Valproic acid increases CAR T cell cytotoxicity against acute myeloid leukemia

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ABSTRACT

The treatment of B cell malignancies has dramatically changed with the introduction of immunotherapy, especially chimeric antigen receptor T (CAR-T) cell therapy. However, only limited efficacy is observed in acute myeloid leukemia (AML). In this study, we detected CD123 and CLL-1 expression on leukemia cells from Relapsed/Refractory AML (R/R AML) patients. Then, we constructed anti-CD123 CAR and CLL-1 CAR with different co-stimulation domains (CD28 or 4-1BB) and detected their anti-AML effects. To increase the efficacy of CAR-T cell therapy, we tested different strategies, including application of combined checkpoint inhibitors and histone deacetylase inhibitors (HDACi) in vivo and in vitro. We found CD123 and CLL-1 were highly expressed on AML cells. The proportions of T cell subsets and NK cells involved in anti-tumor or anti-inflammation processes in AML patients significantly decreased when compared with healthy donors. Both CD123 CAR and CLL-1 CAR displayed specific anti-AML effects in vitro. To improve the lysis effects of CAR-T cells, we combined CAR-T cell therapy with different agents. PD-1/PD-L1 antibodies only slightly improved the potency of CAR-T cell therapy (CD123 CAR T 60.92% ± 2.9087% vs. 65.43% ± 2.1893%, 60.92% ± 2.9087% vs. 67.43% ± 3.4973%; 37.37% ± 3.908% vs. 41.89% ± 5.1568%, 37.37% ± 3.908% vs. 42.84% ± 4.2635%). However, one HDACi (valproic acid [VPA]) significantly improved CAR-T cell potency against AML cells. In vitro, CD123 CAR T 34.97% ± 0.3051% vs. 88.167% ± 1.5327%, p < 0.0001; CD123 CAR T 26.87% ± 2.7010% vs. 82.56% ± 3.086%, p < 0.0001 in MV411; CLL-1 CAR T 78.77% ± 1.2061% vs. 93.743% ± 1.2333%, p < 0.0001; CD123 CAR T 64.10% ± 1.5130% vs. 94.427% ± 0.142%, p = 0.0001 in THP-1). Combination therapy prolonged the overall survival of mice when compared with single CD123 CAR-T cell therapy (median survival: 180 days vs. unfollowed). A possible mechanism is that activated CD8+ T cells upregulate natural-killer group 2 member D (NKGD2), and VPA upregulates NKGD2 ligand expression in AML cells, contributing to NKGD2-mediated cytotoxicity of CAR-T cells against tumour cells. In conclusion, CD123 and CLL-1 are promising targets for AML CAR-T cell therapy. A combination of VPA pre-treatment and CAR-T against AML exhibits synergic effects.

BACKGROUND

In recent years, preclinical studies of chimeric antigen receptor T (CAR-T) to treat acute myeloid leukemia (AML) have yielded promising results. Several tumor antigens targeted to AML have been explored, including CD33, CD123, C-type lectin-like molecule-1 (CLL-1), CD44v6, and CD7. Among them, CD123 and CLL-1 remain the most promising targets for AML. However, the short persistence of CAR-T cells and immune escape may result in a relapse of AML.

Natural-killer group 2 member D (NKGD2) is normally expressed in all natural killer (NK), NK T cells (NKT), CD8+ T cells, and subsets of γδ T cells. NKGD2 recognizes a family of major histocompatibility complex (MHC) I chain-related molecules A and B and a family of six UL16-binding proteins 1-6 (ULBP1-6), which are widely expressed in tumor cells. Recent studies have shown that NKGD2L on tumor cells can be upregulated by histone deacetylase inhibitors (HDACis), which may make tumor cells sensitive to immune cells mediated cytotoxicity. NKG2D/NKG2DL interaction could significantly activate T cells and NK cells, which enhances antitumor activity. HDACi can influence tumor immunogenicity, the microenvironment, and the functional activity of specific immune cells. Moreover, HDACi was reported to upregulate NKGD2L in AML. Therefore, it is likely that a combination of CAR-T cell therapy with agents involved in T cell activation could be a novel potency to treat AML by activating T cells.

In the present study, we constructed anti-CD123 CAR and CLL-1 CAR, which displayed specific anti-AML effects in vivo and in vitro. To improve the therapeutic effect and overcome immune escape, we tested the combined CAR-T cell therapy with HDACi and programmed cell death protein-1 (PD-1) or its ligand PD-L1 antibodies to treat AML. Interestingly, one HDACi, valproic acid (VPA), could significantly synergize with CAR-T cell therapy to improve antitumor activity. Our work suggests that a combination

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of CAR-T cell therapy with VPA could be a promising strategy to improve the therapeutic efficacy of Relapsed/Refractory AML (R/R AML).

METHODS

T cell sorting and manufacturing of CAR-T cells

Isolation of peripheral blood mononuclear cells (PBMCs) and manufacture of CAR-T cells were as previously described.14

In vitro assay

The phenotype of patients with AML, AML cell lines (THP-1 and MV-411) and B-Acute lymphoblastic leukemia (B-ALL) cell line (BALL-1) were detected to determine the expression of CD123 and CLL-1 using flow cytometry. To test the effect of CAR-T cells, CD123 and CLL-1 CAR-T cells were co-cultured with AML cell lines at different effect/target ratios at 24 hours or 48 hours, then the lysis effects were calculated with Green Fluorescent protein (GFP) and CD3 by flow cytometry.

To determine the lysis effects of the combination treatment of HDACi or PD-1/PD-L1 antibodies (Ultra-LEAF Purified anti-human Antibody, Biologend) and CAR-T cells, co-culture was performed in three different ways: (1) Add the drugs to the co-culture medium; (2) Pretreat tumor cells with drugs for 24 hours or 48 hours, wash the medium, and then co-culture with CAR-T cells; and (3) Pretreat the tumor cells with drugs for 24 hours or 48 hours and then directly co-culture with CAR-T cell and drugs. The lysis effect was then performed using flow cytometry.

In addition, cytokine levels, including IL-2, IL-10, and granulocyte-macrophage colony-stimulating factor (GM-CSF) in the co-culture supernatants, were quantified by BD cytometric bead array (BD bioscience) according to the manufacturer’s instructions. All experiments were repeated three times.

In vivo experiment

Non obese diabetic (NOD)/ShiLtJGpt-Pkdenci26Cd52Il2rg
s20c122/Gpt (NCG) mice (Gempharmatech, China) were injected intravenously (i.v.) with 1.0×10⁶ THP-1-fluc tumor cells via the tail vein. NCG mice were treated with VPA (1.0 mg/kg, n=6; 0.5 mM, n=6) via the tail vein, 100 mg/kg via intraperitoneal injection (n=6) according to Dowdell et al.15 or saline (n=6) on day 7, followed by CAR-T and untreated T cell treatment (i.v. at day 8, day 9, and day 10 after tumor cell inoculation). In vivo imaging systems spectra (IVIS, PerkinElmer) were used to monitor leukemia progression every 7 days.

Estimation of gene signatures and immune cell subtype

To identify the characteristics of immune environment between AML and healthy donors. The RNA sequencing data of patients with AML and healthy donors were obtained from the The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expressio (GTeX) databases.16 The different gene expressions and the proportion of immune cells between AML and healthy donors were performed on the online tool Gene Expression Profiling Interactive Analysis (GEPIA) (http://gepia.cancer-pku.cn) using Cell-type Identification By Estimating Relative Subsets Of RNA Transcripts (CIBERSORT) calculation method. To further track the tumor immunotype progress of patients with AML, we analyzed the status of anticancer immunity and the proportion of tumour-infiltrating immune cells using the ‘single-sample Gene set enrichment analysis (ssGSEA)’ and ‘CIBERSORT’ package on the online bioinformation analysis tool Tracking Tumor Immuno-phenotype (TIP) (http://biocc.hrbmu.edu.cn/TIP/) based on the TCGA–AML RNA-seq database. Correlations between normalized expression of T cell activation/inhibition genes and immune cell subtype infiltration were assessed by Spearman’s correlation analysis.

Statistical data analysis

Statistical analyses were performed using GraphPad Prism (V.8.0). The differences between groups were analyzed using Student’s t-test or one-way analysis of variance. The correlation between groups was assessed with the Spearman correlation coefficient. Kaplan–Meier survival curves were compared using the log-rank test. Values of p<0.05 were considered statistically significant.

RESULTS

CD123 and CLL-1 CAR-T cells show anti-AML activity in vitro

To confirm the CD123 and CLL-1 expression in patients with AML, we isolated bone marrow mononuclear cells and PBMCs from patients with AML (n=40) and detected the CD123 and CLL-1 expression by flow cytometry using AML cell lines and the B-ALL cell line as positive and negative controls, respectively. The results indicate that CD123 and CLL-1 were significantly expressed in most patients with AML (figure 1A, online supplemental figure S1A and table S1). In addition, mRNA of CD123 and CLL-1 was upregulated on the TCGA database (online supplemental figure S1B), which is consistent with previous reports. However, the expression pattern of CD123 and CLL-1 could be variable in different patients with AML, which may indicate targeting each tumor antigen alone could easily lead to immune escape. CD123 and CLL-1 could be detected in THP-1 and MV411, but were not expressed in BALL-1 (figure 1B).

To assess the cytotoxicity of CD123 and CLL-1 CAR-T cells against AML cell lines, we co-cultured CAR-T cells of different designs (figure 1C) with AML cell lines (THP-1, MV411) and the BALL-1 cell line as the controls. Both CD123 and CLL-1 CAR-T cells showed potent antitumor
Figure 1  Expression of CD123 and CLL-1 on AML cells and function of CD123 and CLL-1 CARs. (A) Percentages of CD123 and CLL-1 expression on PBMCs/BMMCs of patients with AML (n=40) and healthy donors (n=5) detected by flow cytometry. The percentages of CD123 and CLL-1 are significantly upregulated in patients with AML. The mean fluorescence intensity (MFI) of CD123 and CLL-1 differ for each patient with AML (Student's t-test, bars represent means±SEM, and dots represent means of the individual). (B) Expressions of CD123, CLL-1, CD33, and CD3 on AML cell lines (THP-1, MV411) and the B-ALL cell line (BALL-1). (C) Structure of CD123 and CLL-1 CARs; both CARs consist of a CD28/CD3ζ or 4–1BB/CD3ζ intracellular signaling domain, truncated epidermal growth factor receptor (tEGFR), and a single-chain variable fragment (scFv) of a specific antibody of CD123 and CLL-1. (D) Specific lysis effect of CD123 and CLL-1 CAR-T cells evaluated by flow when co-cultured with AML cell lines THP-1 and MV411 at an effector/target (E/T) ratio of 1:1. Data are pooled from three independent experiments and represented as the mean±SEM for each treatment group. Values of p for (D) were calculated using two-sided one-way ANOVA. (E) Immune cells involved in the antitumor or anti-inflammation process, including activated CD4+ memory T cells, follicular helper T cells, and activated NK cells are significantly decreased in patients with AML when compared with healthy donors. (F) Heatmap of semantic similarity between T cell activation/inhibition genes and cancer-immunity cycle seven steps/immune cell infiltration (left panel) in patients with AML using the TIP method. The y-axis and x-axis of the heatmap correspond to T cell activation/inhibition genes and cancer-immunity cycle seven steps/immune cell infiltration, respectively. The heatmap is partitioned into distinct clusters based on semantic distance; black squares denote significant associations between genes and immune cells/steps (annotated on the left); the right-hand panel shows a scatter plot of the relationship between NKG2D and PD-1 with CD8+ memory T cells. ANOVA, analysis of variance; AML, acute myeloid leukemia; BMMC, bone marrow mononuclear cells; CAR, chimeric antigen receptor; NKG2D, natural-killer group 2 member; PBMCs, peripheral blood mononuclear cells.
effects on AML cell lines at a 1:1 effector/target (E/T) ratio (figure 1D) and no response against control BALL-1 cells. In this assay, we found that CAR-T cells with CD28/4-1-BB co-stimulation displayed similar cytotoxicity against AML cell lines compared with untreated T cells. (figure 1D).

**RNA expression profiles indicate immune protection defects in AML patients**
To explore potential strategies for improving the therapeutic effect of CAR-T cells against AML, we investigated differential gene expressions between patients with AML and healthy donors on the GEPIA website and characterized the tumor immune environments in terms of the proportion of T cell subsets and NK cells. As expected, cells involved in antitumor or anti-inflammation processes, including activated CD4+ memory T cells, follicular helper T cells, and activated NK cells, were significantly decreased in patients with AML (figure 1E), reflecting immune dysfunction in AML. To further track tumour-infiltrating immunophenotypes, the cancer-immunity cycle seven steps/immune cell infiltration in patients with AML was evaluated by the TIP method. The data suggest that T cell inhibition genes like PD-1 are negatively associated with CD8+ memory T cells in patients with AML, which may lead to T cell dysfunction. Interestingly, T cell activation positively associated with the gene KLRK1 (NK2D) was involved in CD8+ memory T cells in patients with AML (figure 1F), which suggests that the NK2D–NK2DL axis may play an important role in T cells’ memory and cytotoxicity against AML immune environments.

**PD-1/PD-L1 blockade does not significantly enhance CAR-T cell cytotoxicity against AML cells**
Since the PD-1/PD-L1 axis contributes to the tumor immune suppressive microenvironment, we detected PD-1/PD-L1 expression on CAR-T cells. Surprisingly, both PD-1 and PD-L1 were upregulated in activated T cells as well as CAR-T cells after co-culture with THP-1 and MV411 (figure 2A,B). Interestingly, PD-L1 was also upregulated on THP-1 and MV411 after co-culture with CAR-T cells compared with co-culture with non-treated T cells (figure 2C).

To test whether PD-1/PD-L1 blockade could improve CAR-T cell antitumor activity, PD-1 and PD-L1 antibodies were added to the co-culture assay of CAR-T cells and tumor cells individually. Unexpectedly, PD-1 antibodies blocked the expression of PD-L1 on the tumor cells (figure 2D), while both PD-1 and PD-L1 antibodies only slightly improved the cytotoxicity of CAR-T cells against AML cells (figure 2E).

**VPA upregulates NKG2DL expression on AML cells and significantly enhances CAR-T cell cytotoxicity against AML cells**
The ssGSEA and CIBERSORT analyses indicated that T cell persistence and memory may be associated with the NKG2D/NKG2DL axis. NKG2D was expressed on CD8+ T cells but not on CD4+ T cells, and was upregulated on activated T cells. Interestingly, NKG2D was significantly upregulated on CD8+ CAR T cells when co-cultured with THP-1 and MV411 (figure 3A). This suggests that inducing expression of NKG2DL on AML might enhance the susceptibility of AML cells to CAR-T cell-mediated cytotoxicity. Recent studies have indicated that HDACi could be a promising candidate to upregulate NKG2DL. HDACi can regulate tumour-associated aberrant epigenetic status and exhibits antitumor activity in several types of carcinomas by upregulating NKG2DL on tumor cells.17 We tested three types of HDACi: VPA, chidamide, and SB. To avoid the direct cytotoxic effect of these HDACis on AML cells, the CCK-8 assay was carried out to detect the IC50 of VPA on AML cell lines (online supplemental figure S2A). The results indicated that low dose ranges of 0.5–1 mM (VPA), 0.1–0.25 µM (Chidamide), and 0.3–0.6 µM (SB) had no pro-apoptotic effects on AML cells (online supplemental figure S2C). We found VPA could upregulate NKG2DL (ULBP1,2,3) on AML cell lines (THP-1, and MV411; figure 3B), which is in accordance with previous studies.13 18 19

THP-1 and MV411 were pretreated with a low dose of HDACi for 24 hours or 48 hours; then, the washed tumor cells were co-cultured with CAR-T for 24 hours or 48 hours at a 1:5 E/T ratio, followed by flow cytometry analysis. The results indicate that VPA pretreatment significantly enhanced the cytotoxic effects of CAR-T cells against AML (figure 3C), but SB and chidamide treatment had little or no effect (online supplemental figure S3A–D). NKG2DL was not or only slightly upregulated on AML cell lines when treated with SB or chidamide (online supplemental figure S4A). The cytokine release assay also showed that VPA treatment could improve cytokine production (interleukin (IL)-2, GM-CSF, IL-10) of CAR-T cells, which suggests that VPA-treated AML cells may be sensitive to trigger T cell activation and cytokine release (figure 3D). We observed that the AML cell line pretreated with VPA was connected with dose-dependent HDAC2 decrement and H4 expression (online supplemental figure S4B).

**VPA treatment enhances CAR-T cell antitumour activity in a tumour model**
To validate whether VPA could enhance CAR-T cytotoxicity in vivo, we generated an AML xenograft model using the THP-1 cells expressing firefly luciferase (THP-1-FFluc) by tail vein injection. NCG mice were engrafted with 1×10^6 THP-1-FFluc cells 7 days before treatment. Then, the xenograft mice were treated with 1×10^7 CD123 CAR-T cells (with or without two concentrations of VPA) or non-treated T cells (figure 4A). The tumor in the mice was monitored every 7 days using bioluminescence imaging (online supplemental figure S5A,B,D and E). All three groups of CD123 CAR-T cells exhibited a therapeutic effect in vivo compared with the non-transduced T cells. Interestingly, CD123 CAR-T cells with 0.5 mM
Figure 2  PD-1/PD-L1 may not be the main mechanism of immune escape in AML. (A) T cells upregulate PD-1 and PD-L1 when stimulated with CD3/CD28 microbeads. (B) CD123 CAR-T cells and CLL-1 CAR-T cells also upregulate PD-1 and PD-L1 when co-cultured with AML cell lines (THP-1 and MV411) compared with untreated T cells. (C) PD-L1 is significantly upregulated on MV411 and THP-1 when co-cultured with CD123 and CLL-1 CAR-T cells. (D) PD-L1 expression on THP-1 is upregulated when co-cultured with CAR-T cells but can be blocked when combined with the PD-1 antibody; however, the lysis effect of CAR-T cells is only slightly improved (E). MB: CD3/CD28 microbeads. All graphed data are pooled from three independent experiments and represented as the mean±SEM for each treatment group. P values were calculated using two-sided one-way ANOVA (for B–E) or unpaired Students’ t-test (for A). ANOVA, analysis of variance; AML, acute myeloid leukemia; CAR, chimeric antigen receptor T
Figure 3  VPA upregulates NKG2DL expression on tumor cells and enhances lysis effects on CAR-T cells. (A) CD8+ CAR T cells upregulate NKG2D when co-cultured with THP-1 and MV411. (B) NKG2D ligands (ULBP1, ULBP2,5,6, and ULBP3) on THP-1 are upregulated when treated with valproic acid (VPA). (C) THP-1 co-cultured with CAR-T cells and a minute dose of HDACi (VPA 0.5 mM and 1.0 mM) in a 1:5 effect/target (E/T ratio could improve the lysis effect of CAR-T cells. (D) Cytokine levels increase when CAR-T cells are combined with VPA for the treatment of tumor cells. Data are pooled from three independent experiments and represented as the mean±SEM for each treatment group. P values were calculated using two-sided one-way ANOVA (for C–D). ANOVA, analysis of variance; CAR, chimeric antigen receptor T; HDACi, histone deacetylases inhibitor; NKG2D, natural-killer group 2 member D.
**Figure 4** HDACi can improve the lysis effect and persistence of CAR-T cells in vivo. (A) Schematics of the THP-1 xenograft model. NCG mice were injected with $1 \times 10^6$ THP-1 cells that expressed firefly luciferase and GFP label (THP-1-Ffluc) via the tail vein. Then, 1 week later, mice were infused with CD123 CAR-T cells and untreated T cells (CON) with or without VPA treatment (n=6 for each group). (B) Leukemic progression was monitored using an in vivo imaging system (IVIS) every week. THP-1-Ffluc was eliminated in CD123 CAR-T cell therapy, especially in CD123 CAR-T cells combined with VPA treatment. The data of mice leukemia burden are represented with the color scale bars indicating maximum and minimum bioluminescence (BLI) thresholds at different time points. (C) Kaplan–Meier analysis of percentage survival for each treatment group (CON+VPA 100 mg/kg n=6; CD123 CAR-T+PBS n=6; CD123 CAR-T+VPA 0.5 mM n=6; CD123 CAR-T+VPA 100 mg/kg n=6; CD123 CAR-T+VPA 0.5 mM twice daily n=6). All p values were calculated compared with the CON+VPA group. (D) Average BLI data from the THP-1 xenograft model demonstrated that leukemia cells were rapidly grown in vehicle-treated (blue) mice, whereas leukemia cells disappeared in CD123 CAR-T cell-treated (red) mice, and combination therapy-treated mice (green, purple, and orange). The graphed data in D are represented as mean±SEM. The p value in (C) was calculated using a two-sided Mantel–Cox test (log-rank). CAR, chimeric antigen receptor; HDACi, histone deacetylases inhibitor.
VPA treatment significantly enhanced the antileukemia effect and improved OS vs CD123 CAR-T cell treatment alone (online supplemental file 5C,F). However, the 1.0 mM VPA treatment did not prolong the survival of mice. Considering the clinical usage of VPA and other in vivo animal models, the 1.0 mM group was modified to 0.5 mM VPA twice a day or 100 mg/kg by intraperitoneal injection. Then, leukemia progress was monitored by IVIS every 7 days as described above (figure 4B,C). The CAR-T cells combined with VPA showed robust anti-AML effects and significantly prolonged the overall survival of the mice (figure 4D).

**DISCUSSION**

Immunotherapy offers promising opportunities with the potential to induce sustained remissions in tumor patients. CD19-targeted CAR-T cells have achieved remarkable clinical success in certain types of B-cell malignancies, and substantial efforts aimed at translating this success to myeloid malignancies are currently underway. The primary challenge limiting the use of CAR-T cells in myeloid malignancies is the absence of a dispensable antigen, as myeloid antigens are often co-expressed on normal hematopoietic stem/progenitor cells, depletion of which would lead to intolerable myeloablation.

Besides the limitation of tumor antigen, checkpoint inhibition and the immunosuppressive tumor microenvironment represent major barriers to effective tumour-specific T cell responses to cancer. A combination of current strategies with CAR-T cell therapy may generate a promising response; for example, the application of PD1/PDL-1 antibodies, HDACi, or DNA methyltransferase (DNMT) inhibitor. Blockade of immune checkpoint inhibitors could reverse tumor cell-mediated dysfunction of antitumor effector cells and consequently of tumor escape from the host immune system. However, we found that PD-1/PD-L1 antibodies can only slightly improve the cytotoxicity of CAR-T cells against AML cell lines. Pretreatment of leukemia cells with DNMT inhibitors (DNMTis) upregulated the expression of CD123 on AML cells and increased the number of CTLA-4-negative anti-CD123 CAR-T cells, thereby enhancing the targeting and killing of AML cells by CD123 CAR-T cells. The combination of DNMTis and CD123 CAR-T therapy suggests new possibilities for improving the outcomes of immunotherapy.

HDAC is involved in diverse cellular regulatory mechanisms including non-canonical functions outside the chromatin environment. Several publications have demonstrated that selective HDACi can influence tumor immunogenicity, the microenvironment, and the functional activity of specific immune cells. Moreover, HDACis have shown antileukemia effects by promoting cell death, autophagy, apoptosis, or growth arrest in AML preclinical models when in combination with other drugs.

Recent studies have indicated that NKG2DL on tumor cells can be upregulated by HDACi, which may make tumor cells sensitive to immune cell-mediated cytotoxicity. Furthermore, studies have indicated that only inhibition of HDAC1 and HDAC2 can effectively induce the expression of NKG2D ligands in lung cancer. VPA was reported to inhibit the activity of HDAC1/2 in several diseases. Moreover, Gottlicher et al found that VPA could cause hyperacetylation of the N-terminal tails of H3 and H4 histones by inhibiting the catalytic activity of class I HDACs and inducing proteasomal degradation of HDAC2 in the AML model. VPA is a traditional antiepileptic drug for certain types of seizures; it has proven benefits and low cost. VPA is effective, with a favorable safety profile and low potential for drug–drug interactions in polymedicated elderly patients with epilepsy. Recently, VPA has been used for treating various cancers, including AML, either alone or in combination with other antitumor drugs.

We observed that the AML cell line pretreated with VPA was connected with dose-dependent HDAC2 decrement and H4 expression, suggesting that VPA might upregulate the expression of NKG2DL on AML cell lines by inhibiting HDAC2. Hence, HDAC2 inhibitors like VPA may be a novel strategy in the immunotherapy of AML therapy. Moreover, VPA could upregulate NKG2DL on different types of tumor cells and AML cell lines as such, CAR-T cell therapy combined with VPA could suit other types of cancer. These findings pave the way for other CAR-T cell therapies to improve cytotoxic effects.

In summary, CD123 and CLL-1 are promising targets for AML CAR-T cell therapy. VPA upregulates NKG2D ligand expression and enhances the susceptibility of AML cells to CAR-T cell-mediated cytotoxicity in vitro and in vivo. A combination of VPA pretreatment and CAR against AML exhibits synergistic effects, which may provide a promising strategy to treat R/R AML.

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Patient consent for publication Consent obtained directly from patient(s)

Ethics approval The study was approved by the Fujian Medical University Union Hospital Review Board with informed consent obtained from all subjects in accordance with the Declaration of Helsinki (202039695). All animal experiments were approved by the Fujian Medical University Animal Care and Use Committee (FJMU IACUC 2021-0334).

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Figure S1 The expression of CD123 and CLL1 on AML patients

(A) The different gene expression between AML and health donor was performed on online bioinformational analysis tool GEPIA (http://gepia.cancer-pku.cn/index.html). AML RNA sequencing profiles were got from TCGA database and health donor RNA sequencing data was from GTEx database. One-way ANOVA was performed for differential gene expression analysis using disease state (Tumor or Normal) as variable for calculating differential expression. CD123 and CLL-1 were highly expressed on AML patients. (B) The peripheral blood mononuclear cells (PBMC) and bone marrow mononuclear cells from AML patients were tested for CD123 and CLL-1 expression using flow cytometry. CD123 and CLL-1 were frequently expressed on AML patients.
Figure S2  Low dose of HDACi has little effects on tumor cells

(A,B,C) THP-1 and MV411 cells were seeded in 96-well plates and treated with different doses of VPA, SB, or Chidamide. Then the IC50 values were calculated by GraphPad Prism 8.0 software. (D) The cell cycle distribution was measured by flow cytometry after THP-1 and MV411 cells were treated with low doses of HDACi. Low doses of HDACi had little effect on the tumor cell cycle. (E) Low doses of HDACi did not induce apoptosis in THP-1 and MV411 cells. All graphed data are pooled from three independent experiments and represented as the mean ± SEM for each treatment group. p-values were calculated using two-sided one-way ANOVA (D-E).
Figure S3 The effect of low doses of SB and Chidamide on the lysis effects of CAR-T cells
(A) Low doses of SB slightly improved the lysis effects of CD123 CAR-T cells when co-cultured with THP-1 and MV411 cells. (B) Low doses of Chidamide slightly improved the lysis effects of CD123 CAR-T cells when co-cultured with THP-1 and MV411 cells. (C) Low doses of SB slightly improved the lysis effects of CLL-1 CAR-T cells when co-cultured with THP-1 and MV411 cells. (D) Low doses of Chidamide slightly improved the lysis effects of CLL-1 CAR-T cells when co-cultured with THP-1 and MV411 cells. All data are pooled from three independent experiments and represented as the mean ± SEM for each treatment group. p-values were calculated using two-sided one-way ANOVA (A-D).
Figure S4 The NKG2DL expression on MV411 and THP-1 cells

(A) Low doses of SB (sodium butyrate) and Chidamide (Chida) had little or no effect on upregulating the expression of NKG2DL on THP-1 and MV411 cells, whereas low doses of VPA increased the expression of NKG2DL on MV411 cells. (B) VPA also induced a decrease in HDAC2 and an increase in H4 expression. (C) The schematic model shows how VPA might increase the lysis effects of CAR-T cells. Data are pooled from three independent experiments and represented as the mean ± SEM for each treatment group in A.
Figure S5 The in vivo model of CD123 CAR-T cell therapy combined with VPA.

(A) NCG mice were injected via tail vein with $1 \times 10^8$ THP-1 cells that expressed firefly luciferase and GFP (THP-1-Ffluc). One week later, the mice were infused with different treatments: untreated T cells plus VPA 1.0 mM (n=3), CD123 CAR-T cells alone (n=3), CD123 CAR-T cells plus VPA 0.5 mM (n=3), or CD123 CAR-T cells plus VPA 1.0 mM (n=3). Leukemia progression was monitored by IVIS, THP-1-Ffluc was eliminated by CD123 CAR-T cell therapy, especially when combined with VPA treatment. (B) Average bioluminescence (BLI) data from the THP-1 xenograft model demonstrated that leukemia cells were grew rapidly in vehicle-treated (blue) mice, CD123 CAR-T cells treated (red) mice, whereas leukemia cells were disappeared in CD123 CAR-T cells and VPA treated mice (green and purple). (C) The survival analysis of NCG mice treated with CD123 CAR-T cells with or without VPA combination, and non-transduced T cells depicted as Kaplan-Meier curves; The same results were also observed in the second time animal experiments. Mice were randomly assign to 4 groups, untreated T cells combined with VPA 1.0mM (n=3), CD123 CAR-T (n=3), CD123 CAR-T cell combined with VPA 0.5mM (n=4), CD123 CAR-T cell combined with VPA 1.0mM (n=4) (D,E,F). (G) The survival analysis of two times animal experiments.
Table S1 The percentage of CD123/CLL-1 expression on PBMC/BMMC surface of AML patients

<table>
<thead>
<tr>
<th>Antigen</th>
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<td>CD123</td>
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<td>40/42</td>
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PBMC: peripheral blood mononuclear cells; BMMC: bone marrow mononuclear cells