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Peripheral leukemia burden at time of apheresis negatively affects the clinical efficacy of CART19 in refractory or relapsed B-ALL

Biping Deng,[1] Jing Pan,[2] Zhaoli Liu,[1] Shuangyou Liu,[2] Yunlong Chen,[1] Xiaomin Qu,[1] Yu'e Zhang,[1] Yuehui Lin,[2] Yanlei Zhang,[3] Xinjian Yu,[4] Zhongxin Zhang,[1] Xuansha Niu,[1] Rong Luan,[1] Ming Ma,[1] Xiaomei Li,[1] Tingting Liu,[1] Xi'ai Wu,[1] Huan Niu,[1] Alex H. Chang,[3] and Chunrong Tong[2]

[1]Cytology Laboratory, Beijing Boren Hospital, Beijing 100070, China; [2]Department of Hematology, Beijing Boren Hospital, Beijing 100070, China; [3]Clinical Translational Research Center, Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai 200433, China; [4]Medical Laboratory, Beijing Boren Hospital, Beijing 100070, China

Our previous clinical study achieved complete remission (CR) rates of >90% following chimeric antigen receptor T cells targeting CD19 (CART19) treatment of refractory/relapsed B cell acute lymphoblastic leukemia (r/r B-ALL); however, the influence of the leukemia burden in peripheral blood (PB) blasts remains unclear. Here, we retrospectively analyzed 143 patients treated with CART19 (including 36 patients with PB blasts) to evaluate the effect of peripheral leukemia burden at the time of apheresis. One hundred seventeen patients with high disease burdens achieved 91.5% CR or incomplete count recovery CR and 86.3% minimal residual disease-negative CR, and 26 patients with low disease burdens obtained 96.2% MRD^- CR. Collectively, 9 of 36 (25%) patients with PB blasts and 2 of 107 (1.87%) patients without PB blasts did not respond to CART19 therapy. The leukemia burden in PB negatively influenced *ex vivo* cell characteristics, including the transduction efficiency of $CD3^+$ T cells and their fold expansion, and *in vivo* cell dynamics, including peak CART19 proportion and absolute count, fold expansion, and persistence duration. Further studies showed that these patients had higher programmed death-1 expression in CART19 products. Our data imply that PB blasts negatively affected CART19 production and the clinical efficacy of CART19 therapy in patients with r/r B-ALL.

INTRODUCTION

Genetically modified T cells expressing chimeric antigen receptors targeting CD19 (CART19) are effective in patients with relapsed and chemotherapy-refractory B cell acute lymphoblastic leukemia (r/r B-ALL). Clinical trials with CART19 therapy have recently shown 70%–90% complete remission (CR) rates in patients with r/r B-ALL.[1–3] Approximately 10%–20% of patients fail to achieve remission after receiving CART19 therapy.[3–6] This lack of clinical response is in large part due to T cells that result in a poor clinical product that either fails to cultivate or does not proliferate in the patient.[7–9] We hypothesized that the leukemia burden in peripheral blood (PB) negatively influences CART19 production and *in vivo* persistence, thereby impeding the response to CART19 therapy.

A high disease burden, mainly in the bone marrow (BM), markedly influences the outcome of CART19 treatment of B cell leukemia, including *in vivo* chimeric antigen receptor (CAR)-T cell function and expansion.[7,10–12] It has also been shown to influence *in vitro* and *in vivo* CAR-T cell functionality and therapeutic outcomes in chronic lymphocytic leukemia (CLL) and diffuse large B cell lymphoma.[13,14] To date, no studies have tested the hypothesis that the leukemia burden in PB influences the CART19 outcome in B-ALL. Therefore, in the present study we analyzed 143 patients treated with CART19 products, including 36 patients treated with PB blasts and 107 patients without PB blasts, to assess the influence of the leukemia burden on PB.

Most research groups initiate T cell cultures by using bulk PB mononuclear cells (PBMCs) obtained from apheresis products without T cell selection, and some groups use an upfront selection of T cell subsets.[15] Sorting T cells before activation adds both cost and complexity to the manufacturing protocol; as such, researchers have sought a feasible method to manufacture suitable CAR-T cells without complicated selection protocols.[2,16] Our team has manufactured CAR-T cells without upfront T cell purification in most patients, obtaining favorable clinical activity[1,17–19] comparable with that of other groups who initiated $CD3^+$ T selection to manufacture CAR-T cells.[3,6] CART19 is activated by encounters with $CD19^+$ tumor or normal $CD19^+$ B cells, resulting in the proliferation of

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Correspondence: Alex H. Chang, Clinical Translational Research Center, Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai, China.
E-mail: alexhchang@yahoo.com
Correspondence: Chunrong Tong, Department of Hematology, Beijing Boren Hospital, No. 6, South Zhengwangfen, Fengtai District, Beijing 100070, China.
E-mail: tongcr@borenhospital.com

Table 1. Major differences *in vitro* and *in vivo* between the two groups

Groups/*ex vivo* CART19	Median leukemia cells (%)	Median $CD3^+$ T cells (%)	Median transfection efficiency (%)	Median cell viability (%)	Median cell expanding fold	Median TCM CART (%)	Median TEM CART (%)	Median $PD1^+$ CART (%)
Positive group (36 cases)	32.7 (3.23–83.2)	94.1 (80.3–98.5)	35.6 (4.82–56.1)	92.9 (52.6–97.5)	2.27-fold (0.24- to 15-fold)	80.1 (39.7–97.5)	0.01 (0.0–1.98)	7.31 (3.73–19.2)
Negative group (107 cases)	0	96.6 (80.1–99.5)	52.4 (9.44–82)	92.1 (64.7–98.5)	5-fold (0.38- to 33.9-fold)	81.06 (58.6–97.6)	0.13 (0.0–0.66)	5.26 (1.28–19.4)
p value	0.000	0.08	<0.001	0.087	0.0055	0.92	0.31	0.008

Groups/*in vivo* CART19	Median CART19 infusion dose	Median peak of CART19/lymphocyte cells (%)	Median peak of CART19 fold expansion	Median persistence time of CART19 (days)	Median peak of absolute CART19 count	Median occurrence time of peak cell count	Non-responders
Positive group (36 cases)	3 (0.33–20.7) × 10^5 cells/kg	9.67 (0–82)	5.6-fold (0- to 6,432-fold)	17 (0–71)	1.19 × 10^7/L blood (0–1.23 × 10^{10}/L)	day 11 (6–34)	9 cases
Negative group (107 cases)	5 (0.3–42.8) × 10^5 cells/kg	13.6 (0–71.4)	19.4-fold (0- to 2,920-fold)	29 (0–99)	9.92 × 10^7/L blood (0–3.8 × 10^9/L)	day 11 (6–35)	2 cases
p value	0.53	0.042	0.045	0.0001	0.044	0.38	<0.0001

CART19, chimeric antigen receptor T cells targeting CD19; TCMs, $CD45RO^+CD62L^+CCR7^+$; TEMs, $CD45RO^+CD62L^-CCR7^-$.

CAR-T cells and lysis of the target cell.[10] Prior research has demonstrated that the presence of malignant CLL cells during production might negatively affect CAR-T cell products;[20] however, whether $CD19^+$ leukemia cells in PB negatively influence *ex vivo* or *in vivo* cell characteristics and the subsequent clinical responses must be determined.

In the present study, we evaluated factors, namely the percentage of $CD3^+$ T cells, transduction efficiency, cell viability, fold expansion, memory phenotype, and programmed death-1 (PD-1) expression in the final CART19 products, and investigated the *in vivo* CART19 expansion, persistence, and subsequent clinical response in 143 r/r B-ALL patients.

RESULTS

The major differences between the two study groups are shown in Table 1.

Preclinical evaluation of CD19-directed CAR-T cell therapy

A lentiviral vector was used to carry a second-generation CD19-directed CAR with 4-1BB co-stimulatory and CD3ζ signaling domains (Figure 1A). A preclinical evaluation demonstrating the cytotoxicity of CAR-T cells in immune-deficient SCID/beige mice has been reported.[1] For each vector batch, we randomly selected two B-ALL patients to test the CART19 transduction efficiency and B cell elimination capacity before clinical use. We obtained a high transduction efficiency by using our vector in B-ALL patients (56%, Figure 1B, upper panel), and our CART19 demonstrated strong B cell elimination capacity in B-ALL patients (Figure 1B, lower panel).

Patient grouping and baseline characteristics

From patients treated between June 2017 and June 2019, 143 B-ALL patients were included in the analyses. A total of 117 patients had hematological relapsed/refractory B cell malignancies while undergoing chemotherapy or could not achieve CR by ≥1 cycle of chemotherapy after relapse, and 26 patients experienced continuous positive minimal residual disease (MRD^+) for more than 3 months. No patient had previously undergone allogeneic hematopoietic stem cell transplantation. The 143 patients were divided into two groups on the basis of flow cytometry (FCM) results before apheresis: positive group—36 patients with $CD19^+$ leukemia cells in PB, and negative group—107 patients without leukemia cells in PB. All 36 patients in the positive group had a high leukemia burden (≥5% BM blasts), whereas 81 patients had a high leukemia burden (≥5% BM blasts) and 26 patients had a low leukemia burden (<5% BM blasts) in the negative group. The positive group had a higher BM leukemia burden ($p < 0.0001$) and a higher proportion of complex chromosome abnormalities ($p = 0.01$) and *TP53* gene mutations ($p = 0.015$). Other baseline characteristics, including age, sex, weight, previous therapy period, and extramedullary disease (EMD) distribution, were not different between the two groups (Table 2).

Influence of PB tumor burden on the percentage of $CD3^+$ T cells, cell viability, fold expansion, and transduction efficiency in the final products

Beginning with the collection of PBMCs by apheresis, fresh cells were processed using density gradient centrifugation. To further evaluate the proportion of leukemia cells in total nucleated cells before culture, we assayed the separate products by using FCM. We found that 36 patients had a median of 32.7% (3.2%–83.2%, identified as $CD45^{-/dim}CD3^-CD10^+CD19^+$ population) $CD19^+$ leukemia cells (positive group), and 107 patients had no leukemia cells (negative group) in PB. We first determined whether the PB leukemia burden affected the percentage of $CD3^+$ T cells, cell viability, cell expansion, and transduction efficiency of the CART19 products. Compared with the negative group, the positive group demonstrated no significant difference in the percentage of $CD3^+$ T cells and cell viability at the time of the final CART19 harvest. The median

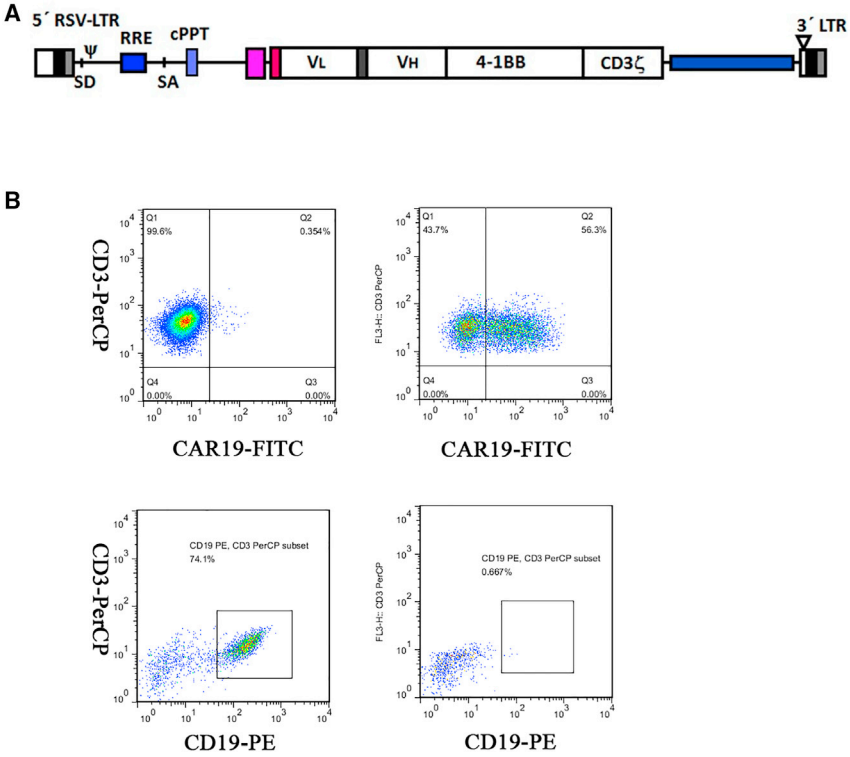

Figure 1. Preclinical evaluation of CD19-directed CAR-T cell therapy

(A) Schematic diagram of a CAR construct: a lentiviral vector carrying a second-generation CD19-directed CAR with 4-1BB co-stimulatory and CD3ζ signaling domains. (B) Representative FCM analysis of CAR expression in $CD3^+$ T cells and B cell elimination capacity (left, non-transduced T cells; right, transduced T cells). PBMCs were activated for 24 h, transduced with the indicated second-generation CD19-directed CAR-T cells or left untreated, and cultured for another 5 days. Transduction efficiency was determined by the ratio of $CD3^+CAR19^+$ T cells to $CD3^+$ T cells, and B cell elimination was determined by $CD3^-CD19^+$ cell analysis. FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin-chlorophyll protein.

percentages of $CD3^+$ T cells were 94.1% (80.3%–98.5%) and 96.6% (80.1%–99.5%) in the positive and negative groups, respectively ($p = 0.08$, Figure 2A). Cell viability in the positive and negative groups was 92.9% (52.6%–97.5%) and 92.1% (64.7%–98.5%, $p = 0.087$; Figure 2B), respectively. In contrast, the positive group had significantly lower transduction efficiency and cell fold expansion. The median transduction efficiency in the positive and negative groups was 35.6% (4.82%–56.1%) and 52.4% (9.44%–82%), respectively ($p < 0.001$, Figure 2C), and the cell expansion was 2.27-fold (0.24- to 15-fold) and 5-fold (0.38- to 33.9-fold) ($p = 0.0055$, Figure 2D), respectively.

T cell subpopulation and exhaustion marker evaluation of CAR-T cell production

Less-differentiated T cells with a high expression of lymphoid homing markers, such as CD62L and CCR7, can persist and engraft long term *in vivo*.[21,22] We tested 17 cases of CART19 in the positive group and 36 cases in the negative group (other products had been infused before analysis) to assess the differences in central memory-like T cells (TCMs, $CD45RO^+CD62L^+CCR7^+$) and effector memory-like T cells (TEMs, $CD45RO^+CD62L^-CCR7^-$). Figure 3 shows no significant differences in median TCM CART19 cells (with versus without PB tumor burden: 80.1% [39.7%–97.5%] versus 81.06% [58.6%–97.6%], respectively [$p = 0.92$, Figure 3A]) and median TEM-CART19 cells (with versus without PB tumor burden: 0.01% [0.0%–1.98%] versus 0.13% [0.0%–0.66%], respectively [$p = 0.31$, Figure 3B]) between the two groups. PD-1 on T cells might cause low response rates of CAR-T cells in hematological malignancies[23–25] and regulatory T (Treg) cells also decrease the antitumor activity of CAR-T cells.[26,27] Therefore, we tested PD-1 expression in CART19 products and the Treg cell ratio in the final products of these 53 cases. The results showed no obvious Treg cells in our culture system ($p > 0.99$, Figure 3C); however, PD-1 expression in CART19 products was significantly higher in the positive group than in the negative group (with versus without PB tumor burden: 7.31% [3.73%–19.2%] versus 5.26% [1.28%–19.4%], respectively [$p = 0.008$, Figure 3D]).

Functional evaluation of CART19

For all 143 patient-derived products, both normal B and leukemia cells were undetectable at the time of the final CART19 harvest. To verify whether these B cells were excluded by the culture system suitable for T cell growth or were specifically eliminated by CART19, we randomly chose three B-ALL patients with $CD19^+$ leukemia cells in PB and three B-ALL patients without leukemia cells in PB to culture non-transduced (control group) and transduced (test group) T cells for 6 days. Both $CD19^+$ leukemia and normal B cells were analyzed using FCM at the time of final CART19 harvest; a representative example from each group is shown in Figure 4 (sample 1. patients with PB blasts; sample 2, patients without PB blasts). Although $CD19^+$ B cells were mostly excluded from the culture system (from 89.4% to 3.75% in sample 1 and from 6.11% to 2.42% in sample 2 after 6 days in culture), the remaining $CD19^+$ B cells were completely eliminated by CART19 (from 3.75% to 0% in sample 1 and from 2.42% to 0% in sample 2 after 6 days in culture). Our data showed that although a few B cells were present in the culture system in the control group, normal B and leukemia cells were completely eliminated in the test group. Concurrently, the two representative examples showed high transduction efficiencies in our culture system (48% versus 27.7% in patients with or without PB blasts, respectively). Our data showed the negative group have statistically higher transduction efficiency than positive group (Figure 2C); however, the representative example with PB blasts had even higher transduction efficiency than the

Table 2. Baseline characteristics of patients in the two groups

	Positive group (n = 36)	Negative group (n = 107)	p value
Male	25	72	1.0
Median age (years)	13 (10 months to 42 years)	7 (10 months to 65 years)	0.15
≤18 years	25	81	0.51
>18 years	11	26	0.51
Weight (kg)	49.2 (7.5–115)	28 (8.9–105)	0.08
Previous therapy period (months)	10 (3–75)	16 (2–84)	0.3
Primary refractory	2/36	6/107	>0.999
Relapse	34/36	101/107	>0.999
EMD distribution	5/36	28/107	0.17
CNSL	4	21	0.32
TL	1	4	>0.999
Others	0	3	0.57
Leukemia burden			
Blasts in BM by morphology (%)	74 (13–97.5)	15.5 (<5–97)	<0.0001
<5% BM blasts	0/36	26/107	0.0003
≥5% BM blasts	36/36	81/107	0.0003
Blasts in BM by FCM (%)	60.4 (8.9–95.1)	9.76 (0.01–83.1)	<0.0001
Blasts in PB by morphology (%)	17 (1–65)	None	<0.0001
Blasts in PB by FCM (%)	16 (2–55)	None	<0.0001
Complex chromosome aberration	25/33	48/96	0.01
Fusion genes	16/33	55/97	0.43
BCR-ABL	6/33	12/97	0.4
Gene mutations	26/28	51/60	0.49
TP53	11/28	9/60	0.015

EMD, extramedullary disease; CNSL, central nervous system leukemia; TL, testicular leukemia; BM, bone marrow; FCM, flow cytometry.
Not all patients had available complex chromosome aberration, fusion genes, and gene mutations.
"Others" refers to knee joint, pleura, pancreas, etc.

representative example without PB blasts. The likely reason may be age (the former is 10 years old and the latter is 42 years old), as age has been observed to influence the transduction efficiency in our study (data not shown).

In vivo characteristics of CAR-T cells in the two groups

The duration of CAR-T cell culture ranged from 5 to 7 days, and median CART19 infusion doses in the positive and negative groups were 3 (0.33–20.7) × 10^5 cells/kg and 5 (0.3–42.8) × 10^5 cells/kg (p = 0.53, Figure 5A), respectively. The efficacy of CAR-T cell therapy is associated with *in vivo* proliferative capacity and sustained persistence.[28] Thus, we subsequently monitored CART19 *in vivo* in all patients. After CART19 infusion, peak CART19/lymphocyte cells (%), cell fold expansion, cell persistence duration, peak CART19 count, and occurrence time were compared between the two groups. The peak CART19 percentage in PB circulation was lower in the positive group than in the negative group, with a median peak of CART19/lymphocyte cells of 9.67% (0%–82%) versus 13.6% (0%–71.4%), respectively (p = 0.042, Figure 5B). The peak CART19 fold expansion *in vivo* was lower in the positive group than in the negative group, with a median peak expansion of 5.6-fold (0- to 6,432-fold) versus 19.4-fold (0- to 2,920-fold), respectively (p = 0.045, Figure 5C). The median persistence time of CART19 was 17 days (0–71 days) in the positive group versus 29 days (0–99 days) in the negative group (p = 0.0001, Figure 5D). In addition, the median peak of the absolute CART19 count was 1.19 × 10^7/L blood (0–1.23 × 10^{10}/L) in the positive group and 9.92 × 10^7/L blood (0–3.8 × 10^9/L) in the negative group (p = 0.044, Figure 5E). The peak CART19 count occurred on day 11 (day 6–34) and day 11 (day 6–35) in the positive and negative groups, respectively (p = 0.38, Figure 5F).

Product quality and clinical response

Of the original 143 products, three (2.1%) failed the first manufacture because the number of CART19 did not meet the release criterion for production of 3 × 10^4 cells/kg. All three cultures were initiated from the positive group. Following failure, a second manufacture was performed. We greatly increased PBMCs in the starting material and shortened the culture time in these three patients, and these adjustments allowed for greater harvest to meet the release criterion. On day 30 post-CART19 infusion, all 143 patients were evaluated. From the 117 patients with a high disease burden (≥5% BM blasts, including 36 cases in the positive group and 81 cases in the negative group), 91.5% (107/117) achieved CR or incomplete count recovery (CRi) and 86.3% (101/117) achieved MRD^- CR, whereas of the 26 patients with a low disease burden (<5% BM blasts) in the negative group, 96.2% (25/26) achieved MRD^- CR. A total of 75% (27/36) in the positive group and 98.1% (105/107) in the negative group achieved a CR or CRi response (Figure 6A); therefore, the positive patient group had a significantly lower response rate to CART19 therapy than the negative group (p < 0.0001, Figure 6B).

There was no difference in CART19 infusion doses between the two groups (Figure 5A), and an infusion dose <1 × 10^5 cells/kg was a significant factor influencing clinical response (Figure 6B, p = 0.0001), mainly in the positive group (p = 0.0018) but not in the negative group (p = 0.14). Six of the 11 patients with no response (54.5%) were infused at < 1 × 10^5 cells/kg because their cultures failed to expand. Overall, five cultures were initiated from the positive group and one was initiated from the negative group. Two non-response patients in the negative group were *BCR-ABL* fusion gene positive. CART19 expansion *in vivo* was notably related to clinical response (Figure 6C, p < 0.001), mainly in the positive group (p < 0.0001) but not in the negative group (p = 0.09). Of the 11 patients who did not respond, six of nine in the positive group and one of two in the negative group had no detectable CART19 in PB after infusion for 3 months. Among the five non-response patients who

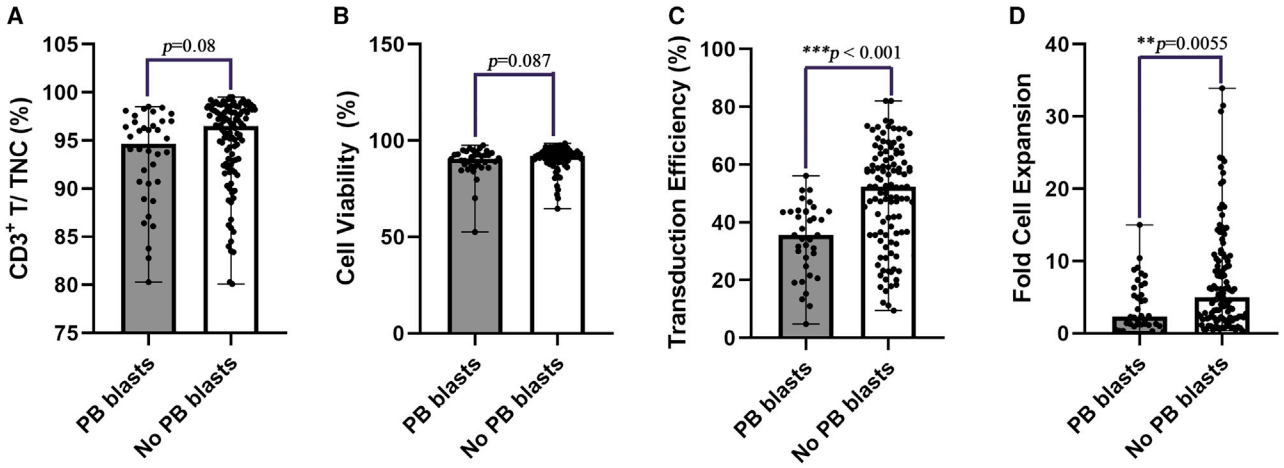

Figure 2. Influence of PB tumor burden on the percentage of CD3$^+$ T cells, cell viability, cell expansion, and transduction efficiency

CART19 generation was performed using PBMCs from B-ALL patients with PB blasts (positive group, n = 36; gray column) or without PB blasts (negative group, n = 107; white column). PBMCs were activated on day 0, transduced with a second-generation CD19-directed CAR on day 1, and cultured for 5–7 days. The ratio of CD3$^+$ T cells in total nucleated cells (A), cell viability (B), transduction efficiency (C), and fold cell expansion (D) were assessed at the time of the final CART19 harvest. These parameters were determined by trypan blue staining or FCM. An unpaired two-tailed Mann-Whitney test was used. Each symbol indicates an individual patient, the middle line denotes the median, and whiskers indicate the minimum and maximum. **p < 0.01, ***p < 0.001.

received $<1 \times 10^5$ cells/kg in the positive group, three had no detectable CART19 *in vivo*.

DISCUSSION

Despite the marked success of CART19 therapy in B cell leukemia, data on whether leukemia burden in PB influences CART19 outcome in r/r B-ALL patients are currently lacking.

Collecting an apheresis product from a patient with high leukemic blasts can create adverse culture conditions when the blasts are not removed upfront.[15] Untreated CLL patients with high PB leukemic blasts remain below 10% of CD45RA$^+$CCR7$^+$ naive T cells within CAR-T cell products.[20] The upfront selection of T cell subsets before CART manufacture might be an option.[11,29] However, selecting T cells for transduction adds complexity and expense to the cell manufacturing and culture process. We achieved 91.5% CR or CRi and 86.3% MRD$^-$ CR without upfront T cell purification in patients with high leukemia burden, which was higher than that achieved by other research groups who initiated T cell subset selection to manufacture CAR-T cells.[3,6,11] Our data showed that PB blasts during CART culture did not influence the final percentage of CD3$^+$ T cells and cell viability. Moreover, PB blasts during CART culture did not influence the cytotoxicity toward CD19$^+$ normal or abnormal B cells *in vitro*. We have demonstrated that CART19 in our culture system completely eradicated leukemia cells at the time of cell harvest; thus, the infusion of leukemia cells into patients could be avoided. A total of $\geq$80% CD3$^+$ T cells, $\geq$2% CAR-positive cells in CD3$^+$ T cells, $\geq$60% cell viability, $\geq$30,000 cells/kg CART19, and complete eradication of B cells were used as the release criteria for production. If B cells could not be completely eradicated or T cells had no effect on transduction, these products could not be infused in patients. In the present study, we did not exclude any patients based on a low absolute lymphocyte count or cell viability and an unsatisfactory CART19 cells/kg count at the time of infusion. One patient with cell viability <60% was included as a compassionate treatment.

Our culture system achieved high TCMs and low TEMs at the final CAR-T cell product, even when PB blasts were present. TCMs are associated with superior CAR-T cell antitumor efficacy.[30] Implementation of interleukin-7 (IL-7)/IL-15 and IL-21 into the CAR-T cell generation protocol might be one of the reasons for the high TCM and favorable clinical activity in our system.[31–33] Therefore, our CART19 products without upfront T cell purification were of good quality.

Our data also showed that PB leukemia burden led to a significant increase in PD-1 expression in CART19 at the time of infusion, similar to reports from other studies regarding CLL.[20,34] No Treg cells were detected in our culture system, regardless of whether a PB leukemia burden was present. One of the limitations of our study is that a relatively small number of products was evaluated for PD-1 expression, as other samples had already been infused in the patients prior to analysis. The PB leukemia burden hampered the transduction efficiency and expansion of CART19 products in our culture system, which was likely related to high PD-1 expression. A similar conclusion has been reported for CLL patients.[20] Whether PD-1 inhibition during CAR-T cell culture or the selection of T cells before CART manufacture constitute a promising treatment strategy in B-ALL patients with PB leukemia burden require further investigation.

The *in vivo* efficacy of CAR-T cells is linked to their proliferative capacity and long-term persistence for sustaining sufficient antitumor activity.[28,35] Treatment failure with CAR-T cells can be at least

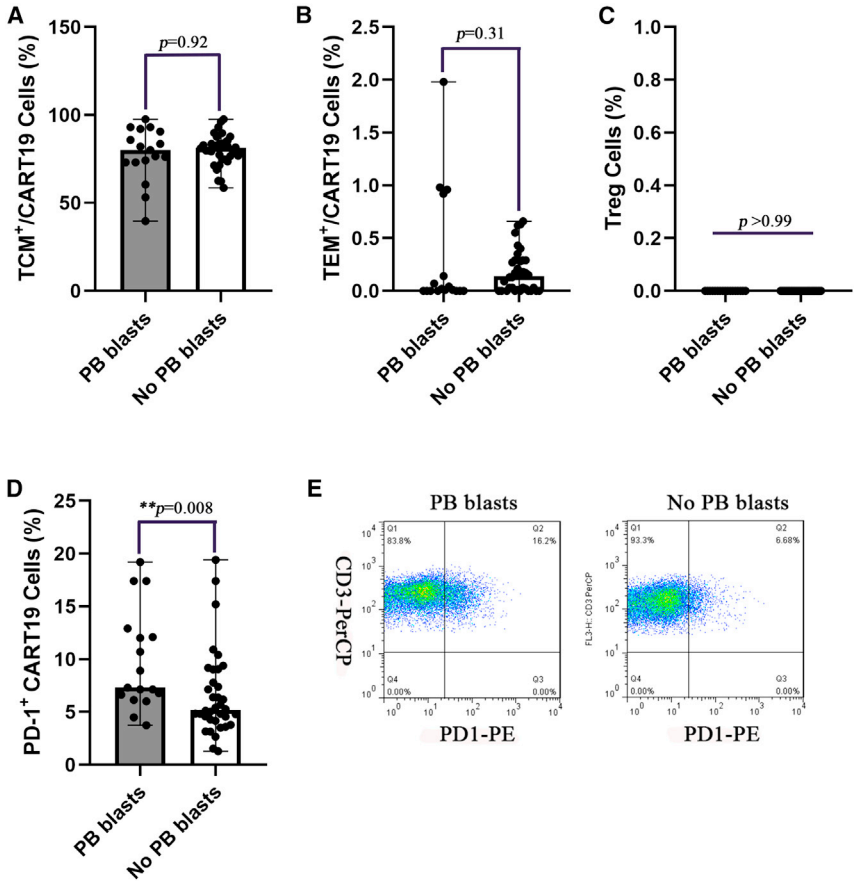

Figure 3. T cell subpopulation and exhaustion marker evaluation in the final products

TCMs were defined as $CD45RO^+CD62L^+CCR7^+$ cells (A), TEMs were defined as $CD45RO^+CD62L^-CCR7^-$ cells (B), and Treg cells were defined as $CD4^+CD25^+POXP3^+$ cells (C). (D) The exhaustion marker PD-1 was assessed in CART19. (E) Representative FCM display of PD-1 in CART19 (left, a positive group patient; right, a negative group patient). TCM-CART19, TEM-CART19, Treg cells, and PD-1^+ CART19 percentages were assessed in the final products from B-ALL patients either with PB blasts (positive group, n = 17; gray columns) or without PB blasts (negative group, n = 36; white columns). These data were measured using FCM. Unpaired two-tailed Mann-Whitney tests were used. In (A) to (D), each symbol indicates an individual patient, the middle line denotes the median, and whiskers show the minimum and maximum. **p < 0.01.

partially explained by their inadequate ability to proliferate and persist *in vivo*.[36] In the present study, PB leukemia cells at the time of apheresis hampered peak CART19/lymphocyte cells, absolute CART19 count, and peak CART19 fold expansion and persistence time *in vivo*. In contrast, many studies have reported that CART expansion is positively associated with disease burden at the time of CART infusion because of sufficient antigenic stimulation to drive it.[11,37] We were not able to conduct an assessment of peripheral disease burden at the time of infusion due to the short interval between apheresis and infusion, although the overall BM disease burden was higher in the cohort with PB leukemia burden at the time of apheresis. After CART19 infusion, patients with PB blasts at the time of apheresis secreted higher levels of cytokines than those without PB blasts (Table 3), suggesting that those patients with PB blasts at the time of apheresis had higher disease burden at the time of infusion. Because many studies have shown that cytokine release is positively correlated with disease burden,[2,37] our data showed that patients with PB blasts at the time of apheresis had weaker CART expansion *in vivo*; therefore, the negative influence of PB blasts at the time of apheresis could not be reversed by the positive influence of disease burden at the time of infusion. Antigen-driving expansion requires that the tumor burden be accounted for in the CAR-T cell growth equation. Our results demonstrated that impaired *ex vivo* characteristics affected the *in vivo* kinetics of CART19 cells. Nevertheless, CART expansion is heterogeneous because cellular kinetics are complicated.[38,39] The range was much greater for some patients in the positive group, reaching a much higher fold expansion than in the negative group, possibly because antigen-driven expansion was stronger than the negative influence of PB blasts in these patients (Figure 5).

In addition, the short CAR-T cell detection time in our study might in part be due to the low sensitivity of FCM relative to qPCR.[40] Additionally, the analysis of T cell persistence in the present study was compromised by the fact that these patients underwent subsequent CD22-CART therapy and allogeneic hematopoietic stem cell transplantation relatively soon after infusion with CAR-T cells (1–3 months).

In the present study, the baseline characteristics, including age, sex, weight, previous therapy period, and EMD distribution, did not differ between the two groups; therefore, these interference factors were excluded. One of the limitations of our study was the relatively small number of patients with PB blasts. Nevertheless, patients with PB blasts had a higher non-response ratio. Impaired CART19 products are thought to result in insufficient cell dose (less than 1×10^5 cells/kg) and absent expansion capacity *in vivo*, ultimately inducing a negative influence on clinical response. Additionally, patients with PB blasts have a higher proportion of complex chromosomal aberrations and *TP53* mutations, which negatively influences the therapeutic efficacy of CAR-T cells.[1,41] In patients with complex chromosomal aberrations and *TP53* mutations it might be more difficult to reduce PB leukemia burden by using previous therapy; therefore, these adverse factors indirectly influence *ex vivo* and *in vivo* CAR-T cell characteristics and ultimately influence therapeutic efficacy. In our study, two

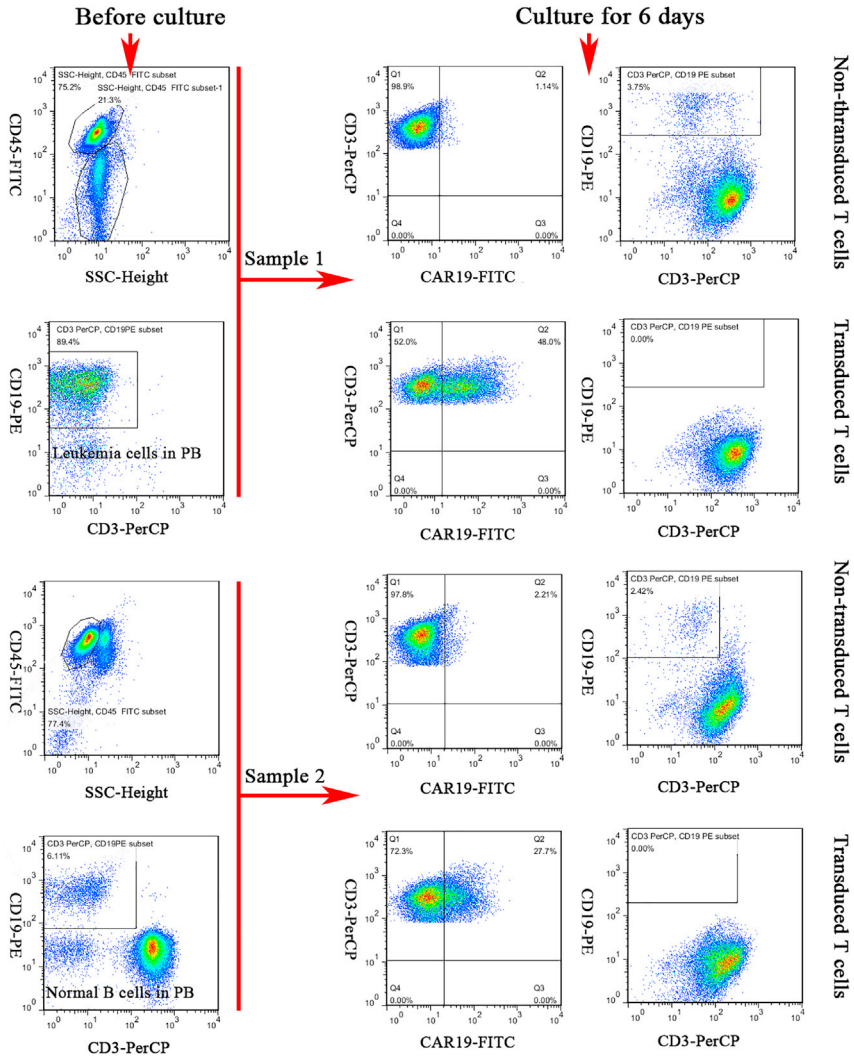

Figure 4. Functional evaluation of CART19

Functional evaluation of CART19 as determined by B cell elimination assays in the final products. A representative example from a B-ALL patient with PB blasts (Sample 1) or from a B-ALL patient without PB blasts (Sample 2) is shown. The ratio of normal B cells (identified as the $CD45^{positive}CD3^{-}CD19^{+}$ population) or abnormal B cells (identified as the $CD45^{-/dim}$ $CD3^{-}CD19^{+}$ population) was analyzed using FCM before culture and at the final CART19 harvest, respectively. PBMCs were activated on day 0 and non-transduced (control group) or transduced (test group) with a second-generation CD19-directed CAR on day 1, and cultured for 6 days. Transduction efficiency was determined by the ratio of $CD3^{+}CAR19^{+}$ T cells to $CD3^{+}$ T cells, and B cell elimination was determined according to the proportion of $CD3^{-}CD19^{+}$ cells. FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin-chlorophyll protein.

non-responders without PB blasts were both *BCR-ABL* fusion gene positive, implying that the *BCR-ABL* fusion gene might impede the efficacy of CAR-T cell therapy.[42] Collectively, complex chromosome aberrations, harmful genes, and leukemia burden in PB might be associated with resistance to CAR-T cell therapy in r/r B-ALL patients.

Conclusions

Our culture procedure without upfront T cell purification is a promising system for obtaining high CR or CRi in r/r B-ALL patients. However, the leukemia burden in PB negatively influences the CAR19 culture and its clinical efficacy in r/r B-ALL patients. Whether $CD3^{+}$ T cell selection prior to culture and PD-1 inhibition during culture might be helpful requires further investigation. To the best of our knowledge, the present study is one of the most extensive reported series of r/r B-ALL patients treated with CART19 and is the first to demonstrate the adverse effects of tumor burden in PB at the time of apheresis.

MATERIALS AND METHODS

Patients' characteristics

A retrospective data analysis was performed on 143 patients treated with CART19 products from r/r B-ALL patients manufactured at the Cytology Laboratory, Beijing Boren Hospital (Beijing, China) between June 2017 and June 2019. These treatments were approved by the Beijing Boren Hospital Institutional Review Board, and informed consent was obtained from all patients. The study was conducted following the principles of the Declaration of Helsinki. All patients met the diagnostic criteria for r/r B-ALL based on the World Health Organization classification and completed a morphological evaluation, immunophenotype analysis by using FCM, cytogenetic analysis by routine G-banding karyotype analysis, and leukemia fusion gene screening by using multiplex nested RT-PCR. The patients with B-ALL were treated with CART19 if they were previously heavily treated and failed reinduction chemotherapy after relapse or had continuous positive MRD for more than 3 months. All patients had positive CD19 expression on leukemia blasts upon FCM analysis (>95% CD19). Other baseline characteristics of the patients in the two groups are shown in Table 2.

Lentiviral construction and preclinical evaluation of CAR-T cells

A lentiviral vector was used to carry a second-generation CD19-directed CAR with 4-1BB co-stimulatory and CD3ζ signaling domains provided by Shanghai YaKe (Shanghai, China). The activity of the CAR-T cells based on this construct was evaluated *in vitro* using a cytotoxicity assay against $CD19^{+}$ B cells from B-ALL patients. The PBMCs were activated for 24 h with anti-CD3 (clone, OKT3) and anti-CD28 (clone, 9.3) monoclonal antibodies (T&L Biotechnology, Beijing, China), then transduced with or without the indicated

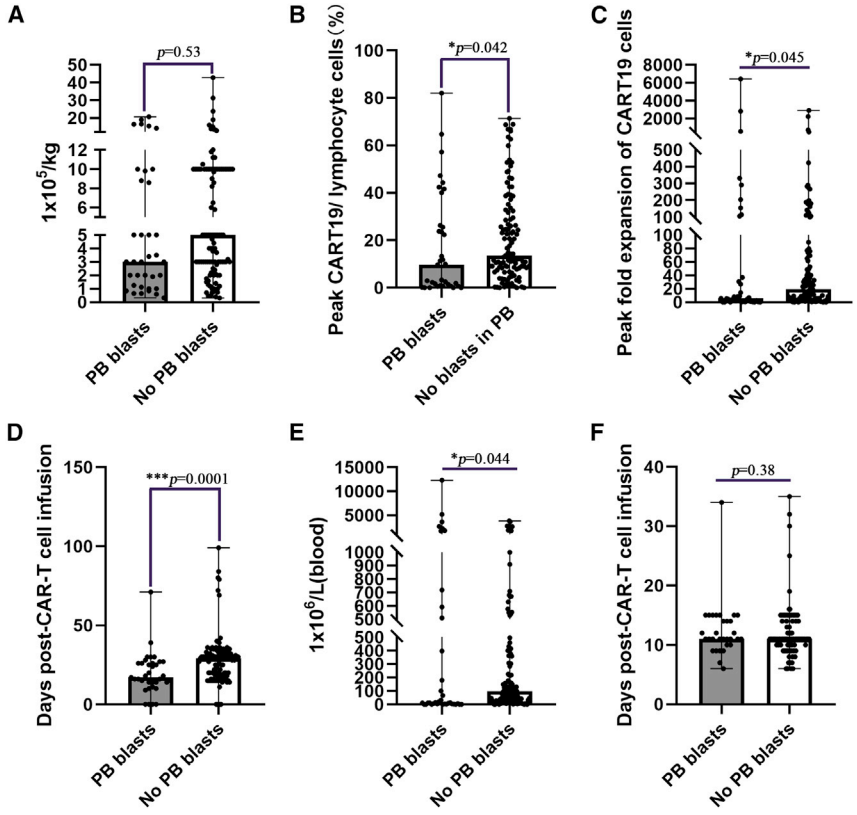

Figure 5. Comparison of *in vivo* characteristics of CAR-T cells in two groups

(A) There was no difference in CART19 infusion doses between the two groups. (B–F) Peak CART19 levels in lymphocyte cells (B), peak fold expansion of CART19 (C), CART19 persistence duration *in vivo* (D), peak CART19 count (E), and time to peak CART19 count (F), after CART19 infusion in the positive (n = 36; gray column) or negative (n = 107; white column) groups. Parameters were analyzed using a cell counter and FCM. Unpaired two-tailed Mann-Whitney test was used. Each symbol indicates an individual patient, the middle line denotes the median, and whiskers show minimum and maximum. *p < 0.05, ***p < 0.001.

second-generation CAR19 with 300 IU/mL IL-2 (Beijing Four Rings Biopharmaceutical, Beijing, China) and 5 ng/mL IL-7, IL-15, and IL-21 (PeproTech, Rocky Hill, NJ, USA), and cultured for another 5 days. The transduction efficiency and B cell elimination capacity were determined using FCM after 6 days of culture.

Generation of CAR-T cells

After apheresis, the PBMCs were treated with lymphocyte separation liquid (MD Pacific Technology, Tianjin, China) to remove granulocytes, red blood cells, and platelets. The manufacture of CAR-T cells from PBMCs commenced on the day of leukapheresis and was completed within 5–7 days. In brief, PBMCs collected from patients were stimulated with anti-CD3 and anti-CD28 monoclonal antibodies for 24 h, then transduced with the lentivirus encoding anti-CD19-CD3ζ-4-1BB CAR and cultured for another 4–6 days. On the next day, transduction was performed at a multiplicity of infection ratio of 1:5. The transduced cells were cultured in TexMACS Good Manufacturing Practices medium and animal-derived component-free and serum-free T cell culture medium (Miltenyi Biotec, Bergisch Gladbach, Germany) with 300 IU/mL IL-2 and 5 ng/mL IL-7, IL-15, and IL-21 for the duration of cell culture. The medium was replaced every 2 days. Five cases in the positive group and nine cases in the negative group were cryopreservation-thawed PBMCs, and two cases in the positive group and two cases in the negative group were cryopreservation-thawed CART19, using the cryopreservation medium CELLBANKER 2 (AMS Biotechnology, Abingdon, UK). Transduction efficiency and cell viability were examined at the time of cell infusion. In addition, CART19 cultures were tested for possible contamination with fungi, bacteria, mycoplasma, or endotoxins.

FCM and cell viability

Patients with or without PB blasts were determined using multicolor FCM assays, and the leukemia cell staining and gating strategy were determined using the clinical diagnosis standard.[1] The standard antibody panels consisted of CD45/CD34/CD10/CD19/CD22/CD79a/CD38 to determine PB blasts. $CD45^{bright}CD3^{-}CD19^{+}$ normal B cells and $CD45^{-/dim}CD3^{-}CD19^{+}$ abnormal B cells were monitored before culture and for the final CART19 products. The presence of CAR-T cells and the phenotype of the T cells were detected and quantified using multiparameter FCM of the PB from all patients. Surface marker staining was performed to assess the corresponding subpopulation markers on Treg cells ($CD4^{+}CD25^{+}FOXP3^{+}$), TCMs ($CD45RO^{+}CD62L^{+}CCR7^{+}$), and TEMs ($CD45RO^{+}CD62L^{-}CCR7^{-}$). Transduction efficiency was defined as the ratio of CAR-T cells to $CD3^{+}$ T cells determined using FCM using a proprietary anti-CD19 CAR-T cell-specific detection reagent (Shanghai Yake). The other antibodies were purchased from BD Pharmingen (San Diego, CA, USA), Miltenyi Biotec, Beckman Coulter, (Brea, CA, USA), and eBioscience (San Diego, CA). Cell viability and cell count were determined using trypan blue exclusion and a Cellometer Auto 2000 (Nexcelom Bioscience, Lawrence, MA, USA). The antibody clones included CD34 (clone, 8G12), CD45 (clone, 2D1) or CD45 (clone, HI30), CD3 (clone, SKT) or CD3 (clone, UCHT1), CD19 (clone, 4GT) or CD19 (clone, J3-119), CD10 (clone, HI10a), CD22 (clone, SHCL1), CD79a (clone, HM47), CD38 (clone, HB-7), CD45RO (clone, UCHL1) or CD45RO (clone, A07787), CCR7 (clone, G043H7), CD62L (clone, 145/15), CD4 (clone, SK3), CD25 (clone, 2A3), Fox P3 (clone, PCH101), and PD-1 (clone, NAT105).

Clinical response evaluation

Following leukapheresis, treated patients briefly received lymphodepleting chemotherapies comprising fludarabine (30 mg/m^2) and

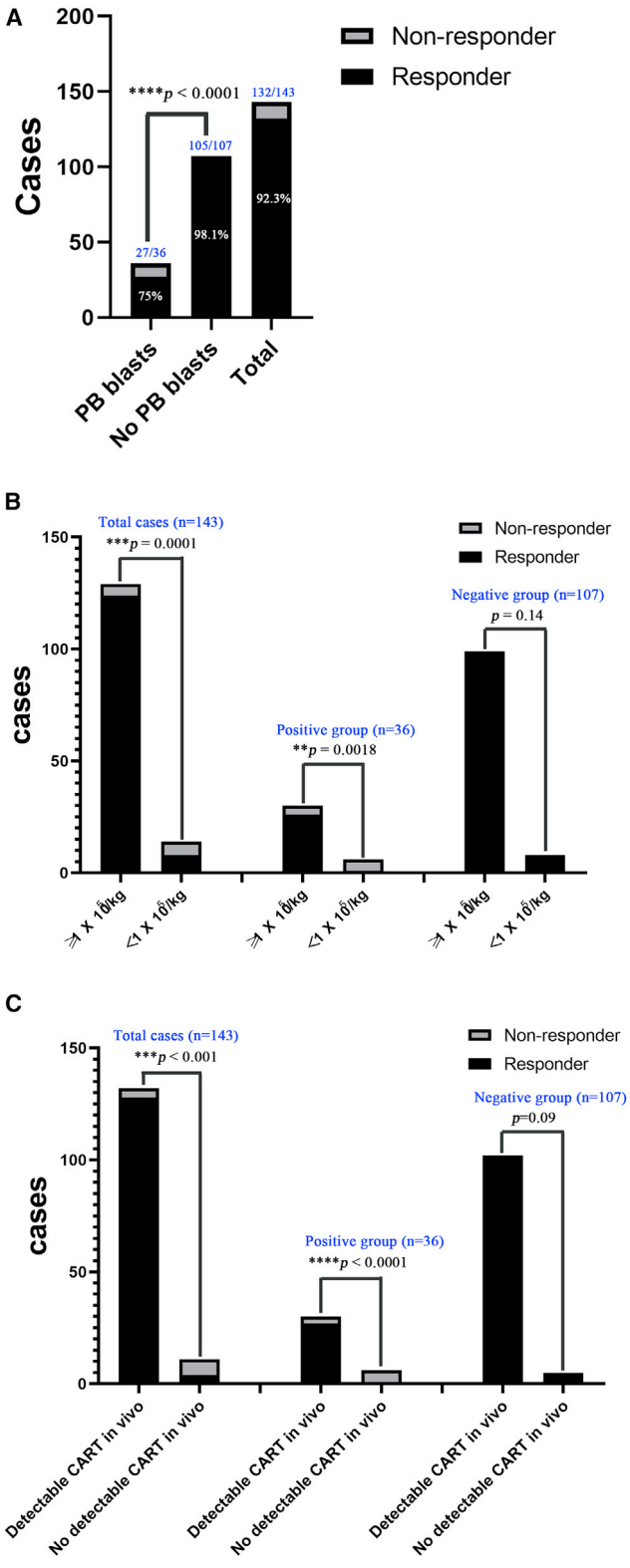

Figure 6. Clinical response

(A) The response rate in all evaluated patients was determined using morphological evaluation and immunophenotype analysis on day 30 after CART19 infusion. (B) Cases responding to CAR-T cell therapy in all patients (n = 143) and in positive (n = 36) or negative (n = 107) patients after receiving $\geq 1 \times 10^5$/kg or $<1 \times 10^5$/kg CAR-T cells. Correlation between CAR-T cell infusion dose and occurrence of an objective response. (C) Cases responding to CAR-T cell therapy in all patients (n = 143) or positive (n = 36) or negative (n = 107) patients, with or without detectable CAR-T cells in the PB after 3 months of infusion. Correlation between CAR-T cells *in vivo* and the occurrence of an objective response. A two-tailed Fisher's exact test was used for statistical analysis. **p < 0.01, ***p < 0.001, ****p < 0.0001.

cyclophosphamide (250 mg/m^2) on days −5, −4, and −3. On day 0, these patients received CART19 infusion. After infusion, PB CAR-T cell numbers in the patients were monitored using FCM. CAR-T cells in PB were measured on days 0, 7, 11, 15, and 30, or as necessary. All patients underwent BM biopsy examination and immunophenotype analysis using multicolor FCM on day 30 to determine their response and remission status. CR was defined as <5% BM blasts, absence of circulating blasts, and no extramedullary sites of disease, regardless of cell count recovery. CRi was defined as a complete response with ongoing cytopenia. A negative status for MRD was defined as less than 0.01% BM blasts, as assessed by multiparameter FCM.

Statistical analyses

The differences between two groups were analyzed using the unpaired two-tailed Wilcoxon-Mann-Whitney test. The correlation between the influencing factors and response rates was analyzed using a two-tailed Fisher's exact test. Statistical analyses were performed using IBM SPSS Statistics (IBM, Armonk, NY, USA) and GraphPad Prism software (GraphPad Software, San Diego, CA, USA). A threshold of $p < 0.05$ was considered statistically significant for all analyses.

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AUTHOR CONTRIBUTIONS

C.T. and B.D. contributed to the conception and design of this study. C.T. contributed to the clinical protocol management. J.P., S.L., and Y.L. were responsible for the recruitment and treatment of patients. A.H.C. contributed to the lentiviral vector design. Y.Z. contributed to the manufacture of lentiviral vectors. Z.L., X.Q., Y.Z., Z.Z., X.N., X.L., and T.L. contributed to the manufacture of CAR-T cells. Y.C., M.M., X.W., and H.N. contributed to CAR-T cell detection and quality control. R.L. contributed to the data acquisition and interpretation. X.Y. contributed to the FCM diagnostics and data analysis. B.D. wrote the initial draft of the manuscript. C.T. revised the manuscript. All authors take responsibility for the accuracy and completeness of the data. All authors have reviewed and approved the final manuscript.

Table 3. Cytokine release in the two groups

Group	Median of peak sCD25 (pg/mL)	Median of peak IL-10 (pg/mL)	Median of peak TNFα (pg/mL)	Median of peak IL-6 (pg/mL)	Median of peak IFNγ (pg/mL)
Positive group (n = 36)	10,132 (1,416–26,352)	120.4 (5.9–737.1)	31 (9.64–223)	81.3 (3.86–3,244)	132.4 (11.24–1,335.85)
Negative group (n = 71)	5,568 (1,128–31,696)	68 (8.48–769)	19.8 (0.47–219.9)	20.86 (1.5–898.7)	54.1 (5.42–1,380.7)
p value	0.0055	0.0074	0.0015	0.0005	0.0067

TNFα, tumor necrosis factor α; IFNγ, interferon-γ.
Only 71 cases in the negative group had cytokine release data.

DECLARATION OF INTERESTS

A.H.C. is a founding member of Shanghai YaKe Biotechnology Ltd., a biotechnology company focused on the research and development of tumor cellular immunotherapy. Y.Z. is an employee of Shanghai YaKe Biotechnology Ltd. The remaining authors declare no competing interests.

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