



# Treatment with Humanized Selective CD19CAR-T Cells Shows Efficacy in Highly Treated B-ALL Patients Who Have Relapsed after Receiving Murine-Based CD19CAR-T Therapies

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## Abstract

**Purpose:** CD19 chimeric antigen receptor (CAR)-T therapy has shown impactful results in treatment of B-cell malignancies. However, immune recognition of the murine scFv may render subsequent infusion(s) ineffective. Also, nonselective expansion of both CAR-transduced and nontransduced T cells during the production stage affects the yield and purity of final products. Here, we aim to develop a humanized selective (hs) CD19 CAR to solve the above problems.

**Experimental Design:** A CD19 hsCAR was designed, which incorporated a short selective domain between the humanized heavy chain and light chain. The CAR was examined for its property, and then trialed in 5 highly treated B-ALL patients.

**Results:** hsCAR possessed around 6-fold higher affinity to CD19 versus murine CAR (mCAR). Incubation with selective domain-specific mAbs (SmAb) selectively expanded CAR-

transduced T cells, and led to a higher proportion of central memory T cells in the final products. SmAb-stimulated CD19 hsCAR-T cells exhibited superior antitumor cytotoxic functions *in vitro* and *in vivo*. Autologous ( $n = 2$ ) and allogeneic donor ( $n = 3$ , with hematopoietic stem cell transplantation) hsCAR-T cells were infused into 5 patients who had relapsed after receiving mCAR-T treatments. Two patients received mCAR-T treatments twice previously but the second treatments were ineffective. In contrast, subsequent hsCAR-T treatments proved effective in all 5 patients and achieved complete molecular remission in four, including one with extramedullary disease with central nervous system involvement.

**Conclusions:** hsCD19 CAR-T treatment shows efficacy in highly treated B-ALL patients who have relapsed after receiving CD19 mCAR-T therapies.

## Introduction

Clinical trials using CD19 chimeric antigen receptor (CAR)-T cells have shown impressive results in treatment of B-cell malignancies such as acute lymphoblastic leukemia (ALL; refs. 1–3), chronic lymphocytic leukemia (CLL) (4), and non-Hodgkin lymphoma (5, 6). The scFv sequences used in these published

clinical trials were based on murine antibody sequences (FMC63- or SJ25C1-mAbs; refs. 7–9). Recent studies have confirmed that host immune responses can recognize the epitopes of the murine scFv and rendered subsequent infusions ineffective (10–14).

Another problem with current technology is that generation of CAR-T cells before infusion normally involves nonselective expansion of all CD3<sup>+</sup> T cells. Patient-derived T cells showed a large variation in the proportion of CAR-transduced T cells (5%–50%) in the final products (15, 16). The variation in CAR transduction rates, and the nonselective expansion of both CAR-positive and -negative T cells prior to infusion, added uncertainty to quality control and interpretation of clinical results, sometimes even leading to failure of CAR-T manufacture. To address this issue, a selective domain can be incorporated in the linker sequence between the heavy chain and light chain. LaSS-B, a stress-dependent shuttling autoantigen containing 408 amino acids, is a natural protein that exists in human cell nucleus (17, 18). A short peptide derived from LaSS-B, an epitope termed E-tag, can be incorporated into the linker area of two particular CARs recognizing prostate stem cell antigen (PSCA) or CD33, without affecting their functionality (19).

In this study, we developed a humanized CD19 CAR and incorporated the 10-amino acid E-tag as a selective domain between the heavy and light chains of scFv. Through activation with selective domain-specific mAbs, SmAb, CAR-transduced T cells were selectively expanded that led to a higher purity and a greater proportion of T central memory (T<sub>cm</sub>) cells in the final

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## Translational Relevance

In this study, a humanized selective (hs) CAR targeting human CD19 was developed. The hsCAR shows around 6-fold higher affinity to human CD19 than the murine counterpart. Incorporation of a selective domain between the heavy and light chains has several-fold merits. First, CAR-transduced T cells can be selectively expanded during the production process; second, stimulation with selective domain-specific mAb promoted central memory T-cell differentiation; third, hsCAR-T cells possessed a superior antitumor function *in vitro* and *in vivo*. The results led to a clinical trial on five highly treated patients who had relapsed after receiving murine CD19 CAR-T, and achieved complete molecular remission in four. The study offers a solution to treat relapsed patients with previous murine CD19 CAR-T therapies; future large cohort study may help to determine whether hsCAR could replace murine CAR as a treatment option.

product. Humanized selective (hs) CD19-specific CAR T cells were used to treat 5 B-ALL patients who had previously received murine scFv CD19 CAR-T therapies.

## Materials and Methods

### CD19 hsCAR design and virus production

CD19 hsCAR comprised a Kozak consensus ribosomebinding sequence, a human CD8 signal peptide (SP), the humanized sequences encoding the VH and VL regions referring to US7635472B2 with the order of VH and VL reversed, and a 20-residue linker peptide containing a 10-amino acid selective domain derived from human nuclear protein, LaSS-B. The humanization of the VH and VL sequences was confirmed by using a web-based program at [www.abysis.org](http://www.abysis.org). The CAR also included a hinge domain, a transmembrane region of the human CD8a molecule, and an intracellular signaling domain containing 41BB used as a costimulation domain, and CD3 $\zeta$  domains used as activation regions. CD19 hsCAR gene was cloned into pLenti6.4 lentiviral vector backbone (Thermo Fisher Scientific) for production of the third-generation lentivirus.

CD19 hsCAR lentivirus was generated and stored at  $-80^{\circ}\text{C}$ . Concentrated viral titers were determined by a limiting dilution method on HT1080 cell line using flow cytometry, as described previously (20).

### Binding affinity assay

The binding affinity of CD19 hsCAR was assayed by using microscale thermophoresis (MST) following the protocol as described previously (21). Briefly, purified recombinant extracellular domains of human CD19, CD19 hsCAR and murine-based CAR (derived from FMC63 clone, named as CD19 mCAR) were obtained by expression in HEK293T, and then purified by going through Ni-NTA columns. The extracellular domain of CD19 was labeled by NT-647 using Mo-L001 MonolithNT<sup>MT</sup> protein labeling kit RED-NHS (NanoTemper) following the manufacturer's instructions, and was added into the reaction system as ligand; then purified extracellular domains of CD19 hsCAR and CD19 mCAR were incubated with target for binding affinity test at room temperature. Affinity value was measured by Monolith NT.115

(NanoTemper). The results were analyzed by MO Affinity analysis software (version 5).

### Cell lines

The cell lines used in this study were obtained from the following sources. HEK293T was a kind gift from Palmer lab at Stanford University (Stanford, CA). Raji, a human Burkitt lymphoma cell line expressing CD19, and HT1080 cell line were purchased from TongPai Biotechnology Co., Ltd (Shanghai, China). Raji cells constitutively expressing luciferase (Raji-Luc) was generated by Beijing Vitalstar Biotechnology Co., Ltd. The cell lines were tested to be Mycoplasma-free by using Rapid Mycoplasma Test Kit (Cellapbio). HEK293T was cultured in DMEM. Raji, Raji-Luc, and HT1080 were maintained in RPMI1640. DMEM and RPMI1640 media were supplemented with 2 mmol/L L-glutamine, 10 mmol/L HEPES, and 10% heat-inactivated FBS. After thawing, the cell lines were used within 5 passages for the experiments in this study.

### CAR-T manufacture and quality control

Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque gradient centrifugation. CD3<sup>+</sup> T cells were purified and activated by CD3/CD28 magnetic beads (Gibco) at the ratio of 1:2. Purified T cells were then seeded into T75 culture flasks with a density of  $1.0 \times 10^6/\text{mL}$  for activation in X-VIVO 15 medium (Lonza) containing 200 IU/mL IL2 (XinLuoEr). The transduction was implemented by adding CD19 hsCAR- or CD19 mCAR-lentivirus with MOI of 25 within 24 to 48 hours. Transduction efficiency was evaluated by flow cytometric assay at day 5. For SmAb-induced restimulation, SmAb mAb (Sino Biological Inc.) was diluted by PBS and precoated in the culture flask at a concentration of 20  $\mu\text{g}/\text{mL}$  overnight at  $4^{\circ}\text{C}$ . Expanded CD19 hsCAR-transduced T cells were transferred into SmAb-coated flask for restimulation on day 6 or day 7, according to the cell proliferation status. Final product was harvested from day 9 to day 12, depending on the cell proliferation status and yield, and was subjected to quality control for subsequent experiments or clinical administration (Supplementary Fig. S1A). For proliferation assay and population analysis, T cells were cultured for up to 15 days *in vitro*. PBMCs isolated from three healthy donors were used in preclinical tests for CAR-T manufacturing and functional evaluation.

Quality control for in-process product and final product was conducted according to the previous description (16). In general, sterility was tested by a 3-day cultivation prior to final product harvest. *Mycoplasma* test was performed by using Rapid Mycoplasma Test Kit (Cellapbio) following the manufacturer's instructions. Transduction efficiency and constitution were examined by using flow cytometry.

### Flow cytometry

The following anti-human antibodies were purchased from BioLegend (BioLegend): anti-human CD3-FITC, anti-human CD19-PE, anti-human CD8-PerCP, anti-human CD4-APC, anti-human CD56-PerCP, anti-human CD14-APC, anti-human CD45RO-PE, anti-human CD197-APC, anti-human CD27-FITC, anti-human CD45RA-FITC, anti-human PD-1-APC, streptavidin-PE. Biotin-labeled protein-L was obtained from ACROBiosystems (ACROBiosystems) for CAR-positive T-cell measurement, and detection of biotinylated protein-L was implemented by adding streptavidin-PE (22). Flow cytometry was performed by using

FACSCalibur (BD), and data were analyzed by using FlowJo (Treestar).

### ***In vitro* cytotoxicity assay**

The cytotoxic activity on Raji was conducted by standard lactate dehydrogenase (LDH) release method. Target Raji cells ( $1 \times 10^4$ ) were incubated with T cells transduced by CD19 hsCAR or CD19 mCAR at various effector/target (E/T) ratios (from 1:1 to 25:1) in 96-well microplates. After 12-hour cultivation at 37°C, the supernatant was harvested and cytotoxicity measured by using CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega), following the manufacturer's instructions.

### **Cytokine release assay**

Cytokines were measured by ELISA or electrochemiluminescence (MSD) assay (Meso Scale Discovery). *In vitro* cell-mediated cytotoxic cytokines, including IL2, IFN $\gamma$ , and TNF $\alpha$ , were tested by ELISA. Briefly, samples of supernatants were collected and measured using ELISA Kits (Neobioscience). Cytokines in serum samples from patients and animals were measured by Cytokine Panel 1 (human) Kit of MSD MULTI-SPOT Assay system (Meso Scale Diagnostics). ELISA data were acquired by using VARIOSKAN FLASH (Thermo Fisher Scientific), and MSD assay data acquired by using QuickPlex SQ120 system (Meso Scale Diagnostics).

### **Animal study**

Six- to 8-week-old female NOD/SCID IL2R $\gamma$ <sup>-/-</sup> NSG mice were obtained from the Beijing Vitalstar Biotechnology Co., Ltd. Mice were injected intravenously with  $1.0 \times 10^6$  Raji-luc tumor cells through the tail vein, and received injections of  $1.0 \times 10^6$  CAR-modified or control T cells intravenously 3 days after tumor inoculation. Tumor progression and distribution were evaluated by serial bioluminescence imaging at indicated time points. Mice were given intraperitoneal injections of luciferin substrate (Promega) resuspended in PBS (15 mg/kg), and were anesthetized with chlorine ammonia ketone (100 mg/kg). Imaging was performed by using an IVIS Lumina K Serial III System (Caliper) from 12 to 14 minutes after luciferin injection in small binning mode at an acquisition time of 1 s to 1 min to obtain unsaturated images. Luciferase activity was analyzed by using Living Image Software (Caliper) and the photon flux analyzed within regions of interest that encompassed the entire body of each individual mouse. The animal studies have been approved by the local institutional review board.

### **Anti-CAR reaction assay using patient sera**

ELISA assay was used to determine anti-serum response targeting CD19 CAR containing murine-based scFv or humanized scFv. Serum was collected from patients who previously received murine-based CD19 CAR-T infusions. Recombinant extracellular domain of CD19 mCAR and CD19 hsCAR (1 mg/mL in PBS) were diluted to 4  $\mu$ g/mL using 0.1 mmol/L PBS, and were coated to the bottom of 96-well ELISA plates. Test wells were blocked for 30 minutes at 37°C using 1% BSA. Samples (100  $\mu$ L) were added into wells and incubated for 1 hour at 37°C. HRP-labeled goat-anti-human antibodies specific for IgA, IgG, or IgM (Beijing Zhuang-Meng Biotechnology Co., Ltd) were added into wells after 5 washes. TMB substrate solution was used for color development, and measurement performed by using microplate reader at 450 nm. Positive results were set at an OD450 value  $\geq 0.2$ .

### **Clinical trials using hsCAR-T cells**

Phase I clinical trials (ChiCTR1800014761 and ChiCTR1800017439) were conducted to evaluate the safety and efficacy of CD19 hsCAR-T treatment. The following major inclusion criteria were used: (i) age < 75 years; (ii) patients with CD19<sup>+</sup> refractory/relapse B-cell lymphoma, acute B lymphocytic leukemia, and chronic B lymphocytic leukemia, including patients who had previously been administered murine-based CD19 CAR-T cells; and (iii) patients with MRD-positive refractory/relapse acute B lymphoblastic leukemia, B-cell lymphoma, and chronic B lymphoblastic leukemia. All clinical investigations were conducted according to the ethical guidelines of Declaration of Helsinki. The clinical studies were performed after approval by the Institutional Review Board. The investigators have obtained written consent from all patients enrolled and treated in this trial.

Prior to CAR-T cell infusion, patients were treated with a fludarabine/cyclophosphamide (FC)-based lymphodepletion chemotherapy scheme consisting of intravenous administration of fludarabine (30 mg/m<sup>2</sup> for 3 days) and cyclophosphamide (250 mg/m<sup>2</sup> for 3 days) to remove endogenous lymphocytes. On day 0, the patients received a single dose of CD19 hsCAR-T infusion at a dosage of 0.3 to  $3 \times 10^6$  cells/kg body weight. The hsCAR-T dosage was determined by tumor burdens, previous murine CAR-T dosage used, and side effects, particularly cytokines release syndrome (CRS) grades following previous murine CAR-T treatment(s). Patients 1 and 2 were treated with autologous CAR-T cells, and patients 3, 4, and 5 with CAR-T cells produced from hematopoietic stem cell transplantation (HSCT) donors' PBMCs. Safety and efficacy were assayed according to the criteria of the National Comprehensive Cancer Network (NCCN) guideline, Acute Lymphoblastic Leukemia (Version 1. 2018).

### **CAR-T proliferation and persistence *in vivo***

Blood samples were obtained from patients before and at intervals after CD19 hsCAR-T infusions, and flow cytometry was performed to analyze the persistence of CAR-T cells. Circulating CD19 hsCAR-T cell numbers per 100  $\mu$ L were calculated on the basis of the absolute CD3<sup>+</sup> T lymphocyte counts. In addition, CAR DNA copies were assessed by qPCR (23) using primers specific to the scFv region of CD19 hsCAR. The primer sequences were listed below:

Forward primer, 5'-GTCTGAACCGGCTGGGGATG-3';  
Reverse primer, 5'-TGGATGCAGCCCGCCATTAG-3'.

### **Statistical analysis**

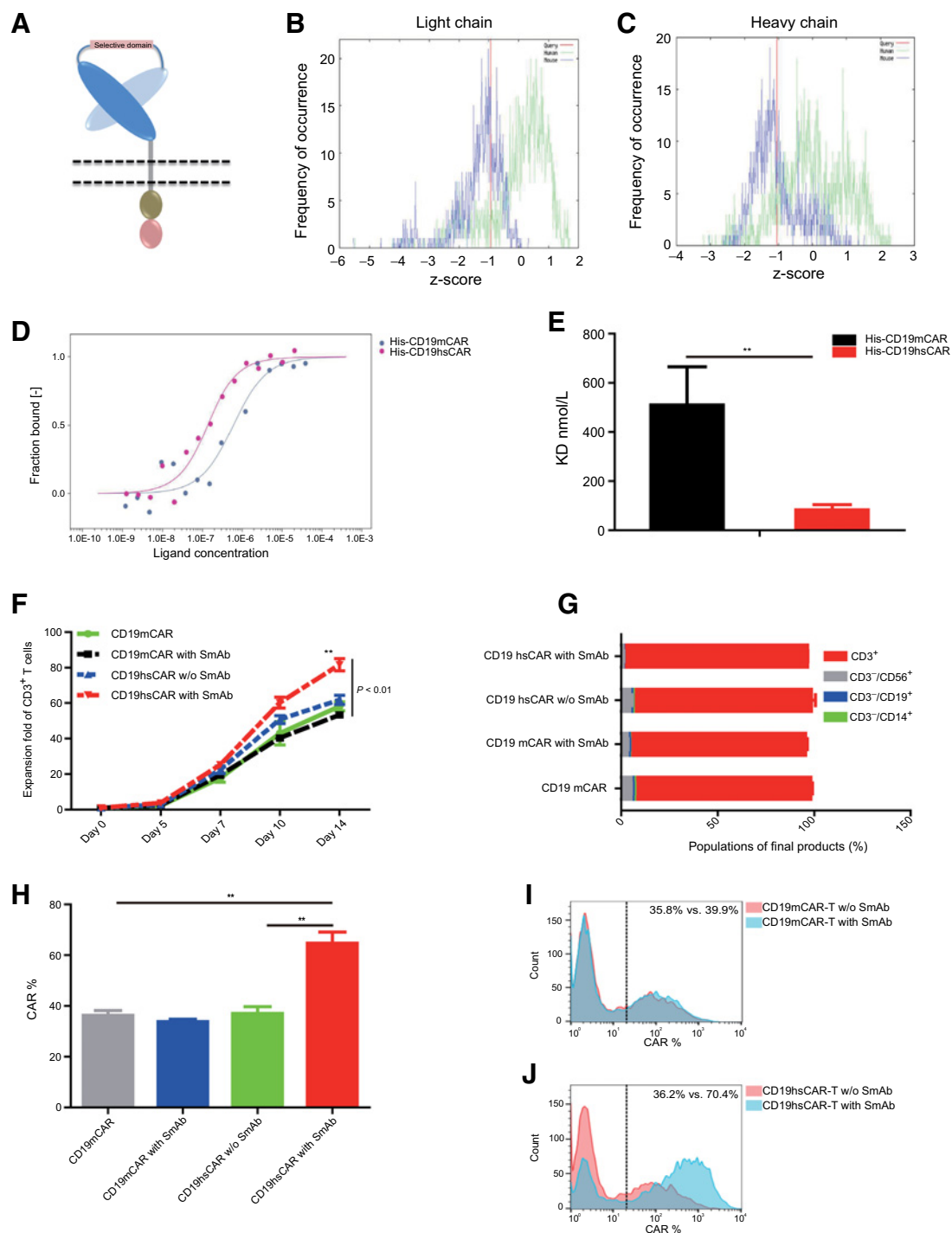
Data were presented as mean  $\pm$  SEM. Statistical analyses were conducted using Prism Software (GraphPad Software). For comparison between two groups, Student *t* test was conducted as a two-sided paired test with a confidence interval of 95%. For comparisons of three groups and above, the analysis was performed by using ANOVA. Results with a *P* value less than 0.05 were considered significant.

## **Results**

### **Design and property of humanized selective CD19 CAR**

The design of humanized selective CAR was described in Materials and Methods and illustrated in Fig. 1A.

An improvement to the traditional CD19 CAR was the humanized sequence of the scFv region (Fig. 1B and C). Murine CD19

**Figure 1.**

Design and evaluation of CD19 hsCAR. **A**, Schematic representation of CD19 hsCAR construct containing scFv regions and a selective domain in the linker sequence. **B** and **C**, The level of humanization for VH and VL were evaluated. **D** and **E**, The binding affinity of CD19 hsCAR with human CD19 extracellular domain was measured by MST assay. FMC63, murine-based CD19 CAR (CD19 mCAR) was used as a control. KD of CD19 hsCAR was 6-fold smaller than that of CD19 mCAR. **F**, SmAb, a monoclonal antibody targeting the selective domain, could re-stimulate hsCAR-transduced T cells, and significantly increased the yield (measured by total CD3<sup>+</sup> cells) compared with that of CD19 hsCAR-engineered T cells without SmAb re-stimulation and that of CD19 mCAR-engineered T cells. **G**, Composition of subpopulations in the final products prepared by using different conditions. **H–J**, SmAb restimulation resulted in significantly higher proportion of CAR-positive T cells in CD19 hsCAR-T group compared with CD19 mCAR-T group with or without SmAb exposure, and with group of CD19 hsCAR-T without SmAb exposure. Three independent assays were performed using 3 healthy donor blood samples, and each value represented mean  $\pm$  SEM in this and all the other figures of this study. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  for this and other figures in this study.

scFv sequence may be recognized by the host immune system and rendered the second murine CD19 CAR-T cell infusion ineffective (10, 14, 24). The humanization of the scFv region was in agreement with the prediction of the online software at [www.abysis.org](http://www.abysis.org) (Fig. 1B and C). An important determinant of the quality of CAR is the specificity to antigen. Using purified human CD19 protein as target antigen, we tested the affinity of murine CD19 CAR and CD19 hsCAR. The kinetics of binding between murine and humanized selective CARs to the ligand (hCD19) was plotted by using MST assay (Monolith NT.115, NanoTemper; Fig. 1D), and the KD values for CD19 mCAR and hsCAR were  $509.4 \pm 89.8$  and  $83.4 \pm 12.2$  nmol/L, respectively (Fig. 1E), suggesting that hsCAR possessed an affinity that was about 6-fold greater than that of mCAR.

Also included in the design of CD19 hsCAR was the selective epitope E-tag (17). Selective domain-specific mAb, SmAb, was coated to the bottom of plates during the *ex vivo* expansion stage. Compared with CD19 mCAR-transduced PBMCs with or without SmAb stimulation and CD19 hsCAR-transduced PBMCs without exposure to SmAb, CD19 hsCAR-transduced PBMCs exposed to SmAb showed a significantly greater proliferative capacity. On day 14, hsCAR-infected PBMCs exposed to SmAb expanded  $81.6 \pm 3.38$  times, while hsCAR-infected PBMCs without exposure to SmAb expanded  $61.7 \pm 2.58$  times, mCAR-infected PBMCs without SmAb stimulation expanded  $58.2 \pm 2.55$  times and  $53.26 \pm 1.79$  times with SmAb treatment (Fig. 1F).

On day 14, among the final product, more than 95% of the total cells were CD3<sup>+</sup> T cells; a small percentage of the total cells (<5%) were CD3<sup>+</sup>/CD56<sup>+</sup> natural killer (NK) cells, and few CD3<sup>+</sup>/CD19<sup>+</sup> B cells or CD3<sup>+</sup>/CD14<sup>+</sup> cells were detected by flow cytometry (Fig. 1G). Among the CAR-positive cells, the proportions of each population were presented in Supplementary Fig. S1B.

Evaluation of CAR-positive cells among the final products revealed that, on day 14,  $64.73\% \pm 4.4\%$  of total cells were positive for CAR in the "CD19hsCAR + SmAb" group, significantly higher than those of the other three groups (Fig. 1H–J). The results confirmed that the selective domain can be used to specifically expand the CAR-transduced cells.

#### CD19 hsCAR-T cells show improved antitumor cytotoxicity *in vitro*

CAR-T cells of different groups (CD19 mCAR, CD19 mCAR with SmAb, CD19 hsCAR w/o SmAb, CD19 hsCAR with SmAb) were incubated with Raji cell line at various E/T ratios. At the four ratios tested (1:1, 6.25:1, 12.5:1, and 25:1), CD19 hsCAR with SmAb all showed superior cytotoxicity versus the other three groups (Fig. 2A). Interestingly, at lower E/T ratios (6.25:1), CD19 hsCAR w/o SmAb also showed a greater cytotoxicity compared with CD19 mCAR-T cells with and without SmAb restimulation. Accordingly, CD19 hsCAR with SmAb, when incubated with Raji cells at E/T ratio 25:1, led to increased cytokine release of IL2, IFN $\gamma$ , and TNF $\alpha$  in the medium, as detected by ELISA (Fig. 2B).

It is interesting to observe that prior activation with SmAb resulted in an improved cytotoxic function of T cells *in vitro*. We next examined whether such activation led to changes in proportions of T memory cell subpopulations. Compared with CD19 mCAR, CD19 mCAR with SmAb and CD19 hsCAR w/o SmAb groups, exposure of CD19 hsCAR group to SmAb led to a greater proportion of central memory T (Tcm) cell subpopulation and a smaller proportion of effector memory T-cell subpopulation

(Fig. 2C–E). The proportions of each subpopulation among the CAR-positive cells were presented in Supplementary Fig. S1C and S1D.

To investigate which downstream signaling pathways had contributed to the enlarged Tcm subpopulation, we examined various pathways associated with T-cell differentiation, and found that ERK1/2, STAT3, P38, and AKT pathways were all activated by SmAb activation (Fig. 2F–K). The results indicated that SmAb restimulation could potentially induce T-cell differentiation via regulation on different signaling pathways.

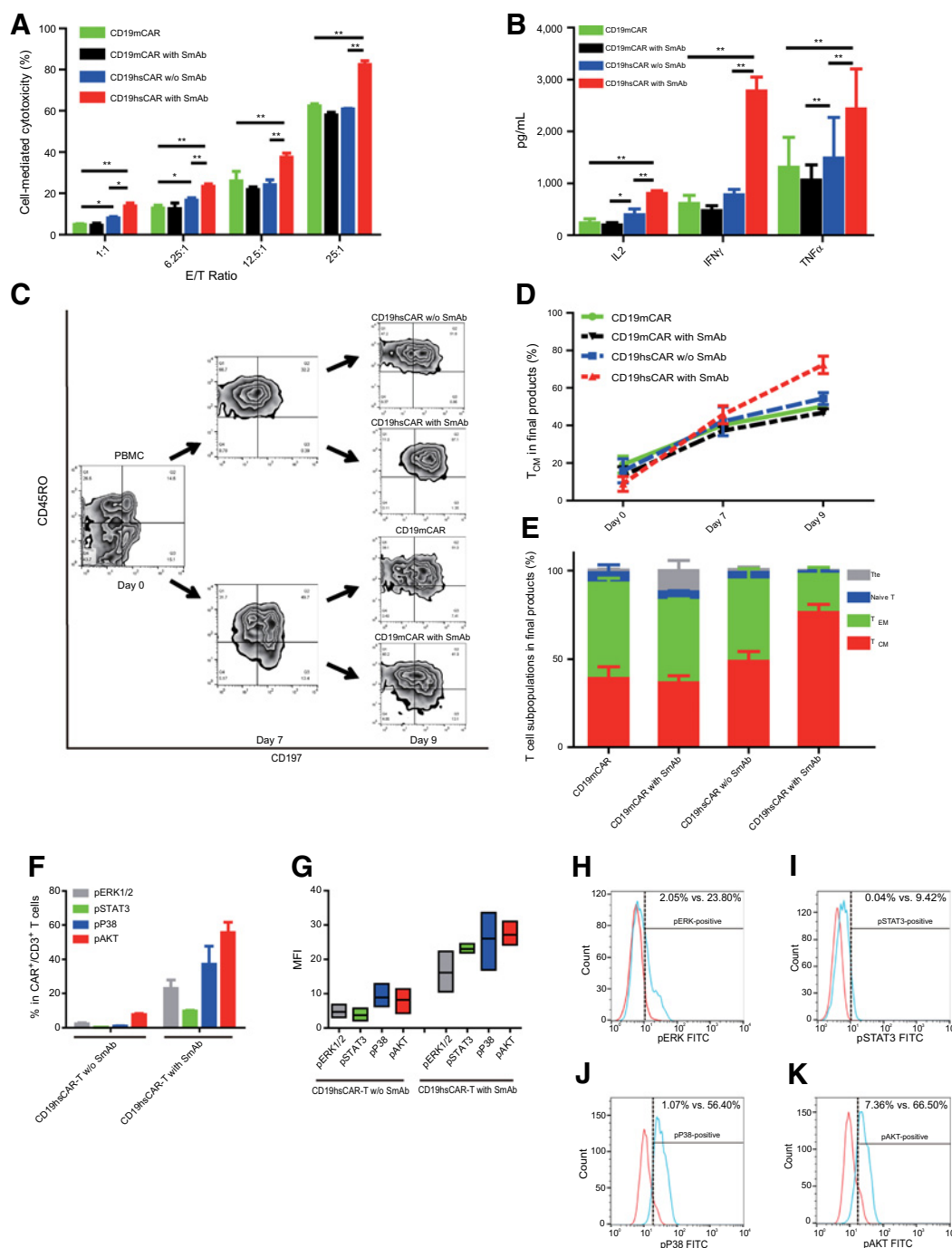
#### Improved efficacy in animal studies

Raji cells that expressed luciferase were intravenously infused into the immunodeficient (NOD/SCID IL2R $\gamma$ <sup>−/−</sup> NSG) mice to establish the leukemia mouse models ( $n = 5/\text{group}$ ). Normal healthy human PBMCs were used to generate various groups of CD19 CAR-T cells, which included mCAR, hsCAR, and a control hsCAR Stopper CAR (CD19 hsCAR lacking intracellular costimulation domain). PBS or each group of the CAR-T cells were intravenously infused into the leukemia mouse models 3 days after Raji cell injection ( $1 \times 10^6/100 \mu\text{L}$  Raji cells/mouse). T cells engineered by lentiviral transduction of EGFP were used as a mock group. CAR-positive T cells ( $1 \times 10^6/100 \mu\text{L}$ ) were infused into each mouse in different CAR-T treatment groups;  $1 \times 10^6/100 \mu\text{L}$  EGFP-positive T cells were infused for mock group, and equal volume of PBS for PBS group. Tumor load and distribution were monitored prior to treatment and 7, 14, 21, 35, 49, and 70 days after treatment (Fig. 3A; Supplementary Fig. S2). Mice treated with hsCAR-T cells activated with SmAb showed significantly longer survival than those with hsCAR-T cells w/o SmAb, and than those with mCAR-T cells (Fig. 3B–D). Analysis of mouse sera from 20 to 50 days posttreatment revealed a higher concentration of proinflammatory cytokines IFN $\gamma$ , IL2, TNF $\alpha$ , IL6, and a lower concentration of IL15 (Fig. 3E–I).

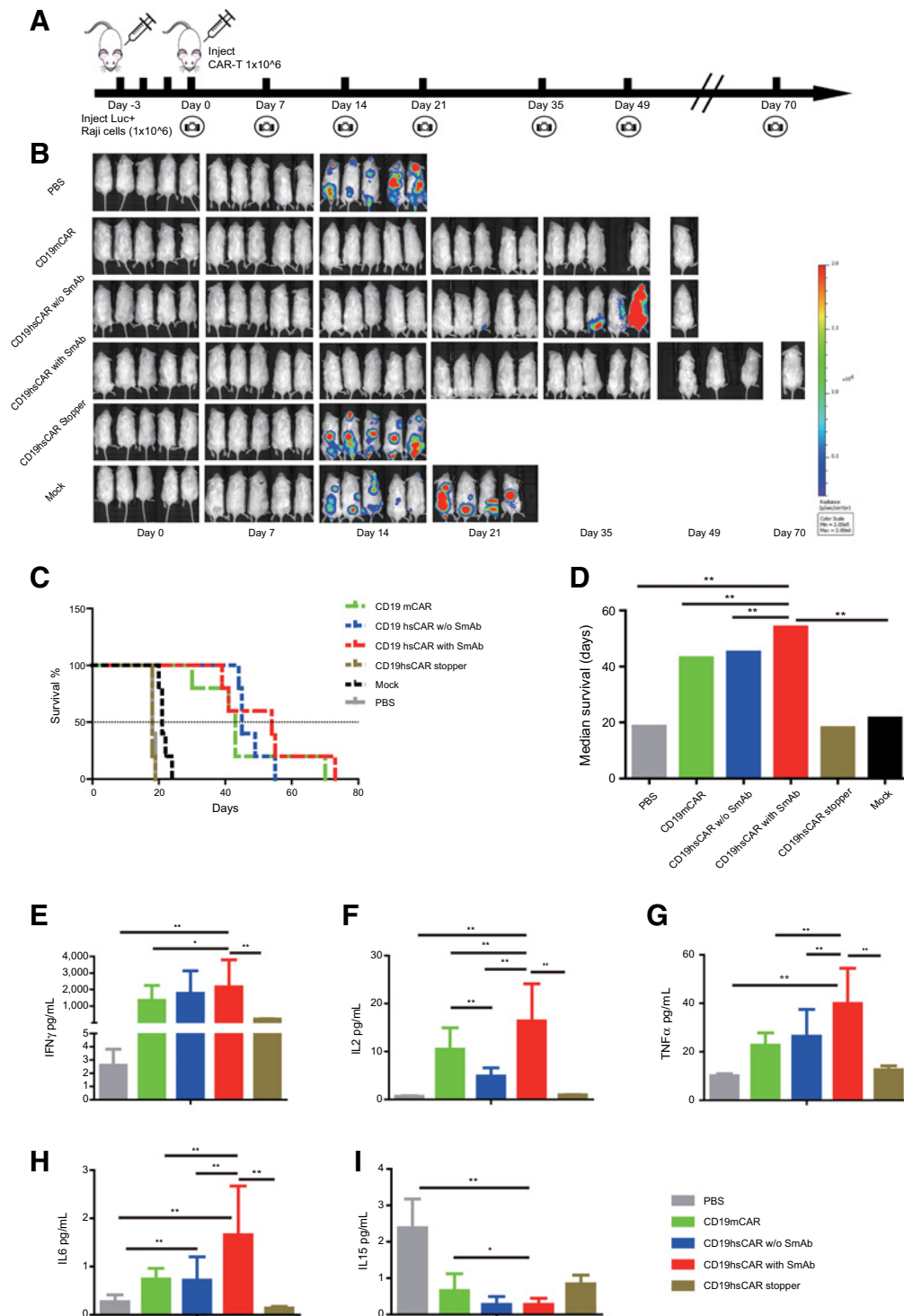
#### Safety and efficacy of hsCAR-T after infusion into patients with previous treatment of murine CAR-T cells

Five refractory/relapsed B-ALL patients were included in the trial who had received previous murine CAR-T treatments and then relapsed with CD19<sup>+</sup> B lymphoblastic cells (Supplementary Tables S1 and S2). Patient 1 was a 9-year old male as in 2018, who was diagnosed of B-ALL with a fusion genotype of E2A-HLF in September 2017 (treatment history listed in Supplementary Table S2). In May 2018, patient 1 received hsCAR-T therapy following lymphodepletion. Grade 1 CRS was observed after hsCAR-T treatment. On day 14, patient 1 achieved CMR (MDR-) and remained in CMR (MDR-) on day 28. Two months later, patient 1 received allo-HSCT and remained in CMR as of March 2019, when the manuscript was prepared. This case proved the concept that humanized CAR-T cells may effectively treat patients who relapsed after murine CAR-T therapy.

Patient 2 was a 14-year-old male patient as in 2018, and, in June 2017, he was diagnosed of B-ALL with a fusion genotype E2A-HLF and a complex chromosome karyotype (Supplementary Table S2). This patient received mCAR-T treatment twice but did not respond to the second murine CAR-T treatment (NR, Supplementary Table S2), which did not reduce the tumor load in bone marrow at any time point examined posttreatment and the disease remained progressive (lymphoblastic cells in bone marrow: day 0 before infusion of the second mCAR-T, 0.5% by morphology, 0.53% by flow cytometry, E2A-HLF fusion gene

**Figure 2.**

SmAb-mediated restimulation targeting the selective domain of CD19 hsCAR enhances the biological function and improves the ratio of central memory T cells in the final product. **A**, CD19 CAR-T cells prepared by different means were co-cultured with Raji, a human B lymphoblastoma cell line at different E/T ratios. The ratios were determined by the number of CAR-positive T cells to Raji cells. The cytotoxicity was measured by LDH release assay after a 12-hour incubation. **B**, Cytokines released during CAR-T mediated cytotoxicity were measured by ELISA assays. **C**, SmAb restimulation reshaped the composition of memory T-cell subpopulations. T cells isolated from PBMCs were transduced by CD19 mCAR or CD19 hsCAR. SmAb was added on day 6 posttransduction. Different memory T-cell subpopulations were quantitated at indicated time points. **D**, SmAb treatment favored proliferation of central memory T cells. The plot represented three independent assays. **E**, Compositions of different T-cell subpopulations in the final product. T<sub>te</sub>, terminally differentiated T cells; T<sub>em</sub>, effector memory T cells; T<sub>cm</sub>, central memory T cells. **F**, SmAb restimulation activated multiple signaling pathways, which are involved in the differentiation of memory T cells. hsCAR-transduced T cells positive for pERK1/2, pP38, pSTAT3, and pAKT were increased in the group with SmAb treatment. **G-K**, Flow cytometric results showing the MFI values and histograms of each pathway. The gated cells in figures **H-K** were those that were double positive for CAR and CD3. Three independent assays were performed using 3 healthy donor samples.

**Figure 3.**

Antitumor function mediated by CD19 hsCAR-T *in vivo*. **A** and **B**, Schematic representation of the animal study. NOD/SCID IL2Rγ<sup>-/-</sup> mice were intravenously injected with  $1 \times 10^6$  Raji cells that constitutively expressed luciferase. After 3 days, mice were subjected to bioluminescence imaging, and grouped by different treatments. Mice were intravenously infused with  $1 \times 10^6$  CD19 mCAR-T, CD19 hsCAR-T without SmAb restimulation, CD19 hsCAR-T restimulated by SmAb, CD19 hsCAR-T without intracellular domain (Stopper), T-cell transduced by lentivirus-expressing EGFP (Mock), or equivalent volume of PBS ( $n = 5$ /group). Tumor progression was monitored by serial bioluminescence imaging at indicated time points. **C** and **D**, Survival rates and median survival time were measured for different treatment groups. **E–I**, Various cytokine levels in sera were measured by MSD assay and compared between different groups. Serum samples were collected from different treatment groups before animals were sacrificed.

0.94%; day 15, 1.5% by morphology, 0.6% by flow cytometry, E2A-HLF fusion gene 13.3%; day 36, 30% by morphology, 46.81% by flow cytometry, E2A-HLF fusion gene 119.58%). In May, 2018, patient 2 received CD19 hsCAR-T treatment. Considering the high tumor load, the dosage of hsCAR-T cells was lowered to  $0.3 \times 10^6/\text{kg}$  body weight, to avoid possible side effect of severe CRS. On day 0 prior to hsCAR-T infusion (after lymphodepleting conditioning), the tumor loads in the bone marrow was 36% according to morphology evaluation, and 34.86% by flow cytometry. On day 15 posttreatment, tumor loads in bone marrow evaluated by morphology and flow cytometry were reduced to 10.5% and 14.98%, respectively; B-cell percentage in peripheral blood was also brought down to almost zero on day 15 (Supplementary Fig. S3B); but on day 30, the tumor loads in bone marrow bounced back to 82% (morphology) and 71.84% (flow cytometry), respectively. The results suggested that hsCAR-T cells might have been effective but exhausted after day 15 possibly due to the low dosage infused and the relative high tumor load. Indeed, the proportion of hsCAR-T cells in peripheral blood was brought down sharply after day 15 and they almost disappeared on day 30 (Fig. 5C). Unfortunately, when we were trying to infuse hsCAR-T cells for another time with a higher dosage, patient 2 quitted the trial by some unknown reasons.

Patient 3 was a 17-year-old male patient as in 2018, who was diagnosed of B-ALL with a fusion genotype BCR-ABL1 in the end of 2014. He had an extramedullary disease with the central nervous system (CNS) involvement, and received targeted therapy (Imatinib followed by dasatinib) and chemotherapy. After achieving CMR, the patient underwent haploidentical HSCT from his mother as donor in May 2015 (Supplementary Table S2). In June, 2017, patient 3 relapsed with extramedullary disease with CNS involvement with a tumor load of 8.96% in the cerebral spinal fluid (CSF). In August, 2017, patient 3 received CD19 mCAR-T treatment, and achieved CMR after experiencing grade 3 CRS and neurotoxicity. In March, 2018, patient 3 received another CD19 mCAR-T as a preventive measure (at this time, the patient had not relapsed). In late April, 29 days after the second time mCAR-T treatment, the patient relapsed with 66.13% lymphoblastic cells of the recipient origin in CSF and 0.02% in BM (evaluated by morphology). In June, patient 3 received CD19 hsCAR-T treatment following intrathecal chemotherapy and lymphodepleting conditioning. On day 5, the patient experienced grade 1 CRS. On day 15, CMR (MRD-) was achieved in bone marrow, peripheral blood, and the CNS (Supplementary Tables S1 and S2). On day 63, the patient relapsed with extramedullary disease with CNS involvement but still remained in CR in bone marrow and peripheral blood. Considering that patient 3 was

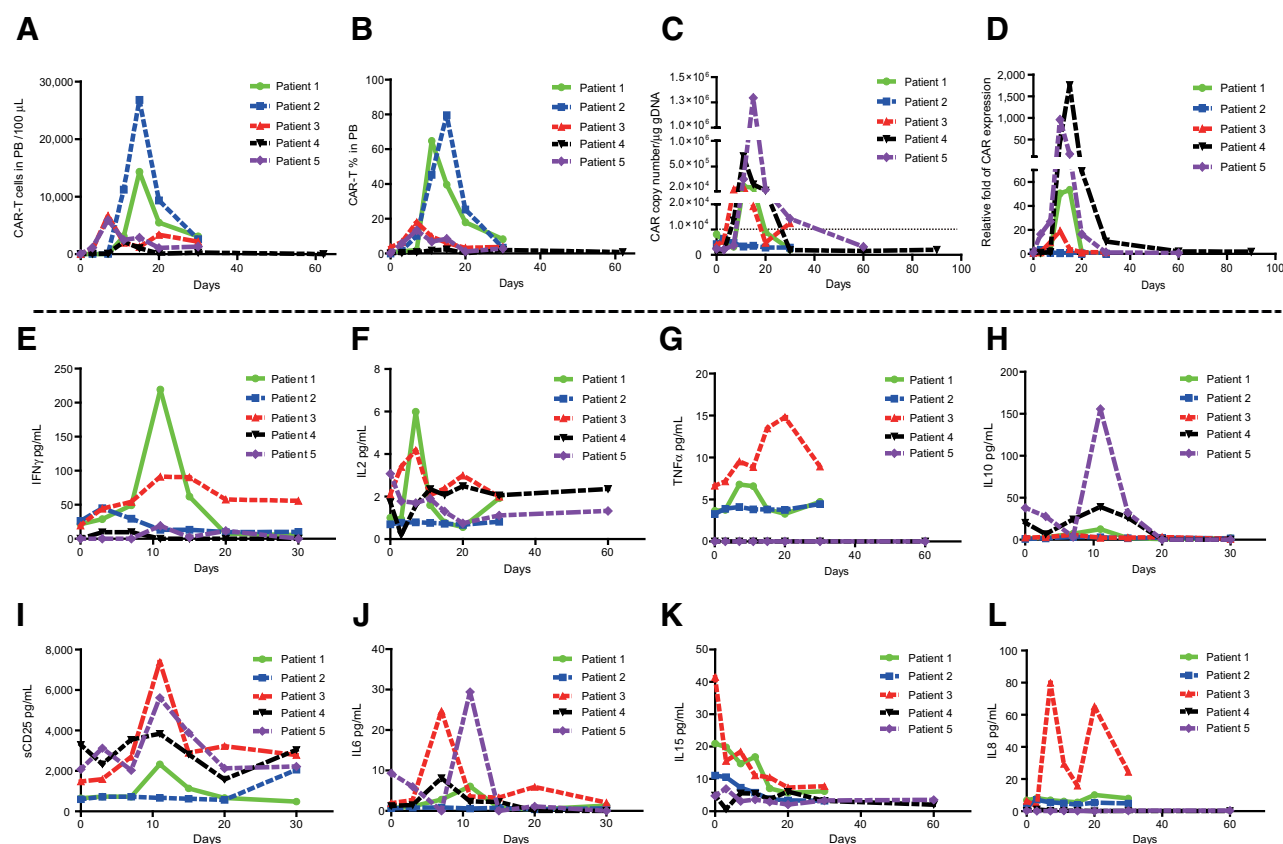
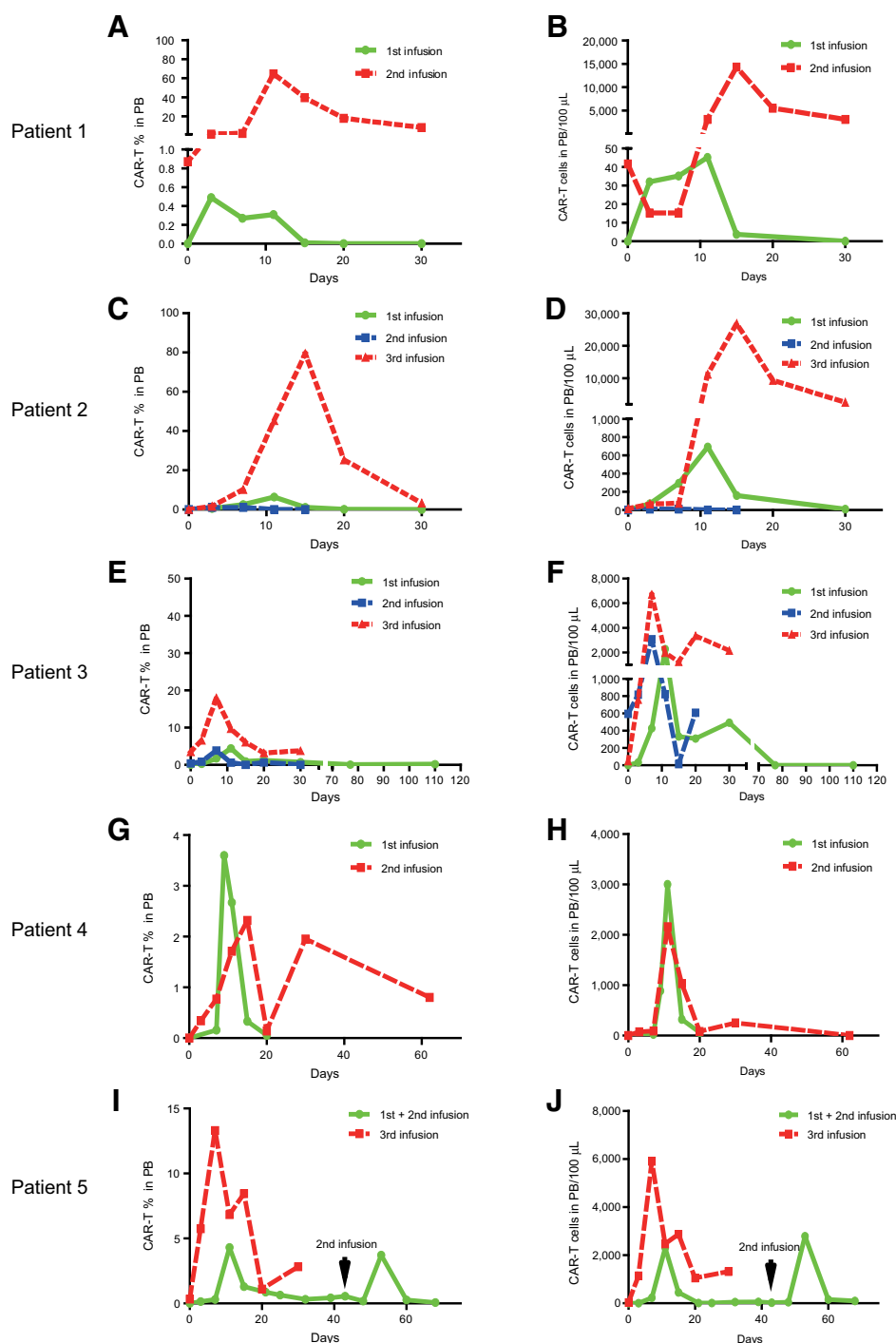


**Figure 4.**

CD19 hsCAR-T cell infusion, proliferation, and persistence in patients. **A** and **B**, Counts and percentages of CD19 hsCAR-T cells in peripheral blood were measured by flow cytometry before and after infusion in patients. **C** and **D**, Copy numbers and the relative fold of expansion of CD19 hsCAR in genomic DNA were tested by qPCR before and after infusion in patients. **E-L**, Various cytokine levels in the peripheral blood of patients were measured by MDS assay at indicated time points after infusion.



clear of cancer cells in bone marrow and peripheral blood, he was given another haploidentical HSCT after intrathecal chemotherapy. As of March, 2019, patient 3 remained in CR in bone marrow, peripheral blood, and CNS.

Patient 4 was a 14-year-old female as in 2018 and was diagnosed of B-ALL in 2016 (Supplementary Table S2). She received CD19 mCAR-T treatment bridging to HLA-identical HSCT from her sister as donor. In August 2018, she relapsed and received CD19 hsCAR-T treatment in September 2018 (Supplementary

Tables S1 and S2); the patient remained in CMR as of March 2019, when the manuscript was prepared.

Patient 5 was a 21-year-old female as in 2018 and was diagnosed of B-ALL in 2016. Similar to patient 4, patient 5 received mCAR-T treatment bridging to haploidentical HSCT from her father as donor. In September 2018, patient 5 relapsed and received CD19 hsCAR-T treatment in October 2018. This patient remained in CMR as of March 2019 (Supplementary Tables S1 and S2).

After infusion of hsCAR-T cells, various levels of expansion of hsCAR-T cells were detected in the peripheral blood of all 5 patients within 3 weeks postinfusion (Fig. 4A–D). Accordingly, the number and proportion of B cells in peripheral blood remained close to zero on day 30 in patient 1 and patient 3 who achieved CMR. In patient 2, who did not achieve CR, B cells started to be detected after day 20 (Supplementary Fig. S3A–S3C). In patient 4, B cells started to be detected after day 30 (Supplementary Fig. S3D); in patient 5, the proportion of B cells remained close to zero two months after hsCAR-T treatment (Supplementary Fig. S3E). The cytokine concentrations in the peripheral blood were measured and presented in Fig. 4E–L. A surge of IL2, IL6, sCD25, and TNF $\alpha$  levels was detected in the sera of patients 1 and 3, but not in that of patient 2. A surge of IL6 was also detected in patients 4 and 5.

We also compared the expansion of CAR-T cells and cytokine levels after hsCAR-T versus mCAR-T treatments (Fig. 5; Supplementary Fig. S4). Patient 1 received  $0.3 \times 10^6$  mCAR-T/kg body weight and  $1 \times 10^6$  hsCAR-T/kg body weight; prior to the mCAR-T and hsCAR-T treatments, the tumor loads in bone marrow were 5.04% and 4.00%, respectively. After treatments, the proportion of CAR-T cells in peripheral blood reached the highest value of 0.5% on day 3 following mCAR-T versus 61% on day 11 following hsCAR-T treatment (Fig. 5A). The number of CAR-T cells in 100  $\mu$ L peripheral blood reached the highest value of 43 on day 11 following mCAR-T versus 15,000 on day 15 following hsCAR-T treatment (Fig. 5B). Patient 2 received mCAR-T infusions twice and hsCAR-T once, and all the three were at the same dosage of  $0.3 \times 10^6$ /kg body weight. The tumor loads in bone marrow before each of the three infusions were 0.43%, 0.50%, and 46.8%, respectively. The proportion of CAR-T cells in peripheral blood reached the highest value of 6.36% on day 11 following the first time mCAR-T infusion and remained close to zero following the second time mCAR-T infusion (Fig. 5C). In contrast, following subsequent hsCAR-T treatment, the proportion went up to 79.64% on day 15. The number of CAR-T cells in 100  $\mu$ L peripheral blood reached the highest value of 693 on day 11 following the first time mCAR-T infusion versus 26,800 on day 15 following hsCAR-T infusion (Fig. 5D). Very few mCAR-T cells were detected following the second time mCAR-T infusion. As to patient 3, the peak of CAR-T expansion was detected earlier (day 7) following the second time mCAR-T infusion versus following the first time mCAR-T infusion (day 11; Fig. 5E and F). Compared with the two mCAR-T treatments, the peak values of hsCAR-T cells were higher (17.96% vs. 4.45% and 3.85%, Fig. 5E; 6,690/100  $\mu$ L vs. 2,280/100  $\mu$ L and 3,070/100  $\mu$ L, Fig. 5F). Patient 4 and 5 both received mCAR-T treatments before HSCT and hsCAR-T after. The percentages of CAR-T cells in peripheral blood and the numbers of CAR-T cells in 100  $\mu$ L peripheral blood in patient 4 were comparable following mCAR-T versus hsCAR-T treatments (Fig. 5G and H). Patient 5 showed a higher degree of CAR-T expansion following hsCAR-T treatment compared with mCAR-T treatment (Fig. 5I and J).

The cytokine levels in the sera of the 5 patients following each treatment were presented in Supplementary Fig. S4A–S4T. With regard to patient 1, after mCAR-T treatment and relapse, hsCAR-T treatment still elicited proinflammatory cytokine response, such as IL6 and IFN $\gamma$  (Supplementary Fig. S4A and S4F); and the level of anti-inflammatory cytokine, IL10, was lower (Supplementary Fig. S4K). As to patient 2, the second time mCAR-T treatment induced a surge in both IL6 and IL10. hsCAR-T treatment caused a

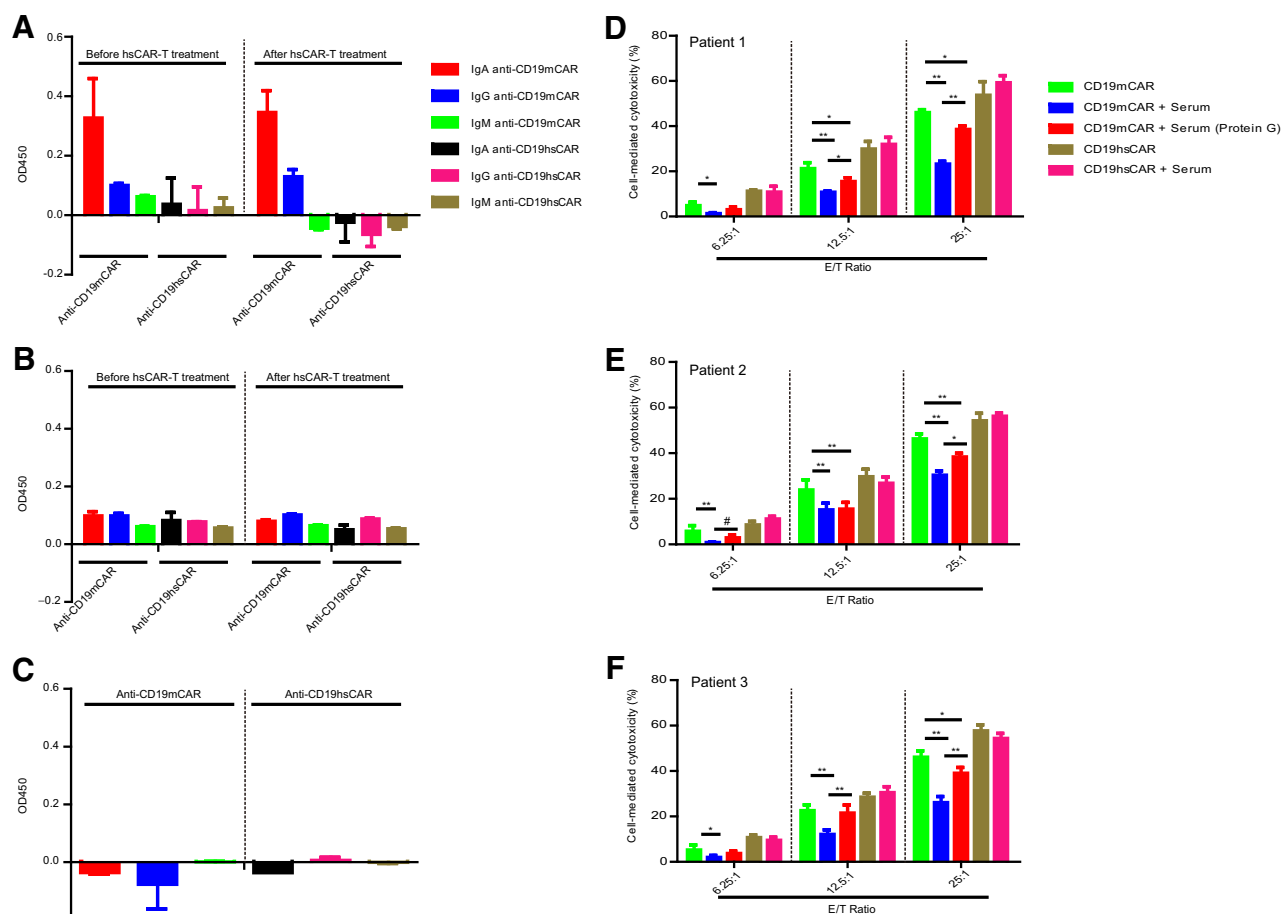
higher expression of IFN $\gamma$ , compared with mCAR-T results. No data of sCD25 after the second time mCAR-T treatment were available for patient 2 (Supplementary Fig. S4B, S4G, S4L, and S4Q). As to patient 3, after mCAR-T infusion for the second time, although the CAR-T cells seemed to have expanded to some extent (Fig. 5E and F), proinflammatory cytokine response was dampened compared with that after the first time mCAR-T treatment (Supplementary Fig. S4C and S4H). However, hsCAR-T treatment following the two mCAR-T treatments elicited proinflammatory cytokine response (Supplementary Fig. S4C and S4H) and reduced the level of anti-inflammatory cytokine, IL10 (Supplementary Fig. S4M). In patient 4 and 5, levels of proinflammatory cytokines IL6 and IFN $\gamma$  were both lower following hsCAR-T versus mCAR-T treatments (Supplementary Fig. S4D, S4I, S4E, and S4J), possibly due to the suboptimal reconstruction of the hematopoietic system following HSCT (Supplementary Table S3), which might have subsequently affected the cytokine responses.

One of the reasons that might underlie the diminished efficacy following the second murine CAR-T treatment in patients 2 and 3 was that the patients may have developed antibodies that recognized the murine scFv sequences. To test this hypothesis, we analyzed the sera isolated from the 5 patients (Fig. 6A and B) and two healthy control people (Fig. 6C). The serum was tested for the reaction to the extracellular domains of murine and humanized CD19 CARs before and after hsCAR-T treatment. Patients 1–3 who received murine CD19 CAR-T cell treatment showed a positive IgA reaction to murine CAR; after treatment with hsCAR-T, no immunoglobulins reactive to humanized CAR was detected in the sera of the three patients (Fig. 6A). Similarly, sera from patients 4 and 5 did not show immunoglobulins reactive to humanized CAR (Fig. 6B, no blood samples available before HSCT). The data of reactivity to murine and humanized CD19 CARs from each individual patient were presented in Supplementary Table S4.

To further confirm the inhibitory effects, T cells derived from patients 1–3 were infected with murine CAR or hsCAR, and cocultured with Raji cell line at different E/T ratios with or without patient serum, to test the cytotoxic functions. In one experimental group, protein G was added together with the serum to block the effects of immunoglobulins (Fig. 6D–F). The cytotoxic functions of mCAR-T cells were inhibited by the patient serum, and incubation with protein G could rescue the inhibitory effects. In contrast, the cytotoxicity of hsCAR-T cells was not inhibited by patient serum (Fig. 6D–F).

## Conclusions/Discussion

One issue associated with the current CAR-T production process is that both CAR-transduced and nontransduced T cells are expanded. In an attempt to enhance the purity and yield, we developed the selective CD19 CAR. After activation with coated SmAb, CAR-transduced T cells in the final product were increased from 32.2% to 64.7% (Fig. 1H); the percentage was almost doubled. The yield of total CD3<sup>+</sup> T cells on day 14 was also significantly greater, achieving around 80-fold expansion with SmAb stimulation versus around 60-fold expansion without (Fig. 1F). Theoretically, hsCAR-transduced T cells can be further purified by magnetic sorting using beads-conjugated SmAb, an approach that can be used to address whether CAR-negative T cells affect efficacy. Another merit of the selective CAR was that stimulation with SmAb during expansion stage can reshape the composition of T-cell subpopulations, leading to a higher

**Figure 6.**

Anti-CAR response and attenuation of mCAR-mediated cytotoxicity by patients' sera. **A–C**, Anti-CAR immunoglobulins, including IgA, IgG, and IgM, were detected by ELISA assay. Sera were collected from patients ( $n = 5$ ) who had been previously treated with CD19 mCAR-T cells at least once (**A**, patients 1–3; **B**, patients 4 and 5) and from healthy donors (**C**,  $n = 2$ ). Purified his-tagged extracellular domains of CD19 mCAR or hsCAR were coated on ELISA plates, and incubated with the serum samples. HRP-labeled goat anti-human IgA, IgG, and IgM antibodies were used for detection. **D–F**, Cytotoxicity mediated by CD19 mCAR-T cells on Raji cell line was attenuated by sera derived from patients with previous CD19 mCAR-T treatment(s). The inhibitory effect could be reversed by adding immunoglobulin-adsorbing Protein-G.

proportion of Tcm in the product. According to the results, this impact was associated with activation of several signaling pathways, including ERK1/2, AKT, P38, and STAT3, which have been shown to be involved in T-cell differentiation process (25–28). Previous studies have shown that persistence of GD2 CAR-T cells is concordant with the percentage of Tcm in the final product (29). In our study, leukemia mouse models treated with SmAb-stimulated hsCAR-T cells displayed a longer survival compared with the other groups (Fig. 3C and D), suggesting an improved antitumor efficacy associated with SmAb-stimulated hsCAR-T cells. The CD4/CD8 ratios and other subpopulation composition in the final products for the 5 patients were also measured and presented in Supplementary Table S5. It seemed there existed a large variation in the CD4/CD8 ratios between the final products of each individual. The kinetics of CD8 Tcm cells after infusion into the 5 patients were presented in Supplementary Fig. S5.

The other major improved feature of the current CAR design was the humanized scFv sequence. This hsCAR showed a 6-fold

higher affinity to CD19, compared with the murine counterpart. Other groups also reported the design of humanized CD19 CARs (30–32), but the affinity was comparable to the murine counterpart or not measured. At a higher E/T ratio which may lead to a saturated cytotoxic effect, hsCAR-T cells with a higher affinity and mCAR-T cells with a lower affinity showed comparable antitumor cytotoxicity. At a lower E/T ratio, hsCAR-T cells exhibited a better cytotoxicity (Fig. 2A). The comparable efficacies as observed in the animal experiments in mCAR-T group versus hsCAR-T group without SmAb stimulation were probably due to the saturated dosage of CAR-T cells *in vivo* (Fig. 3D). Also, the microenvironments in which humanized CAR-T cells kill human Raji cells were different in mice versus in humans. The mouse milieu may not preferably favor humanized CAR over murine CAR to implement their functions.

Humanized CAR-T cells have a potential to break the barrier caused by immune recognition of murine scFv following repetitive infusions of murine CAR-T cells. The two FDA-approved products, Kymriah and Yescarta, were based on murine scFv

sequences (33, 34). Many of those patients will relapse eventually, even if CAR-T therapy is bridged to allo-HSCT. With an increasing number of patients undergoing such treatments, there is a pressing need to find a new interventional strategy to treat the patients who have relapsed with CD19<sup>+</sup> leukemia cells after receiving murine CAR-T therapies. In this study, we enrolled 5 highly treated, refractory/relapsed, B-ALL patients who had exhausted all available options and who might represent different complex conditions. Patient 2 and 3 both received mCAR-T treatments twice. After the second time, mCAR-T cells failed to expand in patient 2 (Fig. 5C and D); mCAR-T cells in patient 3 expanded to some extent after the second time (Fig. 5E and F) but without triggering proinflammatory cytokine response (Supplementary Fig. S4C and S4H) or influencing B cell percentages in the PB (Supplementary Fig. S3C). Analysis of the sera from the three patients revealed that there were IgA that were reactive to the extracellular domain of CD19 mCAR (Fig. 6A). We further confirmed that there was an inhibitory component in the patient serum that could reduce the cytotoxic effect of mCAR-T cells (Fig. 6D–F), and the inhibitory effect was reversed by pulling down immunoglobulins through adding protein G (Fig. 6D–F). It was unclear whether patient 4 and 5 had developed antibodies to murine CAR before HSCT due to the unavailability of the blood samples prior to transplantation. Even though they had, the HSCT process would have eliminated the B-cell clones that produced those antibodies in the recipient, and reconstructed the hematopoietic system with the cells of donor genotype. The cases of patients 4 and 5 indicated that hsCAR-T may also replace mCAR-T as an earlier line of treatment option. The comparison of efficacies of hsCAR-T versus mCAR-T would require a separate study with a larger patient cohort. In all of the 5 patients, no antibodies reactive to hsCAR were detected in the sera, suggesting that hsCAR-T might be repetitively administered to the patients and remain efficacious.

The 5 patients represented various complex conditions. Patient 2 and 5 exhibited complex chromosomes; patient 3 had Philadelphia chromosome and CNS involvement. All of the above factors are associated with poor prognosis with conventional treatments. Patient 1 and 2 did not undergo HSCT; patient 3 went through HSCT before murine CAR-T treatments; patient 4 and 5 received HSCT after murine CAR-T therapies. For patients 3, 4, and 5, we used the healthy HSC donors' PBMCs for production of CAR-T cells, and no symptoms of graft-versus-host disease (GvHD) were observed. The results suggest that CD19 hsCAR-T may be employed in a wide spectrum of situations involved in B-cell malignancies.

Another noteworthy result was that the 5 patients all showed very mild side effects after hsCAR-T treatment (Supplementary Tables S6 and S7). Only a temporary fever occurred which quickly resolved. The CRS was only at grade 1 for all the 5 patients. One possible reason for the mild side effects might be due to the higher affinity of the scFv region of hsCAR. Compared with other

reported CD19 humanized CARs (30, 31), hsCAR still possessed higher affinity to CD19. The higher affinity may be due to a different humanized scFv sequence used in our study, the reversed order of heavy chain and light chain, and/or incorporation of the selective short peptide, which might have contributed to a change in the three-dimensional configuration of the antigen-recognizing domain of CAR. Further studies are needed to elucidate the underlying mechanisms.

In summary, the humanized selective CD19 CAR is superior to its murine counterpart in terms of antigen-binding affinity, product yield, purity, and *in vitro* antitumor efficacy. The results from the highly treated patients confirmed that hsCAR-T therapy is effective to treat those who have relapsed from murine CAR-T therapies.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

Conception and design: P. Lu, Z. Chen

Development of methodology: Y. Zhao, J. Long, P. Lu, Z. Chen

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Zhao, Z. Liu, X. Wang, H. Wu, J. Zhang, J. Yang, F. Zhang, J. Long, P. Lu, Z. Chen

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Zhao, Z. Liu, X. Wang, H. Wu, J. Zhang, J. Yang, F. Zhang, L. Liu, J. Long, P. Lu, Z. Chen

Writing, review, and/or revision of the manuscript: Y. Zhao, Z. Liu, X. Wang, H. Wu, J. Zhang, J. Yang, F. Zhang, L. Liu, J. Long, P. Lu, Z. Chen

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Zhao, Z. Liu, X. Wang, H. Wu, J. Zhang, J. Yang, F. Zhang, J. Long, P. Lu, Z. Chen

Study supervision: J. Long, P. Lu, Z. Chen

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# Clinical Cancer Research

## Treatment with Humanized Selective CD19CAR-T Cells Shows Efficacy in Highly Treated B-ALL Patients Who Have Relapsed after Receiving Murine-Based CD19CAR-T Therapies

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