or cytoreductive chemotherapy prior to receiving CAR T cell therapy, median OS was significantly longer compared to those who didn’t receive such therapy (16.3 vs 4.3 months). Interestingly, no survival benefit was observed with high-intensity compared to low-intensity bridging chemotherapy, even in patients with high disease burden. In addition, high-intensity regimens were associated with increased rates of infectious complications and therapy-related toxicities. If these findings are replicated in AML patients, opting for less intensive bridging chemotherapy may preserve eligibility for CAR T cell therapy in patients experiencing rapid disease progression.

Recent studies indicate that achieving complete remission (CR) after CAR-T therapy in relapsed/refractory (R/R) patients does not necessarily equate to a cure [319]. CAR T therapy may serve as a bridging treatment or as an alternative to AlloSCT [320]. Most data on CAR-T therapy come from phase I and II studies, which primarily focus on early responses and safety [321]. In comparison to AlloSCT studies, more data are needed on critical aspects of CAR-T therapy. A key challenge is whether allogeneic SCT should be used to consolidate MRD-negative remission in patients who are in remission post-CAR-T. Some CAR T cell therapies can maintain functional persistence *in vivo*, potentially allowing continuous tumor surveillance [322]. Theoretically, if CAR T cells can be utilized to eliminate LSC in MRD-negative patients, better therapeutic outcomes could be achieved.

Moreover, CAR T therapy is often used in combination with small molecule drugs. Exploring the synergy between CAR T therapy and established small-molecule inhibitors (such as HDAC, FLT3, IDH2, and BCL2 inhibitors) represents a promising frontier in AML treatment strategies. HDAC inhibitors have been shown to upregulate NKG2D expression, thereby enhancing the efficacy of CAR-engineered NK cells [323]. At the same time, the use of FLT3 inhibitors enhances the eradication capabilities of FLT3-specific CAR T cells against AML blasts, both *in vitro* and *in vivo* [324]. Venetoclax, a BCL-2 antagonist, has been shown to directly stimulate T cells and enhance their AML-targeted cytotoxicity through the induction of reactive oxygen species [325]. In the context of B-ALL, pre-treatment with venetoclax has been reported to enhance CAR T cell-mediated cytotoxicity, which has been attributed to upregulated expression of CD19 and pro-apoptotic proteins [326]. In addition, the use of DNA methyltransferase inhibitors (e.g azacitidine and decitabine) appears to enhance the antileukemic activity of CD123 CAR T cells in preclinical studies [321-322]. As the field of CAR T cell therapy in AML continues to evolve, the potential for augmentative interactions between targeted small molecule inhibitors and CAR modalities is likely to be further elucidated.

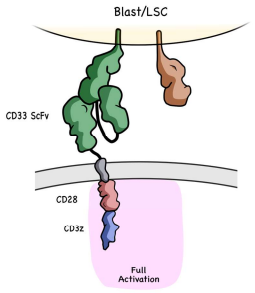
The expanding landscape of AML CAR T cell therapy is poised to reshape our perspective on alloSCT, traditionally the cornerstone of treatment for patients with intermediate or high-risk AML in CR1 [329]. Insights from CAR T cell applications in B-ALL provide valuable precursors for identifying potential beneficiaries of post-CAR SCT in AML. A landmark study on CD19 CAR T cells in B-ALL showed 83% CR rate and 67% MRD negativity. This finding suggests that SCT may not be universally beneficial for patients who achieve MRD negative after CAR T therapy [214], [330]. Conversely, the correlation between improved pre-SCT remission status and prolonged post-SCT survival complicates decision, particularly in high-risk cohorts. In a study of R/R B-ALL patients treated with CD22 CAR T cells, those who achieved CR and proceeded to SCT demonstrated superior EFS and OS compared to their non-SCT counterparts [331]. In addition, high disease burden consistently predicted an increased risk of relapse. An analysis of 185 patients treated with Kymriah underscored the negative impact of high disease burden on CR rates, EFS and OS [332]. The data showed significantly different 1-year EFS rates based on disease burden at the time of CAR T cell administration. Therefore, balancing the risk of relapse following CAR T cell therapy with the non-relapse mortality associated with allogeneic SCT is critical. Optimization of the pre-SCT response, potentially via CAR T cell intervention, is advocated to improve outcomes while recognizing the adverse implications of persistent disease.

Advances in AML CAR T cell therapy represent a breakthrough, transforming the therapeutic approach to a disease that is often resistant to conventional treatments. The achievement of durable and profound remissions in many patients signals a shift toward the possibility of long-term survival and significantly improved quality of life. The future of CAR T therapy in AML appears promising, underscored by rigorous research to refine CAR T cell structures, improve safety measures, and enhance therapeutic efficacy. Key areas of focus include the identification of antigen targets specific to AML, the development of dual-antigen-targeting strategies to circumvent antigen escape, and the engineering of CAR structures to increase specificity and minimize off-target effects on healthy cells. These advances have the potential to expand the applicability of CAR T cell therapy beyond AML to a broader range of hematologic disorders. CAR T cell therapy will hopefully redefine the clinical landscape for AML, offering profound therapeutic benefits to patients who have exhausted all conventional options.

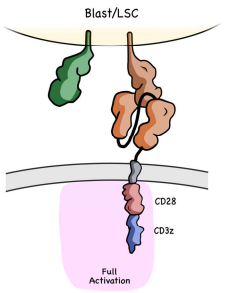
# 7. Research aim

The primary goal of this investigation is to explore efficacy and specificity of dual-targeting CAR T cells directed against the AML-associated target antigens CD33 and TIM3. Different CAR T designs will be generated and characterized in relation to on-target cytotoxicity and on-target-off-leukemia toxicity. We hypothesize that split CAR T cells will preferentially recognize AML cells and spare healthy hematopoiesis, thereby increasing the safety profile and allowing CAR T application outside the allogeneic SCT setting. In contrast, pooled, tandem and compound CAR T cells directed against CD33 and TIM-3 will be highly active against AML cells and have the potential to counteract antigen escape variants. However, these constructs will likely only be applicable in a "bridge to transplant" setting.

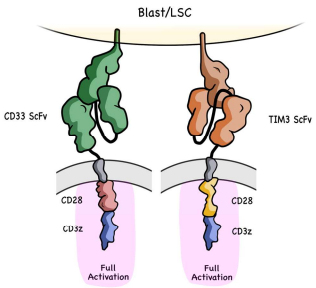
**Figure 1: Schematic representation of CAR T cells binding to AML blast/LSC or HSC/HSPC.**



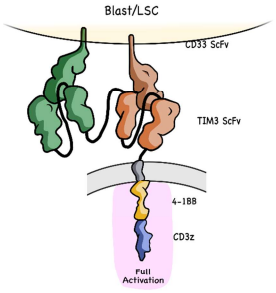

CD33 single CAR T cell



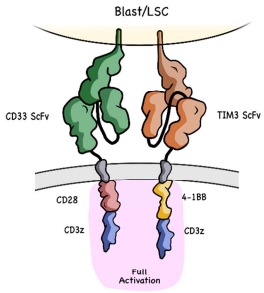

TIM3 single CAR T cell



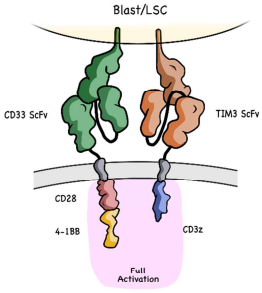

Pooled CAR T cells



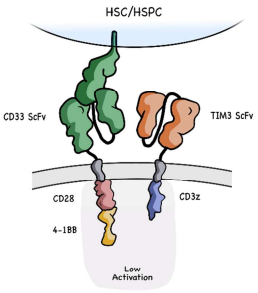

Tandem CAR T cell




Compound CAR T cell




Split CAR T cell




Split CAR T cell

# 8. Material and Methods

## 8.1 Material

### 8.1.1 Cell lines

Cell lines are listed in the table below (Table 9).

**Table 9: Cell lines.**

| Cell line name | Wild type/transduced | Species | Cell line source | Cell culture medium | Provided by |
|---|---|---|---|---|---|
| BA/F3 | wild type | Murine | pro B cell | R10+IL-3 | DSMZ ACC 300 |
| | TIM3 transduced | | | | |
| Kasumi 1 | wild type | Human | AML (FAB M2) | R20 | DSMZ ACC 220 |
| Kasumi 3 | wild type | Human | AML (FAB M0) | R20 | DSMZ ACC 714 |
| HL-60 | wild type | Human | AML (FAB M2) | R10 | DSMZ ACC 3 |
| MV4-11 | wild type | Human | AML (FAB M5) | R10 | DSMZ ACC 102 |
| OCI-AML3 | wild type | Human | AML (FAB M4) | A10 | DSMZ ACC 582 |
| | TIM3 transduced | | | | |
| | CD33 knockout | | | | |
| | CD33 knockout TIM3 transduced | | | | |
| Thp1 | wild type | Human | AML (FAB M5) | R10 | DSMZ ACC 16 |
| | TIM3 transduced | | | | |
| | CD33 knockout | | | | |
| | CD33 knockout TIM3 transduced | | | | |
| KG-1a | wild type | Human | AML (FAB M6) | R20 | DSMZ ACC 421 |
| Jurkat | wild type | Human | AML (FAB T AML) | R10 | DSMZ ACC 282 |
| SKM-1 | wild type | Human | AML (FAB M5) | R20 | DSMZ ACC 547 |
| HNT-34 | wild type | Human | AML (FAB M4) | R10 | DSMZ ACC 600 |
| MS-5 | wild type | mice | C3H/HeNSIc mice | A10 | DSMZ ACC 441 |
| Plat-A | engineered cell line | Human | HeK 293T (NIH3T3) | D10 | Cell Biolabs, INC (RV-102) |
| 293Vec-Galv | engineered cell line | Human | HeK 293T (NIH3T3) | D10 | BioVec Pharma Inc |
| 293Vec-RD114 | engineered cell line | Human | HeK 293T (NIH3T3) | D10 | BioVec Pharma Inc |

| | | | | | |
|---|---|---|---|---|---|
| Primary T cell | primary cell | Human | Healthy donor | T cell medium | Healthy donor |
| Primary AML cell | primary cell | Human | AML patient | Blast medium | AML patient |
| Primary BM cell | primary cell | Human | Healthy donor | BM medium | Healthy donor |
| XL-1 blue | - | E. coli | - | LB | Agilent |

### 8.1.2 Buffers and solutions

Buffers and cell culture medium are listed in the table below (Table 10).

**Table 10: Composition of buffers and cell culture medium.**

| Buffer/solution | Composition |
|---|---|
| R20 | 80% RPMI 1640 (PAN-Biotech)<br>20% h.i.FBS (FCS, Gibco)<br>1% Penicillin/Streptomycin/L-Glutamine (Gibco)<br>10% HEPES |
| R10 | 90% RPMI 1640 (PAN-Biotech)<br>10% h.i.FBS (FCS, Gibco)<br>1% Penicillin/Streptomycin/L-Glutamine (Gibco)<br>10% HEPES |
| D10 | 90% Dulbecco Modified Eagle Medium (DMEM, Gibco)<br>10% h.i. FBS<br>1% Penicillin/Streptomycin/L-Glutamine<br>10% HEPES |
| A10 | 90% alpha-MEM (PAN-Biotech)<br>10% h.i.FBS<br>1% Penicillin/Streptomycin/L-Glutamine<br>10% HEPES |
| T cell medium | TexMacs medium (Miltenyi Biotech)<br>1% Penicillin/Streptomycin<br>10ng/mL interleukin-7 (PeproTech)<br>5ng/mL interleukin-15 (PeproTech) |
| Blast culture medium | 90% alpha-MEM (PAN-Biotech)<br>12.5% h.i.FBS (FCS, Gibco)<br>1% Penicillin/Streptomycin/L-Glutamine (Gibco)<br>rh IL-3 20ng/ml (PeproTech)<br>rh G-CSF 20ng/ml (PeproTech)<br>rh TPO 20ng/ml (PeproTech) + β-mercaptoethanol 57.2 µM (Sigma-Aldrich) |
| BM culture medium | Blast medium<br>rh IL-6 20ng/ml (PeproTech)<br>FLT-3 ligand 20ng/ml (PeproTech)<br>rh SCF 20ng/ml (PeproTech) |
| BM CFU medium | MethoCult H4434 medium |
| Transfection medium | Opti-MEM I Reduced Serum Medium (Gibco) |
| LB medium | LB medium (Sigma-Aldrich)<br>Ampicillin 100 µg/mL (Sigma-Aldrich) |
| FACS buffer | PBS (PAN-Biotech)<br>1% BSA (Miltenyi Biotec)<br>0.5M EDTA (Invitrogen) |
| FACS FIX buffer | FACS buffer |

| | |
|---|---|
| | 0.54% Formaldehyde (Sigma) |

### 8.1.3 Antibodies and staining reagents

FACS antibodies and staining reagents are listed in the table below (Table 11).

**Table 11: Antibodies and staining reagents list.**

| Name | Channel | Clone number | Company | Reference number |
|---|---|---|---|---|
| CD2 | APC | RPA-2.10 | BioLegend | 300214 |
| | PE | | BioLegend | 300208 |
| CD3 | PerCP-Cy5.5 | SK7 | Invitrogen | 45-0036-42 |
| | APC-Cy7 | | BioLegend | 344818 |
| CD4 | APC | OKT4 | BioLegend | 317416 |
| | FITC | | BioLegend | 317408 |
| | BV650 | | BioLegend | 317436 |
| CD8 | APC-Cy7 | SK1 | BioLegend | 344714 |
| CD25 | BV421 | BC96 | BioLegend | 302630 |
| CD33 | PE | WM53 | BioLegend | 303404 |
| | APC | WM53 | BioLegend | 303408 |
| CD34 | PE-Cy7 | 581 | BioLegend | 343516 |
| CD38 | PerCP | HB-7 | BioLegend | 356622 |
| hCD45 | BV650 | HI30 | BioLegend | 304044 |
| CD45RA | BV421 | H100 | BioLegend | 304130 |
| CD69 | APC | FN50 | BioLegend | 310910 |
| CD107a | APC-Cy7 | H4A3 | BioLegend | 328630 |
| CD197(CCR7) | APC | G043H7 | BioLegend | 353214 |
| CD223(LAG3) | PE | REA351 | Miltenyi Biotech | 130-126-610 |
| | BV650 | 11C3C65 | BioLegend | 369316 |
| CD279(PD1) | PE-Cy7 | EH12.2H7 | BioLegend | 329918 |
| CD366(TIM3) | PE | F28-2E2 | BioLegend | 345006 |
| | BV421 | | BioLegend | 345008 |
| | BV650 | | BioLegend | 345028 |
| Anti-c-myc | FITC | SH1-26E7.1.3 | Miltenyi Biotech | 130-116-653 |
| Anti-HA | PE | GGG8-1F3.3.1 | Miltenyi Biotech | 130-120-786 |
| IgG isotype FITC | FITC | MoPC-21 | BioLegend | 400108 |
| IgG isotype PE | PE | MoPC-173 | BioLegend | 400212 |
| IgG isotype APC | APC | MoPC-21 | BioLegend | 400120 |
| IgG isotype PerCP | PerCP | MoPC-21 | BioLegend | 400148 |
| IgG isotype PC5.5 | PC5.5 | MoPC-173 | BioLegend | 400252 |
| IgG isotype PC7 | PC7 | | BioLegend | |
| IgG isotype BV421 | BV421 | MoPC-21 | BioLegend | 400158 |
| IgG isotype BV650 | BV660 | MoPC-21 | BioLegend | 400164 |
| Far-red | - | - | Thermo Fisher | C34564 |
| CFSE | - | - | Thermo Fisher | C34554 |
| TIM3 protein IgG | - | H5258 | ACRO Biosystems | TIM3-H5258 |
| CD33 protein IgG | - | hP67.6 | Absolute Antibody | Ab00283-13.0 |
| Live/Dead | - | - | Thermo Fisher | L34968 |

### 8.1.4 Vector/plasmid

The pMP71 plasmid was kindly provided by AG Kobold (Ludwig Maximilian University of Munich).

### 8.1.5 Kits

Commercial kits are listed in the table below (Table 12).

**Table 12: Commercial kits list.**

| Name | Purpose | Company | Lot number |
|---|---|---|---|
| Gibson assembly kit | Seamless clone | New England BIolabs | E5510S |
| T4 DNA ligase kit | Sticky ends clone | New England BIolabs | M0202 |
| Plasmid isolate kit (Mini) | Plasmid isolation | Macherey-Nagel | 740588.50 |
| Plasmid isolate kit (Midi) | Plasmid isolation | Macherey-Nagel | 740420.50 |
| Gel and PCR clean-up kit (Mini) | DNA fragment clean up | Macherey-Nagel | 740609.50 |
| Phusion High-Fidelity PCR kit | PCR clone | Thermo Fisher | F553L |
| PCR ladder 1KB | PCR product test | Thermo Fisher | SM0311 |
| Pan T cell isolation kit | CD3+T cell sorting | Miltenyi Biotech | 130-096-535 |
| TransAct CD3/CD28 beads | Active T cells | Miltenyi Biotech | 130-128-758 |
| CCK-8 | Cell proliferation assay | Sigma-Aldrich | 96992-100TESTS-F |
| Poly-L-lysine | facilitate cell adhesion to chip | Thermo Fisher | A3890401 |
| Lipofectamine 3000 | Transfection | Thermo Fisher Scientific | L3000001 |
| Polybrene | Transduction | Sigma-Aldrich | TR-1003 |
| RetroNectin | Transduction | TaKaRa | T100B |
| Countess Cell Counting Chamber Slides | Cell counting | Thermo Fisher Scientific | C10283 |
| Cytometric Bead Array (CBA) | Cytokines assay | BD | 551809 |

### 8.1.6 Experimental instruments

Experimental instruments are listed in the table below (Table 13).

**Table 13: Experiment instrument list.**

| Name | Company | Type name |
|---|---|---|
| Biological safety cabinet | Thermo Fisher Scientific | Safe 2020 |
| Centrifuge | Eppendorf | 5920 R |
| | Hettich | Rotina 420R |
| Incubator | Binder | CB60 |
| 4° fridge | Liebherr | GN 3356 |
| -20° freezer | Liebherr | GSND 3323 |
| -150° freezer | Sanyo | MDF-594 |
| -80° freezer | Thermo Fisher Scientific | 5705 |
| Flow cytometer | Beckman Coulter | Cytoflex LX |
| | Beckman Coulter | Cytoflex S |
| S1 cell sorter | BD Biosciences | BDMelody |
| S2 cell sorter | Beckman Coulter | MoFlo Astrios |
| Cell avidity analyzer | Lumicks | z-Movi |

| | | |
|---|---|---|
| Cell Imaging Multimode Reader | Agilent | CY5 |
| X-ray irradiator | Xstrahl | CIX3 |
| Shaking incubator | Eppendorf | Innova 44 |
| Gel imaging machine | Vilber | E box |
| Electrophoresis machine | Bio-rad | Wide Mini-Sub cell GT |
| DNA absorbance reader | DeNovix | DS-11 FX+ |
| Cell counter | Thermo Fisher Scientific | Countess II |

## 8.2 Methods

### 8.2.1 Clinical sample collection

BM aspirate samples from 540 patients diagnosed with AML at the time of initial diagnosis and 24 healthy donors were analyzed using flow cytometric analysis. All samples were collected after written informed consent in accordance with the Declaration of Helsinki and approval by the Institutional Review Board of the Ludwig Maximilian University Munich. The diagnosis of AML was based on cytomorphology, cytogenetics, fluorescence in situ hybridization (FISH), molecular genetics, and immunophenotyping.

### 8.2.2 ScFv screen and generation

The TIM3 antigen was administered to mice to induce an immunogenic response. Subsequently, B cells harvested from the mice were fused with myeloma cells to form hybridomas, specifically producing TIM3 antibodies. These antibodies underwent rigorous evaluation for their TIM3 selectivity using both Enzyme-Linked Immunosorbent Assay (ELISA) and FACS techniques, ensuring high specificity. After identifying the desired antibodies, the DNA sequence encoding the anti-TIM3 scFv was precisely determined by sequencing the TIM3 antibody genetic material.

### 8.2.3 Retroviral vector production

Fragment PCR cloning: Primers specific to the target sequence were designed using Benchling. The reaction mixture was prepared according to the kit's instructions, consisting of 4 µL 5 x Phusion HiFi buffer, 0.4 µL 10 mM dNTPs, the designed primers, template DNA, and Phusion DNA polymerase. The protocol involved an initial denaturation at 98 °C for 30 seconds, followed by 30 cycles of denaturation at 98 °C for 5-10 seconds and annealing/extension at 72 °C. A final extension was performed at 72 °C for 10 minutes, followed by a hold at 4 °C. Upon PCR completion, the products were analyzed using agarose gel electrophoresis to confirm amplification success and specificity. The DNA fragments were then harvested and purified using a DNA purification kit, preparing them for vector cloning.

Seamless cloning: For seamless cloning, the Gibson Assembly method was utilized. Samples were incubated in a thermocycler at 50 °C for 15 minutes to facilitate the assembly of two fragments. After incubation, samples were stored on ice or at -20 °C for subsequent transformation. XL-1 Blue competent E. coli cells were transformed with 2 µL of the assembly mixture following the specified transformation protocol.

Sticky-end cloning: T4 DNA ligase was utilized to facilitate sticky-end cloning. The reaction mixture was assembled in a microcentrifuge tube on ice, containing 2 µL T4 DNA ligase buffer, 50 ng vector DNA, 37.5 ng insert DNA, and nuclease-free water up to 20 µL, with T4 DNA ligase added last. The mixture was mixed gently by pipetting up and down, then briefly centrifuged. It was incubated at 16 °C overnight to allow cohesive end formation, then heat-inactivated at 65 °C for 10 minutes and chilled on ice. Finally, 2 µL of the reaction mixture was transformed into 50 µL of competent cells.

Competent cell transformation: 2 µL of DNA was added to ice-cold XL-1 Blue cells and mixed briefly.

The mixture was incubated for 30 minutes on ice, followed by a heat shock for 1 minute at 42 °C. The cells were allowed to recover for 3 minutes on ice, then 200 µL of LB medium was added. The cells were incubated for 1 hour at 37 °C with shaking at 1000 rpm, plated on LB agar plates containing the appropriate antibiotics, and incubated overnight at 37 °C.

### 8.2.4 Plasmid structure

All CAR sequences were cloned into a pMP71 vector as the Table 14 shows below:

**Table 14: CAR T cell structures.**

| Name | abbreviation | vector number | Detailed structure |
|---|---|---|---|
| CD33 single CAR T cells | 33-z | 1 vector | CD33 scFv-c-myc-CD8α-3ζ |
| | 33-28z | | CD33 scFv-c-myc-CD8α-CD28-3ζ |
| | 33-BBz | | CD33 scFv-c-myc-CD8α-4-1BB-3ζ |
| | 33-28BBz | | CD33 scFv-c-myc-CD8α-CD28-4-1BB-3ζ |
| TIM3 single CAR T cells | TIM3-z | 1 vector | TIM3 scFv-HA-CD8α-3ζ |
| | TIM3-28z | | TIM3 scFv-HA-CD8α-CD28-3ζ |
| | TIM3-BBz | | TIM3 scFv-HA-CD8α-4-1BB-3ζ |
| | TIM3-28BBz | | TIM3 scFv-HA-CD8α-CD28-4-1BB-3ζ |
| Compound CAR T cells | 33-28z-TIM3-BBz | 2 vectors | CD33 scFv-c-myc-CD8α-CD28-3ζ |
| | | | TIM3 scFv-HA-CD8α-4-1BB-3ζ |
| | 33-BBz-TIM3-28z | 2 vectors | CD33 scFv-c-myc-CD8α-4-1BB-3ζ |
| | | | TIM3 scFv-HA-CD8α-CD28-3ζ |
| Split CAR T cells | 33-z-TIM3-28BB | 2 vectors | CD33 scFv-c-myc-CD8α-3ζ |
| | | | TIM3 scFv-HA-CD8α-CD28-4-1BB |
| | 33-28BB-TIM3-z | 2 vectors | CD33 scFv-c-myc-CD8α-CD28-4-1BB |
| | | | TIM3 scFv-HA-CD8α-3ζ |
| Tandem CAR T cells | 33-TIM3-BBz | 1 vector | CD33 scFv-TIM3 scFV-HA-CD8α-4-1BB-3ζ |
| | TIM3-CD33-BBz | 1 vector | TIM3 scFv-CD33 scFv-c-myc-CD8α-4-1BB-3ζ |

### 8.2.5 Plasmid isolation and purification

Plasmid DNA was isolated and purified using a plasmid isolation kit (Macherey-Nagel Plasmid Mini or Midi Kit) according to the manufacturer's instructions. Bacterial cultures containing recombinant plasmids were cultivated in LB medium supplemented with ampicillin (100 µg/mL) to facilitate the selection of plasmid-containing cells. The cultures were incubated at 37 °C with shaking at 1000 rpm until they reached an optical density (OD600) of approximately 0.6. Cells from 1 mL of each culture were harvested by centrifugation at 13,000 g for 2 minutes. The supernatant was then discarded. Subsequently, the cells were lysed by the addition of 200 µL of lysis buffer, and the mixture was gently inverted on several occasions to ensure complete lysis. Subsequently, 150 µL of neutralization buffer was added, and the mixture was immediately centrifuged at 13,000 g for 10 minutes to pellet cellular debris and denatured proteins. The plasmid DNA was purified from the lysate using silica membrane spin columns (Macherey-Nagel Plasmid Mini Kit), following the manufacturer's instructions. The DNA bound to the column was washed twice with wash buffer to remove impurities, and high-quality plasmid DNA was eluted in 50 µL of elution buffer. The concentration and purity of the isolated plasmid DNA were assessed using a NanoDrop spectrophotometer. The yield and quality of the isolated plasmid DNA were further verified by agarose gel electrophoresis.

### 8.2.6 Protein docking prediction with AlphaFold2

CD33 antigen protein structure (PDB ID:5F71) were published in the Protein Data Bank [333]. The PDB100 template, mmseqs2_uniref_env, unpaired paired model, and AlphaFold2-multimer_v2 were selected and executed on an AlphaFold2-multimer server using ColabFold, with other settings configured to automatic [334]. The results were analyzed using PyMOL software for rigid binding analysis.

### 8.2.7 Cell culture

Suspension cells, including AML cell lines, primary T cells, and CAR T cells, were maintained at 37 °C and 5% $CO_2$ and sub-cultured twice per week. The counted cells were gently transferred to a sterile conical tube and centrifuged at 550 g for 5 minutes to pellet the cells. The supernatant was carefully decanted or aspirated, avoiding disturbance of the cell pellet. The cell pellet was gently resuspended in fresh, pre-warmed culture medium and transferred to a new culture flask, which was then returned to the incubator.

Adherent cells, including Plat-A, HEK293Vec-Galv, and HEK293-RD114, were maintained at 37 °C and 5% $CO_2$ and sub-cultured twice per week. To split the cells, each was washed with phosphate-buffered saline (PBS), then treated with 4 mL of 0.05% trypsin/0.5 mM ethylenediaminetetraacetic acid (EDTA) in a 10 cm dish and incubated at 37 °C for 3 minutes. The dish was tapped to detach the cells, which were then transferred to a 50 mL tube containing 10 mL of culture medium. The dish surface was washed three times with 1 mL of Trypsin/EDTA using the same pipette. The cell suspension was then mixed thoroughly and transferred to a 50 mL tube containing 10 mL of medium. The cells were centrifuged at 1400 rpm for 5 minutes. The supernatant was discarded, and the cell pellet was loosened by tapping, followed by gentle resuspension in culture medium. Subsequently, 5 mL of culture medium was added to the cell suspension, which was then gently mixed. An additional 15 mL of culture medium was then added, after which the cells were counted and seeded. Typically, $10^7$ cells were harvested from a T75 flask.

### 8.2.8 General flow-cytometry staining

The cell concentration was adjusted to a minimum of $5\times10^6$ cells/mL. Then cell suspension was centrifuged at 550 g for 5 minutes, after which the supernatant was discarded. Cells were then washed once with FACS buffer. Subsequently, staining solution was prepared by adding the appropriate antibody to 100 µL FACS buffer. Cells were incubated with the staining solution at 4 °C for 15 minutes in the dark. Afterward, the cells were centrifuged at 550 g for 5 minutes, and the supernatant was removed. After that, cells were washed with FACS buffer and fixed with FACS Fix buffer.

### 8.2.9 General FAC-sorting

Cells were incubated with specific antibodies at 4 °C for 15 minutes in the dark then washed with FACS buffer in order to remove any excess antibodies. Subsequently, the stained cells were sorted based on fluorescence using BD Biosciences FACS Melody cell sorter or the Beckman Coulter MoFlo Astrios cell sorter. The instrument settings were optimized for the detection of specific fluorescent markers, and cells were sorted into distinct populations based on their fluorescence intensity. Following the sorting procedure, the cells were collected in 3 mL FACS tubes containing culture medium supplemented with 10% fetal bovine serum, which was used to promote cell viability. Subsequently, the cells were cultured in a 37 °C and 5% $CO_2$ incubator.

### 8.2.10 Phosphoflow flow cytometry staining

CAR T cells were cultured overnight in a medium enriched with IL-7 and IL-15. Following overnight culture, CAR T cells and target cells were stained with a Live/Dead reagent. Subsequently, $1 \times 10^6$ CAR T cells were co-cultured with $5 \times 10^6$ OCI-AML-TIM3 target cells to initiate stimulation. Following this, the cell mixture was centrifuged at 200 g for 30 seconds and then incubated at 37 °C for 15 minutes. Following the cessation of stimulation, the cells were fixed by the addition of 4 mL of PhosphoFlow Lyse/Fix Buffer (BD Biosciences) and incubation at 37 °C for 10 minutes. The cells were then washed and subsequently permeabilized by the addition of 3 mL of PhosphoFlow Perm Buffer III (BD

Biosciences), followed by incubation on ice for 30 minutes. Subsequently, the cells were incubated with specific antibodies (Biolegend or BD Biosciences) for 20 minutes at 4 °C.

### 8.2.11 T cell isolation and activation

Blood samples were diluted with an equal volume of PBS and carefully layered over Ficoll medium in a centrifuge tube to minimize mixing. The mixture was centrifuged at 800 g for 30 minutes at room temperature. After centrifugation, the mononuclear cell layer was transferred to a new centrifuge tube and washed thoroughly with PBS. The suspension was centrifuged at 550 g for 5 minutes to pellet the cells.

T cells were isolated from the PB mononuclear cells of healthy donors using a Pan T cell isolation kit (Miltenyi Biotech). These cells were activated with TransAct CD3/CD28 colloidal polymeric nanomatrix (Miltenyi Biotech) beads at a 1:1 bead-to-cell ratio in T cell medium, maintaining a density of $5 \times 10^5$ cells/mL. The culture medium was refreshed every 2-3 days while culturing T cells at 37 °C in a 5% CO2 incubator.

### 8.2.12 Transfection and transduction

Transfection of 293-Galv or Plat-A cells: HEK293-Galv or Plat-A cells were transfected using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's protocol. Briefly, DNA and Lipofectamine 3000 reagent were diluted separately in Opti-MEM I Reduced Serum Medium (Gibco) and then combined to form DNA-lipid complexes. After 15 minutes of incubation at room temperature, the complexes were added to the cells, which were subsequently cultured at 37 °C and 5% $CO_2$ incubator. FACS was used to test transfection efficiency.

Transduction of 293-RD114 cells: Following the transfection of CAR plasmids into HEK293-Galv cells, the virus-containing supernatant was harvested 48 hours post-transfection and filtered through a 0.45 µm filter. The HEK293-RD114 cells were subsequently exposed to the viral concentrate in 8 µg/mL polybrene (Sigma-Aldrich) to enhance infection efficiency. After 24 hours, the medium was replaced with fresh culture medium, and HEK293-RD114 cells were further cultured at 37 °C and 5% $CO_2$ incubator for 72 hours to test CAR gene expression. Flow-cytometry was used to test transduction efficiency.

T-cell transduction: T cells were isolated as described above. Following isolation, $CD3^+$ T cells were cultured in a specific CAR T Medium (Miltenyi Biotec), supplemented with 10 ng/mL interleukin-7 (IL-7) and 5 ng/mL interleukin-15 (IL-15). On the second day, these activated T cells were harvested and subjected to transduction using a retrovirus on RetroNectin-coated plates (TaKaRa). The culture medium was refreshed every 3-4 days until the 14th day of cultivation under controlled conditions of 37 °C and 5% CO2 incubator. Over the course of the cultivation, total cell counts were monitored periodically using trypan blue exclusion. The efficiency of the transduction was subsequently assessed by flow-cytometry.

### 8.2.13 CRISPR/Cas9 mediated CD33 antigen knockout

CRIPSR/Cas9-mediated gene editing has been extensively described elsewhere [335]. OCI-AML3 and THP-1 AML cell lines were subjected to electroporation with a complex comprising TrueCut Cas9 protein (Integrated DNA Technologies) and guide RNA targeting CD33. Following electroporation and verification of CD33 knockout, cell sorting was employed to isolate CD33-negative AML cell lines.

### 8.2.14 CCK-8 cell proliferation assay

Cells were plated in 96-well plates at a density of $1 \times 10^5$ cells per well. Following transfection for 0, 24, or 48 hours, cells were treated with 10µL of CCK-8 solution (Merck) well and incubated in a humidified 37 °C incubator with 5% CO2 for 4 hours. Optical density (OD) values were determined at 450 nm using a Cytation cell imaging multimode reader (Agilent).

### 8.2.15 Cytotoxicity assay

CAR and un-transduced (Mock) T cells were added at multiple effector-to-target (E: T) ratios. After 24-48 hours of incubation, the supernatant was harvested for cytokine release assay. AML cell lines were stained with flow-cytometry antibodies (e.g. CD2-BV421, CD33-APC, TIM3-BV660, anti-c-myc-FITC, anti-HA-PE, all Biolegend; Live/Dead regent, Thermo Fisher), target cell counts were detected with a CytoFLEX S/LX flow cytometer (Beckman Coulter) The formula to calculate % specific lysis is as follows:

$$CAR\ T\ cell\ specific\ lysis = \left(1 - \frac{Target\ cell\ count\ in\ CAR\ T\ group}{Target\ cell\ count\ in\ Mock\ T\ group}\right) \times 100\%$$

### 8.2.16 Cytokine release assay

The concentrations of IFN-γ and IL-2 were measured using Cytometric Bead Array (CBA) kits, according to the manufacturer's guidelines (BD Biosciences). The CBA analysis was conducted using a Cytoflex S/LX flow cytometer (Beckman Coulter).

### 8.2.17 Avidity measurement

OCI-AML3-TIM3 cells were seeded in a z-Movi microfluidic chip (Lumicks, Amsterdam, Netherlands) coated with poly-L-lysine (Thermo Fisher) to enhance cell adhesion and cultured overnight. The next day, CAR-T cells were introduced into the chips and co-incubated with the target cells for five minutes to facilitate binding, followed by a three-minute linear force ramp. During the force ramp, the z-Movi avidity analyzer (Lumicks) continuously captured images using its integrated bright-field microscope. The acoustic force levitated the detached cells towards the acoustic nodes, enabling XY position tracking. Changes in the z-position altered the diffraction pattern, distinguishing between cells adhered to the substrate and those levitated to the acoustic nodes. This data was used to correlate specific rupture forces with cell detachment events. Data analysis was performed using the Oceon software, which calculated the median acoustic forces. The results are presented as the median acoustic force (rForce), calibrated against 10 µm polystyrene beads, representing the relative force required for cell detachment. The affinity was assessed by counting the percentage of CAR T cells binding to target cells under varying intensities of the acoustic force field.

### 8.2.18 Colony formation unit assay with HSCs

HSCs were isolated from the bone marrow of healthy donors using CD34 microbeads (Miltenyi Biotech). Following isolation, the $CD34^+$ HSCs were co-incubated with either CAR or non-transduced T cells, maintaining an E:T ratio of 10:1 for 6 hours. Post-incubation, the cell suspensions were plated in duplicates using MethoCult H4434 medium, enriched with recombinant cytokines (Stemcell Technologies). Following 14 days incubation period, colonies were enumerated under an inverted microscope, calculating the median total number of colonies for each condition.

### 8.2.19 Cytotoxicity assays with primary AML samples

Prior to the thawing of the primary AML samples, the MS-5 cells were irradiated with 60Gy, seeded in a 6-well plate at 3 x $10^5$ cells per well, and incubated for 24 hours to form a monolayer. Primary AML cells were co-cultured with 6 x $10^5$ cells/mL in blast culture medium over the MS-5 monolayer for a period of two days. Cytotoxicity assays were conducted with primary AML samples in 96-well round-bottom plates using blast medium. CAR T cells or mock T cells were cocultured with precultured AML blast cells at an E:T ratio of 1:5, with 1.2 x $10^5$ cells/well for 24 hours in a 37 °C and 5% $CO_2$ incubator. After 24 hours, the cells were gently aspirated from the monolayer surface and then stained according to the previously described FACS protocol. The analysis was performed using a CytoFlex S/LX flow cytometer.

### 8.2.20 Cytotoxicity assay with primary cell and health BM cells mixture

Before thawing the primary AML and healthy donor BM samples, MS-5 cells were irradiated with 60 Gy, seeded in a 6-well plate at 3 x $10^5$ cells per well, and incubated for 24 hours to form a monolayer.

Primary AML cells and healthy donor marrow cells were co-cultured at 6 x $10^5$ cells/mL in blast culture medium over the MS-5 monolayer for two days. The primary AML and healthy BM cells were pre-mixed in a 1:1 ratio before cytotoxicity assay. Cytotoxicity assay using the cell mixture were conducted in 96-well round-bottom plates filled with blast medium. CAR T cells or mock T cells were cocultured with the precultured cell mixture at an effector-to-target (E:T) ratio of 1:5, using 1.2 x $10^5$ cells per well, in a 37 °C, 5% $CO_2$ incubator. After 24 hours, cells were gently aspirated from the monolayer surface and stained for flow cytometry analysis according to a previously described FACS protocol. The analysis was conducted using a CytoFlex S/LX flow cytometer.

### 8.2.21 Antigen long term exposure assay

For the repeat antigen stimulation assay, on day 0, TIM3 transduced OCI-AML3 were irradiated with 70 Gy, then plated in 6-well plates with 3 × $10^5$/ml. On day 1, target cells mixed with 3 × $10^5$ viable CAR-T cells and un-transduced T cells in 24-well plates with T cell medium. On day 4, new target cells were treated as on day 0, viable CAR-T cells were counted, and 3 × $10^5$ CAR T cells from the 24-well plates that expanded were remixed with 3 × $10^5$ irradiated TIM3 transduced OCI-AML3 cells as on day 1. This process was repeated until day 28. Fold expansion after each stimulation was calculated as (viable CAR T or Mock T cells on day 4)/ (3 × $10^5$), whereas the cumulative fold expansion was regulated by fold expansion times.

## 8.3 Data Analysis

Flow-cytometry data analysis was performed with FlowJo v10.5.3 (BD Life Sciences). Plasmid construction, primer design, and sequencing result comparisons were performed using the online version of Benchling (Benchling.com). The analysis and visualization of protein tertiary structures were conducted using PyMOL software (pymol.org). All diagrams were created using Affinity Designer iPad version (Affinity). Calculations and statistical analysis were performed with Microsoft Excel 2019 (Microsoft Corporation) and GraphPad Prism v9.1.0 (GraphPad Software).

Unless otherwise stated, all data are representative of at least three independent experiments. All data are presented as mean ± SEM. Significant differences were analyzed by t test or two-way ANOVA. P-values are represented as either not significant (ns), *p < 0.05, **p < 0.01, ***p < 0.001, or ****p < 0.0001.

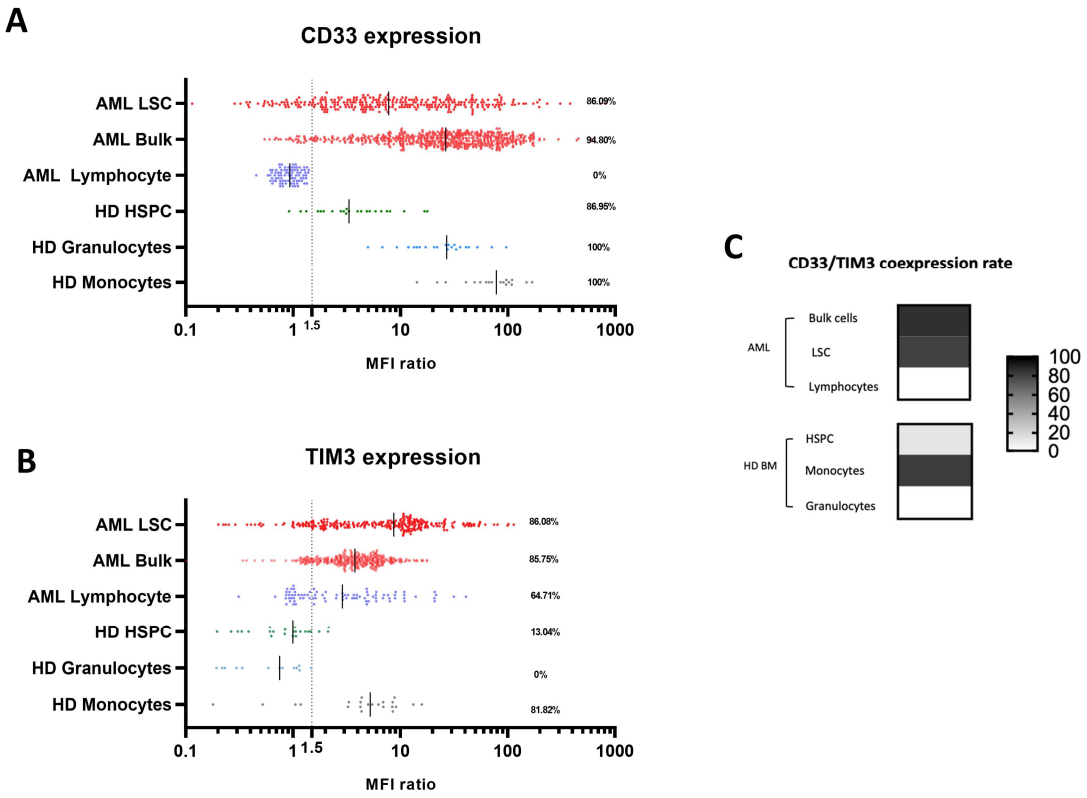
# 9. Results

## 9.1 CD33 and TIM3 were highly expressed in AML patients

To assess the expression of CD33 and TIM3 in AML patients, multi-color flow cytometry data from newly diagnosed non-APL AML patients (n=85-540) were analyzed. AML bulk cells showed the highest CD33 MFI ratio (median = 26.42, n=540). The MFI ratio for AML lymphocytes was below 1.5 (median = 0.93, n=85), all other cell subsets exhibited ratios above 1.5 (AML LSCs median = 7.75, HD monocytes median = 78.20, HD granulocytes median = 26.91, HD HSPCs median = 3.31). However, the MFI ratios of TIM3 on bulk and LSC cells remained lower compared to those of CD33 (3.78 and 4.68 vs 26.42 and 7.75, $p<0.0001$, paired t test). LSCs exhibited the highest TIM3 MFI ratio (median = 4.68) compared with other cell subsets (AML bulk cells median = 3.78, monocytes median = 5.24, lymphocytes median = 2.88).

CD33 and TIM3 were considered positive in the majority of cells if their MFI ratio exceeded 1.5. In AML patient samples, the single antigen positive rates on AML bulk cells were 94.80% (CD33) and 85.75% (TIM3), while on LSC cells they were 86.09% (CD33) and 86.08% (TIM3). Notably, CD33 was also highly expressed on normal hematopoietic cells, such as HSPCs (86.95%), granulocytes (100%), and monocytes (100%). However, TIM3 expression was elevated in a limited subset of cells, specifically monocytes (81.82%) and lymphocytes (64.71%) (Fig. 2 A and B). Furthermore, CD33 and TIM3 co-expression was observed on blasts and LSC cells in over 80% of patients, whereas this phenomenon was absent in the majority of normal cells (Fig. 2 C). These results align closely with our previous finding [271].

**Figure 2: CD33 and TIM3 were highly expressed in AML patients.**

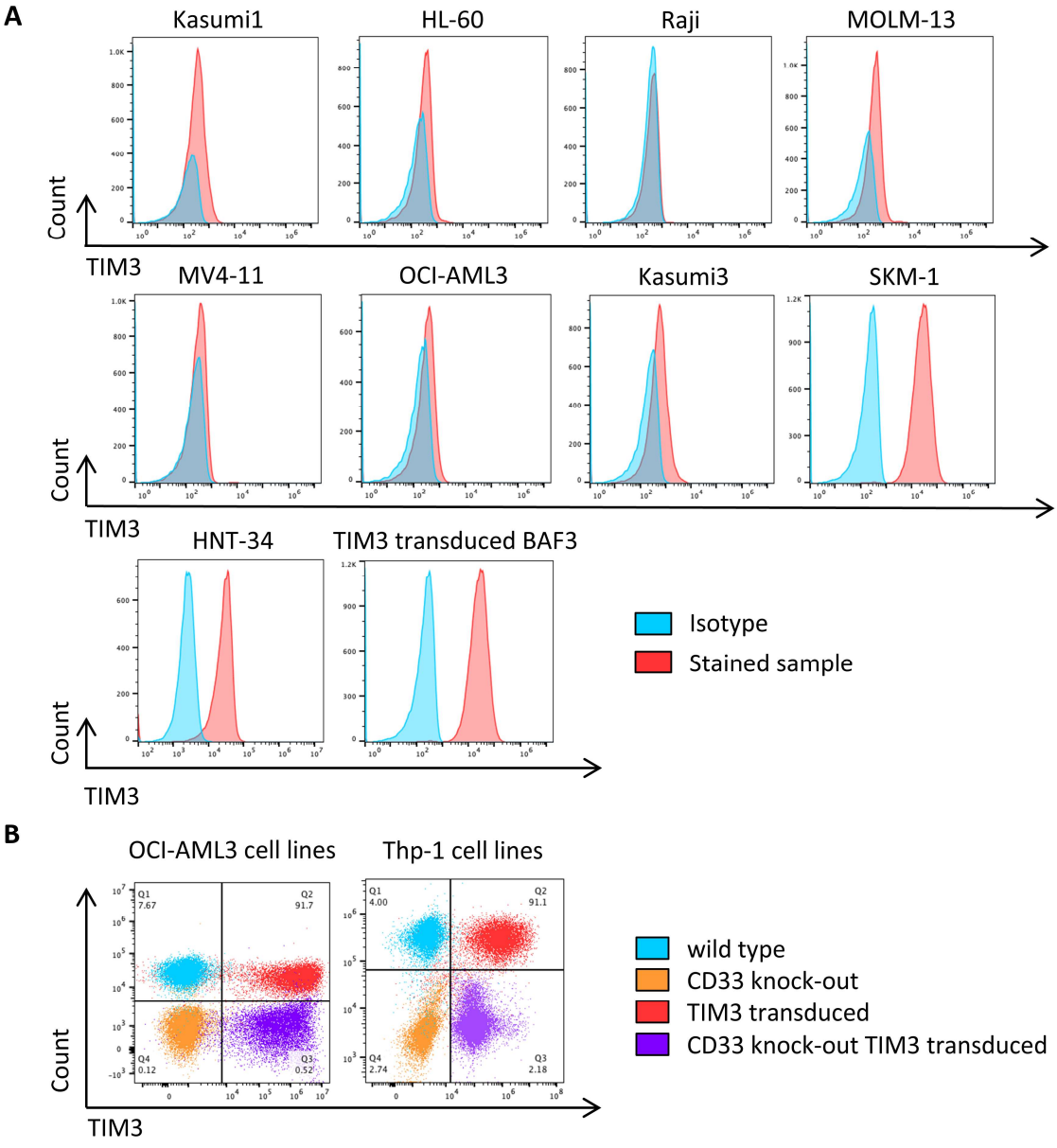



**A, B** CD33 and TIM3 MFI ratio of cell subsets in AML patients and healthy donors (AML patients: n=85-540, healthy donors: n=13, each dot represents an independent sample). **C** Percentage of co-expression of CD33 and TIM3 in AML patients vs healthy donors (AML patients: n=354, healthy donors: n=13, each dot represents a biological replicate).

## 9.2 TIM3 expression was limited to a few selected AML cell lines

According to the literature, TIM3 expression on AML cells was predominantly associated with undifferentiated cells [272], [336]. Consequently, in the initial phase of this study, we assessed the expression of TIM3 across commonly used AML cell lines based on the FAB classification: Kasumi-3 (M0), HL-60 (M2), OCI-AML3 (M4), THP-1 (M5), and MOLM-13 (M5a), using TIM3 transduced BA/F3 cells as a positive control. TIM3 expression was absent in all cell lines (Fig. 3 A). Building on these findings, the human TIM3 cDNA sequence was cloned into a pMP71 vector and transfected into Plat-A cells. The resulting retrovirus produced by Plat-A cells was used to transduce OCI-AML3 and THP-1 cell lines. Subsequently, the CRISPR-Cas9 system was employed to knock out the CD3 3 gene, establishing cell lines with no expression, single expression, and dual expression of CD33 and TIM3 for further study (Fig. 3 B). In the later stages of the study, guided by literature reports, two wild-tye TIM3 expressing AML cell lines were identified (SKM-1 and HNT-3) [337].

**Figure 3: TIM3 expression was limited to a few AML cell lines.**

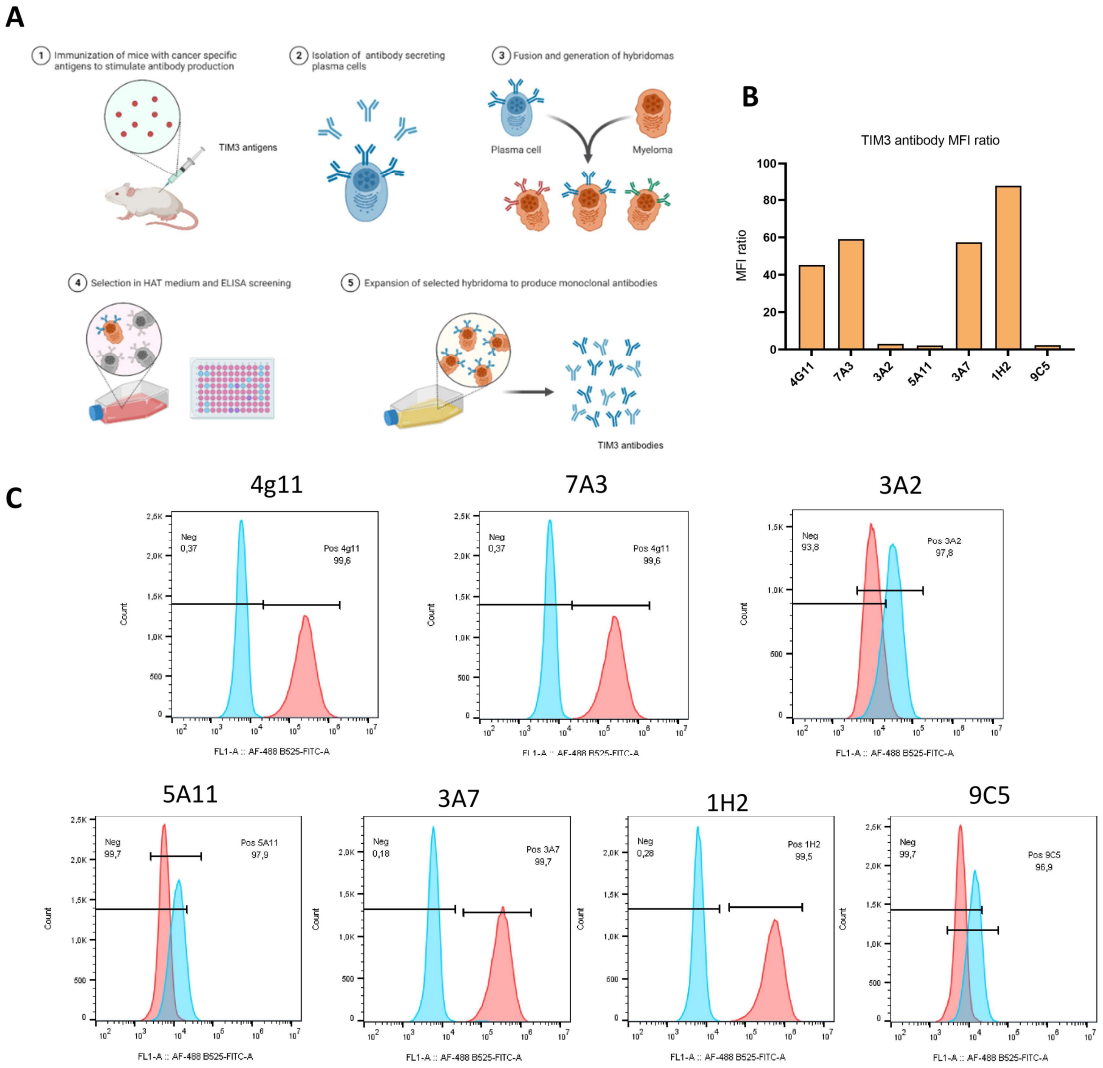



**A** TIM3 expression on AML cell lines. **B** Antigen expression of engineered OCI-AML3 and THP-1 cell lines. CRISPR-Cas9 was used to knock-out CD33. One of a representative example in 3 independent experiments is shown.

## 9.3 TIM3 antibody screen and TIM3 scFv DNA sequencing

Following the detection of CD33 and TIM3 expression and considering the scarcity of available TIM3 antibodies, we developed our own antibody and derived the anti-TIM3 scFv DNA sequence (Fig. 4 A). Initially, TIM3 antigen protein was injected into mice. Then plasmacytoid cells were isolated from the spleen of these mice and fused with myeloma cells. After selecting cells that specifically produced anti-TIM3 antibodies, seven candidates were identified (Fig. 4 B). Ultimately, through flow cytometric analysis, antibody clone 4G11 was selected as our TIM3 antibody, and its corresponding DNA sequence was determined by myeloma cell DNA sequencing.

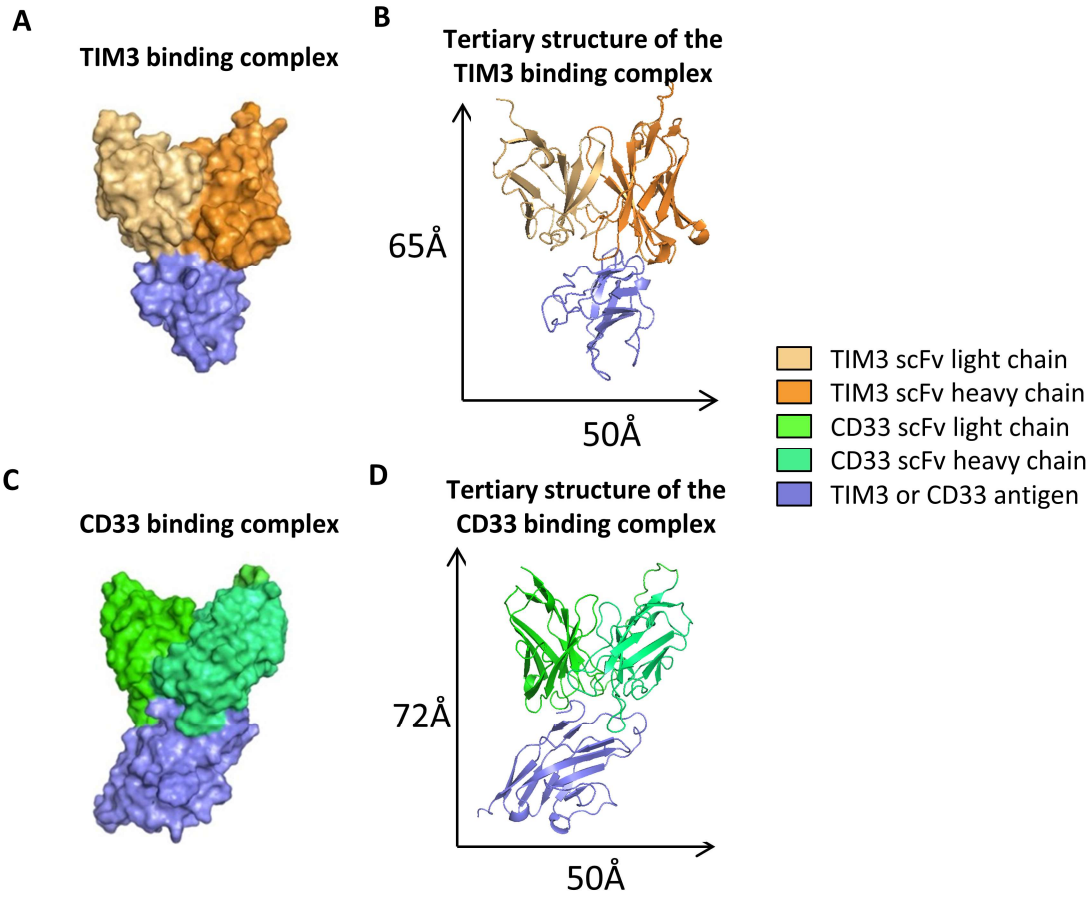
**Figure 4: TIM3 antibody screen and TIM3 scFv DNA sequencing.**




**A** Representation of the pipeline used for TIM3 antibody generation. **B** MFI ratio of TIM3 antibody candidates on BaF3-TIM3⁺ cells. **C** Representative example of TIM3 binding capacity of different TIM3 antibody candidates on BaF3-TIM3⁺ cells. The gating strategy in flow cytometry is based on isotype.

We then used AlphaFold2 to predict and confirm the interaction between the protein encoded by the TIM3 scFv sequence and the TIM3 antigen (PDB ID: 5F71) (Fig. 5 A and B). Similarly, the binding complex of CD33 scFv sequence from gemtuzumab ozogamicin (clone hP67.6) and CD33 protein (PDB ID: 6D48) was modeled by using structural simulation. This analysis aimed to characterize their interaction (Fig. 5 C and D). From the perspective of the binding complex model, both CD33- and TIM3-scFv effectively bound to their respective antigens. TIM3 scFv demonstrated a higher affinity for its antigen compared to CD33 scFv by analyzing the interaction surfaces of the tertiary structures (Fig. 5 B and D).

**Figure 5: AlphaFold2-based prediction of the scFv bound to its antigen.**




**A** Surface view of TIM3 scFv and antigen binding complex. **B** Tertiary structure of TIM3 binding complex. **C** Surface view of CD33 scFv and antigen binding complex. **D** Tertiary structure of CD33 binding complex.

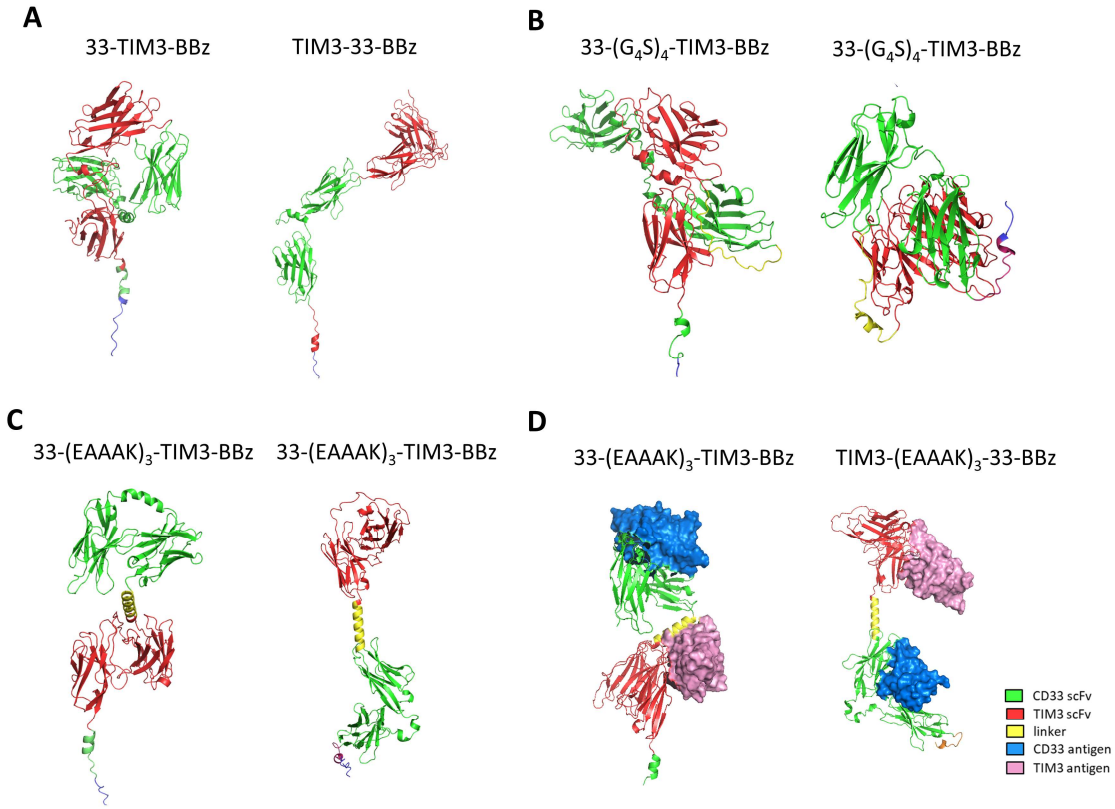
## 9.4 The rigid linker $(EAAAK)_3$ demonstrated a superior binding complex structure in tandem CAR design

Having determined the sequences for CD33 and TIM3 scFvs, the dual CAR T cells were designed. Dual CARs were mainly classified into four structures based on the scFv pairing: pooled CARs, compound CAR, split CAR and tandem CAR (Fig. 1). The first three structures function through two separate CAR structures, whereas a connecting method needed to be designed between the scFvs of the tandem CAR. Connecting methods can be categorized into two main groups: direct (no linker) and linker-mediated connecting. The latter included rigid (e.g. $(EAAAK)_3$) and flexible linkers (e.g. $(G_4S)_4$). It is of the utmost importance to avoid mismatches between the heavy and light chains of the different scFvs in tandem CAR design [338], [339].

Therefore, the AlphaFold2 platform was employed to simulate the three protein structures (no linker, $(G_4S)_4$ linker and $(EAAAK)_3$ linker) in order to ensure that the CD33 and TIM3 scFvs were correctly linked without causing mismatch of the light and heavy chains (Fig. 6A-C). All predictions were ranked according to the pLDDT confidence value, with the result with the highest confidence value being selected as the output. In both, there was some mismatch of heavy and light chains in the “no linker” and “flexible linker” $(G_4S)_4$ structures, whereas this was not observed using the rigid linker $(EAAAK)_3$ (Fig. 6 A and B). Consequently, the rigid linker was selected for further evaluation, and AlphaFold2 was used to model their interaction with their respective antigens (Fig. 6 D). 4-1BB was selected as the co-stimulatory domain, given the significant success of 4-1BB in tandem CAR studies [340], [341],

[342].

**Figure 6: Binding structure of different linkers in tandem CAR design.**

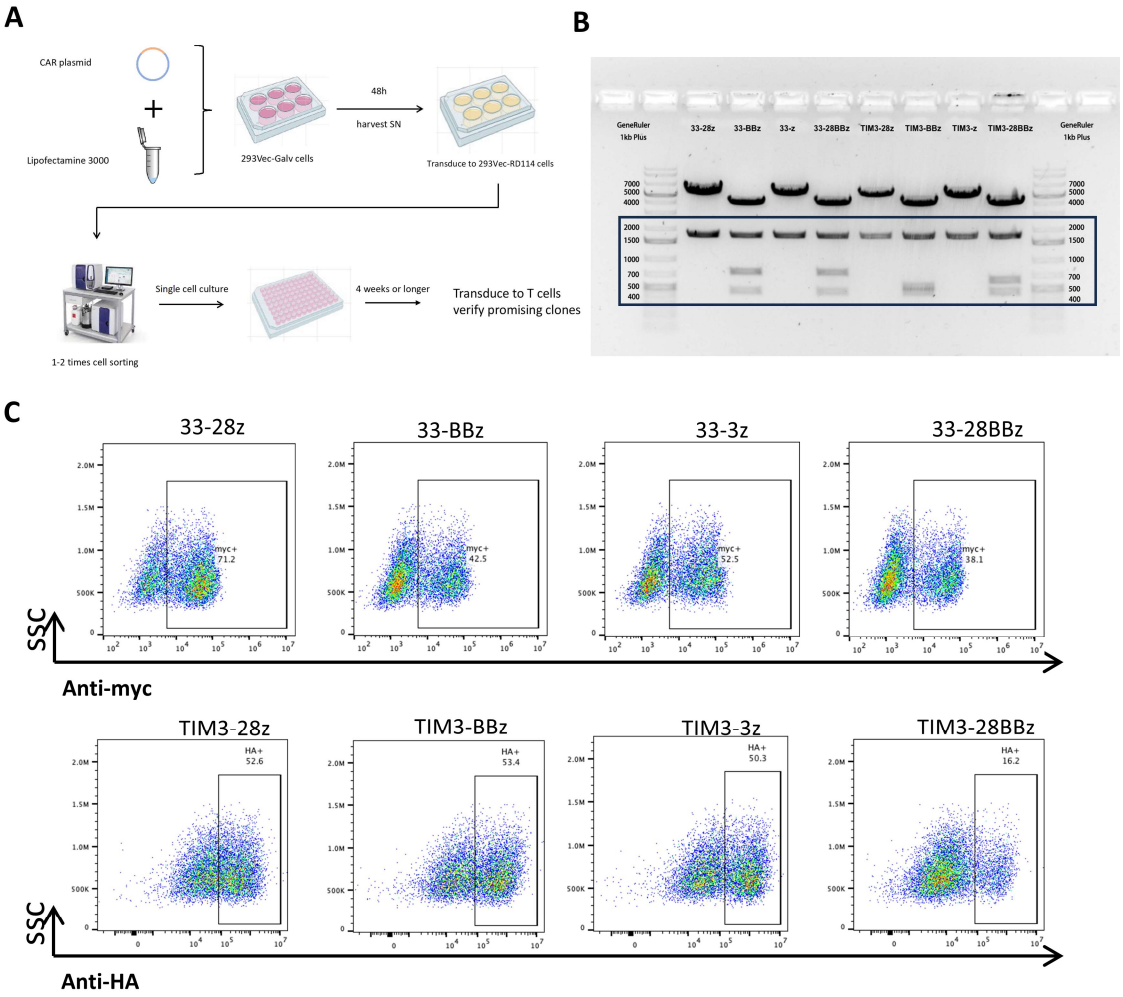



**A** Tertiary structure of the "no linker" tandem CAR ectodomain. **B** Tertiary structure of the flexible $(G_4S)_4$ linker tandem CAR ectodomain. **C** Tertiary structure of the rigid $(EAAAK)_3$ linker tandem CAR ectodomain. **D** Tertiary structure of the rigid $(EAAAK)_3$ linker tandem CAR ectodomain bound to the respective antigens.

## 9.5 Establishment of CAR virus production platform

Once the structure of the different CAR constructs had been determined (Fig. 1), all necessary CAR DNA fragments were synthesized via molecular cloning (Fig. 7 A). These fragments were subsequently incorporated into the pMP71 plasmid using Gibson assembly (Fig. 7B). The plasmid was then transfected into HEK293T-GALV cells. Subsequently, HEK293T-RD114 cells were infected with supernatant containing the virus generated by HEK293T-GALV cells, to establish a virus producer cell line for CAR production. Following the implementation of FACS-sorting and monoclonal cell culture, CAR producer cell lines were successfully established (Fig. 7 A). The CD33 CAR structures included a c-myc as a reporter gene following the transmembrane domain, whereas the TIM3 CAR structures incorporated the HA as a reporter gene. The variations in CAR structures and fragment lengths had no significant impact on the establishment of the CAR virus producer cell lines (Fig. 7 C).

**Figure 7: Establishment of CAR virus production system**

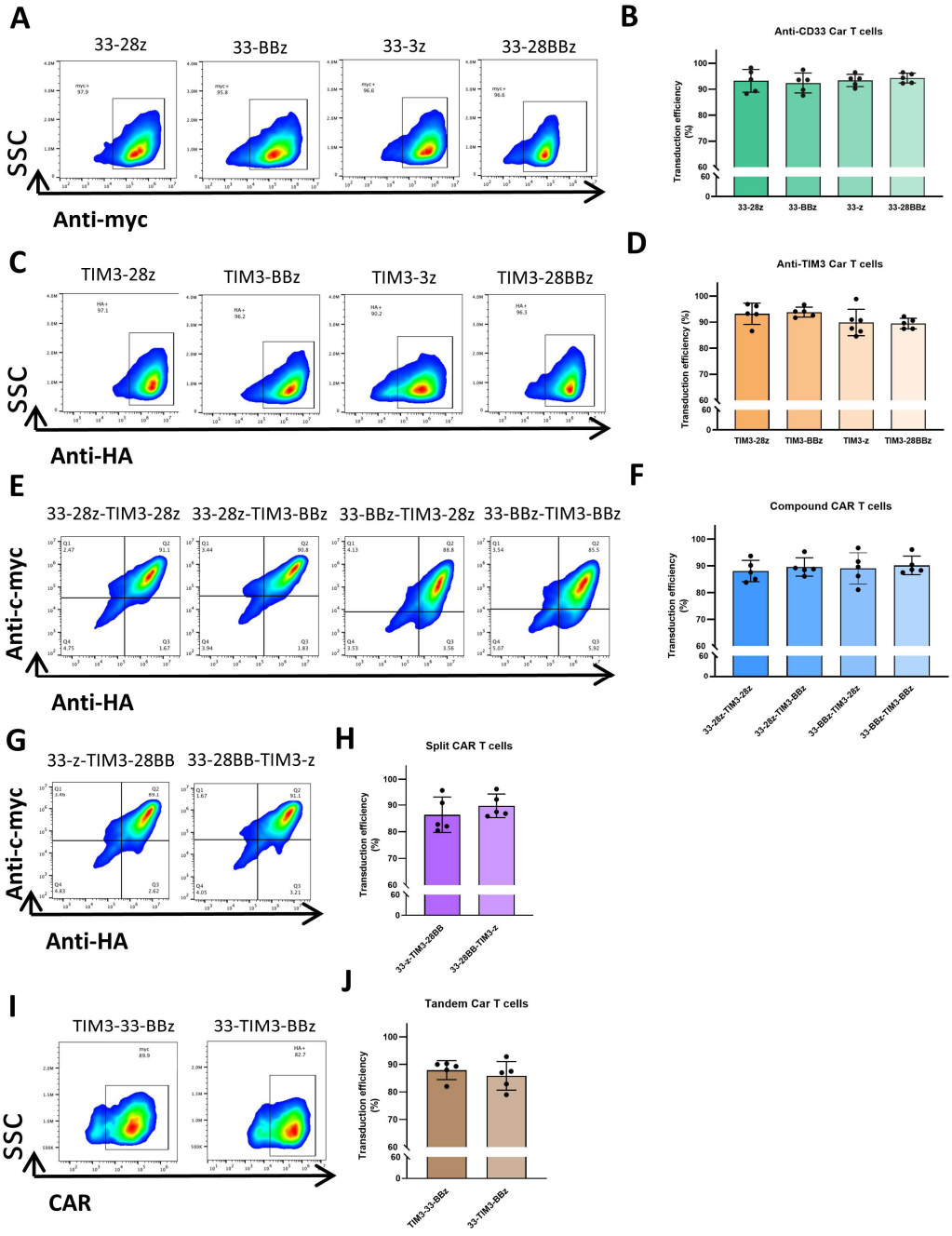



**A** Flowchart of CAR virus producer cell line generation. **B** Vector insert different fragment sizes of CD33 and TIM3 single CAR structures. **C** Expression of CAR tag proteins (c-myc or HA) on CAR virus producer cell lines with different co-stimulation domains . Representative example of an independent experiments is shown.

Following the successful establishment of the CAR virus producer cell lines, the transduction efficiencies were evaluated using T cells from different healthy donors (Fig. 8 A-J). A major challenge in CAR T cell production is the reduced transfection efficiency associated with excessively long insertion sequences, which diminishes T cell efficacy [343]. In CD33 single CAR T cell production, despite the inclusion of the co-stimulatory domains 4-1BB and CD28, transfection efficiency remained high on day 14 of *in vitro* expansion (33-3ζ: 93±1%, 33-28z: 93±2%, 33-BBz: 92±2%, 33-28BBz: 94±1%, Mean ± SEM, n=5), with no significant differences observed between the constructs (Fig. 8 B, $p > 0.05$, paired t test). This pattern was similarly noted in the production of TIM3 single CAR (TIM3-3ζ: 90±2%, TIM3-28z: 93±2%, TIM3-BBz: 94±1%, TIM3-28BBz: 89±1%, Mean ± SEM, $p > 0.05$, paired t test, n=5-6; Fig. 8 D).

For dual CAR T cell production, no single positive cell populations from co-transduction method were detected (Fig. 8 E and G). No significant differences were observed in the transfection efficiencies among the compound CAR constructs (33-28z-TIM3-28z: 88±2%, 33-28z-TIM3-BBz: 90±2%, 33-BBz-TIM3-28z: 89±3%, and 33-BBz-TIM3-BBz: 90±2%; Mean ± SEM, n=5, paired t-test, p > 0.05, Fig. 8 F). Concurrently, the transduction efficiencies for split CARs via co-transduction were achieved (33-3ζ-TIM3-28BB: 86±3%, 33-28BB-TIM3-z: 90±2%, Mean ± SEM, n=5, Fig.8 H). Notably, tandem CARs demonstrated the lowest transduction efficiencies within the dual CARs (TIM3-33-BBz at 88±2%, 33-TIM3-BBz at 85±2%, Mean ± SEM, n=5, Fig. 8 J). Although the transfection efficiency of dual CARs was marginally lower than single CAR T cells, the label proteins c-myc and HA were successfully identified.

**Figure 8: CD33 and TIM3 single and dual CAR T-cell transduction efficiency.**




CAR T-cell transduction efficiencies were determined 14 days post T-cell transduction **A, B** CD33 single CAR T-cell transduction efficiency (n=5). **C, D** TIM3 single CAR T-cell transduction efficiency (n=5-6). **E, F** Compound CAR T-cell transduction efficiency (n=5). **G, H** Split CAR T-cell transduction efficiency (n=5). **I, J** Tandem CAR T-cell transduction efficiency (n=5). Gating was set based on an isotype control. For each construct a representative example is shown. Bars represent the mean ± SEM, each dot represents a biological replicate.

## 9.6 TIM3 CAR T cells did not show fratricide due to autoantigen expression

A critical consideration in designing CAR T cells was the prevention of fratricide, due to target antigen expression on the CAR T cells themselves [344]. This issue was reported for CD5 or CD7 CAR T cells, which can thereby lose their cytotoxic capabilities [345]. In this study, the investigation of TIM3 CAR T cells is pivotal since TIM3 was expressed not only on leukemia cells but also significantly on activated and exhausted T cells [269], [270], [346]. Concurrently, elevated TIM3 expression in the early

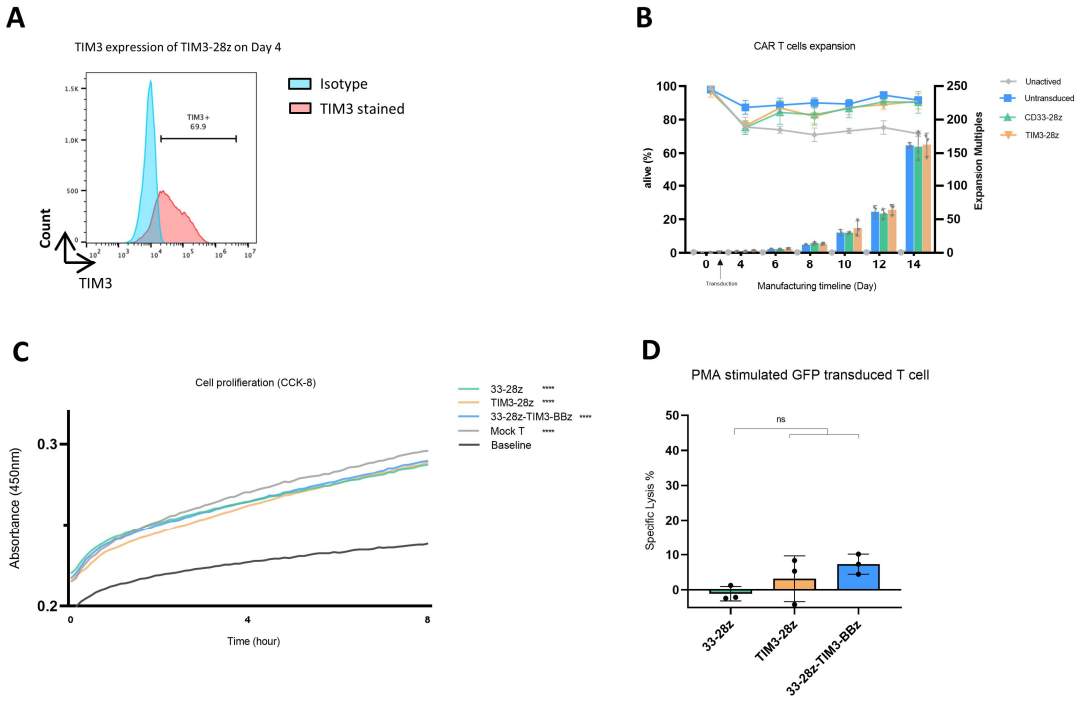
stages of CAR-T cell production was observed due to activation by CD3-CD28 beads (Fig. 9 A).

Furthermore, the expansion and viability of CAR T cells during the manufacturing process were monitored and analyzed to assess the presence of fratricidal effects. Over the 14-day *in vitro* expansion period, TIM3-28z CAR T cell demonstrated no significant differences in growth compared to those with CD33-28z CAR T cell (Fig. 9 B). Notably, there was a decline in the viability of all CAR constructs post-transduction, which improved after day 4 (Fig. 9 C).

To more accurately assess the expansion of CAR T cells, CCK-8 proliferation assay reagent was added on day 4 of the manufacturing timeline. The cells were then cultured *in vitro* for 8 hours, with real-time monitoring of absorbance at 405 nm. The results demonstrated that CAR T cells containing TIM3 scFv demonstrated lower expansion rates than CD33 single CAR T cells ($p < 0.0001$, paired t test, n=3, Fig. 9 D).

Nevertheless, a cytotoxicity assay was performed to accurately evaluate potential fratricide. GFP-transduced T cells were cultured *in vitro* with PMA (500 ng/mL) and ionomycin (10 µg/mL) for two days to ensure full activation and TIM3 expression. These cells were then co-cultured with TIM3 scFv-expressing CAR T cells at a 1:1 ratio for 24 hours to assess fratricide. Compared to 33-28z, TIM3-28z and 33-28z-TIM3-BBz exhibited slightly enhanced specific lysis, recording 6 ± 3% and 5 ± 5%, versus 33-28z: -1 ± 3%. However, the differences were not statistically significant ($p > 0.05$, paired t-test, n = 3, Fig. 9 E).

**Figure 9: TIM3 CAR T cells did not show fratricide due to autoantigen expression.**



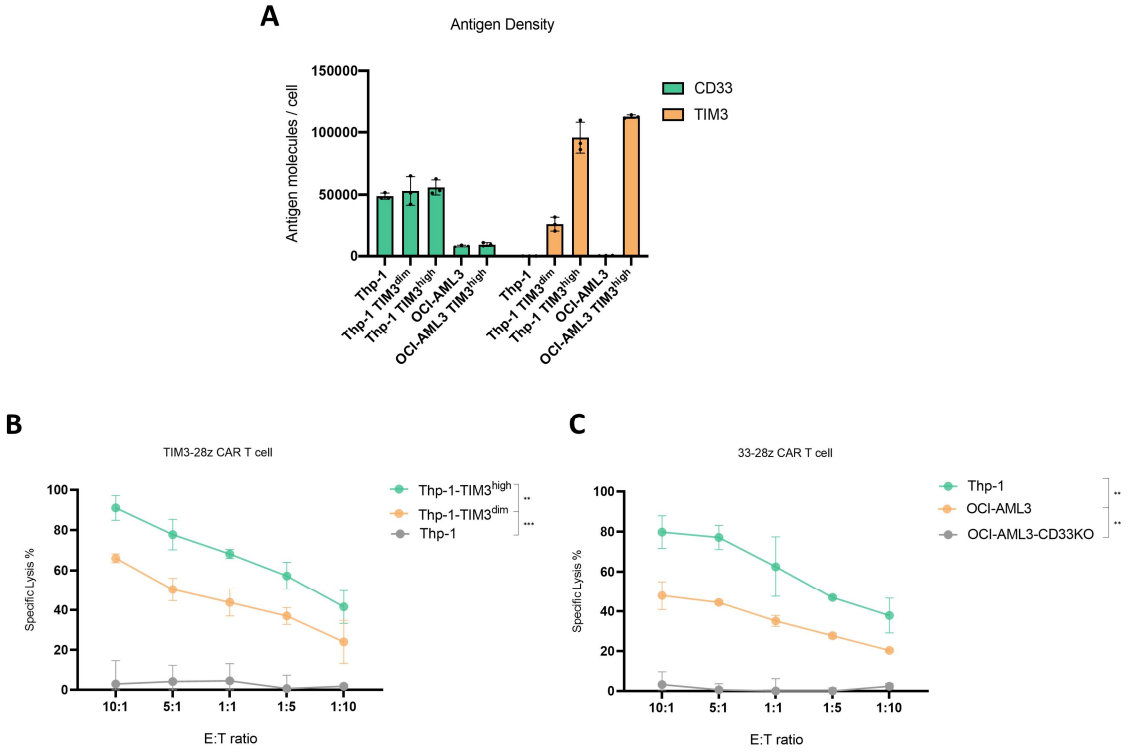

**A** TIM3 expression on TIM3-28z CAR T cells on day 4 of CAR T cell manufacturing. A representative example of 3 independent experiments is shown. **B** Viability and expansion of CD33 and TIM3 single CAR T cells during 14 days of manufacturing. 33-28z and TIM3-28z CAR T cell expansion is shown as fold change and viability during manufacturing time (n=3). Each dot represents a biological replicate and is an average of 3 technical replicates. **C** Proliferation assays of 33-28z, TIM3-28z and 33-28z-TIM3-BBz CAR T cells based on the CCK-8 method. Each line represents the mean of three healthy donor samples and is based on three technical replicates. **D** Cytotoxicity assays using PMA-stimulated and GFP-labeled T cells in co-culture with CAR T cells. Each dot represents an individual healthy donor (n=3). Statistical analysis: paired t test for two group comparisons (ns $p > 0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$). Bars represent mean ± SEM.

## 9.7 CAR T cell cytotoxicity was antigen density depended

Following the successful establishment of a CAR T-cell production system, the *in vitro* efficacy of the CAR T cells was further validated. Variable expression of CD33 antigen across different AML cell lines was observed, and therefore the THP-1 cell line (high CD33 expression) and OCI-AML3 cell line (low CD33 expression) were selected as models for studying the CD33 target. Additionally, THP-1 cells transduced with human TIM3 cDNA were sorted by FACS to isolate populations with low and high TIM3 expression for studies targeting TIM3 (Fig. 10 A). Subsequently, THP-1, THP-1-TIM3$^{dim}$, and THP-1-TIM3$^{high}$ cells, along with TIM3-28z CAR T cells, were co-cultured at varying E: T ratios from 10:1 to 1:10 for 24 hours to assess efficiency (Fig. 10 B). TIM3-28z CAR T cells demonstrated substantial specific lysis against TIM3 transduced THP-1 cell lines compared to the wild-type THP-1 cell line ($p$ <0.001, paired t test, n=3, Fig. 10 B). Moreover, in comparison to the THP-1-TIM3$^{dim}$ cell line, the TIM3-28z CAR T cells exhibited significantly enhanced cytotoxicity against the THP-1-TIM3$^{high}$ cell line at different E:T ratios ($p$ <0.01, paired t test, n=3, Fig. 10 B).

Similarly, CD33 antigen density also correlated to cytotoxicity. The THP-1 (CD33 high expression), OCI-AML3 (CD33 low expression), and OCI-AML3-CD33KO (no CD33 expression) cell lines served as target cells, which were co-cultured with 33-28z CAR T cells at various effector-to-target (E: T) ratios from 10:1 to 1:10 for 24 hours to evaluate efficacy. As with TIM3 antigen expression, the 33-28z CAR T cells demonstrated a higher specific lysis against the THP-1 cell line compared to the OCI-AML3 cell line, and a significantly higher specific lysis compared to the OCI-AML3-CD33KO cell line ($p$ <0.01, paired t test, n=3, Fig. 10 C).

**Figure 10: CAR T cell cytotoxicity was antigen density dependent.**




**A** CD33 and TIM3 antigen density of different cell lines. Each dot represents an individual replicate. **B** Cytotoxicity of TIM3-28z CAR T cells in co-culture with THP-1, THP-1-TIM3$^{dim}$ and THP-1-TIM3$^{high}$ cell lines at E:T ratios of 10:1 to 1:10 after 24 hours. Each dot represents a biological replicate and is an average of 3 technical replicates (n=3). **C** Cytotoxicity of CD33-28z CAR T cells in co-culture with THP-1, OCI-AML3 and OCI-AML3-CD33KO cell lines at E:T ratios of 10:1 to 1:10 after 24 hours. Each dot represents a biological replicate and is an average of 3 technical replicates (n=3). Statistical analysis: two-way ANOVA for two group comparisons (*$p$<0.05, **$p$<0.01, ***$p$<0.001). Bars represent mean ± SEM.

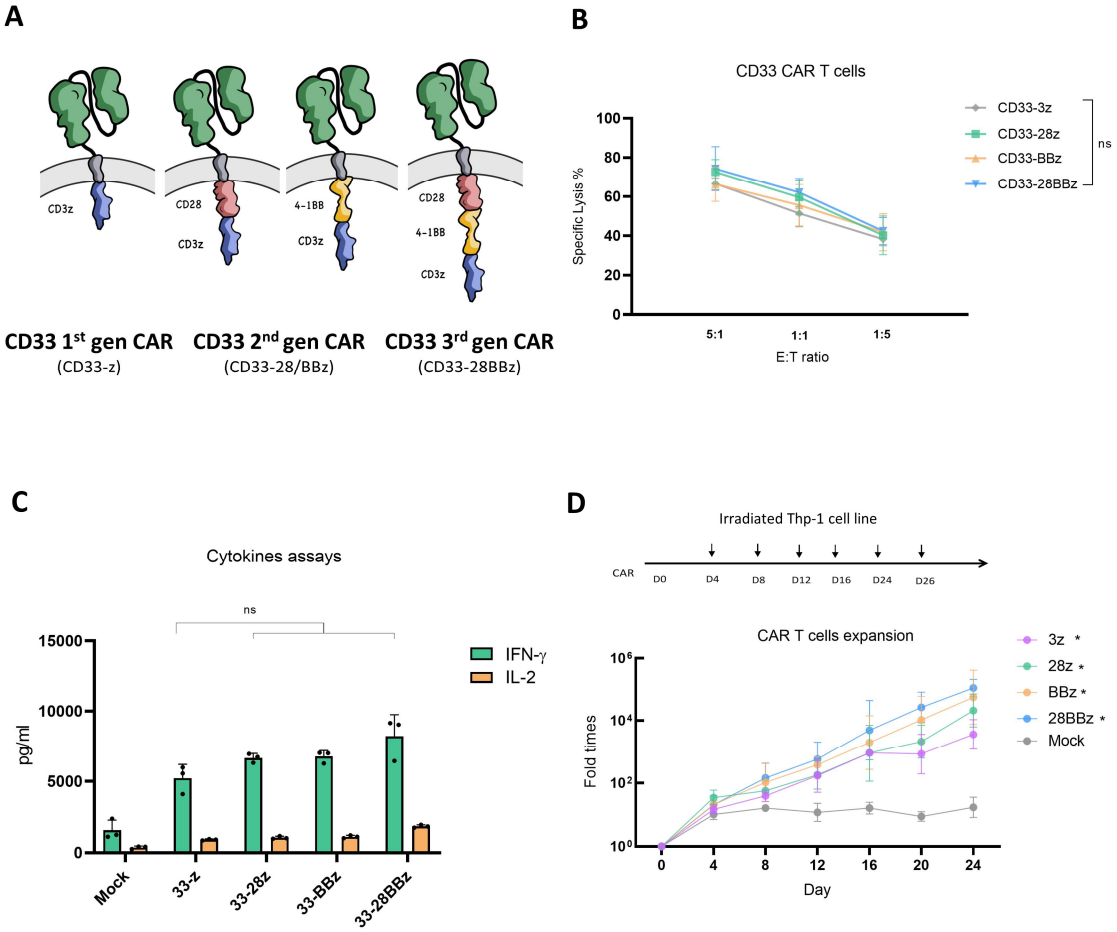
## 9.8 Co-stimulation domain did not affect CAR T cell cytotoxicity in short-term cocultures with AML cells

For the design of the CAR T cells, it was essential to consider not only the role of the scFv but also the impact of the co-stimulation domain. Currently, CAR T cell designs and clinical applications primarily comprise three generations of structures. The first-generation structure features a CD3ζ domain linked behind a transmembrane domain, which activates the CAR T cells directly. The second-generation structure, which is widely used clinically, incorporates a co-stimulatory domain represented by CD28 or 4-1BB before CD3ζ, which enhances proliferation and efficacy. The third-generation structure includes both CD28 and 4-1BB co-stimulatory domains. To provide a reference for the selection of dual CARs, CD33 scFv CAR T cells from first to third generations were designed and produced for comparative evaluation of their efficacy (Fig. 11 A).

Four distinct CAR T cells were evaluated: 33-3ζ, 33-28z, 33-BBz, and 33-28BBz, comprised of varying co-stimulation domains. In order to assess the short-term efficacy of the CAR T cells and to measure the secretion of IFN-γ and IL-2, wild-type THP-1 cells were co-cultured with the CAR T cells at E:T ratios from 5:1 to 1:5 for 24 hours. 33-28BBz CAR T cell demonstrated a trend towards higher specific lysis than the 33-28z CAR T cell and 33-BBz CAR T cells. However, there was no significant differences in cytotoxicity against THP-1 cells among the CAR T cells at varying E:T ratios ($p > 0.05$, two-way ANOVA, n = 3, Fig. 11 B). In addition, the cytokines IFN-γ and IL-2 were measured after co-culture for 24 hours. The highest levels of IFN-γ secretion were observed for the 33-28BBz CAR T cells: 8205 ± 886 pg/ml, followed by the 33-28z CAR T cells: 6649 ± 183 pg/ml and the 33-BBz CAR T cells: 6750 ± 244 pg/ml. The lowest levels were observed in 33-3ζ: 5214 ± 587 pg/ml. Regarding IL-2 secretion levels, the 33-z CAR T cells exhibited the lowest levels: 916 ± 29 pg/ml, while the other CAR T cells exhibited a higher level (33-28z: 1056 ± 59 pg/ml, 33-BBz: 1116 ± 60 pg/ml, 33-28BBz: 1854 ± 57 pg/ml). There were no significant differences between the different CAR T cell generations ($p > 0.05$, paired t-test, n=3, Fig. 11 C).

To assess the proliferation of various CAR T cell constructs under continuous antigen stimulation, a long-term co-culture experiment was designed. Wild-type THP-1 cells were irradiated with X-rays to ensure a consistent antigen presentation without extensive target cell proliferation. These cells were then co-cultured with different CAR T cell constructs at an E:T ratio of 1:1. The number of live cells was enumerated at 4-day intervals over a 24-day period. At the same E:T ratio, irradiated wild-type cells were added to the culture every 4 days to ensure consistency. In long-term culture under irradiated THP-1 cell line stimulation, 33-28BBz CAR T cells exhibited the highest proliferative capacity, followed by 33-BBz and 33-28z. The CAR T cells with the lowest proliferative response was the CD33-3ζ CAR. Significant differences between each group were observed ($p < 0.05$, two-way ANOVA, n=3, Fig. 11 D).

**Figure 11: Influence of the co-stimulation domain on CAR T cell-mediated cytotoxicity.**




**A** Schematic diagram of CD33 CAR T cell designs from first generation to third generation constructs. Figure created with Affinity designer. **B** Cytotoxicity of different generation CD33 CAR T cells in co-culture with THP-1 cell line at E:T ratios of 5:1 to 1:5 after 24 hours. Each dot represents an individual healthy donor (n=3). Two-way ANOVA with Bonferroni post-hoc test for multiple comparisons (ns $p > 0.05$, *$p<0.05$, **$p<0.01$). **C** IFN-γ and IL-2 secretion of different constructs of CD33 CAR T cells. Each dot represents a biological replicate (n=3). Paired t test for two group comparisons (ns $p > 0.05$, *$p<0.05$, **$p<0.01$). Bars represent mean ± SEM. **D** CD33 CAR T cell proliferation assay during long-term co-culture with an irradiated THP-1 cell line. Each dot represents a biological replicate (n=3). Two-way ANOVA with Bonferroni post-hoc test for multiple comparisons (ns $p > 0.05$, *$p<0.05$, **$p<0.01$).

## 9.9 CD33 and TIM3 scFv exchange did not affect the efficacy of compound CAR T cells

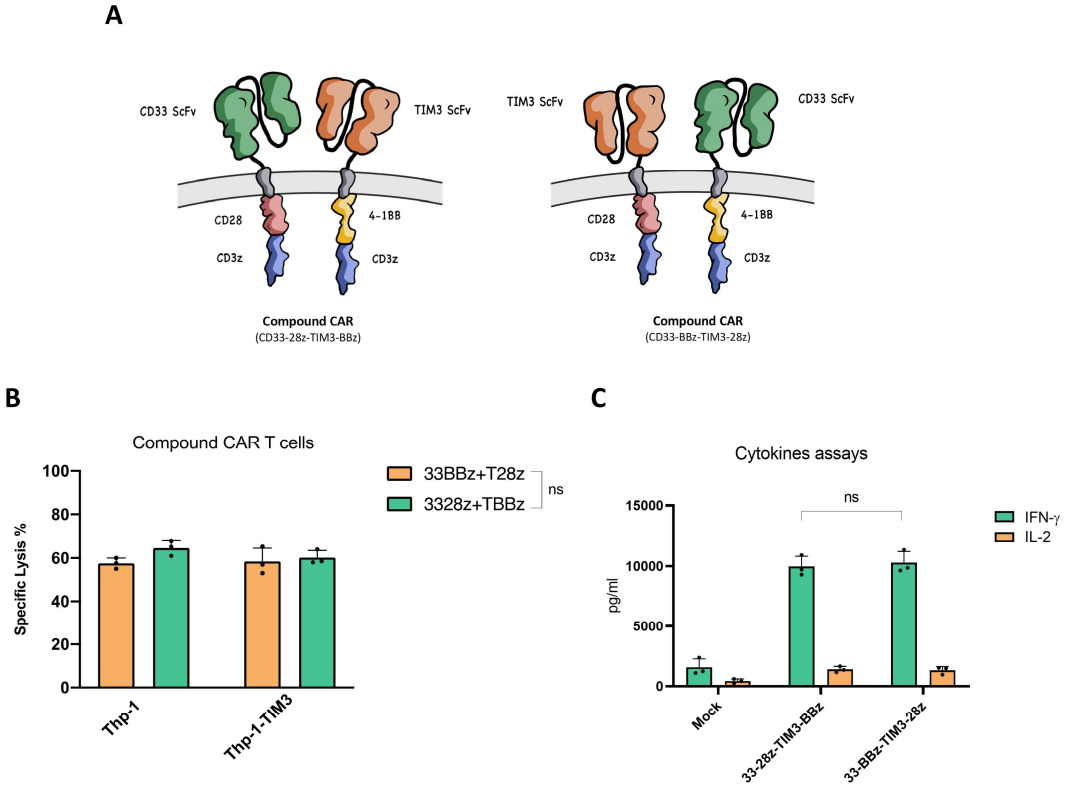
Based on the above findings and current research progress [347], the design of the co-stimulation domain influenced CAR T cell efficacy, dual CAR T cells were designed.

In the design of compound CAR structures, one co-stimulation domain was designated as CD28, while the other was assigned as 4-1BB. Despite previous research indicating that CAR T cell efficacy is influenced by antigen density on target cells [348] , it remained unclear whether exchanging scFv structures affects the efficacy of compound CAR T cells against two antigens differential expressed. To address this question, two compound CAR T cells with exchanged CD33 and TIM3 scFv were developed for comparison of their efficacy (Fig. 12 A).

Two compound CAR T cell structures (33-28z-TIM3-BBz and TIM3-28z-33-BBz) were cultured with both wild-type THP-1 ($CD33^+TIM3^-$) and THP-1-TIM3 cell lines ($CD33^+TIM3^+$) *in vitro* at an E:T ratio of 1:5 for 24 hours. The efficacy of the CAR T cells was assessed using flow cytometry. The results demonstrated no significant differences in efficacy against either THP-1 or THP-1-TIM3 cell lines (THP-1: 64 ± 2% vs 58 ± 1%, THP-1-TIM3: 60 ± 2% vs 59 ± 4%, $p > 0.05$, paired t-test, n = 3, Fig. 12 B). During the 24 hours co-cultures assays using exchanged scFv compound CAR T cells targeting THP-1-TIM3

cells, no significant differences were observed in the secretion levels of IFN-γ or IL-2 (IFN-γ: 9982 ± 478 pg/ml vs 10297 ± 541 pg/ml, IL-2: 1048 ± 145 pg/ml vs 1334 ± 180 pg/ml, $p > 0.05$, paired t-test, n = 3, Fig. 12 C).

**Figure 12: CD33 and TIM3 scFv exchange did not affect the efficacy of compound CAR T cells.**



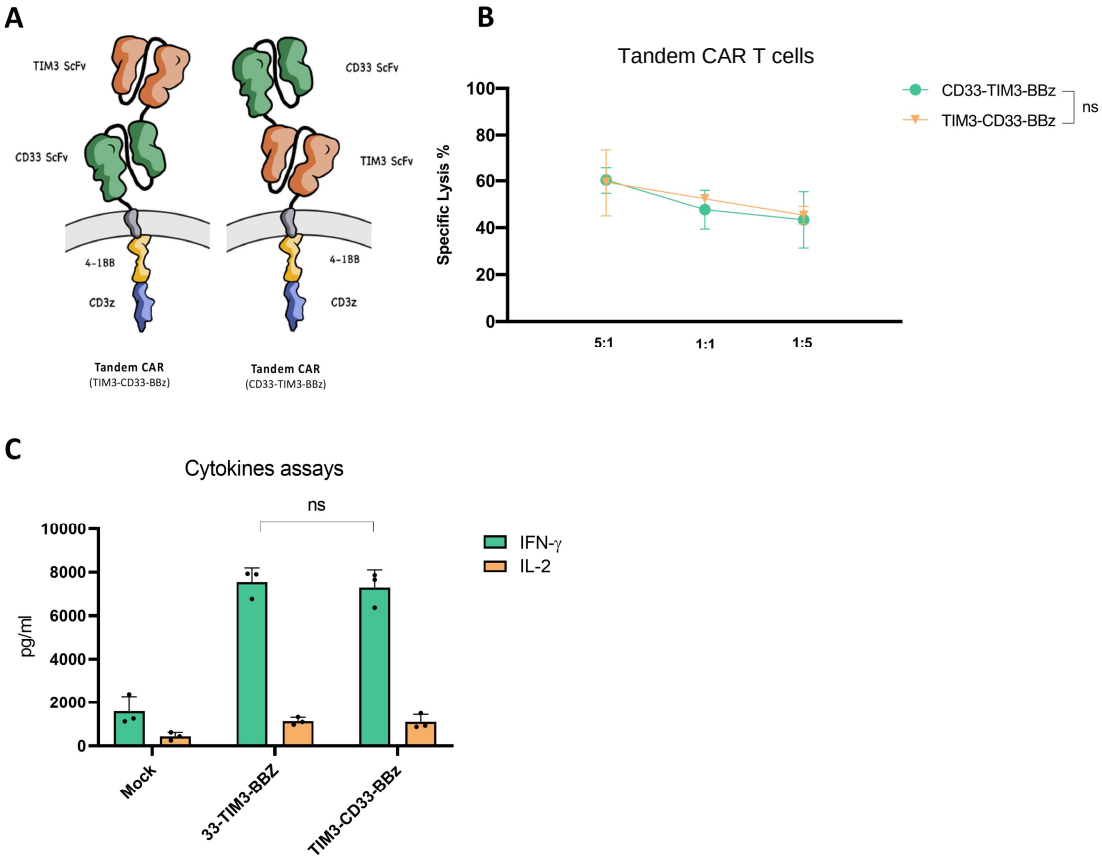

**A** Schematic diagram of two compound CAR T cell constructs. Figure created with Affinity designer. **B** Cytotoxicity of compound CAR T cells in co-culture with THP-1 and THP-1-TIM3 at an E:T ratio of 1:10 after 24 hours. Each dot represents a biological replicate (n=3). **C** IFN-γ and IL-2 secretion by compound CAR T cells. Each dot represents a biological replicate (n=3). Statistical analysis: paired t test for two group comparisons (ns $p > 0.05$, *$p<0.05$, **$p<0.01$). Bars represent mean ± SEM.

## 9.10 The position of CD33 and TIM3 scFv did not affect the efficacy of tandem CAR T cells

Next, the optimal scFv arrangement for tandem CAR T cells was investigated. A rigid linker $(EAAAK)_3$ was selected for the tandem CAR T cells, in order to prevent mismatching of heavy and light chains between CD33 and TIM3 scFvs (Fig. 6 C). 4-1BB was selected because it was reported to have better long-term function and favorable clinical trial results [349], [350], [351]. Consequently, two tandem CAR T cell structures with different positions of the CD33 and TIM3 scFvs were designed (Fig. 13 A).

The two tandem CAR T cell constructs, 33-TIM3-BBz and TIM3-33-BBz, were co-cultured with THP-1 TIM3 cells at E:T ratios from 5:1 to 1:5 for 24 hours *in vitro* to compare their cytotoxicity. No significant differences were observed between the two constructs at different E:T ratios (5:1: 61 ± 3% vs 60 ± 8%,1:1: 51 ± 5% vs 53 ± 4%, 1:5: 44 ± 7% vs 46 ± 2%, $p > 0.05$, two-way ANOVA, n = 3, Fig. 13 B). In addition, IFN-γ and IL-2 secretion was measured after 24 hours of co-culture. No statistically significant differences in cytokine secretion levels were observed between the two types of tandem CAR T cells (IFN-γ: 7534 ± 380 pg/ml vs 7297 ± 465 pg/ml, IL-2: 1131 ± 101 pg/ml vs 1101 ± 198 pg/ml, $p >$ 0.05, paired t-test, n = 3, Fig. 13 C).

**Figure 13: CD33 and TIM3 scFv exchange did not affect efficacy of tandem CAR T cells.**




**A** Schematic diagram of tandem CAR T cell constructs with different arrangements of the CD33 and TIM3 scFvs. Figure created with Affinity designer. **B** Cytotoxicity of two tandem CAR T cells in co-culture with THP-1-TIM3 at E:T ratios from 5:1 to 1:5 after 24 hours. Each dot represents a biological replicate (n=3). Statistical analysis: Two-way ANOVA with Bonferroni post-hoc test for multiple comparisons (ns $p > 0.05$). **C** IFN-γ and IL-2 secretion of the two tandem CAR T cells. Each dot represents a biological replicate (n=3). Statistical analysis: paired t test for two group comparisons, (ns $p > 0.05$). Bars represent mean ± SEM.

## 9.11 Dual CAR T cells showed higher specific lysis than single CAR T cells

The final structures of all dual CAR T cells for further validation (compound CAR, split CAR (see section 9.12 below), tandem CAR) were based on the previous findings. 33-28z-TIM3-BBz was selected as compound CAR T cell structure, 33-28BB-TIM3-3ζ was selected as split CAR T cell structure, and 33-TIM3-BBz was selected as tandem CAR T cell structure. Moreover, considering the dual CAR design strategy, both pooled CAR T cells (33-28z+TIM3-BBz) and single CAR T cells (33-28z or TIM3-28z) also needed to be evaluated (Fig. 14 A). The efficacy of CAR T cells based on different gating strategies requires further validation. For this purpose, dual CAR T cells and single CAR T cells were cocultured with three AML cell lines with different TIM3 antigen density (OCI-AML3, OCI-AML3-TIM3$^{low}$, OCI-AML3-TIM3$^{high}$).

In cytotoxicity assays in which CAR T cells were cocultured with the OCI-AML3 cell line (CD33$^{+}$TIM3$^{-}$) at E:T ratios from 10:1 to 5:1 for 24 hours, dual CAR T cells using an “OR gating” strategy (compound CAR T cell and tandem CAR T cell) demonstrated potent efficacy (Fig. 14 B). However, there was no significant difference compared to CD33 single CAR ($p > 0.05$, two-way ANOVA, n = 3-5). Furthermore, as expected the TIM3 single CAR T cells and split CAR T cells did not show cytotoxicity against the TIM3 deficient OCI-AML3 wild-type cell line. Notably, pooled CAR T cells exhibited less cytotoxicity against the AML cell line compared to compound CAR T cells and tandem CAR T cell ($p < 0.001$, two-way ANOVA, n = 3-5).

Next, CAR T cells were cocultured with TIM3 transduced AML cell lines (OCI-AML3-TIM3$^{low}$ and OCI-

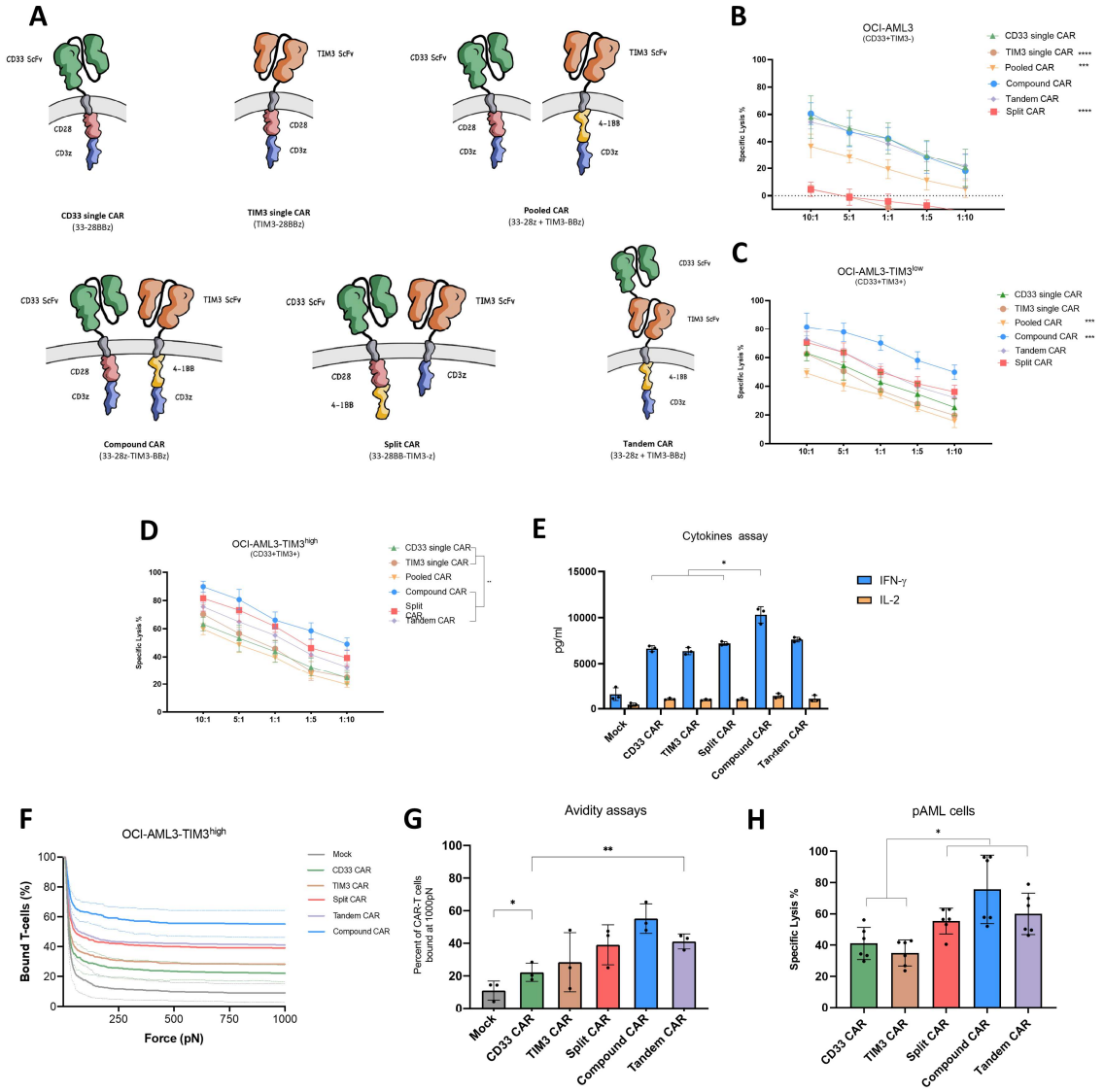
AML3-TIM3$^{high}$) cell line at E:T ratios from 10:1 to 5:1 for 24 hours. All dual CAR T cells (compound CAR T cell, tandem CAR T cell and split CAR T cell) showed higher specific lysis than CD33 or TIM3 single CAR T cells ($p < 0.001$-$0.01$, two-way ANOVA, n = 3-5). Similar to the OCI-AML3 wild-type group, pooled CAR T cells showed the lowest efficacy (Fig. 14 C and D). Notably, the efficacy of dual CAR T cells against OCI-AML3-TIM3 cell lines was influenced by TIM3 antigen density of target cells, consistent with previous finding (Fig. 14 C and D).

Given the lower cytotoxicity of pooled CAR T cells against AML cell lines compared to other dual CAR formats, this combination approach was not investigated further. Subsequently, the secretion levels of IFN-γ and IL-2 were quantified in a 24-hour cytotoxicity assay. Notably, only compound CAR T cells exhibited significantly elevated levels of IFN-γ and IL-2 secretion (10315 ± 510 pg/ml vs 1408 ± 145 pg/ml), demonstrating a pronounced difference compared to the other CAR T cell constructs ($p < 0.05$, paired t-test, n = 3, Fig. 14 E).

To investigate differences in binding avidity between CAR T cells and AML cells, zMovi analyses were performed. Compound CAR T cells demonstrated the highest avidity against OCI-AML3-TIM3, followed by tandem CAR T cells and split CAR T cells (55 ± 5%; 41 ± 3%; 39 ± 7%). Notably, the avidity of all the dual CAR T cells was higher than that of single CD33 or TIM3 CAR T cells (22 ± 3%; 28 ± 10%). There was a significant difference in avidity between CD33 or TIM3 single CAR T cells and other dual CAR T cells (split CAR, compound CAR and tandem CAR) ($p < 0.01$, paired t-test, n = 3, Fig. 14 F and G).

Given the considerable heterogeneity of TIM3 expression observed in AML cell lines and patient samples, BM samples from patients with newly diagnosed AML were employed for co-culture with CAR T cells in order to further assess efficacy. Prior to co-culture with CAR T cells, all samples were cultured on an MS-5 feeder layer, and specific lysis was quantified by flow cytometry. In line with the data generated with AML cell lines, dual CAR T cells demonstrated a significantly higher cytotoxicity against primary AML cells compared to single CD33 or TIM3 CAR T cells ($p < 0.05$, paired t-test, n = 6, Fig. 14 H). Among the dual CAR T cells, the compound CAR T cell exhibited the highest specific lysis (76 ± 9%), followed by tandem CAR T cell (60 ± 5%) and split CAR T cell (55 ± 3%).

**Figure 14: Dual CAR T cells showed higher specific lysis than monospecific CAR T cells.**




**A** Schematic diagram of dual CAR T cell constructs. Figure created with Affinity designer. **B** Cytotoxicity of CAR T cells in co-culture with the OCI-AML3 cell line at E:T ratios of 10:1 to 1:10 after 24 hours. Each dot represents a biological replicate (n=5). **C** Cytotoxicity of CAR T cells in co-culture with the OCI-AML3-TIM3$^{low}$ cell line at E:T ratios of 10:1 to 1:10 after 24 hours. Each dot represents a biological replicate (n=5). **D** Cytotoxicity of CAR T cells in co-culture with OCI-AML3-TIM3$^{low}$ cell line at E:T ratios of 10:1 to 1:10 after 24 hours. Each dot represents a biological replicate (n=5). **E** IFN-γ and IL-2 secretion of CAR T cells. Each dot represents a biological replicate (n=3). **F, G** CAR T cells were incubated with OCI-AML3-TIM3$^{high}$ cells within a z-Movi microfluidic chip. Increasing acoustic force was applied, and the median percentage of bound T cells was determined over time. Each dot represents a biological replicate (n=3). **H** Cytotoxicity of CAR T cells in co-culture with primary AML samples after 24 hours. Each dot represents a biological replicate (n=6). Statistical analysis: paired t test for two group comparisons (Fig. E, G and H), Two-way ANOVA (Fig. B, C and D) with Bonferroni post-hoc test for multiple comparisons (ns $p > 0.05$, *$p<0.05$, **$p<0.01$). Bars represent mean ± SEM.

## 9.12 Split CAR T cells specifically lysed primary AML cells but not hematopoietic cells

Split CAR T cells using a dual "AND" gate strategy were thought to be strongly activated only when both CD33 and TIM3 antigens are co-expressed. In contrast, they showed weaker or no activation when either was antigen expressed alone (Fig. 15 A). Therefore, it was critical to investigate whether split CAR T cells could potentially spare healthy tissue while remaining effective against CD33 and TIM3 double positive cells. In this context, the present study used an engineered OCI-AML3 cell line, previously modified with CRISPR-Cas9 to knock out CD33 and transduced with human TIM3 cDNA, as

a target cell line in co-culture with split CAR T cells. Split CAR T cells were co-cultured with one of four engineered or wild-type OCI-AML3 cells (OCI-AML3-CD33KO, OCI-AML-3, OCI-AML3-TIM3, OCI-AML3-TIM3-CD33KO) at E:T ratios ranging from 2:1 to 1:2 for 24 hours *in vitro* to compare specific lysis (Fig. 15 B). Split CAR T cell demonstrated specific lysis against a single TIM3-expressing cell line or a cell line co-expressing CD33 and TIM3, but not against non-TIM3-expressing cell lines. Notably, split CAR T cell exhibited higher specific lysis against CD33 and TIM3 co-expressing cell line than the TIM3 single expressing cell line (Fig. 15 C and D, $p < 0.001$, paired t-test, n = 3).

Further investigation was warranted to validate the intracellular signaling pathways of split CAR T cells. Our goal was to determine whether both domains are signaling when co-engaged with two antigens. According to the literature, activation of the CD28 co-stimulatory domain in CAR T cells leads to the phosphorylation of upstream PI3K and subsequent activation of the downstream AKT pathway, whereas activation of 4-1BB phosphorylates downstream P38MAPK proteins [352], [353]. Therefore, split CAR T cells were co-cultured with the OCI-AML3-TIM3 cell line for 15 minutes to make sure that both pathways were simultaneously activated by the CD33 and TIM3 co-expressing AML cell line. Dual CAR T cells (compound, split and tandem), mock T cells and non-activated PBMCs served as positive and negative controls, respectively. In this assay, split CAR T cell demonstrated effective activation against OCI-AML3-TIM3 cell line. Notably, compound CAR T cell exhibited a faster activation signal speed than split CAR T cell (Fig. 15 C, $p < 0.01$, paired t-test, n = 3).

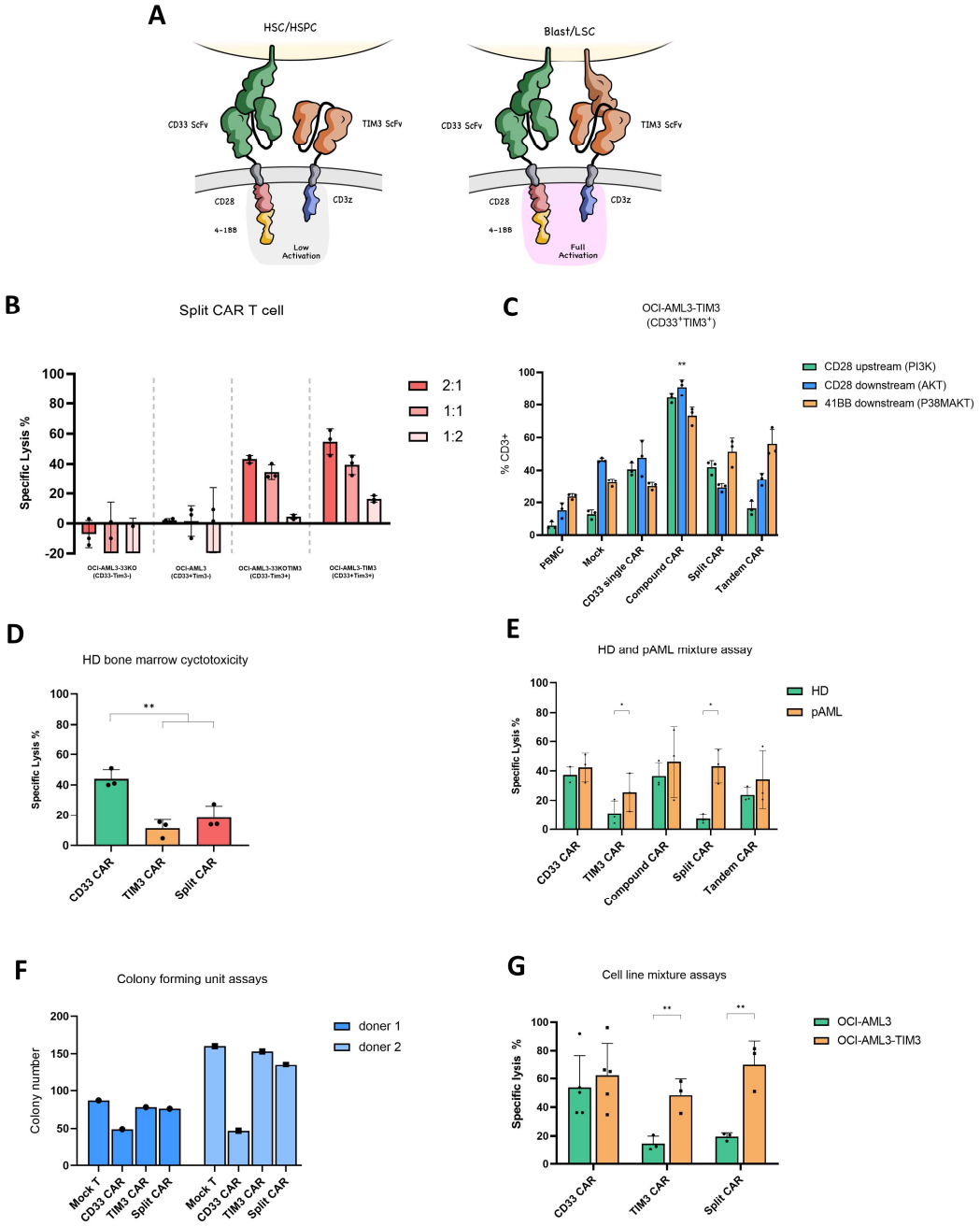
Subsequently, $CD34^+$ cells derived from healthy donor BM were utilized to validate the safety of split CAR T cells. $CD34^+$ cells and split CAR T cells were co-cultured under ex vivo conditions on an MS-5 stromal layer at a 1:1 E:T ratio for 24 hours to assess specific lysis, with CD33 and TIM3 single CAR T cells serving as controls. Split CAR T cells demonstrated lower lysis than CD33 single CAR T cell against $CD34^+$ cells (18 ± 4% vs 44 ± 4%), showing no significant difference compared to TIM3 CAR T cells (Fig. 15 D, $p > 0.05$, paired t-test, n = 3).

CFU assays were then designed to evaluate the specific effects of split CAR T cells on $CD34^+$ cells derived from healthy donor BM. Split CAR T cells were initially co-cultured with $CD34^+$ cells at a 10:1 E:T ratio for 6 hours, after which the mixture was transferred to CFU medium and cultured for 14 days. The resulting hematopoietic colonies were then counted. In both samples, initial co-culturing with CD33 CAR T cells consistently demonstrated inhibition of hematopoietic colony growth, a phenomenon not observed with TIM3 single and split CAR T cells (Fig. 15 E).

Nest, a mixing assay was performed to evaluate the specificity of split CAR T cells. OCI-AML3 and OCI-AML3-TIM3 cells were mixed 1:1 to serve as target cells and then co-cultured 24 h with split CAR T cells to assess specific lysis of OCI-AML3-TIM3 cells and non-specific effects on OCI-AML3 cells.
In the mixed cytotoxicity assay of CD33 single expressing cell line (OCI-AML3) and those co-expressing CD33 and TIM3 (OCI-AML3-TIM3), split CAR T cells exhibited higher specific lysis against the dual-expressing cells (70 ± 10%), while showing limited non-specific lysis against cells expressing CD33 alone (20 ± 2%). This phenomenon was also observed in the TIM3 single CAR T cell group, although the specific lysis achieved was lower than that of split CAR T cells (48 ± 7% vs 70 ± 10%, $p > 0.05$, paired t-test, n =3).

After confirming the specific cytotoxicity of split CAR T cells in mixing assays, primary AML cells derived from clinical patients and $CD34^+$ cells derived from healthy donors were mixed in a 1:1 ratio. The cells were co-cultured with split CAR T cells on a MS-5 feeder layer to further evaluate safety. CD33 or TIM3 single CAR and other dual CAR T cells were selected as controls. Split CAR T cells demonstrated significantly higher lysis against primary AML cells compared to hematopoietic cells (43 ± 7% vs 7 ± 2%, $p < 0.05$, paired t-test, n = 3). This result was also observed with TIM3 single CAR T cells (25 ± 7% vs 11 ± 5%, $p < 0.05$, paired t-test, n = 3). However, the specific cytotoxicity of split CAR T cells was significantly higher than that of TIM3 single CAR T cells (43 ± 7% vs 25 ± 7%). Notably, the other dual CAR T cells exhibited lysis of CD33 expressing healthy hematopoietic cells (Fig. 15 G).

**Figure 15: Split CAR specifically killed AML cells but not hematopoietic cells.**




**A** Schematic diagram of the split CAR T cell activation mechanism. Schematic diagram of dual CAR T cell constructs. Figure created with Affinity designer. **B** Split CAR T cell-mediated lysis against engineered AML cell lines co-cultured at E:T ratios of 2:1 to 1:2 for 24 hours. Each dot represents a biological replicate (n=3). **C** Phosphoflow assay of PBMC, mock, and CAR T cells (CD33 single, compound, split, and tandem CAR) against the OCI-AML3-TIM3 cell line. CD28 upstream pathway PI3K, CD28 downstream AKT, and 4-1BB downstream P38MAKT phosphorylation protein were measured to verify the CAR intracellular signaling pathway. Each dot represents a biological replicate (n=5). **D** Cytotoxicity assay of CD33-, TIM3-, and split-CAR T cells against $CD34^+$ cells derived from healthy donor BM for 24 hours at E:T ratio of 5:1. Each dot represents a biological replicate (n=3). **E** Specific lysis against a mixture of healthy donor $CD34^+$ BM cells and primary AML cells at E:T ratio of 1:5 for 24 hours. Each dot represents a biological replicate (n=3). **F** CFU assay conducted after co-culturing CD33-, TIM3-, and split-CAR T cells for 6 hours with $CD34^+$ healthy donor cells at E:T ratio 10:1. Each dot represents a biological replicate (n=2). **G** CAR T cell-mediated lysis against mixed OCI-AML3 and OCI-AML3-TIM3 cell lines co-cultured at E:T ratio of 1:1 for 24 hours. Each dot represents a biological replicate (n=3-5). Statistical analysis: paired t-test for two group comparisons (Fig. B-G) (*$p$<0.05, **$p$<0.01). Bars represent mean ± SEM.

## 9.13 Split CAR T cells demonstrated similiar proliferative potential during

## long-term antigen exposure

In the late stages of this study, SKM-1 and HNT-34 cell lines were identified as wild-type CD33 and TIM3 co-expressing cells. These cell lines closely mimic the physiological TIM3 expression levels observed in patients, and therefore serve as effective models for predicting and evaluating the function of CAR T cells.

To elucidate the proliferative response and immune checkpoint expression of dual CAR T cells during long-term exposure to antigens, irradiated SKM-1 cells and CAR T cells were co-cultured at a 1:1 ratio. Cytotoxicity, proliferation, and immune checkpoint expression of CAR T cells were evaluated on a four-day interval during 24 days (Fig. 16 A). All CAR T cells exhibited enhanced proliferative capacity in comparison to mock T cells ($p < 0.05$, paired t-test, n=3). Notably, the proliferation of compound CAR T cells was slightly lower than that of other CAR T cells ($p < 0.05$, paired t-test, n=3, Fig. 16 B).

Although some literature reported that clinically applied CD19 CAR T and BCMA CAR T cells persist long-term after patient remission [354], [355], the CAR expression of dual CAR T cells under long-term antigen exposure *in vitro* remains unclear. Consequently, CAR expression was monitored throughout a 24-day long-term antigen exposure experiment. CAR expression remained high and showed no significant differences in all CAR T cells ($p > 0.05$, two-way ANOVA, n=3. Fig. 16 C). Subsequently, CAR T cells cytotoxicity was assessed on day 0 and day 20, showing no significant differences in specific lysis against SKM-1 cells at these time points ($p > 0.05$, two-way ANOVA, n=3. Fig. 16 D).

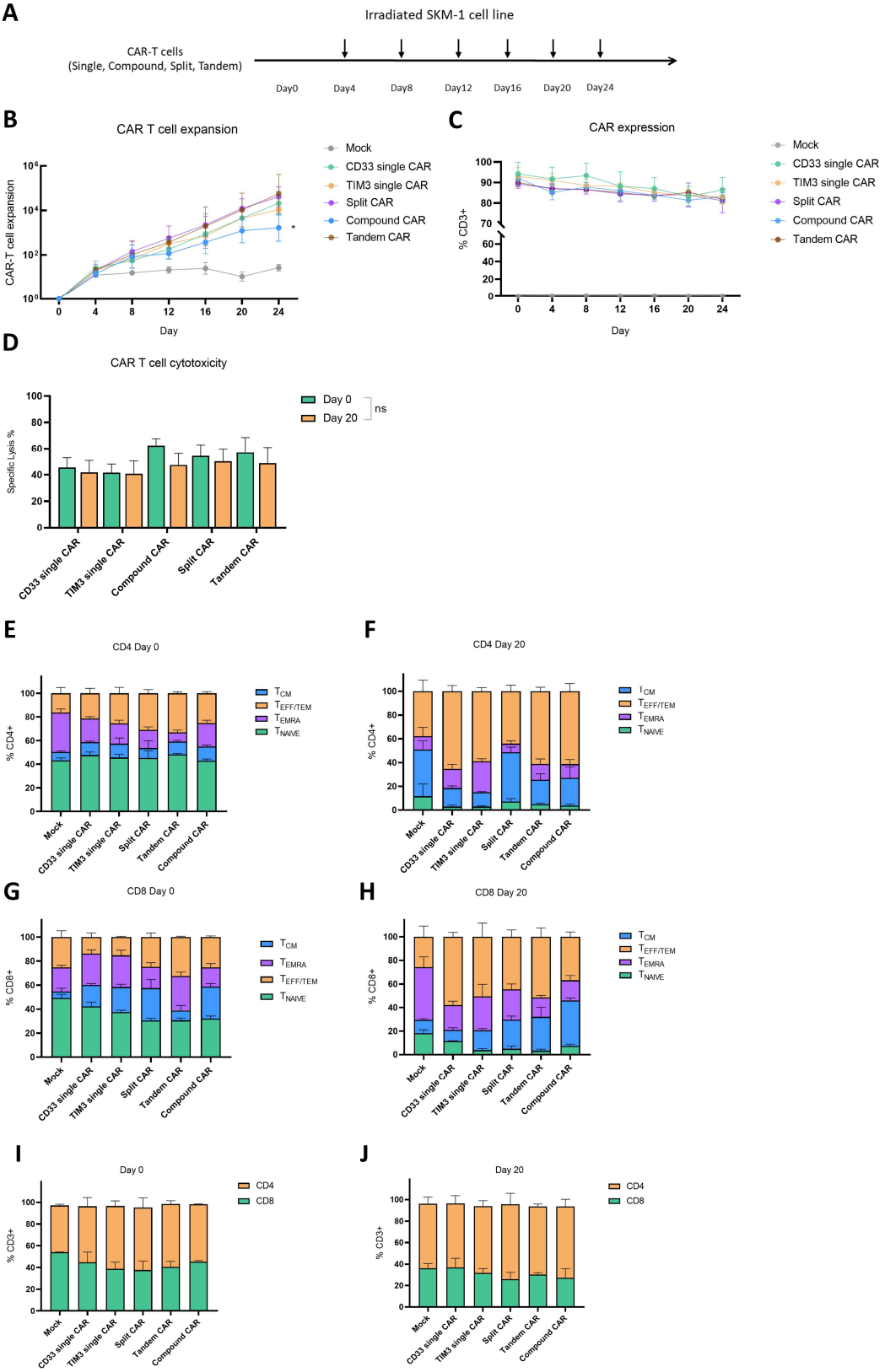
In context of long-term antigen exposure, CAR T cell differentiation and subsets, including naïve ($T_{NAIVE}$), central memory ($T_{CM}$), effector memory ($T_{EFF/TEM}$), and effector memory cells re-expressing CD45RA ($T_{EMRA}$) play a significant role in CAR T cell proliferation. Consequently, these subsets were monitored during the whole exposure period. $CD4^+$ and $CD8^+$ naïve T cells had no significant differences among the different CAR T cells on day 0. The percentage of naïve T cells declined over time. Notably, the split CAR T cells exhibited consistently higher levels of naïve cells compared to the other CAR T cells on day 20 (Fig. 16. E, F, G, H). There were no significant difference in the percentage of $CD4^+$ and $CD8^+$ T cells among all CAR T cell groups. However, the percentage of $CD8^+$ CAR T cells decreased over time (Fig. 16 I, J)

Furthermore, the percentage of check point molecules (TIM3/LAG3 or PD-1) single positive $CD4^+$ and $CD8^+$ CAR T cells was analyzed and compared after five rounds of stimulation. No significant differences were observed among $CD4^+$ CAR T cell types ($p > 0.05$, two-way ANOVA, n =3. Fig. 16 D). However, the percentage of $TIM3^+LAG3^+PD\text{-}1^+$ triple-positive $CD8^+$ CD33 single CAR T cells was lower than for the other CAR T cells constructs (Fig. 16 K, L).

At last, the expression of PD-1, LAG-3 and TIM-3 on CAR-T cells was assessed before and after five rounds of stimulation. Compound CAR T cells expressed highest levels of PD1 and LAG3 after five stimulations compared to the other CAR T cells in both the $CD4^+$ and $CD8^+$ subset. The expression levels of the checkpoint markers on split CAR T cell were similar to those on other CAR T cell types. Interestingly, all CAR T cells with TIM3 scFv constructs showed a lower TIM3 expression than CD33 single CAR T cells (Fig. 16 M-P).

**Figure 16: Split CAR T cells demonstrated similiar proliferative potential during long-term antigen**

**exposure.**


A
Irradiated SKM-1 cell line
CAR-T cells
(Single, Compound, Split, Tandem)
Day0
Day4
Day8
Day12
Day16
Day20
Day24
B
CAR T cell expansion
CAR-T cell expansion
Day
Mock
CD33 single CAR
TIM3 single CAR
Split CAR
Compound CAR
Tandem CAR
C
CAR expression
% CD3+
Day
D
CAR T cell cytotoxicity
Specific Lysis %
Day 0
Day 20
ns
CD33 single CAR
TIM3 single CAR
Compound CAR
Split CAR
Tandem CAR
E
CD4 Day 0
% CD4+
F
CD4 Day 20
G
CD8 Day 0
% CD8+
H
CD8 Day 20
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Day 0
% CD3+
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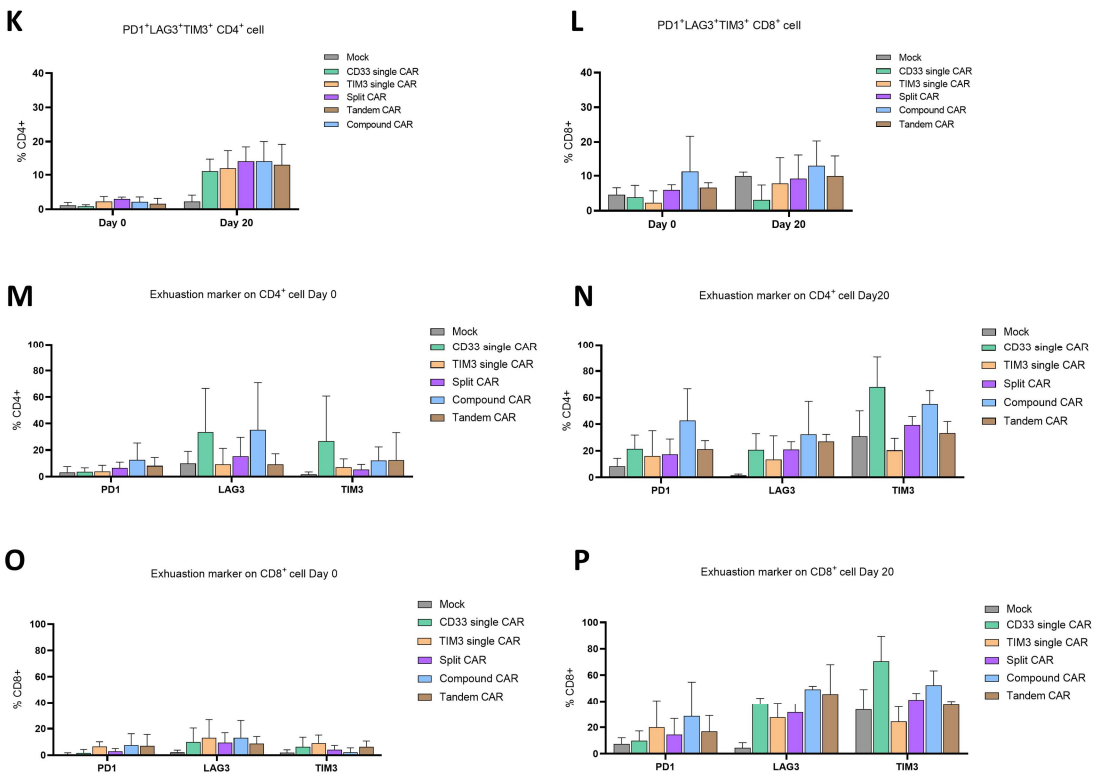
CD8
J
Day 20




**A** Schematic diagram of continuously exposure of irradiated SKM-1 cell line ($CD33^+TIM3^+$). **B** CAR T cell expansion during 24 day long-term antigen exposure given as fold change compared to day 0 (n=3). **C** CAR expression during 24 day long-term antigen exposure (n=3). **D** Cytotoxicity assay of CAR T cells on day 0 and day 20 against SKM-1 cell line at an E:T ratio of 1:5 after 24h (n=3). **E, F** $CD4^+$ CAR T cell subsets on day 0 and day 20 (n=3). **G, H** $CD8^+$CAR T cell subsets on day 0 and day 20 (n=3). **I, J** Percentage of $CD4^+$ and $CD8^+$ CAR T cells on day 0 and day 20 (n=3). **K, L** Percentage of $PD\text{-}1^+LAG3^+TIM3^+$ triple-positive $CD4^+$ and $CD8^+$ CAR T cells on day 0 and day 20 (n=3). **M, N** Percentage of PD-1, LAG3 and TIM3 expressing $CD4^+$ CAR T cells on day 0 and day 20 (n=3). **O, P** Percentage of PD-1, LAG3 and TIM3 expressing $CD8^+$ CAR T cells on day 0 and day 20 (n=3). Statistical analysis: paired t-test for two group comparisons (Fig. D) Two-way ANOVA with Bonferroni post-hoc test for multiple comparisons (Fig. B and C, E-P), (ns $p > 0.05$, *$p<0.05$, **$p<0.01$). Bars represent mean ± SEM.

# 10. Discussion

Throughout the lengthy and complex history of human struggle against AML, treatment methodologies have undergone significant transformations. Initially, researchers and clinicians recognized the importance of detailed subtyping and identification in AML treatment, starting with the initial understanding by FAB and continuing with the continually updated WHO and ICC classifications[5], [6], [7]. These methodologies have influenced subsequent therapeutic approaches, thereby markedly improving patient outcomes. Furthermore, treatment strategies for AML are becoming increasingly precise and personalized [356]. This is evidenced by the targeted identification of AML cells and the use of immunocellular therapy to eradicate AML blasts, which are currently key areas of current research [357].

The development of flow cytometry has facilitated extensive res earch on numerous cell surface targets for AML, including CD33, CLL1, and CD123 [242], [358], [359], [360]. However, a current challenge is that these targets are expressed not only on AML blast cells but also on healthy hematopoietic stem and progenitor cells. A number of case reports have indicated that targeting CD33 can result in life-threatening myeloid depletion in some patients [231]. The primary solution has been the use of gene editing to knock out CD33 expression on progenitor cells, with the aim of preventing off-target effects [347]. Furthermore, the experience with CD19 CAR T cells in treating ALL has demonstrated that many patients face the risk of relapse due to antigen escape, as targeting solely CD19 does not provide sufficient protection [361], [362]. In response, a new cellular therapy strategy has been developed that simultaneously targets CD33 and TIM3, which are highly expressed on AML.

In investigating the potential phenotypes of AML patients, this study identified CD33 and TIM3 as feasible targets for CAR T cell therapy. Through the design and combination of various CAR T cell structures, the study successfully generated dual-targeting CAR T cells utilizing both "AND" and "OR" gating strategies to target CD33 and TIM3, enhancing their efficacy against AML cells *in vitro*. The findings demonstrated that these CAR T cells exhibit superior binding avidity and cytotoxic capabilities towards cells expressing both CD33 and TIM3 antigens, compared to targeting a single antigen. Notably, the split CAR T cell approach effectively eradicated $CD33^{+}TIM3^{+}$ cell lines and primary AML cells, while minimizing the impact on healthy hematopoietic cells.

TIM3 is a validated target with relative tumor specificity and exhibits specific expression in some AML cell lines and patient samples [176], [270], [363]. It is expressed in exhausted T cells, NK cells, and monocytes [364]. Additionally, TIM3 is involved in regulating a range of immune activities, including T cell activation and proliferation, dendritic cell antigen cross-presentation, macrophage TLR stimulation, and mast cell activation [365], [366], [366], [367], [368]. However, it is noteworthy that it shows distinctive expression in AML blasts and LSCs [369], [370]. Despite the existence of several clinical studies targeting TIM3, the results have been suboptimal [176], [177]. Our statistical analysis of clinical samples indicates that although TIM3 is expressed in AML patients, its expression density is lower than that of CD33. The finding matches those observed in our earlier studies [271]. Although TIM3 is reported to be highly expressed on AML LSCs, AML bulk, and healthy donor monocytes, one unanticipated finding from further analysis was that TIM3 was also expressed at higher levels in T cells from AML patients compared to T cells from healthy donors in our patient database [271]. Some studies have also observed the same phenomenon and speculated that these cells are exhausted T cells [371], [372], [373]. Although the TIM3 expression levels in these $TIM3^{+}$ lymphocyte cells are lower than those in LSCs and bulk cells, there is still a risk of fratricide in TIM3 CAR T cells. Additionally, whether CAR T cells targeting TIM3 will kill these lymphocytes and TIM3 expressed NK cells remains to be studied [364]. A study has reported that the activation mechanisms differ between $TIM3^{+}$ exhausted T cells and $TIM3^{+}$ LSCs [374]. TIM3 signaling hijacks the classical Wnt/β-catenin pathway to maintain the stem cell properties of AML cells [374]. Our research found that TIM3 expression in LSC and AML blast cells is higher than in exhausted T cells. Many research groups have confirmed the efficacy of TIM3 CAR T cells in AML, which supports that TIM3 is a target for AML immunotherapy [273], [277], [278], [375].

Additionally, the stable expression of TIM3 in wild-type AML cell lines warrants further investigation.

While some research groups have detected stable expression of TIM3 in KG-1a, Kasumi-3, SKM-1, and HNT-34, our study only found its expression in SKM-1 and HNT-34. Interestingly, both of these cell lines are derived from patients with MDS who progressed to leukemia. This finding is consistent with reports identifying TIM3 as a specific marker for LSCs [272], [363], [370]. The lack of TIM3 expression in other AML cell lines may be related to their differentiation [374]. The underlying mechanisms still require further investigation.

Given this situation, BM or PB samples from AML patients were typically used for research [363], [376]. Unfortunately, patient sample availability is low, the costs are high, and the heterogeneity of leukemia cells among different patients makes this method challenging for early-stage research. Therefore, this study utilized a retroviral system to transduce human TIM3 in OCI-AML3 and THP-1 cells. Some studies have reported that compared to wild-type cell lines, cell lines with aim-gene over-expression cannot fully explain the heterogeneity and chromosomal properties of tumor cells [377]. Additionally, these cells exhibit different signal pathways directly activated and varied expression due to target gene overexpression [377], [378], [379], [380]. This study was limited by the TIM3 expression in wild-type cell lines and thus focused on the investigation of TIM3-engineered cell lines (OCI-AML3-TIM3 and THP-1-TIM3).

Subsequently, we have designed a series of CAR T cells that simultaneously target both TIM3 and CD33. The concurrent engagement of both targets by employing an "OR" gating strategy aimed at enhancing the affinity of CAR T cells for AML cells, thereby increasing their efficacy, avoiding antigen escape and heightening sensitivity to AML cells heterogeneity. This increased precision in targeting reduces the likelihood of missing cancer cells that do not express a single, high-density antigen, leading to a more thorough and effective elimination of cancerous cells. Another significant challenge in CAR T cell therapy is antigen escape, where AML cells lose or downregulate the targeted antigen, rendering the therapy less effective or ineffective. Meanwhile, AML heterogeneity also affects the cytotoxicity of CAR-T cells [381], [382]. By using an "OR" gating strategy, CAR T cells are programmed to recognize and attack cancer cells expressing either of the two target antigens [283], [359], [383], [384]. Several studies already evaluated the safety and feasibility of administering various doses of CD33 CAR T cells to patients with R/R AML [230]. Despite the therapeutic promise, the administration of autologous CD33 CAR T cells in R/R AML has been associated with serious adverse effects and lack of efficacy [384]. Furthermore, AML is characterized by a highly heterogeneous cell population, with different subclones expressing varying levels of antigens [385]. This heterogeneity makes it difficult for single-target CAR T cells to achieve complete and sustained remission, as not all cancer cells will express the target antigen at sufficient levels.

One of many challenges in CAR T cell therapy design is the creation and optimization of target-specific scFv sequences [386]. In the majority of cases, the scFv targeting domains are derived from the variable regions of the antibody heavy and light chains, which are then linked together by a peptide linker of 15 to 20 amino acids. In this study, the CD33 scFv was derived from gemtuzumab, an antibody that has already been approved for clinical use [387]. This CD33 scFv has also been employed in a CD33-targeting BiTE (bispecific T-cell engager) [388]. Subsequently, this study screened six TIM3 antibody candidates through hybridoma technology. Some studies have shown that low-affinity CD19 scFv is beneficial for CAR proliferation and cytotoxicity [389], [390], [391]. Although this study randomly selected a TIM3 antibody as the TIM3 scFv, the affinity of TIM3 scFv and the density of TIM3 still warrant further discussion in the future.

Alphafold2 protein structure prediction model was employed to ascertain that the selected CD33 and TIM3 scFvs were capable of effectively binding to their target antigens [338], [392]. Despite the availability of numerous protein-docking systems, Alphafold2 was selected for its accuracy and reliability [393], [394], [395]. The efficacy of the CD33/TIM3 scFvs was assessed by simulating the binding complex structure with the corresponding antigens. Both TIM3 and CD33 scFvs demonstrated effective binding to their respective antigens, which was consistent with expectations.

One approach to address leukemia cell heterogeneity is to design Tandem CAR T cells based on an

"OR" gating strategy. This method has been highly successful in patients with DLBCL expressing both CD19 and CD20/CD22 [349], [351]. When Tandem CAR T cells encounter immune evasion due to the loss of a single target, the second target can still effectively activate the CAR T cells to exert cytotoxic effects [396]. Moreover, the likelihood of immune evasion occurring due to the simultaneous loss of both targets is extremely low. Therefore, this study designed CD33 and TIM3 Tandem CAR T cells based on an "OR" gating strategy. For this purpose, AlphaFold2 platform was employed to design the structure of Tandem CARs [338] [330]. In contrast to conventional dual CARs, Tandem CARs feature a critical design aspect in the linker between the scFvs of CD33 and TIM3. Theoretically, the linker should match the spatial separation between the scFvs and the antigens for enhanced efficacy [188], [397], [398]. However, due to the fluidity and dynamics of membrane proteins, accurately measuring these distances is challenging [399], [400]. Some studies have demonstrated that different linker structures can significantly impact the efficacy of dual scFvs [340], [401]. Furthermore, there is a potential for mismatch between the leading scFv light chain and the trailing scFv heavy chain. Common linkers in Tandem CARs include the flexible $(G_4S)_4$ and the rigid $(EAAAK)_3$ structures [398]. Flexible linkers permit better freedom between the scFvs, whereas the rigid linkers maintain a fixed distance. Simulation results from AlphaFold2 indicate that the rigid structure is more suitable for linking CD33 and TIM3 scFvs, also mitigating potential mismatches between heavy and light chains. Nevertheless, further validation is necessary to ascertain the optimal arrangement of TIM3 and CD33 in the sequence configuration.

The sequences for CD33 and TIM3 were cloned into the pMP71 plasmid, facilitating the establishment of a cell line carrying the CAR DNA in order to produce virus for transducing T cells. Currently, there are two primary CAR gene insertion systems: the virus-based retroviral and lentiviral systems, followed by the non-viral systems, such as electroporation, the CRISPR-Cas9 system, and the PiggyBac system [402]. Although the virus-based gene editing systems carry a potential oncogenic risk due to the randomness of gene insertion sites, their capability to carry longer fragments makes them more suitable for dual-targeting of lengthy sequences, particularly the single-plasmid tandem CAR constructs [355]. In clinical settings, the lentiviral system is commonly used for transduction [403]. However, a high-efficiency retroviral system was selected due to the dual-targeting plan for CD33 and TIM3 using a two-vector system [404], [405]. Additionally, this approach also facilitates future commercial-scale production. Despite the varying fragment lengths of different constructs, they do not significantly affect the transfection efficiency of CAR T cells. Current studies report a lentiviral transfection efficiency of approximately 60-70%, which is lower than that of the retroviral system used in our study [406]. Due to the high transfection efficiency currently achieved, this study did not utilize FACS for further sorting to enhance transfection efficiency.

TIM3 is expressed not only in AML blasts and LSCs but also in exhausted T lymphocytes. These results are consistent with those of other research groups [374], [407], [408]. Therefore, it is necessary to check whether fratricide occurs in TIM3 CAR-T cells mediated by TIM3. A 14-day *ex vivo* expansion showed that TIM3 expression increased by day 4, and GFP-labeled cytotoxicity assays indicated slight fratricide. However, the 14-day expansion rates and 8-hour proliferation assays for both CD33 and TIM3 CAR T cells showed no significant differences. Compared to the fratricide phenomenon observed in CD5 and CD7 CAR T cells, TIM3 CAR T cells did not exhibit proliferation effects caused by fratricide [345], [409], [410].

Following the successful establishment of the CAR T cell production platform, the expression of CD33 and TIM3 on target cell lines was reconsidered. Subsequent studies confirmed TIM3 expression on leukemia cell lines, although only two lines (SKM-1 and HNT-34) showed low TIM3 expression [176]. Therefore, it was considered crucial to engineer cell lines to overexpress TIM3. Cell lines were engineered using CRISPR-Cas9 and viral transduction systems to knock out CD33 and transduce TIM3. These engineered cell lines will be used to validate the impact of dual-targeting strategies involving CD33 and TIM3 and antigen density on therapeutic efficacy.

In house patient data analyzed in this study indicates that the TIM3 expression level is lower compared to that of CD33. Previous literature has indicated that the efficacy of CAR T cells is influenced

by the expression of antigens [411], [412]. However, it remains unclear whether the efficacy of CD33 and TIM3 CAR T cells is influenced by the density of antigen expression. Consequently, target cells with varying antigen expression densities of CD33 and TIM3 were utilized to assess efficacy. The study findings indicate that the efficacy of both CD33 and TIM3 CAR T cells is dependent on antigen density. These results are also consistent with other studies [386], [413].

Initially, it was unclear whether differences in co-stimulatory domains affect the efficacy of CAR T cells. The evolution of CAR T cell therapies has led to the classification of these treatments based on co-stimulatory domains [414], [415], [416]. The first generation of CAR T cells is characterized by the presence of the CD3ζ activation domain, while the second generation is distinguished by the inclusion of a co-stimulatory domain (e.g. CD28 or 4-1BB), in addition to the CD3ζ domain. The third generation of CAR T cells incorporates two co-stimulatory domains simultaneously [414]. Compared to first-generation CAR T cells, second- and third-generation CAR T cells show marked enhancements in cytokine secretion, CAR T cell proliferation, and overall anti-tumor efficacy [417], [418], [419], [420]. Although a study has shown that BCMA CAR T cells using the CD28 co-stimulatory domain have greater efficacy than those using 4-1BB [421], CD33 CAR T cells revealed no significant differences between them in our study and some literature [347], which may be related to scFv differences [422], [423].

This study evaluated the efficacy of first to third-generation CD33 CAR T cells against $CD33^+$ cell lines, laying the groundwork for the design of dual CAR T cells. Experimental data indicated that, specific lysis and cytokine secretion levels didn't vary among the three generations in 24 h co-culture assays. Although a study has shown that third-generation CD33 CAR T cells exhibit higher viability, increased proliferation, and greater cytotoxicity, these differences only emerge during longer culture after 48 hours [347]. However, third-generation CARs demonstrated enhanced proliferation, while the first-generation had the lowest proliferative capacity in long-term antigen re-stimulation over 24 days in our studies. These findings are consistent with previous literature reports of poorer *in vitro* and *in vivo* performance of first-generation CAR T cell [424], [425].

The efficacy of compound CAR T cells remains uncertain following the swapping of co-stimulatory domains, particularly in the context of combinations of strong and weak antigens such as CD33 and TIM3. Theoretically, CD33 expression is much higher than that of TIM3, and the activation of CD3ζ in CAR T cells is also related to antigen expression. Therefore, altering the position of co-stimulation domain could potentially affect the efficacy of CAR T cells. To verify this hypothesis, this study designed experiments to swap co-stimulatory domains. The results indicate that even after swapping the co-stimulatory domains of CD33 and TIM3, the compound CAR T cells showed no significant differences in 24 h specific lysis or cytokine secretion levels against target cells (Fig. 12 B). This is likely due to the double CD3ζ signaling pathway, which allows for the full activation of the CAR T cells [426].

The arrangement of CD33 and TIM3 on tandem CAR T cells and its impact on efficacy remains unclear. Theoretically, the scFv with higher affinity should be positioned first to enhance the CAR T cells' recognition of target cells [340], [386], [427]. However, this study found that the sequence of CD33 and TIM3 scFv linked by a rigid linker in tandem CAR T cells does not affect their efficacy and cytokine secretion. These findings are also similar to the research results of CD19 and CD22 tandem CAR T cells [428], [429]. Interestingly, this study found that the sequence of CD33 and TIM3 scFv linked by a rigid linker in tandem CAR T cells does not affect their efficacy and cytokine secretion. It may be attributed to the binding sites between the scFv and the antigens. However, the exact mechanisms remain to be further investigated.

After establishing the structures of all dual CARs, this study compared the efficacy of various dual CAR T cells (pooled, compound, tandem, split). Dual CAR T cells demonstrated specific lysis against OCI-AML3 engineered cells, both singly and doubly expressing CD33 and TIM3. Moreover, cytotoxicity of dual CARs was superior to that of single CAR T cells (Fig. 14 B-D). This finding is consistent with those reported in the literatures [428], [430], [431], [432]. Nonetheless, the synaptic area formed during CAR T cell-mediated killing of target cells requires further investigation, particularly when CAR T cells with the same CAR density encounter antigens with high or low expression levels. However,

within the immune microenvironment recognized by CAR-T cells, the sequential targeting and recognition of AML cells co-expressing CD33 and TIM3 versus cells expressing CD33 or TIM3 individually remain to be investigated. Furthermore, it is still unclear what the primary contributions of scFv are to the cytotoxicity mechanism of CD3ζ activation pathways in the context of high CD33 expression and low TIM3 expression [433].

When combining two different types of CAR T cells in equal parts to create pooled CAR T cells, their effectiveness was unexpectedly reduced. This may be because mixing them diluted the number of effective CAR T cells, which in turn lowered their ability to specifically target and kill AML cells. A study has reported that when co-culturing two CAR T-cell populations, one population expands disproportionately compared to the other [434]. This results in a dominant CAR T cell population, potentially diminishing the overall clinical efficacy. On the contrary, some studies have demonstrated that these pooled CAR T cells (also known as cocktail CAR T cells) are effective against antigen escape, however, their safety and off-target effects, especially CRS effects caused by high-dose pooled CAR T cell therapy, still require further investigation [435], [436]. CD33-TIM3 compound CAR T cells showed the highest efficacy among all types, likely due to the simultaneous binding of bilateral co-stimulatory molecules. These findings are consistent with other reports in the literature on compound CAR T cells [254], [437], [438]. Moreover, the efficacy of CAR T cells was also found to be influenced by TIM3 antigen density. Specifically, CAR T cells demonstrated greater cytotoxicity against high TIM3 expressing target cells compared to those with low expression. Additionally, the secretion levels of cytokines IFN-γ and IL-2 were found to correlate with the efficacy of the CAR T cells. The results demonstrated that higher avidity correlated with increased efficacy during the initial stages of target cell recognition and lysis, consistent with the 24 h cytotoxicity results (Fig. 14 G). At last, the efficacy of dual CAR T cells was revalidated *in vitro* using primary AML cells, corroborating the results obtained with cell lines. Some studies have reported that compound CAR T cell therapy might lead to increased tonic signaling and reduced efficacy [439], [440]. Our study also found that under the stimulation of long-term repeated antigen exposure, compound CAR T cells exhibit lower proliferation compared to other dual CAR T cells. This may be due to the simultaneous activation of bilateral CAR pathways leading to T-cell tonic signaling, with the specific mechanisms still requiring further investigation.

This underscores the imperative need for refined safety protocols for CD33 CAR T cell therapies. An "AND" gating strategy is employed to prevent the nonspecific cytotoxic effects of CAR T cells on normal hematopoietic cells [277], [441]. Both these two gating strategies have been employed in preclinical AML immunotherapy [278], [441], [442]. However, the efficacy of targeting CD33 and TIM3 has not been conclusively established. Therefore, we validated the efficacy of split CAR T cells employing an "AND" gate strategy. This logic gate requires recognition of CD33 and TIM3 antigens on the same target cell to trigger complete CAR-T cell activation. We hypothesized that this method can increase efficacy and decrease on-target-off-tumor lysis compared with CD33- or TIM3- single CAR T cells. The way to implement this strategy is to split the CD3ζ signal and the CD28 and 4-1BB co-stimulatory domain into different receptors, with each signaling domain connected to an scFv targeting a different antigen[277], [278], [441], [443]. However, several studies using such split receptor systems have found that the CD3ζ signal alone is sufficient to induce certain T cell effector functions, including the lysis of single-positive cells [443]. To enhance the specificity of split CAR T cells, which aims to accurately identify and kill AML cells which express both CD33 and TIM3, while sparing normal hematopoietic cells. By linking TIM3 with CD3ζ, we ensured the specificity of the split CAR because TIM3 is highly expressed on LSC and AML blast cells than HSCs. The activation mechanism of the split CAR was effectively validated in a mixed cell experiment using primary cells (Fig. 15 E).

In co-cultures with engineered OCI-AML3 cells exhibiting varying expression of CD33 and TIM3, split CAR T cells demonstrated selective cytotoxicity. Split CAR T cell demonstrated selective targeting of cell lines expressing TIM3, with no evidence of nonspecific lysis of cells lacking CD33 expression. Notably, split CAR T cells demonstrated enhanced efficacy against cells co-expressing CD33 and TIM3 compared to their activity against singly expressing TIM3 cells. A study has shown that split CAR T cell can be activated by target cells expressing a single antigen [443], our research found that split CAR T cells also exhibit partial cytotoxicity against cell lines expressing TIM3. This could be due to split CARs

unilaterally activating the TIM3 signaling pathway. In such cases, split CARs function similarly to first-generation CAR T cell (TIM3-3ζ). Based on previous studies indicating this kind of CAR T cell is ineffective and due to the low TIM3 expression on other non-AML cells in patients [191], [192], [193], [194], [271], [396], we do not expect this activation to cause off-target cytotoxicity.

Furthermore, this study sought to elucidate the signaling pathways activated by dual CAR T cells when targeting dual-expressing CD33 and TIM3 cells. This was done with the specific aim of determining the role of the CD33-28BB co-stimulatory domain in CAR T cell activation. The literature indicates that upon activation by a specific antigen, the co-stimulatory domains in CAR T cells induce the phosphorylation of PI3K, subsequently phosphorylating AKT, thereby activating the mTOR signaling pathway [444], [445]. Moreover, the 4-1BB domain phosphorylates TRAF2, which subsequently activates the downstream p38MAPK, thereby activating the ATF2 signal [446]. Our findings indicate that CD33-CD28BB signaling cascade was also activated in split CAR T cells, in comparison to CD33 single CAR T cells and those utilizing an "OR" gating strategy. This was consistent with increased efficacy of split CAR T cells over single CAR T cells. The split CAR T cell approach involves a design that can engage more than one target or activation pathway, which allows it to more effectively recognize and kill AML cells compared to single CAR T cells that target only one molecule. This split CAR T cell mechanism often results in enhanced efficacy because it can integrate multiple signals to strengthen the T cell response.

Subsequently, the study evaluated the safety of split CAR T cells using CFU assays. CD33 single CAR T cells demonstrated a suppressive effect on HSPC proliferation from healthy donors. In parallel, experiments involving a combination of $CD34^+$ cells from healthy donors and primary AML cells demonstrated that split CAR T cells exhibited significantly greater cytotoxicity against primary AML cells than against normal HSPCs compared with CD33- or TIM3- single CAR T cells. This evidence demonstrates the selective cytotoxicity of split CAR T cells towards primary AML cells over healthy hematopoietic cells. Similarly, this phenomenon was corroborated in co-culture experiments involving a variety of cell lines. Thus, our findings confirm that split CAR T cells employing "AND" gate strategy show no nonspecific lysis of CD33 expressing normal hematopoietic cells, and exhibit stronger efficacy against CD33 and TIM3 co-expressing blast cells and LSCs compared to TIM3 single expressing cells. Compared to current CD33 single targeting CAR T cell studies, this approach offers increased specificity and greater safety for AML blast cells and LSCs [226], [244], [247], [441], [447], [448].

After that, this study compared the proliferation, CAR T cell-mediated cytotoxicity, proportion of naive T cells, CD4/CD8 ratios, and the expression of checkpoint markers in dual CAR T cells under long-term antigen stimulation. Although compound CAR T cells exhibited the strongest efficacy, their proliferative capacity remained limited, and they had a lower proportion of naive T cells and higher levels of checkpoint marker expression compared to other CAR T cell configurations. This suggests the potential for T cell exhaustion due to intense cytotoxic activity [449], [450]. In contrast, split CAR T cells demonstrated similar proliferation under long-term antigen stimulation as single-targeting CAR T cells, but exhibited enhanced efficacy and similar checkpoint marker levels. Notably, all CAR T cells incorporating TIM3 scFv exhibited lower TIM3 expression compared to CD33 single CAR T cells, despite previous data confirming the absence of TIM3 fratricide during CAR T cell production and no difference in proliferation between TIM3-targeting CAR T cells and CD33 single-targeting CAR T cells in our study. One research indicates that TIM-3 signaling hijacks the canonical Wnt/β-catenin pathway to maintain cancer stemness in AML [374]. However, this pathway is not activated in TIM3-expressing exhausted T cells [374]. This may affect the specificity of TIM3 CAR T cells in recognizing $TIM3^+$ AML cells by different TIM3 protein splicing variants. Additionally, another research has shown that TIM3 CAR T cells exhibit higher sensitivity to AML compared to $TIM3^+$ expressing T cells, enabling specific targeting and killing of $TIM3^+$ AML cells [273]. The same study observed fratricide *in vitro* [273]. Additionally, our study found that TIM3 CAR T cells exhibit cytotoxic effects against $TIM3^+$ T cells induced by PMA stimulation. Nevertheless, TIM3 CAR T cells still demonstrated specific killing efficacy against AML cells *in vivo* [273], [277], [278]. The reason for the observed reduction in TIM3 expression on T cells in the presence of prolonged antigen exposure remains unclear and thus warrants further investigation.

# 11. Conclusion and outlook

We successfully generated dual-targeting CAR T cells utilizing both "AND" and "OR" gating strategies to target CD33 and TIM3, enhancing their efficacy against AML cells *in vitro*. The findings demonstrated that these CAR T cells exhibit superior binding avidity and cytotoxic capabilities towards cells expressing both CD33 and TIM3 antigens, as opposed to targeting a single antigen. Notably, the split CAR T cell approach effectively eradicated $CD33^{+}TIM3^{+}$ cell lines and primary AML cells, while minimizing impact on healthy hematopoietic cells. The implications of these results suggest that dual CAR T cell configurations might serve as potential bridging therapies before hematopoietic stem cell transplantation. The split CAR T cell particularly offered a potentially safer alternative, possibly obviating the need for transplantation. The efficacy and specificity of CD33- and TIM3-targeting dual CAR T cells need further investigation and validation *in vivo*. These advancements have the potential to markedly alter the therapeutic landscape for AML, offering more precise, effective, and safer options for treatment.

The outlook for these dual CAR T cells is promising, particularly with the potential to extend these findings into humanized mouse models, which could provide crucial insights into their safety and efficacy in a more physiologically relevant context. Furthermore, rigorous assessment of the risk of CRS will be vital as these therapies move closer to clinical application. Although CRS remains a significant challenge in CAR T cell therapy, the precise targeting mechanisms employed by our split CAR T cells may reduce the incidence and severity of such adverse events, thereby improving patient safety. Further research in these areas will be critical to fully realize the therapeutic potential of CD33- and TIM3-targeting dual CAR T cells in AML treatment.

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# Affidavit

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# 16. List of publications

**Wang, Yingshuai**, Wenjie Lu, Lisa Rohrbacher, Heinrich Flaswinkel, Alica Joana Emhardt, Giulia Magno, Sascha Haubner, et al. “CD33-TIM3 Dual CAR T Cells: Enhancing Specificity While Maintaining Efficacy Against AML.” *Blood* 142 (2023): 3449.*

*ASH abstract