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Original Article

Successful application of PD-1 knockdown CLL-1 CAR-T therapy in two AML patients with post-transplant relapse and failure of anti-CD38 CAR-T cell treatment

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Abstract: Patients with relapsed/refractory acute myeloid leukemia (R/R AML) often show resistance to chemotherapy and have dismal outcomes. Therefore, it is urgent to develop new treatment strategies to address this problem. With tremendous achievement of chimeric antigen receptor T cells (CAR-T) therapy against B-cell malignancies, many efforts have been devoted to developing CAR-T therapy for R/R AML but with limited success, in part owing to a lack of specific targets. C-type lectin-like molecule-1 (CLL-1) is highly expressed on AML blasts with no expression on normal hematopoietic stem cells, which makes it an ideal target of immunotherapy for AML. Here, we report 2 R/R AML patients who relapsed after allogeneic stem cell transplantation and failed multiline salvage therapies including anti-CD38 CAR-T therapy, but were successfully treated with PD-1 silenced anti-CLL-1 CAR-T therapy. Both patients achieved molecular complete remission with incomplete hematologic recovery at 28 days of evaluation after CLL-1 CAR-T cell infusion. Cytokine release syndrome in cases 1 and 2 were grade 1 and 2, respectively. At the last follow-up, cases 1 and 2 had maintained continuous remission for 8 and 3 months, respectively. Our results demonstrated that CLL-1 CAR-T cells might be an effective and safe salvage therapy for AML patients with post-transplant relapse.

Keywords: Acute myeloid leukemia, C-type lectin-like molecule-1, programmed cell death protein 1, chimeric antigen receptor, immunotherapy

Introduction

Outcomes for patients with relapsed or refractory acute myeloid leukemia (R/R AML) are dismal, with the 5-year overall survival rate of approximately 10% [1]. Despite the increasing availability of new agents and allogeneic hematopoietic stem cell transplantation (allo-HSCT), a substantial number of patients still show resistance to these therapies [2, 3]. Moreover, there is no standard salvage strategy for post-HSCT relapse. In this situation, novel treatment strategies urgently need to be investigated.

Chimeric antigen receptor T cell (CAR-T) therapy has made marvelous achievements in R/R B-cell malignancies [4]. Some potential antigenic targets of CAR-T cells, such as Lewis Y, CD33, CD123 and CD38, have been explored in preclinical studies and a few clinical trials. However, there are still various difficulties for CAR-T therapy in AML, such as the immunosuppressive milieu, and a lack of optimal target is a major hurdle in CAR-T therapy for AML [5]. C-type lectin-like molecule-1 (CLL-1) can be detected in 32.4%-99.8% of newly diagnosed AML, and unlike other targets such as CD33 and CD123, it is not expressed on normal hematopoietic stem cells and any other tissues [6], but is restricted to the hematopoietic cells of the myeloid lineage. Therefore, CLL-1 could be an ideal target for immunotherapy in AML [7]. CAR

T cells targeting CLL-1 have been applied in limited relapsed AML cases in preclinical and clinical trials [8-11]. Furthermore, considering the PD-1/PD-L1 pathway as an important immunosuppressive mechanism in AML, PD-1 silenced CLL-1 CAR-T cells were constructed and shown to be more effective in killing AML cells [12].

Here, we report the successful application of CLL-1 CAR-T cells with PD-1 knockdown in 2 patients who responded poorly to multi-line salvage therapies including venetoclax and anti-CD38 CAR-T therapy.

Materials and methods

The CAR was encoded by a third-generation lentiviral vector containing a humanized anti-CD38 single-chain variable fragment (scFv), CD28 and 4-1BB. We also developed a CAR with PD-1 silencing which contained a murine anti-CLL-1 scFv, CD28, OX40 and the PD-1 silencing shRNA sequence. CAR-T cells were successfully manufactured and cultured for 14 days before infusion. The clinical trial identification number for the CLL-1 CAR-T therapy trial was NCT04884984.

Results

The first case was a 28-year-old male who was admitted to our hospital because of dizziness and fatigue in June 2012. A diagnosis of CMML-0 was made according to the WHO criteria [13], with intermediate-2 based on the CMML-specific Prognostic Scoring System [14]. He underwent allo-HSCT from a female unrelated donor and remained in complete remission (CR) for almost 8 years. In October 2020, disease relapse was observed at a routine blood test which showed pancytopenia with presence of monocytosis (19% of WBC count). The bone marrow (BM) smear demonstrated 17% blasts. Multiparameter flow cytometry (FCM) revealed 19% blasts, with coexpression of CD13, CD33, CD34, CD38, CD117, CLL-1 and HLA-DR. A complex karyotype was detected with monosomy chromosome 12. Deletion of TP53 was confirmed with fluorescence in situ hybridization (FISH). Reinduction with decitabine and venetoclax was applied, with no response (NR), and the disease quickly progressed to AML. Since a high expression of CD38 (87.8%) was detected on the blasts, the patient was subsequently enrolled in our clinical trial (NCT04351022). Autologous anti-CD38 CAR-T cells were provided by the Unicar Therapy Biomedicine Technology. The third-generation CAR lentiviral construct is shown in Supplementary Figure 1. A total of 2×10^7/kg anti-CD38 CAR-T cells were infused at dose escalation within 4 days after the lymphodeletion regimen (FC) containing fludarabine and cyclophosphamide. No cytokine release syndrome (CRS) or organ toxicity was observed. Unfortunately, he did not achieve CR according to BM evaluation at day 28 post anti-CD38 CAR-T cell infusion with 18.5% marrow blasts.

Reinduction chemotherapy with decitabine, cytarabine, cladribine and granulocyte colony-stimulating factor (D-CLAG) was applied to reduce tumor burden. After chemotherapy, BM morphology still showed 7% of blasts. The expression of CLL-1 on the blasts was 90.8%. Based on these findings, autologous CAR-T cells targeting CLL-1 were prepared by the Unicar Therapy Biomedicine Technology. Structure of the CAR was demonstrated in **Figure 1A**. After lymphodepletion chemotherapy with cyclophosphamide (Cy regimen), a total of 1×10^7/kg CLL-1 CAR-T cells were infused by dose escalation within 3 days (**Figure 1B**). The patient suffered grade 1 CRS, which was manifested as fever that peaked at 39.8°C and lasted for 2 days. The peak of serum IL-6 level was observed on the third day after infusion (**Figure 2A**). White blood cells and neutrophils recovered at day 23 after infusion, but platelets were still transfusion dependent (**Figure 2B**). The expansion of CAR-T cells reached at a peak on day 7 and then gradually declined (**Figure 2C**). At the 28-day evaluation after CLL-1 CAR-T cell infusion, the blasts decreased to 1.6% (**Figure 2D**). FCM revealed that the expression of CLL-1 on the blasts after CLL-1 CAR-T therapy dropped to 25.8% (**Figure 2E**). The karyotype was normal (**Figure 2F**), with full donor chimerism in BM. No TP53 deletion was detected by FISH (**Figure 2G**). No signs of immune effector cell-associated neurotoxicity syndrome (ICANS) were observed. The patient received a second allo-HSCT from a haploidentical relative on day 50 after CAR-T therapy. He had maintained minimal residual disease (MRD) negative CR for 8 months after CLL-1 CAR-T cell infusion.

The second case was a 28-year-old male diagnosed with AML in February 2019, with the

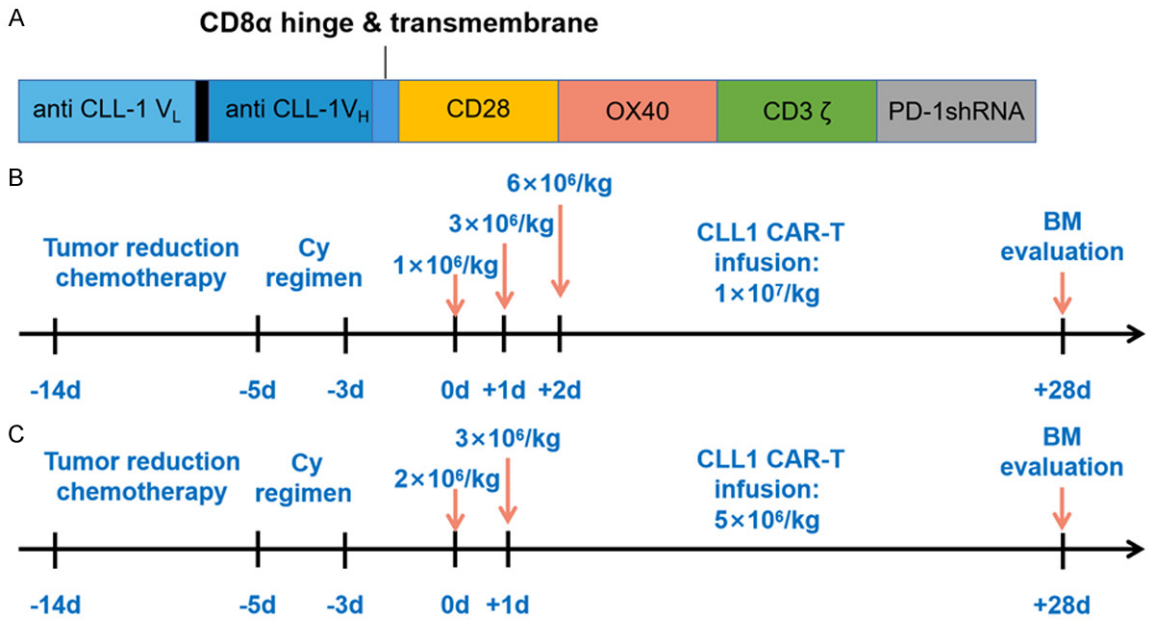

Figure 1. Schematic structure of anti-CLL-1 CAR and CLL-1 CAR-T therapy process. A. Structure of anti-CLL-1 CAR. B. Schematic of CLL-1 CAR-T therapy process of case 1. C. Schematic of CLL-1 CAR-T therapy process of case 2.

chief complaint of scattered petechiae in the skin and bleeding gums. Normal karyotype was detected. He achieved CR after 2 cycles of standard induction chemotherapy including idarubicin and etoposide, idarubicin and cytarabine, which were followed by 2 cycles of consolidation chemotherapy. Relapse occurred at 3 months after discontinuation of chemotherapy. Unfortunately, he failed reinduction combined with chemotherapy with azacitidine, homoharringtonine and low-dose cytarabine and 2 cycles of azacitidine and venetoclax. Then, he was enrolled in the anti-CD38 CAR-T clinical trial. He received cytoreduction chemotherapy with DAC, homoharringtonine, aclarubicin, low-dose cytarabine, granulocyte colony stimulating factor (HAAG) and venetoclax, which was followed by anti-CD38 CAR-T therapy (1×10^7 cells/kg). No CRS was observed. On day 28 of evaluation, he achieved MRD negative CRi. Subsequently, the patient underwent allo-HSCT and maintained in MRD negative CR for 8 months.

Hematologic relapse occurred in 8 months post allo-HSCT, with 27% of blasts in the BM. Flow cytometry analysis demonstrated that 18.51% of blasts had a high expression of CD38 (83.5%) and expression of CLL-1 (27.1%). He received anti-CD38 CAR-T cells from the allo-HSCT donor after reinduction chemotherapy with DAC, HAAG and venetoclax. However, the blasts increased to 53.5% at day 8 after the second CD38 CAR-T therapy. Then, he was enrolled in the CLL-1 CAR-T cell therapy trial (NCT04884984). He received cytoreduction chemotherapy with CLAG, and his marrow blasts dropped to 39% prior to CLL-1 CAR-T cell infusion. Donor-derived CLL-1 CAR-T cells were infused at a total dose of 5×10^6 cells/kg for 2 consecutive days (**Figure 1C**). Grade 2 CRS presented with continuous high fever (maximum to 41°C) and hypotension, without symptoms of ICANS. Following the guidelines, the fever was relieved with dexamethasone and supportive care. The IL-6 level peaked on day 2 after infusion (**Figure 3A**). The patient experienced continuous severe neutropenia and thrombocytopenia (**Figure 3B**). Expansion of CLL-1 CAR-T cells reached the maximum value on day 7 after infusion (**Figure 3C**). The patient obtained a morphological CRi (**Figure 3D**) and MRD negative CRi on day 28. The expression of CLL-1 on blasts after CLL-1 CAR-T therapy could not be analyzed because of too few blast cells (**Figure 3E**).

Discussion

CAR-T therapy is an encouraging treatment option for R/R AML. Our group previously reported the efficacy and safety of anti-CD38

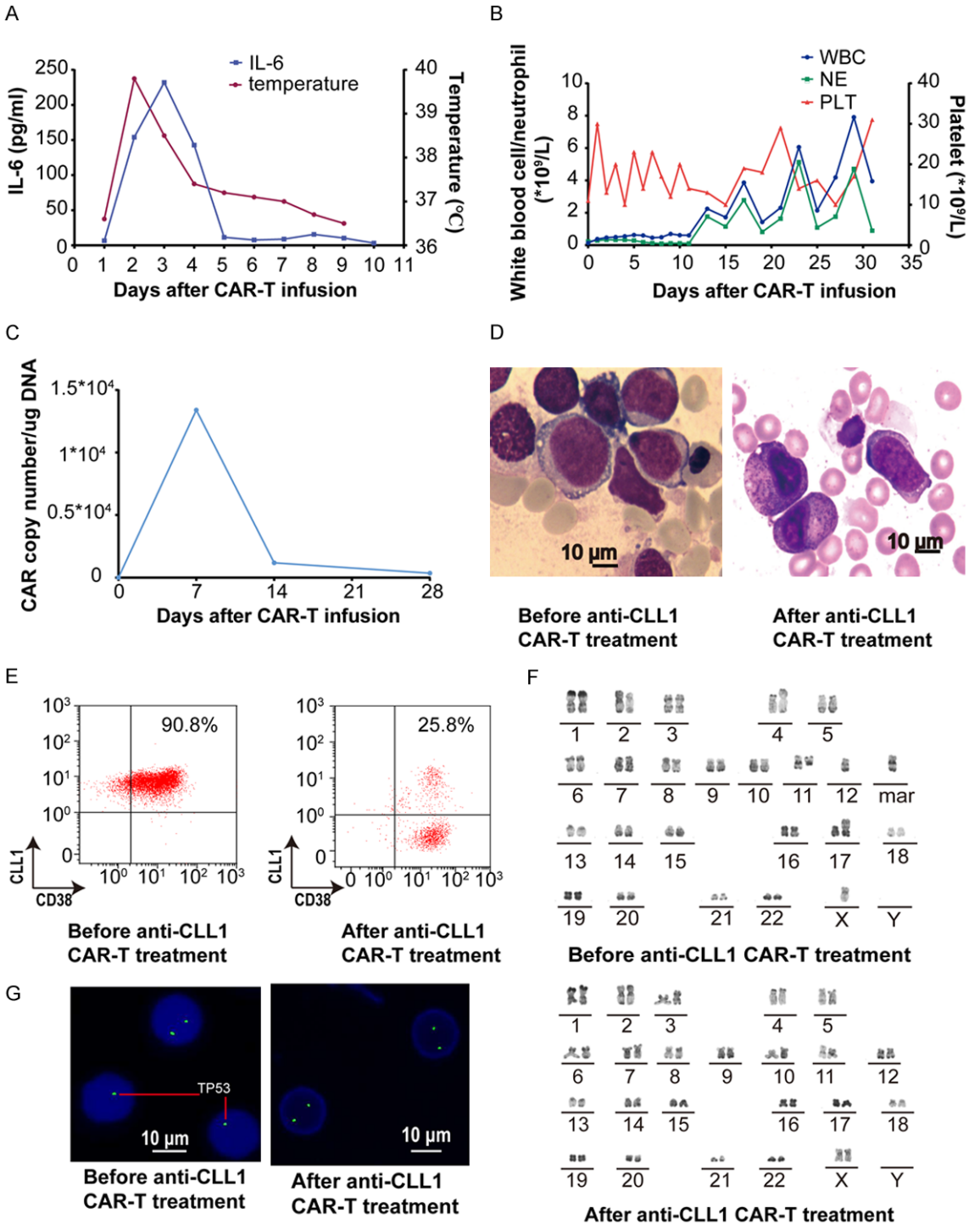

Figure 2. Clinical data of case 1. A. Changes of cytokines and temperature after infusion of CLL-1 CAR-T cells. B. Changes of blood cell counts after infusion of CLL-1 CAR-T cells. C. Changes of copies of CLL-1 CAR-T cells evaluated by qPCR. D. Bone marrow morphology before and after CLL-1 CAR-T cells infusion. The magnification was 1000 times the original size. The scale bar represents 10 μm. E. The expression of CLL-1 before and after CLL-1 CAR-T therapy of case 1. The expressions of CLL-1 on the blasts before and after CLL-1 CAR-T therapy were 90.8% and 25.8%, respectively. F. Karyotype analysis before and after CLL-1 CAR-T cells infusion. The results showed a complex karyotype on BM samples before CLL-1 CAR-T cells infusion. And a normal karyotype was detected on BM samples taken on day 28 post CLL-1 CAR-T therapy. G. FISH results, using probes specific to the TP53 gene. The results showed deletion of TP53 signals on BM samples before CLL-1 CAR-T cells infusion, and normal TP53 signals post CLL-1 CAR-T cells infusion. The magnification was 1000 times the original size. The scale bar represents 10 μm.

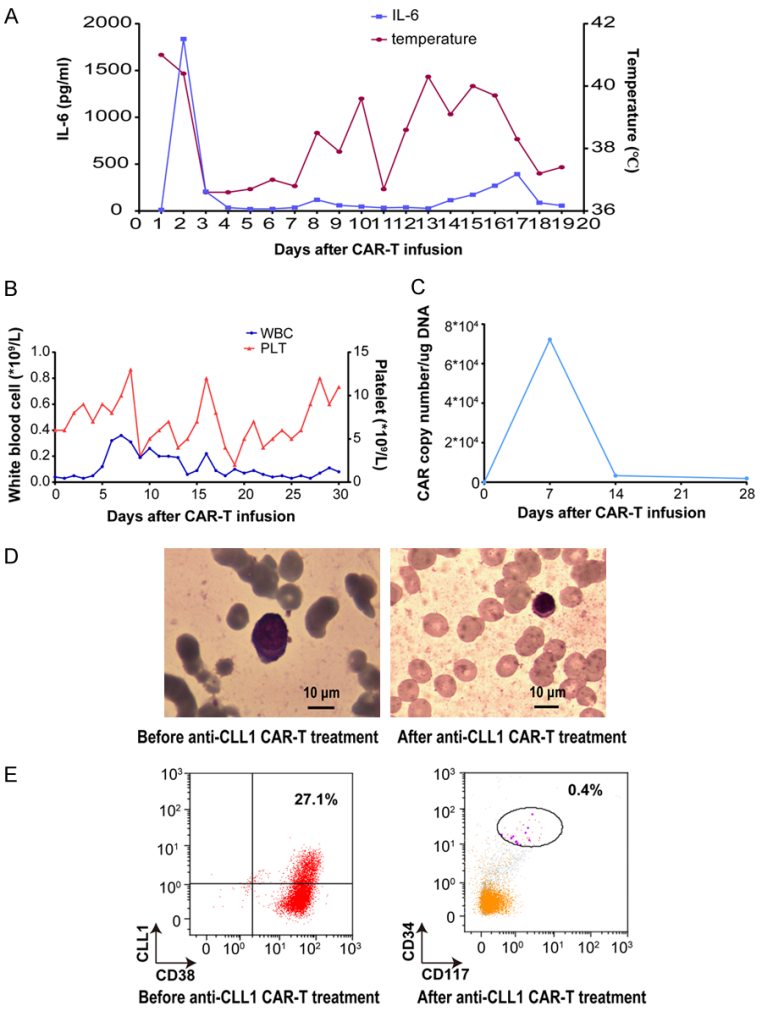

Figure 3. Clinical data of case 2. A. Changes of cytokines and temperature after infusion of CLL-1 CAR-T cells. B. Changes in the white blood cell and platelet count after infusion of CLL-1 CAR-T cells. C. Changes of copies of CLL-1 CAR-T cells evaluated by qPCR. D. Bone marrow morphology before and after CLL-1 CAR-T cells infusion. The magnification was 1000 times the original size. The scale bar represents 10 μm. E. The expression of CLL-1 before and after CLL-1 CAR-T therapy of case 2. The expression of CLL-1 on the blasts before CLL-1 CAR-T therapy was 27.1%, while we can't analyze the expression of CLL-1 on the blast cell because of limited blasts.

CAR-T therapy for AML patients who relapsed post-HSCT, and four of six patients achieved CR or CRi [15]. However, novel therapies are urgently needed for those patients who are resistant to anti-CD38 CAR-T therapy. Mechanisms of failure of anti-CD38 CAR-T cell treatment may be attributed to CAR-T cell exhaustion, immunosuppressive tumor microenvironment and high expression of inhibitory receptors on CAR-T cells such as PD-1.

PD-1 participates in promoting leukemia immune evasion by inhibiting CAR-T cell activation and inducing CAR-T cells dysfunction [16]. The efficacy of CAR-T cells can be limited by the suppressive effect of their own expression of PD-1 in the microenvironment [17]. Moreover, many studies have confirmed that PD-1 blockade can strengthen the antileukemia function of CAR-T cells, prevent CAR-T cells from exhaustion and reverse the immunosuppressive microenvironment [18-20]. According to previous studies [12, 21, 22], PD-1 knockdown CLL-1 CAR-T cells showed a stronger antileukemia effect and fewer side effects than CLL-1 CAR-T cells without PD-1 knockdown in vitro. In addition, PD-1 knockdown prolonged the activation duration, enhanced the proliferation ability, and induced the long-term antileukemia activity of CAR-T cells in a xenograft mouse model. Both patients in our study achieved deep remission after CLL-1 CAR-T therapy. To our knowledge, this is the first report to confirm the efficacy of PD-1 knockdown CLL-1 CAR-T cells in vivo.

CLL-1 CAR-T therapy has been reported in limited cases of AML in the literature [10, 11]. All of them were pediatric patients, who were refractory to 2-5 cycles of chemotherapy. Three of four patients achieved MRD negative CR at one month after CAR-T cell infusion. Compared with the previously reported cases, both cases in our report were extremely high-risk adult AML patients with post-HSCT relapse who achieved fast and deep remission in the situation of failure of 3 lines of salvage therapy, including chemotherapy, venetoclax and CD38 CAR-T cell therapy.

One of these reported patients that received allo-HSCT at 3 months post-CLL-1 CAR-T therapy, no precise data about the recovery of the blood cell counts were provided. In our study,

because of the long-term exposure to chemotherapy combined with CAR-T therapy, they experienced prolonged myelosuppression. Both cases showed persistent thrombocytopenia, and the impact of CLL-1 CAR-T cells on the development of megakaryocytes should be precisely studied in more cases in the future. Case 1 received a second allo-HSCT at day 50 post CLL-1 CAR-T therapy and achieved hematopoietic reconstitution. Including our cases, all patients receiving CLL-1 CAR-T therapy only suffered reversible grade 1-2 CRS, without ICANS and off-target effects.

Conclusion

To the best of our knowledge, this is the first clinical study to illustrate that PD-1 knockdown CLL-1 CAR-T therapy is safe and effective in adult AML patients who relapsed post allo-HSCT and failed anti-CD38 CAR-T cell treatment. Considering the limited sample size and the short follow-up time, a prospective clinical trial (NCT04884984) was carried out to confirm the efficacy, safety, and kinetics of PD-1 knockdown CLL-1 CAR-T therapy at our center.

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Disclosure of conflict of interest

None.

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Supplementary Figure 1. Structure of anti-CD38 CAR.