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Original article

The tandem CD33-CLL1 CAR-T as an approach to treat acute myeloid leukemia

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Background - Acute myeloid leukemia (AML) is characterized by high heterogeneity, poor long-term survival, and a propensity for relapse. Exceptional efficacy in treating recurrent or refractory B-lymphoid malignancies has been demonstrated by Chimeric antigen receptor T cells (CAR-T cells). Given the therapeutic potential of targeting both CD33 and C-type lectin-like molecule-1 (CLL1) in AML, the development of a dual-targeting CD33-CLL1 CAR-T cells assumes significant importance.

Materials and methods - The expressions of CD33 and CLL-1 antigens in peripheral blood cells and bone marrow cells from AML patients was assessed. Subsequently, a Chimeric Antigen Receptor (CAR) incorporating a dual-specific single-chain variable fragment targeting CLL1 and CD33 (CD33-CLL1-CAR-T) was engineered. The anti-tumor efficacy and potential side effects of CD33-CLL1-CAR-T cells were comprehensively investigated in both in vitro and in vivo settings.

Results - The constructed tandem CD33-CLL1 CAR-T exhibited potent cytotoxicity against leukemia cell lines and human primary AML cells *in vitro*. Co-cultivation of AML blasts with CD33-CLL1-CAR-T cells resulted in effective proliferation and the secretion of substantial quantities of GM-CSF and IFN-γ. Importantly, the impact of CD33-CLL1-CAR-T cells on normal hematopoietic stem cells was minimal, ensuring safety in vivo mouse models. Notably, significant anti-leukemic activity was observed in the mouse model, with CD33-CLL1-CAR-T cells leading to tumor eradication and prolonged survival.

Discussion - The tandem CD33-CLL1 CAR-T cells not only efficiently eliminated AML blasts but also exhibited low cytotoxicity toward normal hematopoietic stem cells (HSCs). These findings underscore the potential clinical applicability of the tandem CD33-CLL1 CAR-T cells as an effective and safe treatment strategy for AML, representing a noteworthy advancement in the field of CAR-T cells therapy.

Keywords: *acute myeloid leukemia, C-type lectin-like molecule-1, CD33, chimeric antigen receptor, leukemia stem cells.*

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INTRODUCTION

The prognosis of acute myeloid leukemia (AML) has exhibited limited improvement over the past decade. The cornerstone of successful AML treatment involves intensive

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chemotherapy followed by allogeneic hematopoietic stem cell transplant (HSCT), particularly for individuals with abnormal chromosomal or molecular profiles. However, a subset of patients experiences post-HSCT relapse, leading to a challenging prognosis and constrained therapeutic alternatives. Chimeric antigen receptor T (CAR-T) cell therapy has emerged as a promising therapeutic avenue for patients with relapsed or refractory B-lymphoid malignancies (r/r B-ALL). Despite progress in identifying leukemia antigens such as CD33, CD123, and C-type lectin-like molecule-1 (CLL1) as potential targets over the last decade, their application has not yielded encouraging outcomes[1,2]. This may stem from the limitation of single-antigen targeting, failing to address all AML blasts and posing the risk of fatal "off-tumor, on-target" side effects.

CD33, a prominent therapeutic target for AML, is predominantly expressed in pluripotent bone marrow precursors, mononuclear colony-forming cells, mature granulocytes and monocytes, peripheral granulocytes, and resident macrophages[3-5]. CLL1, a type II transmembrane protein, is exclusively expressed by myeloid cells, particularly prevalent (85-92%) in French-American-British classes M0–M6. Additionally, CLL1 is present on $CD34^+CD38^+$ AML leukemia stem cells (LSCs)[6-8]. Consequently, CLL1 serves as a promising alternative target for AML, specifically addressing leukemia progenitor cells and their progeny while sparing normal bone marrow precursor cells[9].

In this investigation, we constructed CAR-T cells targeting CD33, CAR-T cells targeting CLL1, and the tandem CD33-CLL1 CAR-T cells which simultaneously target two antigens for AML treatment. We attempted to find a specific and targeted CAR-T therapy that could alleviate the cytotoxic effect on normal hematopoietic precursor cells while targeting leukemia cells by analyzing the phenotype of CAR-T cells, their cytotoxicity against leukemia cells *in vitro*, their cytotoxic effects on hematopoietic precursor cells, and evaluating the therapeutic effect of CAR-T cells in an NSG mouse model.

MATERIAL AND METHODS

Patients and samples

Peripheral blood (PB) and bone marrow (BM) samples were acquired from AML patients at The First Affiliated Hospital of USTC between March 1st, 2018, and May 30th, 2020. In accordance with the Declaration of Helsinki, informed consent was obtained from all donors.

Cell lines

Human AML cell lines (Molm-13, THP-1, U937) were sourced from the Genentech Cell Line Repository and cultured in RPMI 1,640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 1% penicillin-streptomycin at 37°C in a 5% CO_2 environment.

Antibody production

Humanized antibodies targeting CLL-1 and CD33 were generated, followed by pharmacologic optimization.

Preparation of CAR and lentivirus

The CLL1 CAR, CD33 CAR, and CD33-CLL1 tandem CAR were generated by linking the CLL1 binder or the CD33 binder-derived single-domain antibody in frame with the hinge and transmembrane domains of CD8 and the cytoplasmic domains of 4-1BB and CD3ζ. The lentivirus packaging plasmid mixture, including pCMV-ΔR-8.47 and pMD2.G (Addgene [Watertown, MA, USA], Cat#12259), was blended with the appropriate CAR-encoding plasmid at a pre-optimized ratio using polyethyleneimine. HEK293 cells were transfected with the mixture, and after overnight culture, the supernatant was collected, centrifuged, and filtered through a 0.45 μm PES filter. The virus particles were pelleted, rinsed, aliquoted, and stored at –80°C. The virus titer was determined by measuring supT1 cell line transduction efficiency via flow cytometric assay.

T cell transduction

Leukocytes were apheresed from healthy donors by apheresis, and peripheral blood mononuclear cells (PBMCs) were isolated using FICOLL-PAQUE™ PLUS Media (Sigma-Aldrich, Burlington, MA, USA). Human T cells were purified with a Pan T cell isolation kit (Miltenyi [Miltenyi Biotec, Bergisch Gladbach, Germany], Cat#130-096-535). The purified T cells were subsequently pre-activated for 48 hours with a human T cell activation/expansion kit (Miltenyi, Cat#130-091-441). Following pre-activation for 48 hours, T cells were transduced with lentivirus stock, cultured, and periodically adjusted. Protein L and rabbit-anti-scFv were used for CAR expression detection on T cells.

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Flow cytometry

Whole blood and bone marrow samples were stained directly with antibody reagents or post-treated with ACK lysis (Thermo Fisher Scientific, Watham, MA, USA) and preincubation with 0.5 mg/mL human IgG for 20 minutes on ice. After 40 minutes of incubation with primary antibodies, data acquisition was performed on a BD LSR Fortessa flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA), with analysis conducted using Flowjo software (Flowjo LLC, Ashland, OR, USA). A list of fluorochrome-labeled antibodies and important experimental materials is provided in the *Online Supplementary* **Table SI**.

In vitro cytotoxicity assay

For rapid evaluation of the anti-tumor activities of CAR-T cells *in vitro*, an LDH (lactate dehydrogenase) assay for cytotoxicity was conducted. On day 5 or day 9 post-transduction, transduced T cells were harvested and co-incubated with target cell lines for 24 hours, respectively. Un-transduced T cells (UnT) from the same batch served as a negative control. The cytotoxicity was calculated by the equation below ($[LDH]_{E+T}$: the LDH released from E/T co-incubation, $[LDH]_E$: the LDH released from Effector only, $[LDH]_{max}$: the LDH released from target cells treated with Triton X-100, $[LDH]_{min}$: the LDH released from UnTreated target cells):

$$\text{Cytotoxicity \%} = \frac{[LDH]_{E+T} - [LDH]_E - [LDH]_{min}}{[LDH]_{max} - [LDH]_{min}} \times 100$$

Long-term cytotoxicity assay

To evaluate the long-term killing efficacy of CAR-T cells, long-term co-culture assays were performed, which mimic the dynamic killing process *in vivo*. AML tumor cell lines (e.g., U937) were labeled with CFSE (Sigma-Aldrich, Cat# 21888-25MG-F). Transduced or non-transduced T cells (1×10^5/well) were co-cultured with tumor cells (CFSE-U937 cells, 4×10^5/well) at an E: T ratio of 1:4 in 24-well plates, in the absence of exogenous cytokines (IL-2). Part of the cells were harvested and stained for CD3 after 2 or 3 days after co-culture. Tumor cells were identified by $CFSE^+$ signal. For serial co-culture assays, the remaining T cells were then re-challenged with fresh CFSE-U937 cells at the same E: T ratio. Co-cultures were carried on until tumor cells were outgrown. The T cell proliferation rate at each time point is calculated by dividing the number of T cells at the time point by the initial number of T cells.

Cytokine release assays

Supernatants from the *in vitro* cytotoxicity assay were collected to assess CAR-induced cytokine release using Homogeneous Time-Resolved Fluorescence (HTRF) assays for IFN-γ and GM-CSF (Cisbio [Bedford, MA, USA], Cat# 62HIFNGPEH).

Mouse experiments

Anti-tumor activities of CAR-T cells were evaluated in a U937-Luc xenograft NSG mouse model. 2×10^6 U937-Luc cells with the firefly luciferase reporter gene expression were implanted subcutaneously on day 0 in NOD/SCID IL-2RγCnull (NSG) mice. Bioluminescent imaging (BLI) was conducted 7 days after tumor inoculation to monitor model development. The animals were randomized based on the BLI photon numbers and animal body weights. After randomization, a single dose (3×10^6 per mouse) of CAR-T cells or UnT cells were infused intravenously. Weekly BLI imaging was performed to record tumor growth.

Statistical analysis

Data were analyzed using Prism 6.0 and Statistical Package for Social Science (SPSS v25.0; IBM Corporation, Armonk, NY, USA). Differences between continuous variables in two independent samples were analyzed using t-tests and chi-square tests. Two-sided p-values <0.05 were considered statistically significant.

RESULTS

Dual-targeting CD33-CLL1 CAR-T cells kill human leukemia cells *in vitro*

Firstly, we detected the expressions of CD33 and CLL1 in human leukemia cells, and the results showed that CD33 was highly expressed in all the cell lines shown, while the expression of CLL1 varied from $CLL1^{low}$ (MLOM-13) to $CLL1^{mid}$ (MV4-11, THP-1) and $CLL1^{high}$ (HL60, U937) (**Figure 1A**). Then we collected samples from 27 patients diagnosed with AML, including PB samples (No.=22) and bone marrow (No.=12). As shown in **Figure 1B**, 19/22 patients showed high expression of CLL-1 and CD33, while 10/12 patients had high expression of CLL1 and CD33 in BM samples (*Online Supplementary* **Table SII**, **Figure S1A** and **B**).

To selectively target $CD33^+CLL1^+$ leukemia cells, we developed CAR-T cells targeting CD33, CLL1, and dual-targeting CD33-CLL1 (*Online Supplementary* **Figure S2A**, **Figure 1C**). The epitope of CD33 binder

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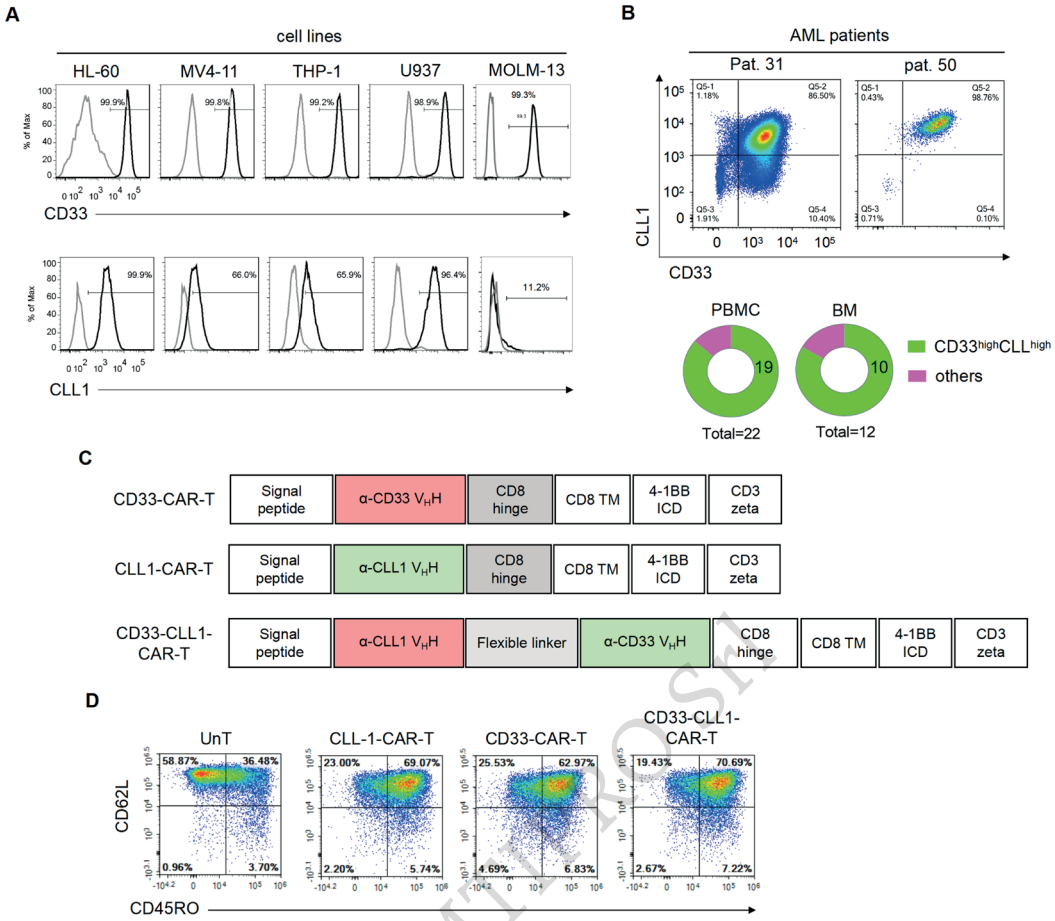
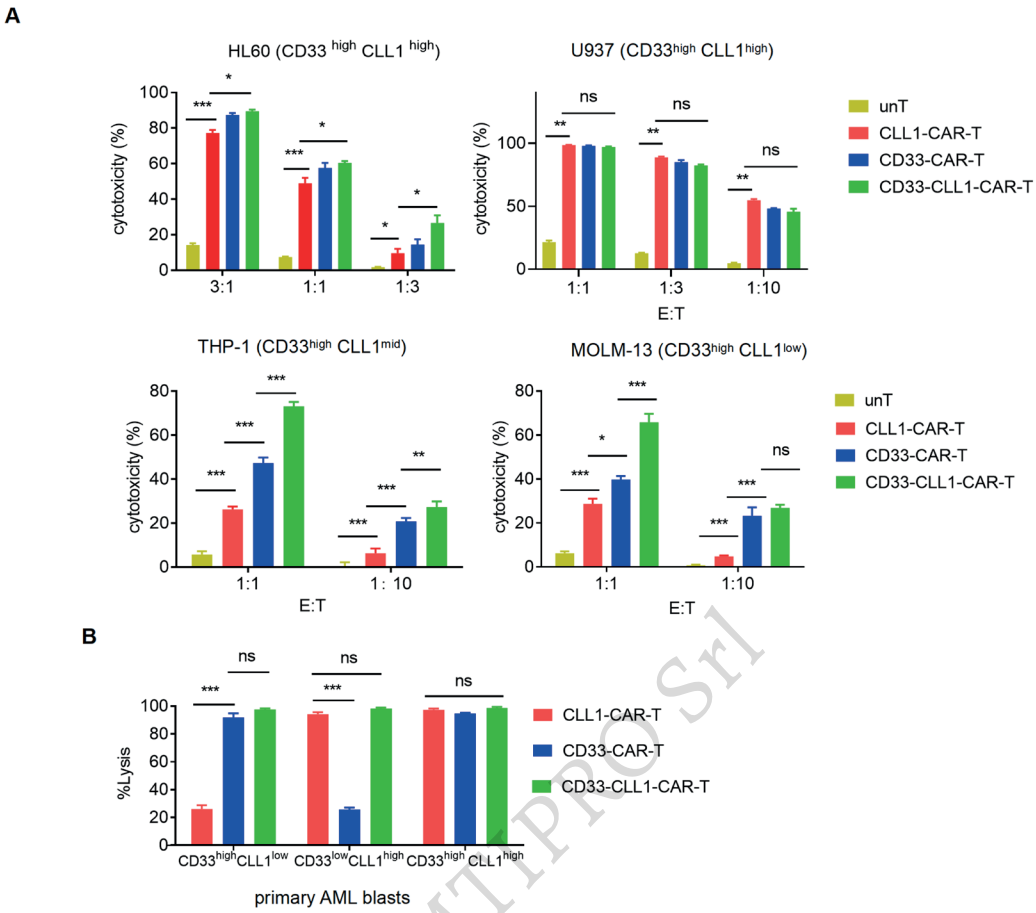


Figure 1 - Constructing the CD33-CLL1 tandem CAR-T
(**A**) Expression of CD33 and CLL1 on the surface of human leukemia cell lines (HL-60, MV4-11, THP-1, U937, MOLM-13) was detected by flow cytometry. The gray line represents the isotype control. (**B**) Expression of CD33 and CLL1 in peripheral blood mononuclear cells (PBMCs, No.=22) and bone marrow (BM, No.=12) of Primary AML patients (down panel). A representative flow plot showed the expression of CD33 and CLL1 in tumor cells from two AML patients (up panel). (**C**) The structure of CD33-CAR-T (targeting CD33), CLL1-CAR-T (targeting CLL1), and CD33-CLL1-CAR-T (targeting both CD33 and CLL1). (**D**) The expression of CD45RO and CD62L on T cells before (un T)and after transduction (CAR-T) were detected using flow cytometry. CAR-T: chimeric antigen receptor T-cells; unT: untransfected T-cells.

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is the membrane-distal V-set domain of CD33. The extracellular part of the CLL1 protein consists of a single extracellular carbohydrate recognition domain. As shown in *Online Supplementary* **Figure S2B**, the transduction efficiency of the CD33-CLL1 tandem CAR is approximately 55%. About 55-60% of the T cells transduced with CAR were $CD8^+$ T cells, and 40-45% were $CD4^+$ T cells, which was similar among the three types of CAR-T cells (*Online Supplementary* **Figure S2C**). We also examined the phenotypes of untransfected T cells (unT) and three types of CAR-T cells and the results showed that approximately 60-70% of CD33-CLL1 tandem CAR-T cells are central memory T cells (T_{CM}, $CD45RO^+CD62L^+$), which is similar to the other two types of CAR-T cells (CD33-CAR-T and CLL1-CAR-T) (**Figure 1D**). T_{CM} has a strong ability to proliferate and secrete IL2, which suggested that CD33-CLL1 tandem CAR-T could rapidly differentiate into effector cells upon contact with the corresponding antigen.

Cytotoxicity assays revealed that CD33-CLL1-CAR-T efficiently killed various leukemia cell lines, outperforming CD33-CAR-T and CLL1-CAR-T, especially when CLL1 expression was variable ($CD33^{high}CLL1^{mid}$ THP-1, and $CD33^{high}CLL1^{low}$ MOLM-13, **Figure 2A**). Notably, CD33-CLL1-CAR-T effectively targeted primary



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Figure 2 - Human leukemia cells were killed by CD33-CLL1 tandem CAR-T
(**A**) Killing percentages of HL60, U937, THP-1, and MOLM-13 cells by unT, CD33-CLL1-CAR-T, CLL1-CAR-T, and CD33-CAR-T cells after 18 h co-culture *in vitro*. E: T ratios mean the ratios of the absolute number of CAR-T cells to target cells. (**B**) Cytotoxic effects of CD33-CAR-T, CLL1-CAR-T, and CD33-CLL1-CAR-T on 3 primary AML blasts (based on the expression of CD33 and CLL1) after 18 h co-culture *in vitro* (E: T=1:4). The data were represented as means ± SD and analyzed by One-way ANOVA and two-tailed unpaired Student's t-test. *p<0.05; **p<0.01; ***p<0.001, ns, no significance.

AML blasts, demonstrating its capability to eliminate leukemia cells regardless of CLL1 expression levels (**Figure 2B**). These results signify the successful construction of CD33-CLL1 dual-targeting CAR-T cells, demonstrating potent antitumor activity against human leukemia cells *in vitro* regardless of high or low expression of CLL1.

CD33-CLL1-CAR-T showed high long-term cytotoxicity to human leukemia cells

To evaluate the long-term cytotoxicity of CD33-CLL1 tandem CAR-T, three types of CAR-T cells were subjected to several rounds of tumor cell stimulation. The results showed sustained killing of U937 cells and persistent proliferation of three types of CAR-T cells (**Figure 3A**, *Online Supplementary* **Figure S3**), accompanied by prolonged secretion of GM-CSF and IFN-γ (**Figure 3B**). These findings suggest that CD33-CLL1-CAR-T cells can continuously proliferate, efficiently secrete cytokines, and sustain antitumor activity *in vitro*, indicating potential applicability *in vivo*.

CD33-CLL1-CAR-T cells were safe for HSCs and mice

Considering the safety of CAR-T cells, we examined the expression of CD33 and CLL1 in $CD34^+$ cord blood stem/progenitor cells of healthy individuals (*Online*

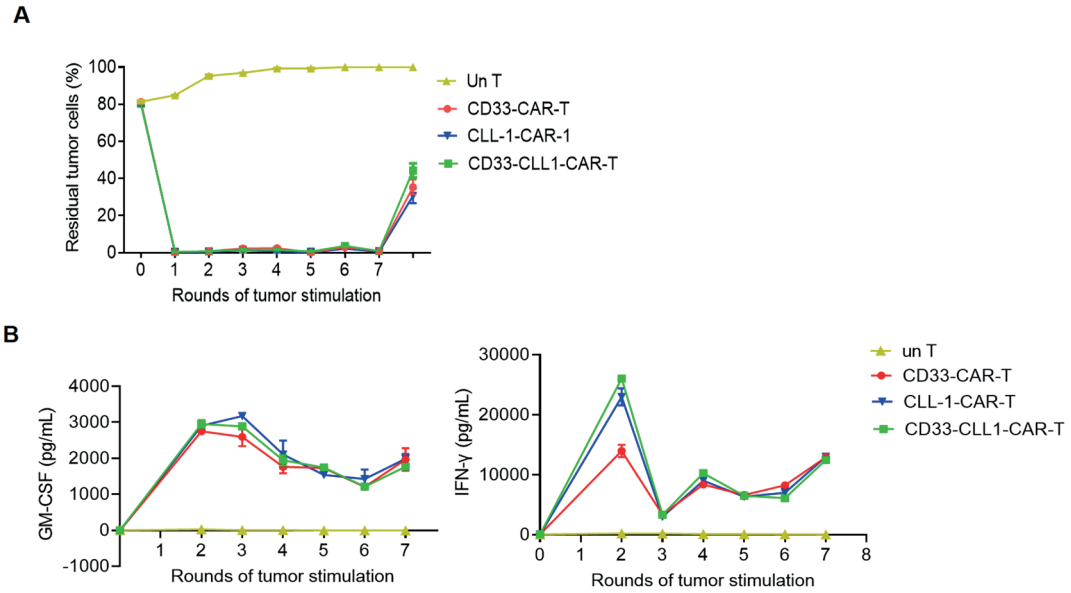


Figure 3 - Long-term cytotoxicity of CD33-CLL1-CAR-T to human leukemia cells
(**A**) The cytotoxicity of the indicated CAR-T on U937 cells (CSFE labeled) after 8 rounds of co-culture at a 1:4 E/T ratio was detected by flow cytometry. (**B**) GM-CSF and IFN-γ concentration in the supernatant after 48 h co-culture of the indicated CAR-T cells with target cells (E:T=1:4). The data were represented as means ± SD.

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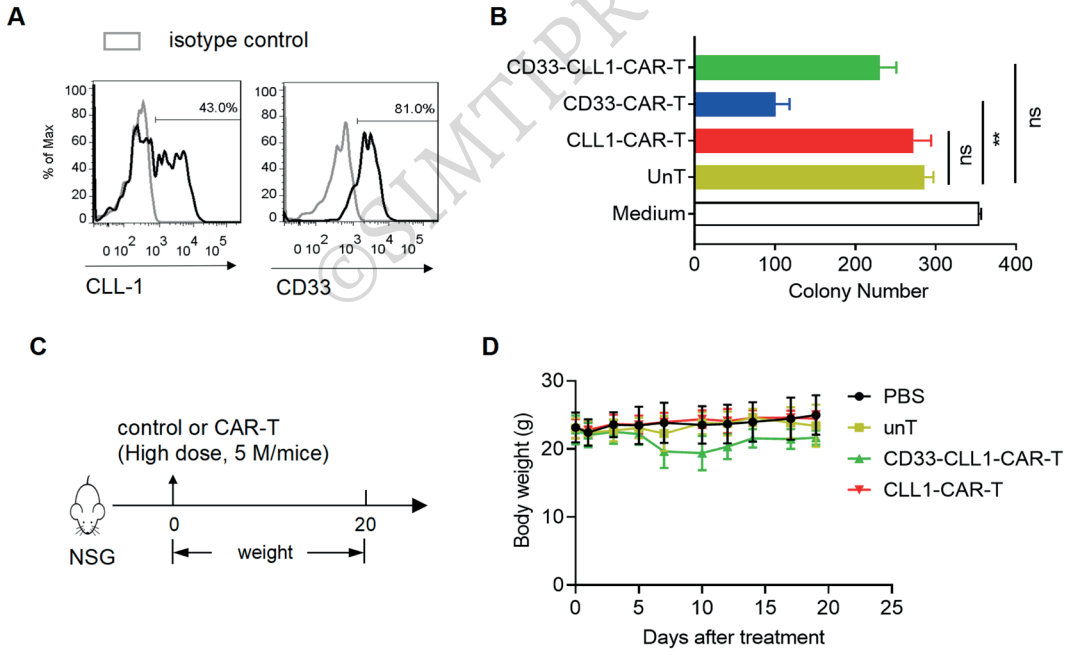


Figure 4 - Effects of CD33-CLL1-CAR-T cells on human hematopoietic stem cells *in vitro* and mice *in vivo*
(**A**) Expression of CLL1 and CD33 on $CD34^+$ HSCs derived from cord blood was detected by flow cytometry. The gray line represents the isotype control. (**B**) Cytotoxicity of CLL1-CAR-T, CD33-CAR-T, and CD33-CLL1-CAR-T on CD34+ cord blood cells. CD34+ Cord blood cells were incubated with CAR-T-cells at E: T=10:1 for 16 h, the mixture was then plated (500 HSCs/plate) in semi-solid media, and colonies were counted for each condition after 6 days. (**C** and **D**) Experimental scheme: NSG mice were injected intravenously with 5×10^6 CAR-T cells (unT, CD33-CLL1-CAR-T, CD33-CAR-T, CLL1-CAR-T) or phosphate-buffered saline (PBS) and the weight was monitored to assess the effect on the NOD/SCID IL-2RγC^{null} (NSG) mice. The data in **B** and **D** were represented as means ± SD and analyzed by One-way ANOVA and two-tailed unpaired Student's t-test. *p<0.05; **p<0.01; ns: no significance.

Supplementary **Figure S4**). As shown in **Figure 4A**, CLL1 was moderately expressed on stem cells, while CD33 was expressed on more than 80% of HSCs. Then cord blood was co-cultured with 3 types of CAR-T cells, and the results showed that compared with untransfected T-cells, HSCs co-incubated with CD33-CAR-T cells produced about 50% fewer cell colonies, while the other two types of CAR-T cells had no significant effect on the growth of stem cell colony formation (**Figure 4B**). Moreover, we evaluated the effects of adoptive transfusions of high-dose CAR-T cells on NSG mice (**Figure 4C**). The results showed that 5 million CLL1-CAR-T or CD33-CLL1-CAR-T cells were injected into the tail vein of each NSG mouse , with no significant effects on mouse weight or survival (**Figure 4D**). These results underscore the safety profile of CD33-CLL1-CAR-T cells for both HSCs and mice.

CD33-CLL1-CAR-T cells eliminated human AML in xenograft models

Further, the anti-leukemic activity of CD33-CLL1-CAR-T cells was evaluated *in vivo* using a xenogeneic AML model. We developed a xenogeneic model of AML using the U937 cells on NSG mice (**Figure 5A**). As shown in **Figure 5B** and **C**, mice treated with UnT cells or PBS exhibited rapid tumor progression and had to be euthanized before the end of the experiment. The mice treated with single-target CAR-T cells had slower tumor growth than the control mice (PBS and unT). Tumor load in mice treated with CLL1-CAR-T cells was significantly increased than that in mice treated with CD33-CLL1-CAR-T and CD33-CAR-T (**Figure 5C**). Moreover, mice treated with CD33-CAR-T and CD33-CLL1-CAR-T achieved long-term survival (**Figure 5D**). These findings substantiate the *in vivo* efficacy of CD33-CLL1 dual-targeting CAR-T cells in eliminating human AML.

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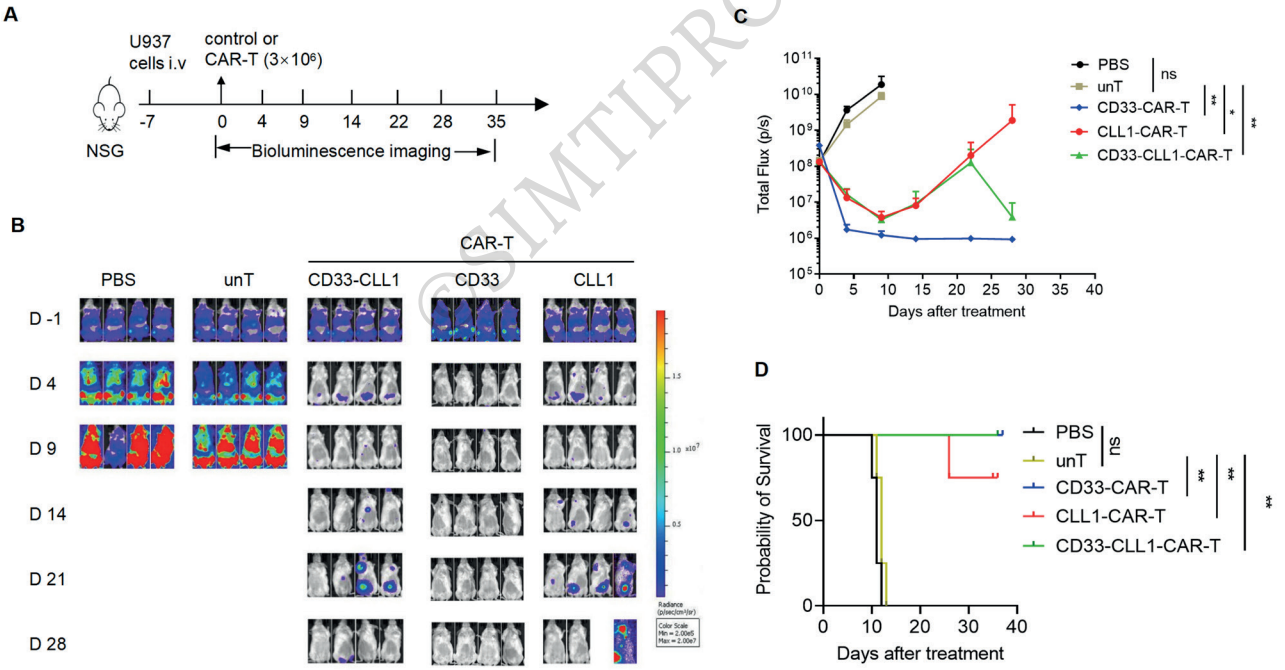


Figure 5 - The anti-tumor activities of CAR-T cells in a U937-Luc xenograft mouse model *in vivo*

(**A**) Experimental scheme: NSG mice were injected intravenously with 1×10^6 U937 cells stably expressing luciferase on day -7. After confirmation of engraftment by Bioluminescence imaging (BLI) on day -1, 3×10^6 CAR-T cells (unT, CD33-CLL1-CAR-T, CD33-CAR-T, CLL1-CAR-T) or PBS were transferred to all the mice via tail vein. Tumor burden was monitored based on the bioluminescence intensity from the IVIS imaging system at the indicated time points. (**B**) BLI of the tumor burden. (**C**) Tumor burden was quantified as the average value of the total flux (p/s). No.= 5 mice per group. (**D**) Kaplan-Meier survival curve of mice bearing U937 cell-derived tumors after indicated treatment. The data in panel C were represented as means ± SD and analyzed by one-way ANOVA with Tukey's multiple comparisons tests. The data in panel **D** were analyzed by log-rank Mantel-Cox test. *p<0.05; **p<0.01; ns: no significance.

DISCUSSION

AML remains a highly heterogeneous hematologic malignancy with a dismal 5-year overall survival (OS) rate of 27%, further compounded by even lower rates of 5% and 10% for elderly and relapsed/refractory (r/r) patients, respectively[10]. The conventional "7+3" regimen, comprising cytarabine and anthracycline, has been the standard of care for AML for four decades, with a dearth of new drugs in its wake[11]. Recently approved agents, including CPX-351, glasdegib ozogamicin, venetoclax, ivosidenib, midostaurin, and gemtuzumab ozogamicin, have provided some breakthroughs, particularly for newly diagnosed patients since 2017[12]. For r/r patients, a combination of potent induction chemotherapy followed by allogeneic hematopoietic stem cell transplant (HSCT) remains the hope to enhance prognosis.

The remarkable success of CAR-T, exemplified by sustained remission over a decade in some cases, has instilled confidence in its potential to perform long-term immune surveillance[13-17]. Target selection principles prioritize antigens expressed persistently in tumor cells while managing on-target and off-tumor activities effectively CAR-Ts targeting CD19 and BCMA have achieved success against B-cell leukemia, lymphoma, and refractory multiple myeloma[16,17]. Notably, myeloid blasts in a significant proportion of AML patients express the CD33 surface antigen, presenting a potential therapeutic target[18,19]. However, due to CD33 expression in normal HSCs and other tissues (e.g., hepatic Kupffer cells), CD33-CAR-T treatment led to cytopenias in human lineage and a decrease in myeloid progenitors in xenograft models of hematopoietic toxicity, suggesting that permanently expressed CD33-specific CART cells would have unacceptable toxicity[20,21]. Therefore, work on optimizing the structure of CD33 CAR in order to reduce toxicity is also ongoing[22].

Leukemia stem cells (LSCs), a minor yet pivotal subpopulation in leukemia patients, contribute to relapse and drug resistance. Treatment goals are shifting towards complete LSC elimination while preserving normal HSCs, such as HSCT and cellular therapies targeting LSC removal[18]. CLL1, expressed in myeloid cells and AML cells, particularly in LSCs, offers a selective target absent in normal HSCs[2]. Some anti-CLL1 antibody-drug conjugates (ADC) exhibit promising antitumor effects with superior safety profiles compared to CD33-ADC[9,23]. Studies on CLL1 CAR-T cells indicate specific anti-leukemic activity, especially against LSCs[6-8]. Targeting both CD33 and CLL1 emerges as a strategy to eliminate AML in the majority of patients while mitigating stem cell toxicity.

In present study, we observed that a majority of AML patients express high levels of CD33 and CLL1, prompting the design of a dual-specific CAR-T cell with enhanced targeting capabilities. *In vitro* experiments validated the superior killing ability of CD33-CLL1-CAR-T across different cell lines compared to single-targeted CLL1-CAR-T and CD33-CAR-T. In this study, the CD33 binder used in CD33-CLL1 tandem CAR T cells recognizes the membrane-distal V-set domain of CD33, and on the other hand, we chose the binding fragment with a weaker affinity for CD33 as the tandem CAR. Moreover, the extracellular part of CD33-CLL1 tandem CAR consists of, in the following order (from N- to C-terminus), CD33 binder, CLL1 binder, and hinger domain. These factors led to significantly low cytotoxicity of CD33-CLL1-CAR-T towards $CD33^+/CLL1^-$ HSCs. As shown in Figure 4B, the toxicity of CD33-CLL1-CAR-T to normal HSC is very weak. Therefore, many models of bridging HSCT after CAR-T cells therapy have been successful[24], CD33-CLL1 tandem CAR-T that eliminates leukemia cells in AML patients may reduce reliance on HSCT.

While co-transfection of CD33-CAR with CLL1-CAR into T cells has shown therapeutic efficacy, our CD33-CLL1-CAR-T structure ensures equal expression of both CARs[25]. In addition, the dual parallel CAR is generally expressed by bicistronic constructs expressing distinct CARs from a single vector, which has a higher CAR density on the cell surface than that of single CAR design. Previous studies have noted that intemperately expressing CAR molecules on the surface of CAR-T runs the risk of creating tonic signaling, which can lead to CAR-T exhaustion[26,27]. Compared with the design of parallel dual CAR, the density of tandem CAR on the cell surface is similar to that of single CAR, which can effectively improve the persistence of CAR-T cells.

Our study has limitations, including the use of AML mouse models constructed with cell lines rather than the typical $CD33^+CLL^-$ and a $CD33^-CLL^+$ mixture leukemia model or primary AML cells. We detected only a limited types of cytokines in the cytotoxicity of CD33-CLL1 tandem CAR-T

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cells on AML cells. Analysis of a more broad panel of cytokines may enable us to elucidate both the impact of inflammatory cytokines in maintaining the response and possible further safety considerations. Using humanized NSG mice reinfused with human CD34 positive stem cells would provide additional insight on both the occurrence of CRS, cytokines milieu and tumor control[28]. Additionally, human clinical trial data are insufficient, although clinical trials are currently underway. Patient data suggested that exposure to high levels of GM-CSF during a cytokine storm may reduce the sensitivity of AML cells to CAR-T cells, indicating that tailor therapy is necessary to minimize the impact of cytokine on AML blasts to improve outcomes with AML-targeted CAR-T cell therapy[29].

CONCLUSIONS

The tandem CD33-CLL1 CAR-T cells demonstrated effective eradication of AML blasts while displaying minimal cytotoxicity towards normal hematopoietic stem cells (HSCs). Our findings suggest the promising clinical utility of tandem CD33-CLL1 CAR-T cells as a safe and efficient therapeutic approach for AML, potentially reducing reliance on transplantation.

Data availability statement

Any additional information required to reanalyze the data reported in this paper is available from the lead contact (Li Zhou, zhouli-324@163.com) upon request.

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AUTHORS' CONTRIBUTION

HW performed the experiments and wrote the manuscript. SF administrated the patients, analyzed and interpreted data. YZ performed the experiments. YZ provided technical advice. ZZ administrated the patients and provided the resources. ZN provided technical advice. XL and PP collected the clinical data. SW and LZ designed the experiments, checked the data, and revised the manuscript.

The Authors declare no conflicts of interest.

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