Bispecific CAR-T cells targeting CD19/20 in patients with relapsed or refractory B cell non-Hodgkin lymphoma: a phase I/II trial

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Methods

Subject inclusion and exclusion criteria

Inclusion criteria:

1. Patients aged ≥14 and ≤75 years with relapsed, refractory B cell non-Hodgkin lymphoma (NHL);

2. Histologically confirmed CD19+ and CD20+ B cell non-Hodgkin lymphoma (NHL), including the following histologies, stage III or IV follicular lymphoma, diffuse large B cell lymphoma and mantle cell lymphoma.

3. Relapsed or refractory disease after the last systemic therapy regimen and meet the following detailed criteria.

We defined chemotherapy-refractory disease as meeting one or more of the following criteria:

- No response to first-line therapy (primary refractory disease).
- No response to second-line or later therapy.
- PD or SD was the best response to the most recent therapy regimen.

4. Performance status (ECOG) between 0 and 2.

5. Expected survival ≥ 3 months.

6. Adequate organ function:

- ALT and AST < 2.5× upper limit of the normal (ULN). alkaline phosphatase < 2.5 x ULN.
- Creatinine clearance ≥ 60 mL/min.
- New York Association (NYHA) classification I or II and Cardiac ejection fraction ≥50%.

7. Successful leukapheresis assessment and pre-culture of T cells.

8. Able and willingness to provide informed consent.

Exclusion criteria:

1. Pregnant or lactating women, male subjects (or their partners) or female subjects have a pregnancy plan during the study period to 6 months after the end of the test, and are unwilling to use a medically approved effective contraceptive measure during the test period (such as intrauterine device or condom);

2. Uncontrolled active bacterial or viral infection (active hepatitis B or hepatitis C infection, HIV infection).

3. History of autoimmune disease or primary immunodeficiency.

4. Patients with active central nervous system (CNS) involvement were not enrolled in this clinical study.

5. Patients with history of allogeneic stem cell transplantation are excluded if they are <100 days' post-transplant, have evidence of active graft-versus-host-disease (GVHD), or are currently on immunosuppression.

6. CD19 CAR-T cell treatment failure or recurrence, detection of a clear human anti-mouse antibody (HAMA) effect, or negative tumour puncture detection of CD20.

7. Class III/IV cardiovascular disability according to the New York Heart Association Classification and a cardiac ejection fraction ≥50%.

8. Requirement for urgent therapy due to tumour mass effects such as respiratory obstruction or blood vessel compression.
9. Any organ failure.
10. Concurrent active malignancy requiring therapy or intervention.
11. Those who need long-term anticoagulation (warfarin or heparin), antiplatelet (aspirin, dose>300mg/d; clopidogrel, dose>75mg/d) treatment.
12. Those who received radiotherapy within 4 weeks before the start of the study (blood sampling).
13. Those who have participated in other clinical trials within 30 days.
14. Subjects considered unlikely to complete all protocol-required study visits or procedures, including follow-up visits, or to comply with the study requirements for participation according to the investigator's judgement.

CAR-T cell manufacturing

Through leukapheresis, peripheral blood mononuclear cells (PBMC) were obtained from the enrolled patients, and then washed and cryopreserved. T cells from thawed PBMC were isolated and activated by human T cell-Activator CD3/CD28 antibody. The medium for T cell culture was X-VIVO 15 supplemented with IL-7/15/21. Two or three days after the activation, T cells were infected with lentiviral vectors carrying the CD19/CD20 CAR. The cells were cultured additional 10 days for expansion. Quality checks are carried out at every step of the manufacturing process. Transduction efficiency was measured by flow cytometry after staining with anti-EGFR and T cell markers (CD3, CD4, CD8). CAR-T cell function was assessed by conducting: (a) LDH assay versus CD19 & CD20 antigen-positive and non-antigen-positive target cells, and (b) Cytokine concentrations secreted after stimulation with the same panel of target cells. The same functional assays were also performed on the final CAR-T cell products.

CAR detection

The CAR structure was combined with EGFR molecular via T2A. The CD3+ and EGFR+ populations are the anti-CD19/20 CAR-T cells. For each blood sample from patients, the red blood cells were removed using a lysing reagent, and white blood cells were collected and blocked with an IgG Fc antibody. The blocked white cells were then stained with anti-CD3 and anti-EGFR antibodies (Biolegend), and then analyzed by flow cytometry.

Soluble factor analysis

Quantification of soluble cytokine factors was performed using an ELISA kit (BioGems, Westlake Village, CA, USA) according to the manufacturer's protocol. The assay was based on the sandwich ELISA principle. Briefly, effector cells were co-cultured with the target cells at an E:T ratio of 5:1 for 18 hours in medium. After the co-culture, the supernatants were collected and diluted according to the instruction, then transferred into a 96-well strip microplate pre-coated with a primary antibody (IFN-γ TNF-α, IL-2, IL-6, IL-8, IL-10 and C-reactive protein (CRP)). Then, the microplate was incubated at 37 °C for 90 minutes and subsequently washed. Following several washes, a color-developing reagent was added to the microplate and maintained in the darkness for 30 minutes. Finally, the reaction was terminated and the absorbance of each well was measured at 450 nm using a microplate reader.

Sequencing data analysis

All libraries were sequenced using Illumina NovaSeq 6000 with 150 bp paired-end reads. Pre-processing, genome alignment, feature counting and expression matrix generation of sc-RNA data were performed using CeleScope (https://github.com/singleron-RD/CeleScope). Cell barcode and UMI were extracted, and STAR v2.6.1a was utilized to map reads to the reference genome GRCh38. FeatureCounts (version 2.0.1) was used to obtain UMI counts and gene counts of each cell, generating expression matrix files for subsequent analysis. TCR clonotype assignment were performed using Cell Ranger (version 4.0.0) VDJ pipeline with GRCh38 as reference. Cells with less than 200 detected genes or with a percentage of mitochondrial reads over 30% were filtered out from subsequent analysis. DoubletFinder was applied to check for doublet, and cells tagged as doublets were excluded from further analysis. Seruat (v4.3.0) was used for normalized gene-cell matrix, highly variable genes identification and cell clustering.

The TCR V(D)J rearrangements and CDR3 sequence were utilized to identify the clonal composition of the CAR-T cells from IP and PB on day 21. The top 9 TCR clonotypes were ranked based on their abundance.

To identify differentially expressed genes (DEGs), we used the Seurat FindMarkers function based on Wilcox likelihood-ratio test with default parameters. The criteria for identifying DEGs with significant differences were as follows: 1) The absolute value of the average log2 fold change (|avg_log2FC|) must be equal to or greater than 1. 2) The adjusted p-value must be less than 0.05. 3) The absolute difference between the two groups must be equal to or greater

than 0.2, while the percentage of genes in the less-expressing group was equal to or less than 0.1. The DEGs were up-regulated or down-regulated according to the avg_log2FC value >1 or <-1. Specific criteria were indicated in the figure legends.

Supplement Table 1. Previous therapy of NHL patients.

Patient No.	Previous therapy
1	R-CHOP*4 (PR); R2-CHOP*2 (PD); ZR2*2; R-VP (PD); R2-CHOPE (PD)
2	R-miniCHOP*2 (PR); R-CHOP*3 (PD); R-GEMnPAC*2(CR); R- GEMnPAC*2 (PD); R2-GemOx*1; R2-GemOx+DXM, ZR2*5 (CR); Zanubrutinib+Lenalidomide (PD)
3	R-CHOP*(CR); R-CHOP*3 (CR); RT (CR); R-IMED*1 (PD); GemOx +Dicitabine+Orelabrutinib*1 (PD)
4	R-CHOP*2 (CR); R-CHOP*4 (CR); Rituximab (PD)
5	R+Peginterferon a-2b*3 (PD)
6	R-CHOP*4(CR); R-CHOP*2(PD); R-CHOP+Zanubrutinib (PD)
7	R-CHOP*4 (CR); R-CHOP*1(PD); CEPP*2 (SD); HDAC/PI3K inhibitor Clinical Trial*6 (CR); HDAC/PIK3 inhibitor Clinical Trial*2 (PD); Orelabrutinib+R-COP*1; Orelabrutinib+ R-CHOP*6 (PD); Orelabrutinib (PD)
8	R-CHOP*5 (CR), R-CHOP*2 (PD)
9	R-CHOP*2 (PR), R-CHOP*1 + CHOP*3 (PD); Bendamustine (SD); G-ICE*2 (SD)
10	R-CHOP*1 (SD); R-CHOPE*1 (SD): R2-CHOP*4 (CR); R-DHAP*2 (CR)
11	R-CHOP *4 (CR); R-CHOP *4 (PD); G-GemOx *2 (PD)

R: rituximab. CHOP: cyclophosphamide, adriamycin, vincristine, prednisone. R2: rituximab, lenalidomide. ZR2: zanubrutinib, rituximab, lenalidomide. VP: etoposide, prednisone. CHOPE: cyclophosphamide, adriamycin, vincristine, prednisone, etoposide. GEMnPAC: Gemcitabine, paclitaxel protein-bound. GemOx: gemcitabine, oxaliplatin. DXM: Dexamethasone. RT: radiation therapy. IMED: Ifosfamide, methotrexate, etoposide, dexamethasone. CEPP: cyclophosphamide, etoposide, prednisone, procarbazine. COP: cyclophosphamide, vincristine, prednisone. HDAC/PI3K: histone deacetylase, phosphoinositide 3-kinase. G: obinutuzumab. ICE: ifosfamide, cisplatin, etoposide. DHAP: cisplatin, cytarabine, dexamethasone. CR: complete remission. PR: partial remission. SD: stable disease. PD: progressive disease.

Supplementary Table 2. The treatment and efficacy of NHL patients received CD19/20 CAR-T cells infusion.

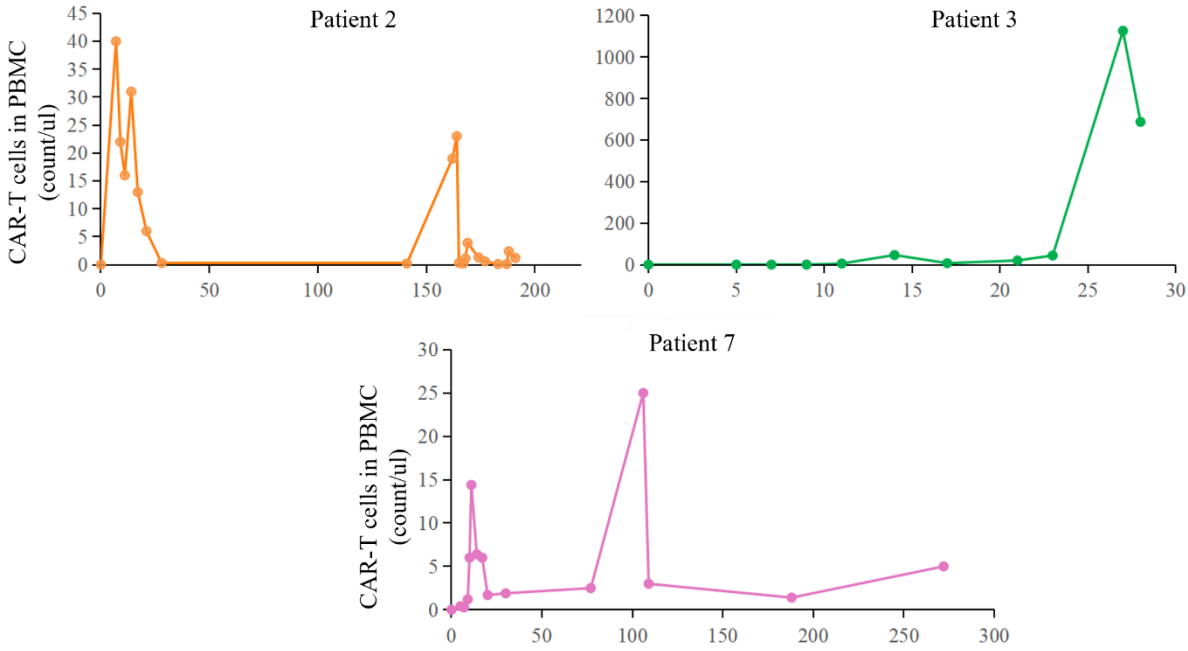
Patient No.	Conditioning regimen	CD19-20·CART cells(*10^6./kg)	CRS grade	symptoms ICANS grade	Best response (duration: months)	Survival
1	F,C	2.62	2	3	CR (15.0)	**Yes**
2	F,C	0.97	1	No	PR (3.7)	**No**
3	F,C	2.00	3	3	NA	**No**
4	F,C	1.38	No	No	CR (13.2)	**Yes**
5	F,C,R	1.09	No	No	CR (8.1)	**Yes**
6	F,C	0.63	No	No	CR (12.4)	**Yes**
7	F,C	0.31	1	No	CR (7.3)	**Yes**
8	F,C	0.40	1	No	CR (9.8)	**Yes**
9	F,C	0.82	No	No	CR (4.8)	**Yes**
10	F,C	0.56	No	No	PR (3.4)	**Yes**

11	G,F,C	0.77	No	No	PD (4.8)	**Yes**

FC: fludarabine, cyclophosphamide; G: obinutuzumab; CR: complete remission. PR: partial remission. SD: stable disease. PD: progressive disease.

Supplementary Table 3. The abundance of top 3 TCR clonotypes of CAR-T cells from infusion product (IP) and peripheral blood (PB) on day 21 after infusion.

TCR clones	Day 0	Day 21
TRBV28, TRBJ2-1 CASSPFLAGELSYNEQFF	0.06%	29.17%
TRBV28, TRBJ2-7 CASSPGEGQVTYEQYF	0.06%	31.55%
TRBV6-5, TRBJ2-5 CASSYSIGEGQETQYF	0.06%	25.60%



Supplementary Figure 1. Absolute counts of CAR-T cells in peripheral blood of three patients were measured using flow cytometry.