

CAR T cells targeting CD99 as an approach to eradicate T-cell Acute Lymphoblastic Leukemia without normal blood cells toxicity

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Abstract

CAR T cell therapy has shown dramatic clinical success in relapsed or refractory (r/r) B-ALL and other haematological malignancies. However, the loss of specific antigens, cell fratricide, T cell aplasia, and normal T cell separation are challenges in treating T cell leukemia/lymphoma with CAR T therapy. CD99 is a promising antigen to target T-ALL and AML as it is expressed on the majority of T-ALL and AML. Here, we isolated a low-affinity CD99 (12E7) antibody, which specifically recognizes leukemia cells over normal bone marrow cells. T cells transduced with an anti-CD99-specific CAR that contained the 12E7 scFv expanded with minor fratricide, maintained their cytotoxic function and mediated powerful antitumour effects. Subsequently, we conducted a pilot clinical study to evaluate the safety and feasibility of therapy with anti-CD99 CAR T cells in 4 patients with r/r T-LBL (n=1), AML (n=2) or myeloid sarcoma (MS) (n=1). The clinical overall response rate (ORR) was 50% (2/4 patients), and 1 patients (25%) achieved complete remission (CR) for 2 month. Mild cytokine release syndrome (CRS) occurred in 2 patients and the CRS no more than grade 2. Together, our results demonstrate that anti-CD99 CAR T cells specifically recognize and efficiently eliminate CD99+ leukemia cells.

Introduction

T. cell acute lymphoblastic leukemia (T-ALL) is an aggressive haematological malignancy accounting for 15% of pediatric and 25% of adult ALL cases^{1,2}. T-ALL patients have high relapse and mortality rates³. The standard treatment of chemotherapy combined with glucocorticoids has significantly improved survival, but up to 20% of pediatric and 40% of adult T-ALL patients are at risk for relapse^{4,5}. Thus, novel optimal therapeutic strategies that could prolong overall survival need to be developed for T-ALL, particularly for relapsed and refractory T-ALL patients.

CAR T cells has exhibited dramatic clinical success when targeting CD19 in B cell acute lymphoblastic leukemia (B-ALL) and other B cell malignancies⁶⁻⁸. Despite recent progress in the understanding of T-ALL oncogenesis, few markers can be used as targets in CAR T cell therapeutics for T-ALL⁹. Because CAR T cells share similar antigens with malignant T cells, translating this approach to T-ALL has been extremely challenging due to fratricidal T-on-T cytotoxicity during CAR T cell manufacture and T cell aplasia caused by eradicating normal peripheral T cells and others normal cells^{9,10}. Various approaches, including targeting antigens that expressed only by a subset of normal T cells, such as CD30¹¹, CD37¹², CCR4¹³, TRBC1¹⁴, CD4¹⁵, CD5¹⁶ and CD1a¹⁷, and using NK cells as effector cells instead of T cells, have been used to overcome this problem⁹. To prevent fratricide among CAR T cells, several groups used the CRISPR/Cas9 system to disrupt the CD7 locus and demonstrated that anti-CD7 CAR T cells retained antitumor activity without fratricide gene-disrupted T cells in pre-clinical and clinical studies (NCT04264078 and NCT04004637)^{18,19}. Although this strategy could circumvent the fratricide of CAR T cells in T cell malignancies, the infused CD7 CAR T cells may retain on-target/off-tumour effects for CD7-positive cells including normal T cells, NK cells and myeloid cells. Hence, new targets and innovative approaches seem to be needed to avoid extensive self-antigen-driven fratricide and T cell aplasia.

CD99 is an O-glycosylated 32-kD type I transmembrane protein that is involved in cell apoptosis, adhesion, extravasation and transmigration processes^{20,21}. CD99 has also been detected in haematologic malignancies and solid tumours, such as acute myeloid leukemia (AML), myelodysplastic syndrome (MDS)²², T-ALL²³, Ewing's sarcoma (ES)²⁴, non-small-cell lung cancer²⁵, glioblastoma (GBM)²⁶, melanoma²⁷ and other types of cancers. Recently, one study found that CD99 is a stem cell marker in AML and that targeting CD99 with a monoclonal antibody (mAb) resulted in significant anti-leukemia activity²⁸. Moreover, CD99 has been demonstrated to have stronger expression in newly diagnosed T-ALL and has also been demonstrated as a new tool for the detection of minimal residual disease (MRD)²³, indicating that CAR T cell therapy based on CD99 expression is a promising therapeutic strategy for T-ALL and AML rooting out. CD99 expressed at very high levels on cancer cells but presented at low levels on several haematopoietic cells, such as NK cells, monocytes, and a fraction of T cells²⁹. Several studies have reported that engineering the scFv with lower affinity could improve the discrimination among cells with varying antigen densities³⁰⁻³⁴. We sought to determine whether fine-tuning the affinity of the scFv could increase the ability of CAR T cells to discriminate tumours from normal tissues expressing the same antigen at different levels.

In this study, we confirmed CD99 expression in multiple cell lines and primary leukemia samples and found that scFv (12E7) had a lower affinity for CD99, which could significantly decrease recognition by normal tissue cells. Based on this scFv, we demonstrated that our anti-CD99 CAR T cells showed robust cytotoxicity specifically against CD99-positive T-ALL and AML cell lines and primary tumour cells *in vitro*; but did not target normal cells and significantly prolonged patient-derived xenograft (PDX) mouse model survival *in vivo*. Moreover, the pilot clinical study also showed that our anti-CD99 CAR T cells were safe and mediated antitumor responses against CD99-positive leukemia.

Results

The 12E7 monoclonal antibody is specific for CD99 in cancer cells

To confirm CD99 is an effective target for T-ALL, AML, and other types of cancers, we evaluated CD99 expression in the Cancer Cell Line Encyclopedia (CCLE). The results showed that CD99 was highly expressed in various cancer cell lines (15 T-ALL and 35 AML), especially in T-ALL, which has the highest expression level among the majority of tumour types that we presented (Supplementary Fig. 1A). To verify these results in patient samples, we downloaded the RNA-seq data of normal samples and leukemia samples retrieved from previous studies (23 normal T cells and 264 T-ALL)³⁵ and flow cytometry was used to measure the protein level. Both CD99 transcript and protein levels were greatly upregulated in the T-ALL and AML samples compared to the normal T cells or bone marrow (BM) cells (Fig. 1A-B and Supplementary Fig. 1B). Importantly, we also found that CD99 expression was high in both primary and recurrent T-ALL but not significantly different between the two disease progression stages (Fig. 1C), indicating that CD99 is a promising target not only for patients with newly diagnosed but also for those with refractory or relapsed acute leukemia .

CD99 is also present at low levels in several particular subtypes of normal cells, such as T cells, B cells, NK cells and endothelial cells²¹. CAR T cells targeting CD99 on tumour cells may recognize and attack normal cells, which causes severe toxicity. Identifying an optimal antibody that could distinguish between cancer and normal cells is important for anti-CD99 CAR T cell therapy. First, three different parental clones of anti-CD99 monoclonal antibodies (mAbs), including 12E7 (Abcam), 1021527 (R&D) and 3B2/TA8 (BioLegend) were evaluated for their binding activity between several normal immune cells and T-ALL cell lines by flow cytometry analysis. All of the anti-CD99 clone mAbs demonstrated staining on the surface of different normal blood cells except 12E7, which specifically recognized CD99 on leukemia cells but did not bind to normal cells (Fig. 1D and Supplementary Fig. 1C-D). We also found CD99 have the high correlation with other T-ALL targets which was confirmed by previous studies by using the RNA-seq data³⁶(Supplementary Fig. 1E). These preliminary observations indicated that 12E7 may be a suitable antibody for CAR T-cell generation.

To systematically investigate the sensitivity of anti-CD99 (12E7) mAb in normal tissue, we have done the immunostain tissue microarrays by this antibody. We observed anti-CD99 (12E7) mAb-positive signals in parts of the thymus, where immune cells accumulate, but not in the spleen, liver, kidney or other important organs (Fig. 1E and Supplementary Fig. 1F). CD7, which was demonstrated to be a suitable target for CAR T cells therapy in T-ALL³⁷ and AML³⁸, was used as control, and the results showed that CD7 were partially positive in extracellular regions in several normal human tissues, such as those of the thymus, spleen, lung, small intestine, colon, tonsil and cervix (Fig. 1E and Supplementary Fig. 1F). Together, the overall results indicate that the 12E7 mAb is an optimal antibody for anti-CD99 CAR T therapy according to its specific target molecule recognition and limited binding to normal cells.

Anti-CD99 CAR T cells specifically targeted CD99-positive cancer cells without normal blood cells toxicity

To test whether the scFv which derived from the anti-CD99 (12E7) mAb has suitable affinity and specificity for CD99, this scFv were evaluated by ForteBio Octet system and Flow cytometry analysis. As shown in Fig. 2A, the anti-CD99 scFv exhibited a binding affinity of 6.97×10^{-8} M, which is lower than the 1021527 (5.76×10^{-9} M) and 3B2/TA8 (1.93×10^{-9} M) (Fig. 2A and Supplementary Fig. 2A-B). And there was a strong positive correlation between the anti-human CD99 mAb and anti-CD99 scFv in the different cell lines according to flow cytometry analysis (Fig. 2B). Next, the 12E7 scFv was incorporated into the lentivirus CAR vector which contained the CD28-4-1BB-CD3 ζ intracellular signaling domains to generated anti-CD99 CAR (Supplementary Fig. 2C).

Following activation and transduction of T cells, anti-CD99 CAR T cells were significantly fewer than the irrelevant anti-CD19 CAR T control (Fig. 2C). Interestingly, the efficacy of transduced CAR positive cells was significantly increased during cell culture, almost 100% in the 12 days (Fig. 2D). We hypothesized that the CAR-negative cells might be killed by CAR-positive cells. The results were consistence with our hypothesis that CD99 induced expression after CD3/CD28 beads activation and could be targeted by anti-CD99 CAR T cells (Fig. 2E-F). To explore whether CD99-based CAR T cells target surface antigens can exhibit fratricide, which is a challenge for many targetable antigens in T-ALL, we purified the CAR positive

cells after 3 days transduction (CAR positive cells range from 20% to 30%, data not show) for the further proliferation evaluation. Interestingly, the antigen of CD99 did not express in anti-CD99 CAR T cells and we also did not find a significant reduction in the proliferation of anti-CD99 CAR positive T-cells compared to anti-CD19 CAR positive T-cells (Fig. 2G,H), suggesting anti-CD99 CAR T cells could not cause significant CAR T-cell fratricide. At the end of manufacturing (12 days). Next, the expression levels of CD25, CD65, CCR7 and CD45RA were measured by flow cytometry to assess CAR T cell activation and differentiation status along with the potential for long-term persistence after culture for 12 days. We observed that anti-CD99 CAR T cells were CD25 and CCR7 positive, indicating that almost all *ex vivo* cultured anti-CD99 CAR T cells retained the activation markers, and most of the CAR T cells showed a central memory phenotype at the end of manufacturing (Supplementary Fig. 2D-E).

Furthermore, to verify the safety of anti-CD99 CAR T cells, cytotoxicity experiments were performed on human normal lymphocyte subpopulations, CD34+ haematopoietic progenitors (LSK), dendritic cells (DCs) and monocytes. The results demonstrated that the anti-CD99 CAR T cells could specifically target the T-ALL cell line MOLT-4 but with minimal killing of normal cells (Fig. 2I). We next assessed the antigen-specificity and cytotoxic activity of anti-CD99 CAR T cells in NIH-3T3 mouse cells and NIH-3T3 mouse cells overexpressing human CD99, and we observed specific killing only in the hCD99 overexpression cell line (Supplementary Fig. 2F-G). In contrast, the antitumor activity was decreased when we knocked down CD99 in the T-ALL MOLT-4 cell line (Supplementary Fig. 2H-I). In addition, calcein-AM-based cytotoxicity assays demonstrated that anti-CD99 CAR T cells induced powerful antitumor activity in several T-ALL (Jurkat, MOLT-4, CUTLL-1 and MOLT-3) and AML (THP-1, SKM-1, MOLM-13 and K562) cell lines at different ratios (Fig. 2 J-K).

In addition to leukemia cell lines, a vast majority of tumour cell lines also express CD99. These include the multiple myeloma (MM) cell line U266B1, the ewing sarcoma (ES) cell lines RD-ES and A-673, the glioma cell lines U251-MG, the breast cancer cell line MCF-7, the cervical cancer cell line HeLa, the ovarian cell lines OVCAR-8 and SK-OV-3, the non-small-cell lung cancer cell lines A549, and the colon adenocarcinoma cell lines SW480 and HCT116. Anti-CD99 CAR T cells could eliminate these CD99-positive tumour cell lines *in vitro* (Supplementary Fig. 2J-K). Taken together, these results demonstrate that anti-CD99 CAR T cells specifically target CD99-positive cells and have no cytotoxicity towards normal cells.

Anti-CD99 CAR T cells exhibited potential cytotoxicity against primary cells and leukemia stem cells

Primary leukemia cells are more complex than cell lines, as they account for the heterogeneity of tumour cells, and the relapse of T-ALL in patients remains a significant clinical problem and is thought to be associated with clonal selection during treatment. To investigate whether CD99 is an optimal target for primary leukemia cells, Six T-ALL and four AML patients were used for CD99 expression and cytotoxic activity analysis. CD45/SSC gating strategy were used to identify leukemia cells from non-leukemia cells by flow cytometry as the previous study described (Supplementary Fig. 3A-B)³⁹. As shown in Fig. 3A and 3C, almost 100% leukemia cells were CD99 positive. Subsequently, we confirmed that the generation of

anti-CD99 CAR T cells had powerful antitumor activity against primary T-ALL and AML cells (Fig. 3B and 3D).

leukemia stem cells (LSCs) are responsible for the initiation, progression, and relapse of leukemia in AML and other leukemias^{40,41}. Recently, several studies showed that the CD99 protein is upregulated in LSCs and enriched in CD34⁺CD38⁻ AML cells compared with normal BM CD34⁺CD38⁻ cells²⁸. We hypothesized that high levels of CD99 expression could make these cells a target for anti-CD99 CAR T cell therapy. To test this hypothesis, LSCs were purified by FACS sorting as previous study described (Supplementary Fig. 3C)⁴², and CD99 expression and cytotoxic activity were analysed. As shown in Fig. 3E and 3F, CD99 was expressed in both LSCs and blast cells, and anti-CD99 CAR T cells exhibited high cytotoxic activity against these cells. According to the above results, we verified that anti-CD99 CAR T cells should be able to eliminate not only T-ALL/AML blast cells but also CD34⁺CD38⁻ LSCs.

Anti-CD99 CAR T cells showed potent antitumor activity *in vivo*

To assess the effect of anti-CD99 CAR T cells against CD99-positive T-ALL cells *in vivo*, we performed experiments using cell line-derived xenografts (CDXs) created from Jurkat and MOLT-4, respectively. NCG (NOD-Prkdcem26Cd52Il2rgem26Cd22/NjuCrI) mouse models were injected with Jurkat cells or MOLT-4 cells engineered to express luciferase and GFP via the tail vein. Three days after tumour engraftment, we injected a single dose of 5×10^6 anti-CD99 CAR T cells or 5×10^6 control non-transduced T cells and monitored the tumour burden by IVIS imaging and flow cytometry analysis (Fig. 4A and Fig. Supplementary Fig. 4A). Anti-CD99 CAR T cells conferred robust protection against leukemia progression and significantly extended the median survival of the mice in the T-ALL model (Fig. 4B-C and Supplementary Fig. 4B-D) but did not induce a significant change in animal body weight in the different treatment groups (Supplementary Fig. 4G). Next, to evaluate the persistence of the anti-CD99 CAR T cells, qPCR was used to measure CAR copy numbers in the peripheral blood (PB) of CDX mice on different days after CAR T cell treatment. The results showed that CARs persisted at a detectable level in the PB for at least 21 days (Fig. 4D and Supplementary Fig. 4E). The tissue architectures of the spleen appeared normal, and infiltrating leukemia cells were rarely detected in the spleens of mice in the CAR T cell treatment groups (Fig. 4E and Supplementary Fig. 4F).

Previous studies showed that the patient-derived xenograft (PDX) model was a highly predictive model that could enhance the translational value of cancer therapy⁴³, so we used the PDX mouse model to examine the antitumor activity of anti-CD99 CAR T cells. Similar to the methods for the CDX models, NCG mice were transplanted with 5×10^6 primary T-ALL blasts. Anti-CD99 CAR T cells administered intravenously at a dose of 5×10^6 cells per PDX model 3 days after demonstrated the ability to clear T-ALL cells. leukemia engraftment was followed up weekly by bleeding and flow cytometry analysis (Fig. 4F). The engraftment of CD99 positive primary T-ALL cells gradually increased over time in the PB of the control T cell groups (Fig. 4G), whereas anti-CD99 CAR T cells significantly delayed leukemia progression, as demonstrated by the nearly complete absence of leukemia cells in the haematopoietic organs (Fig. 4I). More importantly, compared with the normal T cell treatment groups, anti-CD99 CAR T cells significantly

extended the overall survival (OS) of the mice (Fig. 4H) and eliminated infiltrating leukemia cells in the spleen and liver (Fig. 4J-K) without a significant change in animal body weight (data not shown). Taken together, our CDX and PDX models proved that anti-CD99 CAR T cells show significant antitumor efficacy *in vivo* with no significant histological toxicity.

Pilot clinical study to test anti-CD99 CAR T cell safety and antitumor efficacy

We designed a pilot study to assess the safety and feasibility of anti-CD99 CAR T cells in patients with refractory or relapsed (r/r) haematologic malignancies, especially for safety evaluation. A total of 4 cases with CD99-positive disease (AML, n=2; T-LBL, n=1; myeloid sarcoma (MS), n=1) were enrolled, and their clinical and disease subtype characteristics are shown in Table 1. The median age was 30 years (from 26 to 37 years); none of the cases had undergone haematopoietic stem cell transplantation (HSCT) before CAR T cells infusion, and all of the cases had received multiple conventional chemotherapies (Table 1). All the patients enrolled in this clinical study were confirmed to have CD99-positive leukemia cells by flow cytometry analysis and the BM blast percentage ranged from 50.0-80.0%(Fig. 5A).

All cases received the fludarabine and cyclophosphamide (FC) regimen for lymphodepletion as the previous study described⁴⁴ and then treated with a single infusion of anti-CD99 CAR T cells, with a median time from enrollment to infusion of 35 (range, 28-40) days. Clinically applicable CAR T cells were manufactured for all cases via the same process, and the characteristics of the infused products are shown in Table S1. Next, to evaluate the cytotoxicity of anti-CD99 CAR T cells before infusion, the CD99-positive cell line Jurkat, representing the primary cells from CAR T cell-treated cases, were used for further assessment of antitumor activity. The results showed that anti-CD99 CAR T cells of patients lysed over 26.1-43.6% of self-leukemia cells at an E:T ratio of 25:1 (Fig. 5B and Table S1).

The dose of CAR T cells infused was ranged from 1×10^6 /kg to 4×10^6 /kg (Table 2) and there were no toxicity-related deaths after CAR T cell infusion. None of the patients suffered from CAR T cell-related immune effector cell-associated neurotoxicity syndrome (ICANS) (Table 3). Cytokine release syndrome (CRS) occurred in two cases (50%), but the severity was not higher than grade 2, indicating that our anti-CD99 CAR T cells were safe in the patients who enrolled in our study. One patient (Case 3) had fever on the second day after cell transfusion (the highest temperature was 39.0°C) and developed hypotension (80/45mmHg). It returned to normal after using Tocilizumab and dopamine to maintain blood pressure.

The levels of serum IL-6 but not CRP and Serum Ferritin were very consistent with CRS after CAR T cell infusion (Fig. 5C, Supplementary Fig. 5A-B and Table 4). IL-6 levels increased significantly in 2 cases with CRS after infusion of CAR-T cells, and there was no significant change in 2 cases without CRS. The baseline CRP level of 3 cases was higher than the normal value, 2 cases without CRS, one developed CRS, but the increase of CRP for patients with CRS was not as significant as that of IL-6 (Supplementary Fig. 5A and Table 4). Similar results were observed in Serum Ferritin, the baseline value of 2 patients without CRS was much higher than the 2 patients with CRS, and the increase of Serum Ferritin was not significant in 2 patients after developed CRS (Supplementary Fig. 5B and Table 4). The specificity of CRP

and Serum Ferritin is poor, the increase of IL-6 level is more likely to indicate the occurrence of CRS (Table 4).

Furthermore, One patient with acute myeloid leukemia (case 1) achieved complete remission, but relapsed two months after CAR-T infusion and received sibling allogeneic hematopoietic stem cell transplantation, the patient regained complete remission (Table 2). One patient with T lymphoblastic lymphoma (case 4) achieved PR, but progressed one month later and died three months after infusion of CAR T cells. One patient with myeloid sarcoma (case 2) (left thigh) and one patient with extramedullary relapse of acute myeloid leukemia (case 3) (left breast) survived with SD (Table 2).

Importantly, the existence and persistence of anti-CD99 CAR T cells was observed in 4/4 cases (100%) by transgene copy quantitative evaluation and flow cytometry analysis. As shown in the figure 5D, anti-CD99 CAR T cells significantly expanded in most patients, and the median time for the maximal expansion of anti-CD99 CAR T cells was 11 days post-infusion (range, 9–15 days). The maximal expansion reached approximately 3.8×10^5 transgene copies per μg DNA and 6.2% CAR positive T cells in the PB (Fig. 5D). In addition, during the approximately 1-month follow-up after anti-CD99 CAR T infusion, the number of patients' lymphocyte cells, mononuclear cells, neutrophilic cells and red blood cells did not change based on the routine blood examination (figure 5E-H). These results indicate that anti-CD99 CAR T cell therapies could be safe and did not deplete the major part of the blood cells.

Taken together, these findings indicate that anti-CD99 CAR T cells hold great potential for the treatment of CD99-specific r/r leukemia under our manufacturing technology. This is the first study to provide evidence demonstrating that anti-CD99 CAR T cells are safe and mediate antitumor responses against CD99-positive tumours. Nevertheless, the efficacy and safety of anti-CD99 CAR T cell therapy are related to many other factors that call for further investigations.

Discussion

Our data demonstrated that CD99 is an attractive target for the immunotherapy of CD99-positive tumours. CD99 was aberrantly expressed on T-ALL and AML cells but absented or expressed at low levels on normal cells. Here, we first developed a detailed and systematic strategy to evaluate the safety, feasibility and efficacy of anti-CD99 CAR T cells from cell lines and mouse models to human clinical studies. Our results showed that anti-CD99 CAR T cells eliminated various CD99-positive cancer cell lines, primary cells and LSCs *in vitro* (Figs. 2 and 3). The utilized CDX and PDX models proved that anti-CD99 CAR T cells were activated, expanded and mediated significant antitumor efficacy *in vivo* (Fig. 4). Furthermore, the pilot study results showed that our CAR T cell therapy was successful in 50% of the patients, and 2 (50%) patients developed no more than grade 2 CRS, 2 patients have no CRS, indicating that anti-CD99 CAR T-cell treatment was safe for patients and that toxicity was relatively mild (Fig. 5 and Tables).

The tumour antigens are often slightly expressed on the normal cell surface, which increases the potential risk of significant and discourage CAR T therapy strategy from being used for extensive clinical applications⁴⁵. CAR-T cells can also react to low-density antigens and injury to major organs such as cardiovascular system, lung and brain⁴⁶⁻⁴⁸. There was a case report that one patient with metastatic colon cancer died 5 days after anti-ERBB2 CAR T infusion. The cause of death was that CAR-T cells attacked to the epithelial cells of the lung, which expressed low level of ERBB2⁴⁶. Recently, several studies also demonstrated that the high affinity antigen-specific CAR T cells markedly improved the *in vivo* and *in vitro* function of CAR T, however these improvements in function were associated with lethal “on-target, off-tumor tissue” toxicity^{49,50}. In contrast, *in vitro* study showed that the lower binding affinity of anti-CD123 CAR is a safe target antigen for AML therapy³³. Similarity, the *in vivo* studies suggested that reducing the affinity of scFv by mutagenesis and replacing it with another lower-affinity scFv were efficient strategy method to overcome the problem of “on-target, off-tumor” toxicity^{31,51,52}. Furthermore, recently clinical data demonstrated that CAR with lower affinity could enhanced cell amplification and showed less exhaustion compared with higher affinity anti-CD19 CAR T cells⁵³. These results highlight that the binding affinity of a CAR is not only related to efficacy but also the safety, and that the affinity is not the higher the better. In our study, to avoid T cell fratricide and potential on-target, off-tumor effects, we first identified a lower affinity anti-CD99 mAb (12E7) that specifically recognizes CD99-expressing human leukemia/cancer cells but not normal cells (Fig. 1). Next, we also demonstrated that anti-CD99 CAR T cells can efficiently be produced without measurable fratricide and can specifically eliminate leukemia cells but not normal blood cells (Fig. 2).

Although our preliminary results in the pilot clinical study supported that anti-CD99 CAR T cells are safe, the therapeutic effects were modest compared to those achieved with anti-CD19 CAR T cells. However, all of the patients enrolled in this trail with refractory or relapsed, especially one patients suffered from extramedullary relapse to the breast and one patients diagnosis as myeloid sarcoma involved in the left thigh which the diseases phenotype were similar to the solid tumor and presents as rapidly progressive⁵⁴. Both of the patients were responded to our anti-CD99 CAR T cells, indicating that our products have the potential antitumor activity and may use for others CD99 positive solid tumours. Meanwhile, we should be aware that none of patients finally achieved CR for a longer time. In addition, several important factors, such as the capacity for CAR T cell expansion, disease histology and probably disease-intrinsic factors that predispose tumours to resistance associated with antigen loss or downregulation, were reported to affect the outcome of tumours responding to CAR T cell therapy in a clinical study⁵⁵. Together, It is difficult to make conclusions with such a small sample size and more patients should be enrolled to evaluate the efficacy in the further study..

In summary, we established anti-CD99 CAR T cells as a promising therapeutic approach against CD99-positive disease and showed that they were a highly effective immunotherapeutic modality for T-ALL and AML *in vitro* and *in vivo*. As our clinical study only recruited 4 patients, the results are too preliminary to make undoubtable conclusions. To a certain extent, our study demonstrated that anti-CD99 CAR T cells are effective in the treatment of r/r CD99-positive disease and do not cause neurological toxicity or severe

CRS. Importantly, future clinical trials will need to assess the on-target/off-tumour toxicity of CAR T cell therapy.

Declarations

Authorship

Contributions: Haichuan Zhu, Jiangzhou Shi Zijian Zhang and Tongcun Zhang designed the experimental plans; Jiangzhou Shi, Zijian, Zhang, Han Wu, Shangkun Zhang, Yingqi Leng, Anqi Ren, Xiyu Liu, Zijie Zhang, Xiqin Tong and Jinjue Liang performed the experiments. Jiaxing Liu performed the bioinformatic and statistical analyses; Hong Cen, Zhe Li, Fulin Zhou, You Qin, Kunyu Yang collected the clinical samples and data analysis. Hong Cen and Liang Huang were in charge of the clinical studies. Haichuan Zhu, Jiangzhou Shi and Zijian Zhang wrote the manuscript with input from all authors.

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Conflicts of Interest

Tongcun Zhang is a copartner of the Wuhan Bio-Raid Biotechnology Co., Ltd. Others authors declare no competing financial interests.

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Tables

Tables 1-4 are available in the Supplementary Files section.

Figures

Fig.1

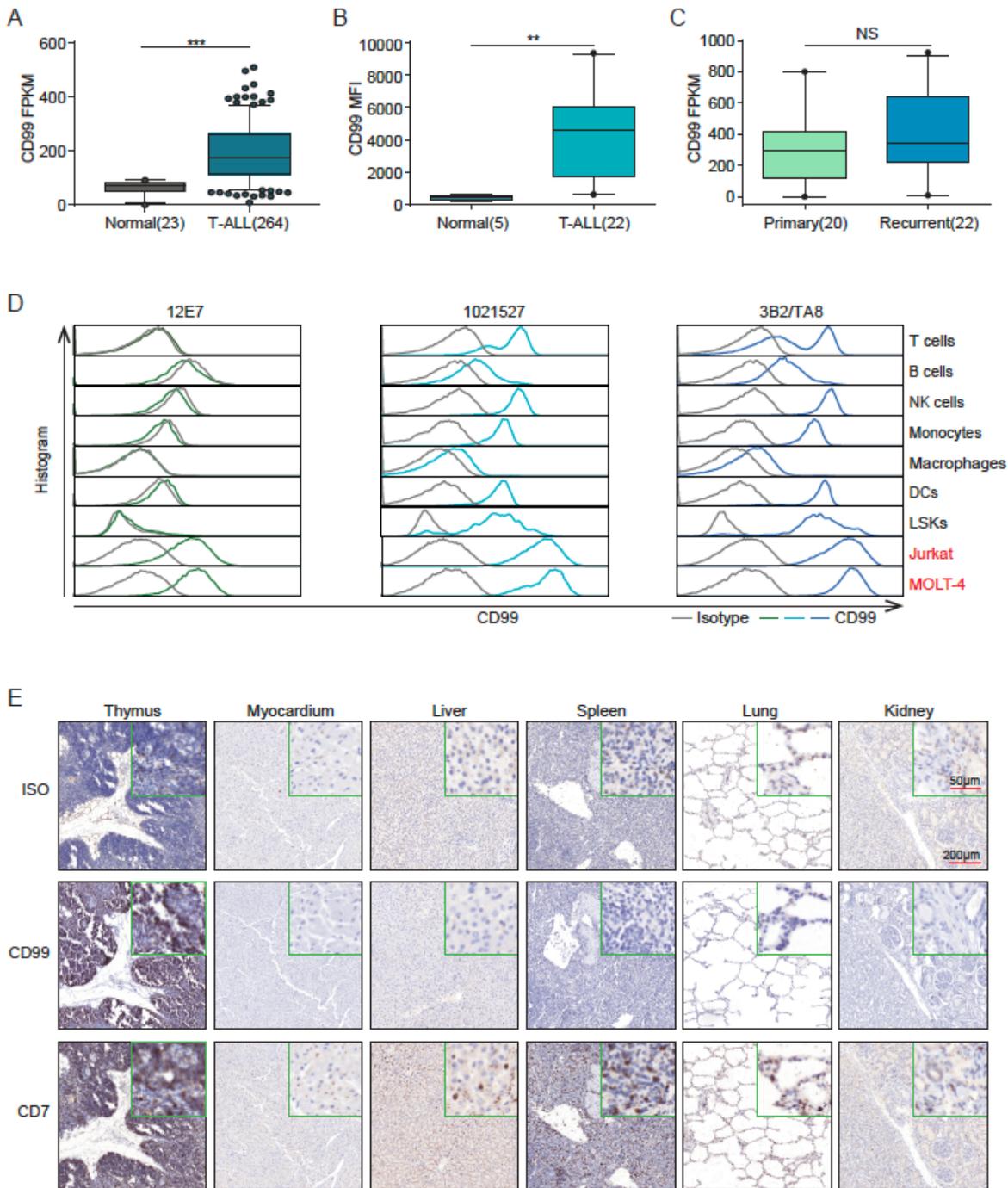


Figure 1

The CD99 (12E7) mAb specifically recognized leukemia cells. (A) Relative CD99 expression was calculated as CD99 fragments per kilobase of transcript per million mapped reads (FPKM) on T-ALL samples (n=264) and normal PBMC samples (n=23). Data from NCBI BioProject: PRJNA252189 (Dvinge H et al. Proc Natl Acad Sci USA, 2014). (B) The relative CD99 protein level was calculated as the CD99 median fluorescence intensity (MFI) on T-ALL samples (n=22) and normal T cell samples (n=5) by flow

cytometry; (C) Relative CD99 expression was calculated as CD99 FPKM on recurrent (n=22) and primary T-ALL samples (n=20). The data from NCBI BioProject: PRJNA534488. (D) CD99 recognizing ability of three anti-CD99 mAbs (12E7, 1021527 and 3B2/TA8) in normal blood cells and leukemia cells by flow cytometry. (E) Representative immunohistochemistry (IHC) images of normal paraffin tissue sections with the CD99 (12E7) mAb and CD7 mAb. IgG as the negative control,***p≤0.001,**p≤0.01,NS=no significant Scale bar, 50 μm or 200μm. Histological staining was quantified using Image J.

Fig.2

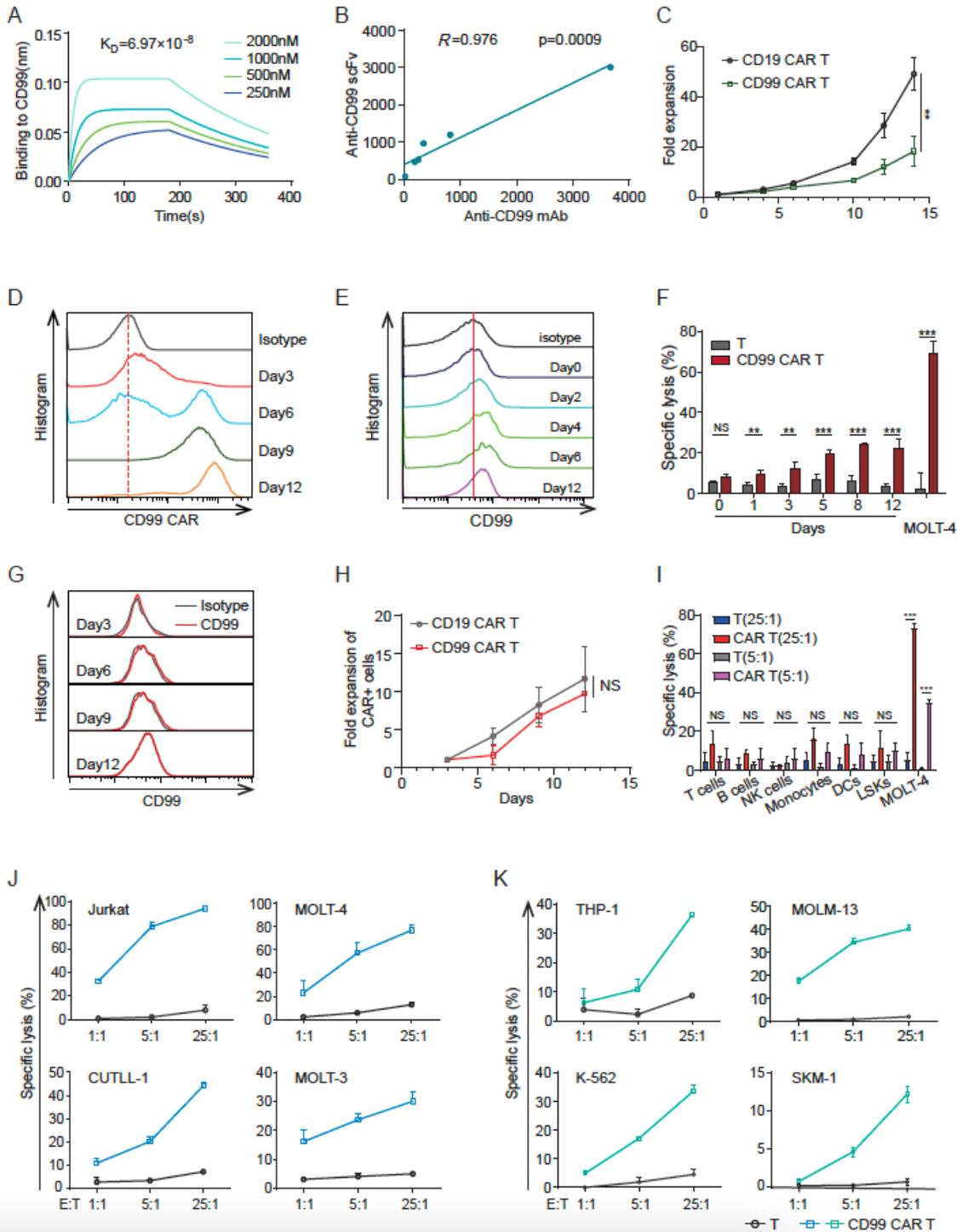


Figure 2

Anti-CD99 CAR T cells mediated potent killing against CD99-positive cells in vitro. (A) Binding kinetics of anti-CD99 scFv with CD99 protein. Analysis of the interaction between the 12E7 scFv and CD99 protein using BLItz biolayer interferometry. The binding signal was measured at 250/500/1000/2000 nanometers (nm) as a function of time (seconds) ($K_D = 6.97 \times 10^{-8}$). (B) The 12E7 antibody and the anti-CD99 scFv showed a strong correlation in different cell lines. (C) Expansion fold change of total T cells transduced with CD19 CAR or CD99 CAR for 14 days. (D) Percentage of CAR positive cells measured by flow cytometry using anti-Strep II antibodies during the CAR T cells in vitro culture. (E) The expansion of anti-CD99 CAR positive cells compared to anti-CD19 CAR positive cells. (F) Expression of CD99 in normal T cells activated by CD3/CD28 beads. (G) Cytotoxic activity of anti-CD99 CAR T cells against normal T cells which activated by CD3/CD28 beads in different days determined by calcein-based cytotoxicity assay at the ratios of 25:1 after 2-3 h co-culture. (H) Expression of CD99 in CAR positive cells by flow cytometry. (I) In vitro cytotoxic activity of anti-CD99 CAR T cells against different normal blood cells. (J-K) Cytotoxic activity of anti-CD99 CAR T cells against T-ALL (Jurkat/ MOLT-4/ CUTLL-1/ MOLT-3) and AML (THP-1/ MOLM-13/ K-562/SKM-1) cell lines as determined by calcein-based cytotoxicity assay at different target ratios (1:1,5:1,25:1) after 2-3 h co-culture. IgG as the negative control, *** $p \leq 0.001$, ** $p \leq 0.01$, NS=no significant.

Fig.3

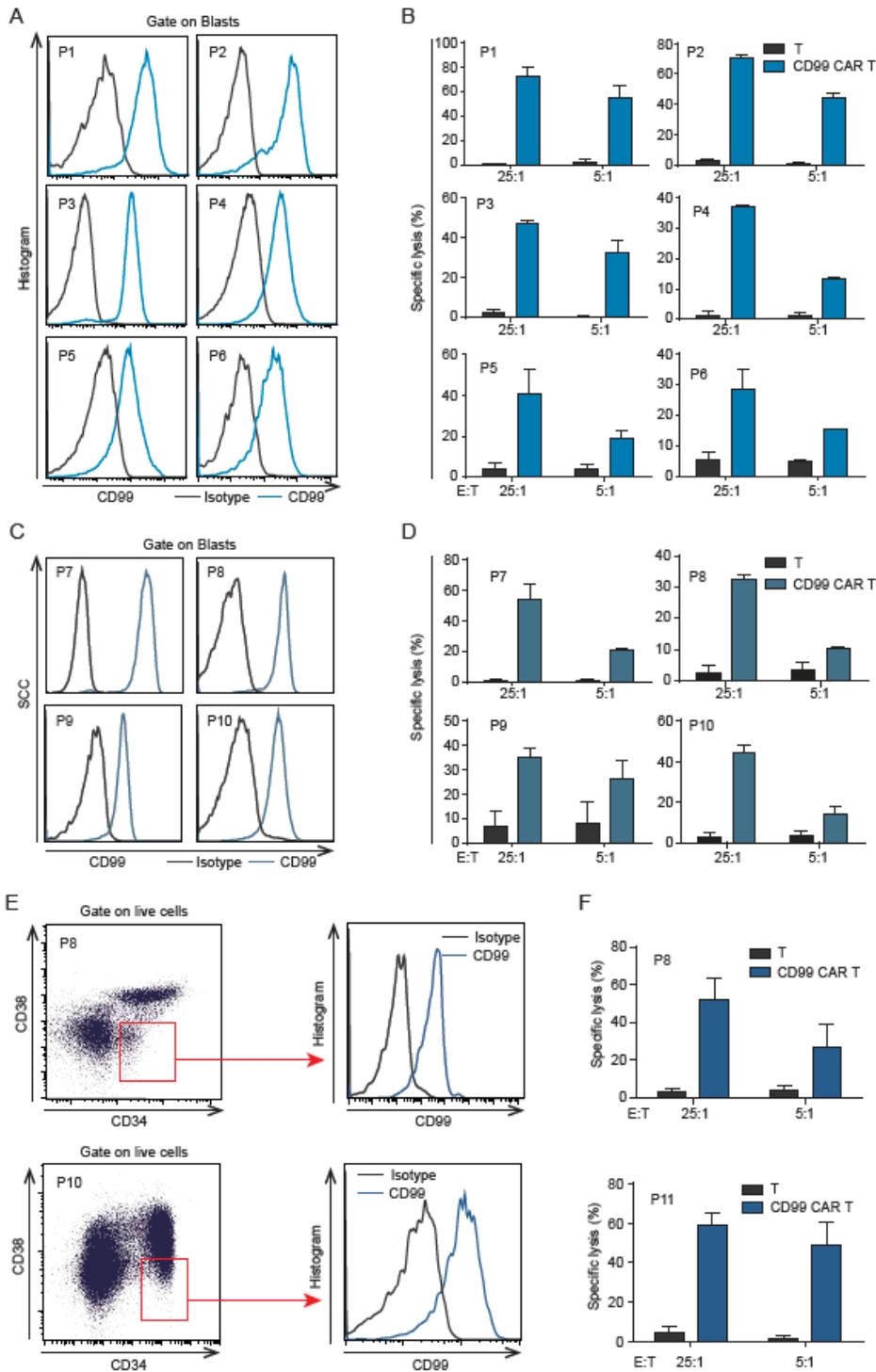


Figure 3

Anti-CD99 CAR T cells effectively eliminated primary leukemia cells and LSCs. (A) Flow cytometry showing CD99 expression in blast cells from different T-ALL patients (Patient 1 to 6). (B) Blast cells from different T-ALL patients were co-cultured with anti-CD99 CAR T cells for 2-3 h at different E:T ratios (1:1,5:1,25:1), and specific lysis was assessed. (C) Flow cytometry showing CD99 expression in blast cells from different AML patients (Patient 7 to 10). (D) The cytotoxic activity of anti-CD99 CAR T cells

against Blast cells, which from different AML patients, at different E:T ratios (1:1,5:1,25:1). (E) Flow cytometry showing CD99 expression in LSCs from different AML patients (CD34+CD38-). (F) The specific lysis of anti-CD99 CAR T against LSCs from different AML patients were carried out at different E:T ratios (1:1,5:1,25:1). IgG as the negative control.

Fig.4

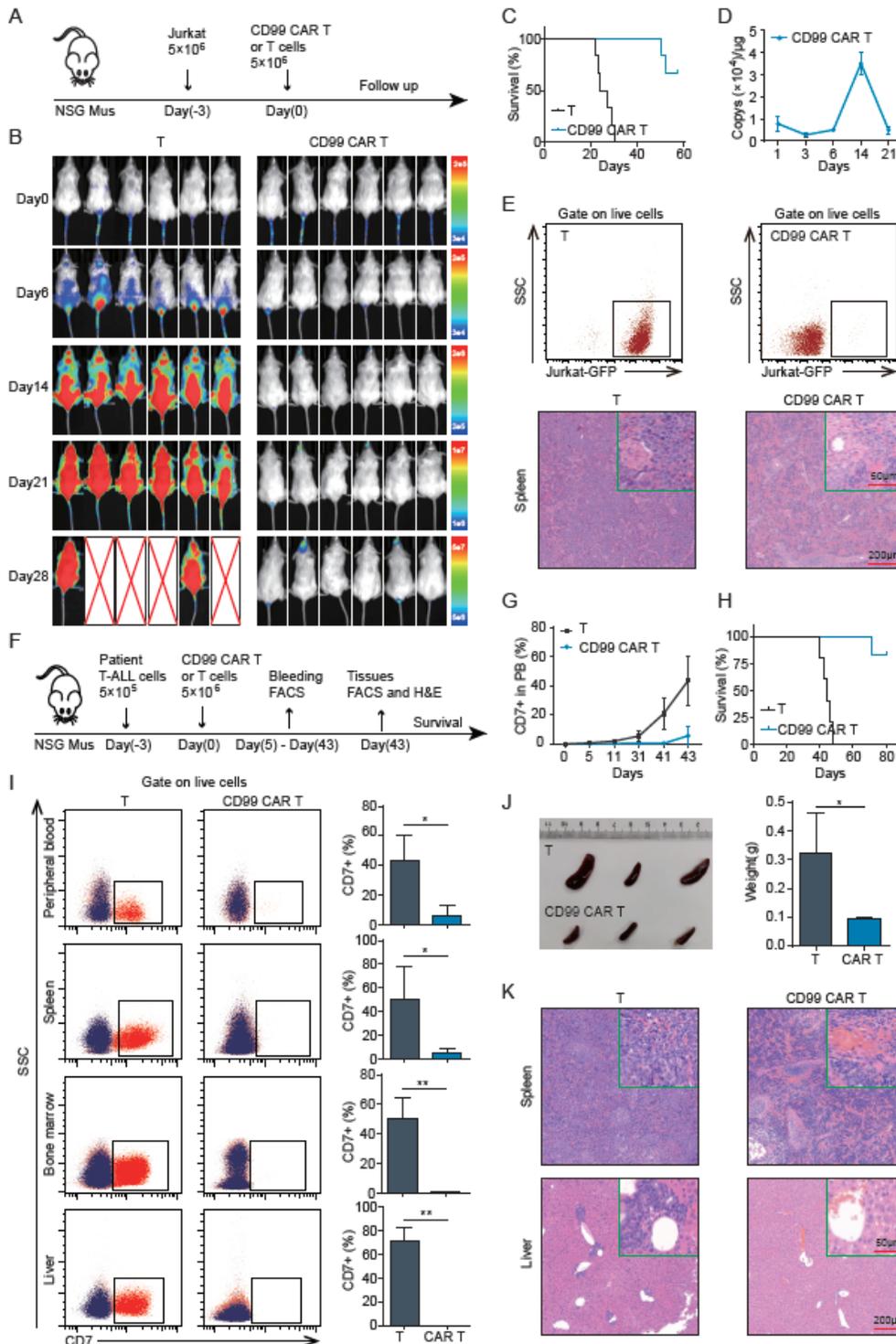


Figure 4

Efficacy and specificity of anti-CD99 CAR T cells in Jurkat xenograft mouse models and a PDX mouse model of T-ALL. (A-B) Schematic outline of the mouse model experiment. NCG mice (n=6 per group) were i.v. injected with GFP-Fluc Jurkat cells and administered anti-CD99 CAR T cells at a dose of 5×10^6 CAR T cells or 5×10^6 T cells per mouse at day 3 following Jurkat cell injection. Tumour burden was monitored weekly by BLI using IVIS imaging. (C) Kaplan-Meier survival curves of mice injected with T cells or anti-CD99 CAR T cells. (D) Anti-CD99 CAR T cell expansion and persistence in vivo. Assessment of the copy numbers of CARs in whole blood by q-PCR on different days. (E) Upper: The proportion of GFP positive leukemia cells in the spleen in the T cell or anti-CD99 CAR T cell treatment groups according to FACS analysis. Lower: Histological features of the spleens of the T cell or anti-CD99 CAR T cell treatment groups according to H&E staining. (F) Schematic outline of the PDX experiment. NCG mice (n=6 per group) were i.v. injected with primary T-ALL cells and administered anti-CD99 CAR T cells at a dose of 5×10^6 CAR T cells or 5×10^6 T cells per mouse at day 3 following cell injection. Tumour burden was monitored every week by FACS analysis. (G) The proportion of human CD7 positive cells in PB from day 0 to day 43. (H) Kaplan-Meier survival curves of PDX model mice injected with T cells or anti-CD99 CAR T cells. (I) The proportion of human CD7 positive cells in the whole living cells of different organs (PB, BM, spleen and liver). (J) Spleens from different treatment groups were weighed and photographed. (K) Histological features of the spleen and liver in the T cell and anti-CD99 CAR T cell treatment groups according to H&E staining.

Fig.5

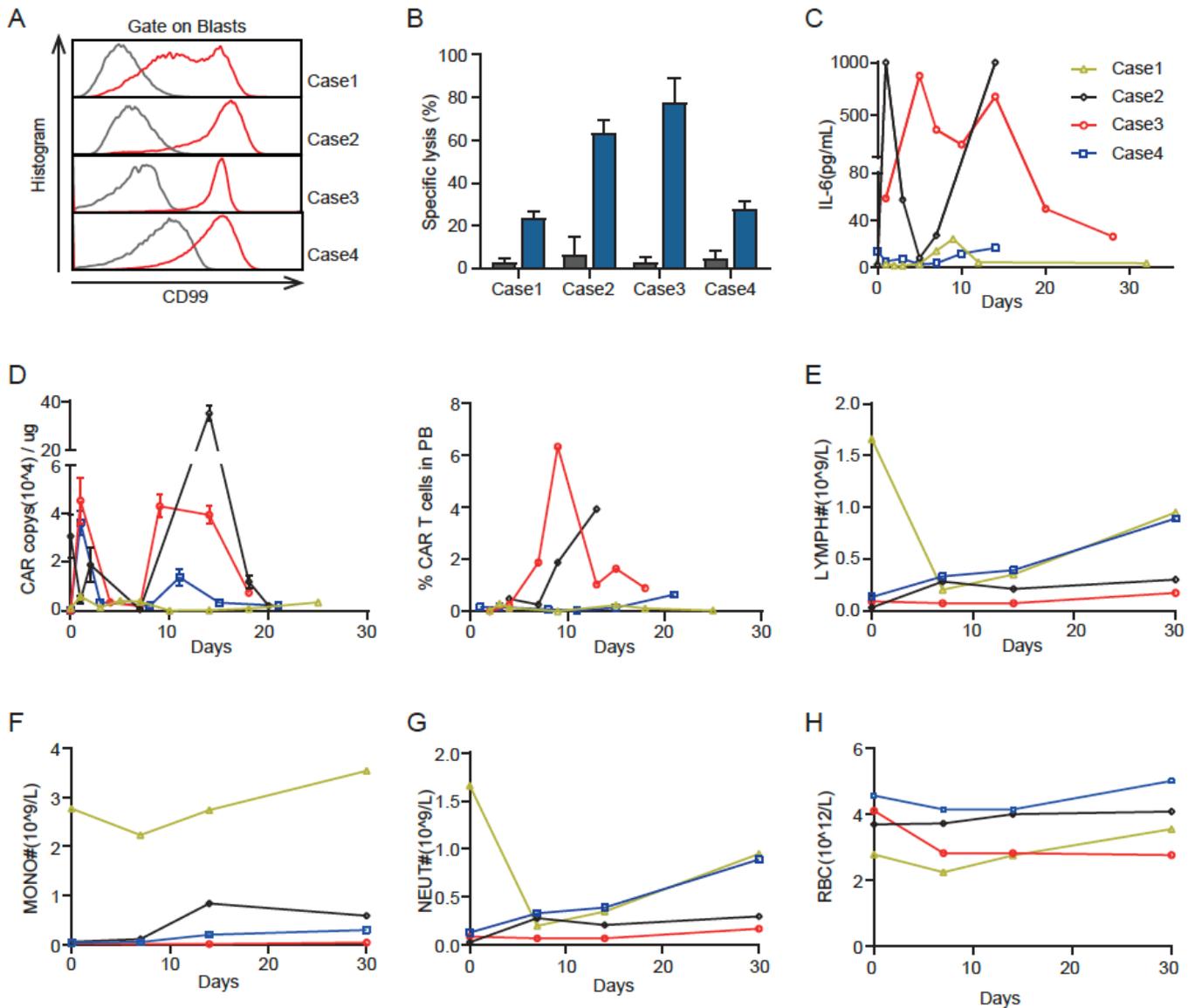


Figure 5

Clinical efficacy and safety of anti-CD99 CAR T-cell infusions. (A) CD99 expression in primary blast cells from different patients with Flow cytometry. (B) Cytotoxic activity of anti-CD99 CAR T cells derived from patient's T cells against Jurkat cell line as determined by calcein-based cytotoxicity assay at target ratios (25:1) after 2-3 h co-culture. (C) Cytometric bead arrays analysis of serum cytokine IL-6 levels in different patients. (D) Left: The copy numbers of the integrated CD99-CAR transgene per μg DNA obtained from PBMCs in different patients. Right: Flow cytometry showing CAR positive cells after infused in patients PB. (E-H) The routine blood examination of patients' lymphocyte cells, mononuclear cells, neutrophilic cells and red blood cells as the time indicated.

Supplementary Files

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