## Supplemental Methods

### Eligibility and exclusion criteria

All eligible patients were of age > 18; had histologically confirmed B-cell Non-Hodgkin's lymphoma according to the WHO classification [1], no history of primary central nervous system lymphoma; Eastern Cooperative Oncology Group (ECOG) scores < 2; adequate cardiac, hepatic and renal functions; and PET-positive lesions. The included patients were previously treated with anti-CD20 monoclonal antibody and anthracycline-containing regimens for at least two cycles or had experienced disease relapse or progression after autologous hematopoietic stem cell transplantation. Patients were excluded if they met one of the following criteria: pregnant, breastfeeding, or expecting to conceive; history of malignancy other than in situ non-melanoma skin carcinoma; or active hepatitis B, hepatitis C or human immunodeficiency virus infection.

### Study procedures and treatment

Patients were enrolled after screening and confirmation of eligibility. They then underwent leukapheresis so peripheral blood mononuclear cells (PBMCs) could be obtained to manufacture CAR-T cell products in a central cell processing facility. The general composition of the CD28z CAR-T and 41BBz CAR-T included the same single-chain variable fragment (FMC63) and CD3z for intracellular signaling but with a different combination of transmembrane and costimulatory domains[2]. Activated T cells were transduced with a γ-retroviral vector (CD28z CAR-T) or self-inactivated lentivirus vector (41BBz CAR-T) containing the CAR gene and expanded in culture.

39 The final CAR-T cell product was washed; tested for identity, potency, sterility,
40 adventitious agents, and cryopreserved. After meeting the acceptance criteria, the
41 product was transported to the clinical sites using a validated cryo-shipper.

42 Lymphodepletion preconditioning regimen consisted of fludarabine 30 mg/m$^2$/day
43 and cyclophosphamide 500 mg/m$^2$/day on day -4 to day -2 in the CD28z CAR-T study,
44 and fludarabine 25 mg/m$^2$/day and cyclophosphamide 250 mg/m$^2$/day on day -4 to Day
45 -2 in the 41BBz CAR-T study, followed by CAR-T cell infusion on Day 0. Complete
46 blood cell counts, serum biochemistry, coagulation panels, and cytokine levels were
47 monitored after infusion. DLTs were evaluated from day 1 to day 29. Primary tumor
48 response was assessed by PET-CT and CT on day 29. Patients entered the follow-up
49 phase after day 29, and the safety, response and persistence of CAR-T cells were
50 evaluated at 3, 6, 9, 12, 18 and 24 months. Duration of response was assessed by CT at
51 months 3, 6, 9, 12, 18 and 24 after a subject achieved CR. PET-CT was required when
52 progression disease was suspected in the follow-up phase. Disease response and
53 duration were determined according to the "Recommendations for Initial Evaluation,
54 Staging, and Response Assessment of Hodgkin and non-Hodgkin Lymphoma: The
55 Lugano Classification"[3].

56

57 **Cytotoxic potency assay**

58 Target Raji cells were labeled with calcein AM (Life Technologies, Invitrogen) for
59 1 hour at 37°C, and then 3000 cells were co-incubated with CAR-T cells for 4 hours at
60 an effector-to-target (E:T) ratio of 20:1. Supernatants were harvested, and calcein
61 release was quantified using a BioTek Synergy 2 plate reader (Ex: 485 nm/Em: 530
62 nm). Cytolytic potency was calculated as % release = (experimental release-

63 spontaneous release)/(maximal release-spontaneous release) 100%, where maximal
64 release was induced by incubation in a 1% Triton X-100 solution.

65

66 **Macrophage differentiation assay**

67 Human CD14-positive monocytes were isolated from PBMCs of healthy
68 volunteers by using magnetic selection (Miltenyi Biotec, Cat. No. 130-042-401)
69 according to the manufacturer's instructions, and seeded at a cell density of $2\text{-}3\times10^5$
70 cells/mL in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% heat-
71 inactivated FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in a
72 humidified 5% $CO_2$ incubator. For differentiation of M2-subtype macrophages, the
73 cytokines CSF (R&D Systems, Cat. No. 216-MC-005), IL-4 (R&D Systems, Cat. No.
74 204-IL-010), IL-6 (R&D Systems, Cat. No. D6050), and IL-13 (R&D Systems, Cat.
75 No. 213-ILB-005) were used at a concentration of 20 ng/mL each.

76

77 **T-cell suppression assay**

78 To determine the ability of M2-subtype macrophages to inhibit T-cell proliferation
79 *in vitro*, carboxyfluorescein succinimidyl ester (CFSE, Thermo Fisher, Cat. No.
80 46410)-stained T cells were stimulated for three days with Dynabeads Human T-
81 Activator CD3/CD38 beads (Thermo Fisher, Cat. No.11131D), and cultured in the
82 absence or presence of M2-subtype macrophages at a 1:1 ratio to the T cells. Cells were
83 harvested, washed, and stained for surface markers, and CFSE dilution was used as a
84 measure of cell proliferation. Samples were collected on a FACSFortessa instrument
85 (BD Bioscience) and data were analyzed using FlowJo software v.10.5.

86 **Single-cell suspension preparation**

87 Collected samples were placed into the GEXSCOPE Tissue Preservation Solution 88 (Singleron Biotechnologies) at 2-8°C. Prior to tissue dissociation, the samples were 89 washed with Hanks balanced salt solution (HBSS) three times and minced into 1-2 mm 90 pieces. The tissue pieces were digested in 2 ml GEXSCOPE Tissue Dissociation 91 Solution at 37°C for 15 minutes. After digestion, a 40-micron sterile strainer (Corning) 92 was used to separate cells from cell debris and other impurities. The cells were 93 centrifuged at 1000 rpm and 4°C for 5 minutes, and cell pellets were resuspended in 1 94 ml PBS (HyClone). To remove red blood cells, 2 ml GEXSCOPE Red Blood Cell Lysis 95 Buffer (Singleron Biotechnologies) was added to the cell suspension and incubated at 96 25°C for 10 minutes. The mixture was then centrifuged at 1000 rpm for 5 minutes and 97 the cell pellet resuspended in PBS. Cells were counted with a TC20 automated cell 98 counter (Bio-Rad).

99

100 **Single-cell RNA sequencing (scRNA-seq) library preparation**

101 The concentration of the single-cell suspension was adjusted to $1\times10^5$ cells/ml in 102 PBS. Single-cell suspensions were then loaded onto a microfluidic chip and scRNA-103 seq libraries were constructed according to the manufacturer's instructions (Singleron 104 GEXSCOPE Single Cell RNAseq Library Kit, Singleron Biotechnologies). The 105 obtained scRNA-seq libraries were sequenced on an Illumina HiSeq X10 instrument 106 with 150 bp paired-end reads.

107

108 **Analysis of scRNA-seq data**

109 Raw reads were processed to generate gene expression matrices by scopetools

110 (https://anaconda.org/singleronbio/scopetools). Briefly, reads without poly T tails at the
111 expected positions were filtered out, and then each read cell barcode and unique
112 molecular identifier (UMI) were extracted. Adapters and polyA tails were trimmed
113 before aligning the second read to GRCh38 with Ensembl version 92 gene annotation.
114 Reads with the same cell barcode, UMI, and gene were grouped together to generate
115 the number of UMIs per gene per cell. The number of cells was then determined based
116 on the inflection point of the number of UMIs versus the sorted cell barcode curve.

**Supplementary Table 1. Patient characteristics**

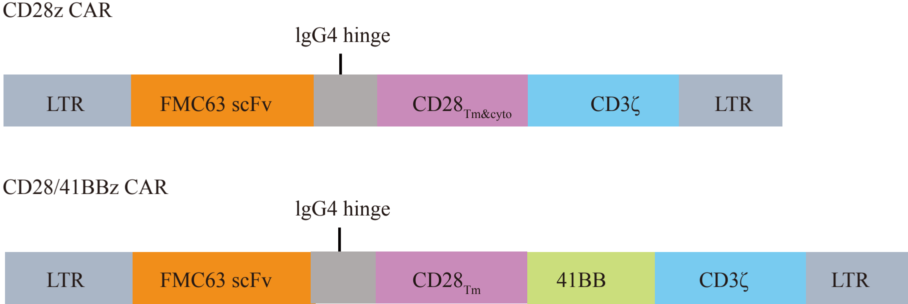
| Characteristics | N (%) |
|---|---|
| **Age (years)** | |
| Median (range) | 56 (29-63) |
| **Sex** | |
| Male | 15/24 (62.5%) |
| Female | 9/24 (37.5%) |
| **Pathologic diagnosis** | |
| Diffuse large B-cell lymphoma | 20/24 (83.3%) |
| Germinal center B-cell | 4/24 (16.7%) |
| Non-germinal center B-cell | 16/24 (66.7%) |
| Transformed follicular lymphoma | 2/24 (8.3%) |
| Primary mediastinal large B-cell lymphoma | 2/24 (8.3%) |
| **Ann Arbor stage** | |
| III or IV | 14/24 (58.3%) |
| **Extranodal lesions** | |
| Yes | 12/24 (50.0%) |
| **Bulky disease (≥7.5 cm)** | |
| Yes | 6/24 (25.0%) |
| **Lactic dehydrogenase** | |
| Elevated | 15†/24 (62.5%) |
| **International prognostic index** | |
| Intermediate (2) | 8/24 (33.3%) |
| High-intermediate (3) | 5/24 (20.8%) |
| High risk (4-5) | 1/24 (4.2%) |
| **Prior lines of therapy** | |
| 1 | 1/24 (4.2%) |
| 2 | 10/24 (41.7%) |
| 3 | 8‡/24 (33.3%) |
| 4 | 4/24 (16.7%) |
| 5 | 1‡/24 (4.2%) |

† Median (range): 267 IU/L (149-895 IU/L)
‡ Patient relapsed after autologous stem cell transplantation.

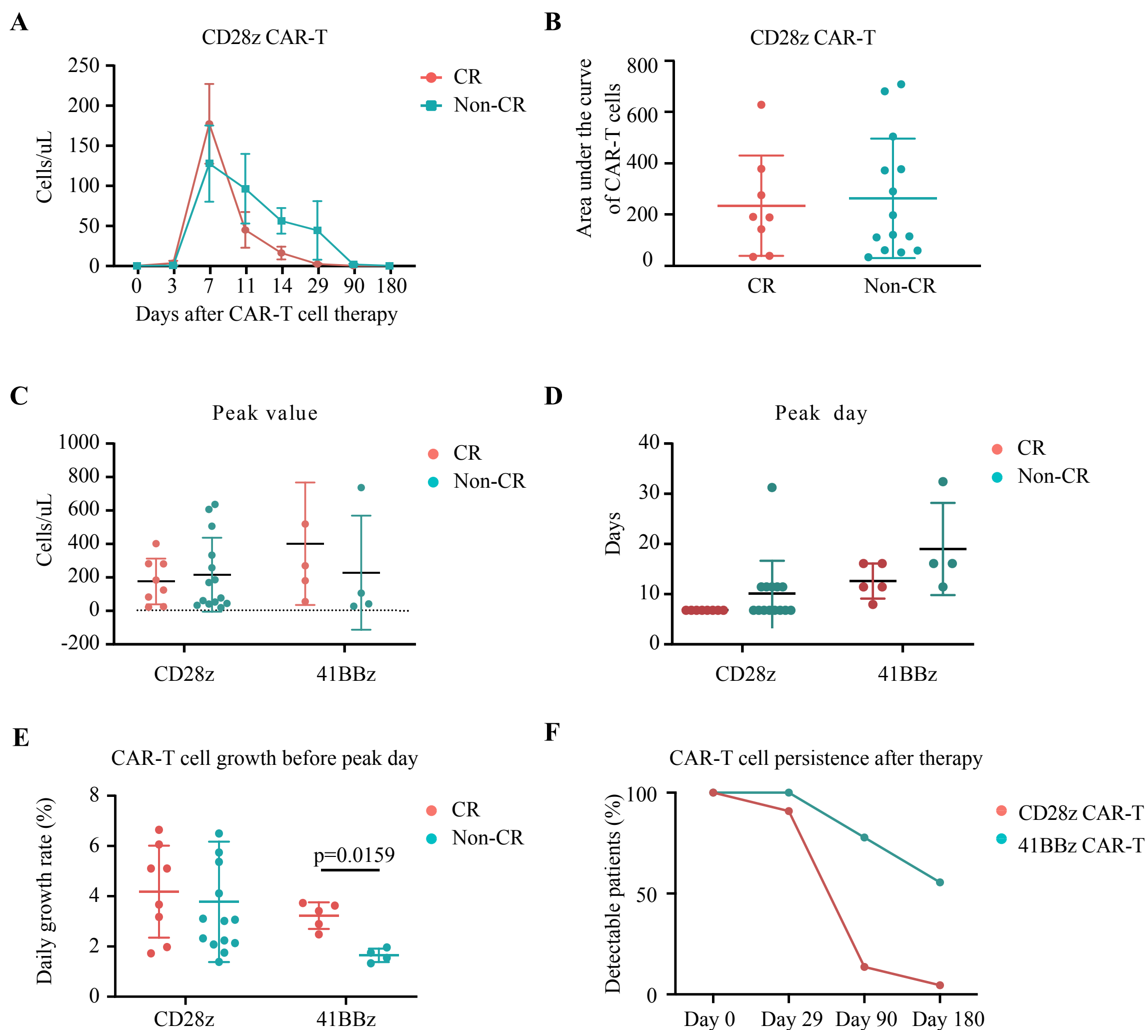
**Supplementary Table 2: Adverse Events**

| Adverse event | Any | Grade ≥3 |
|---|---|---|
| Pyrexia | 24/24 (100%) | 8/24 (33.3%) |
| Leukopenia | 24/24 (100%) | 23/24 (95.8%) |
| Neutropenia | 24/24 (100%) | 23/24 (95.8%) |
| Anemia | 21/24 (87.5%) | 11/24 (45.8%) |
| Thrombocytopenia | 19/24 (79.2%) | 13/24 (54.1%) |
| Hepatotoxicity | 14/24 (58.3%) | 2/24 (8.3%) |
| Muscle soreness | 13/24 (54.2%) | 3/24 (12.5%) |
| Hypoimmunoglobulinemia | 11/24 (45.8%) | 1/24 (4.2%) |
| Hypotension | 12/24 (50.0%) | 1/24 (4.2%) |
| Hypofibrinogenemia | 11/24 (45.8%) | 2/24 (8.3%) |
| Fatigue | 8/24 (33.3%) | 0/24 (0%) |
| Cytokine release syndrome | 24/24 (100%) | 1/24 (4.2%) |
| Neurologic event | 2/24 (8.3%) | 1/24 (4.2%) |

**Supplementary Figure 1. Schematic diagram showing the CAR-T constructions of CD28z CAR-T and CD28/41BBz CAR-T**

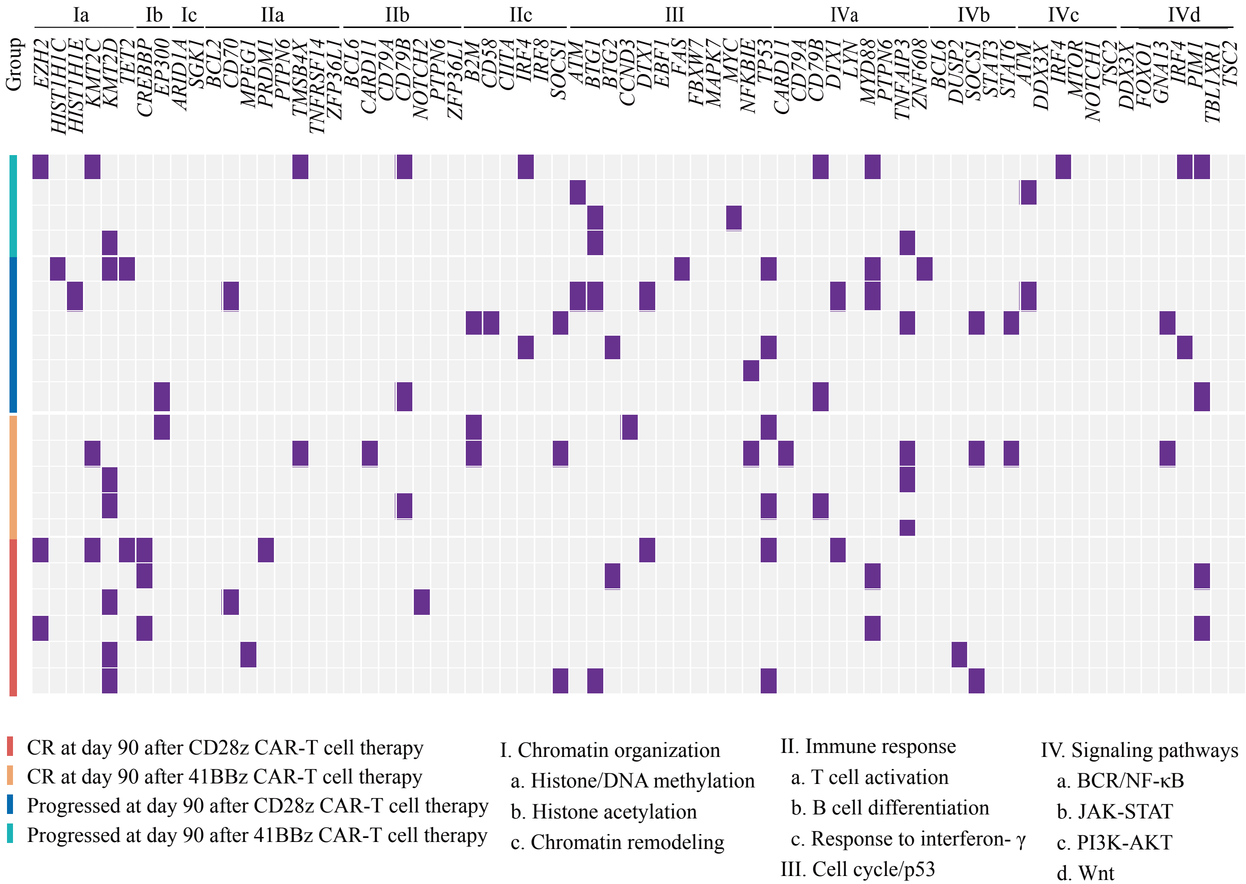


**Supplementary Figure 2. Pharmacokinetics of CAR-T cell expansion.**

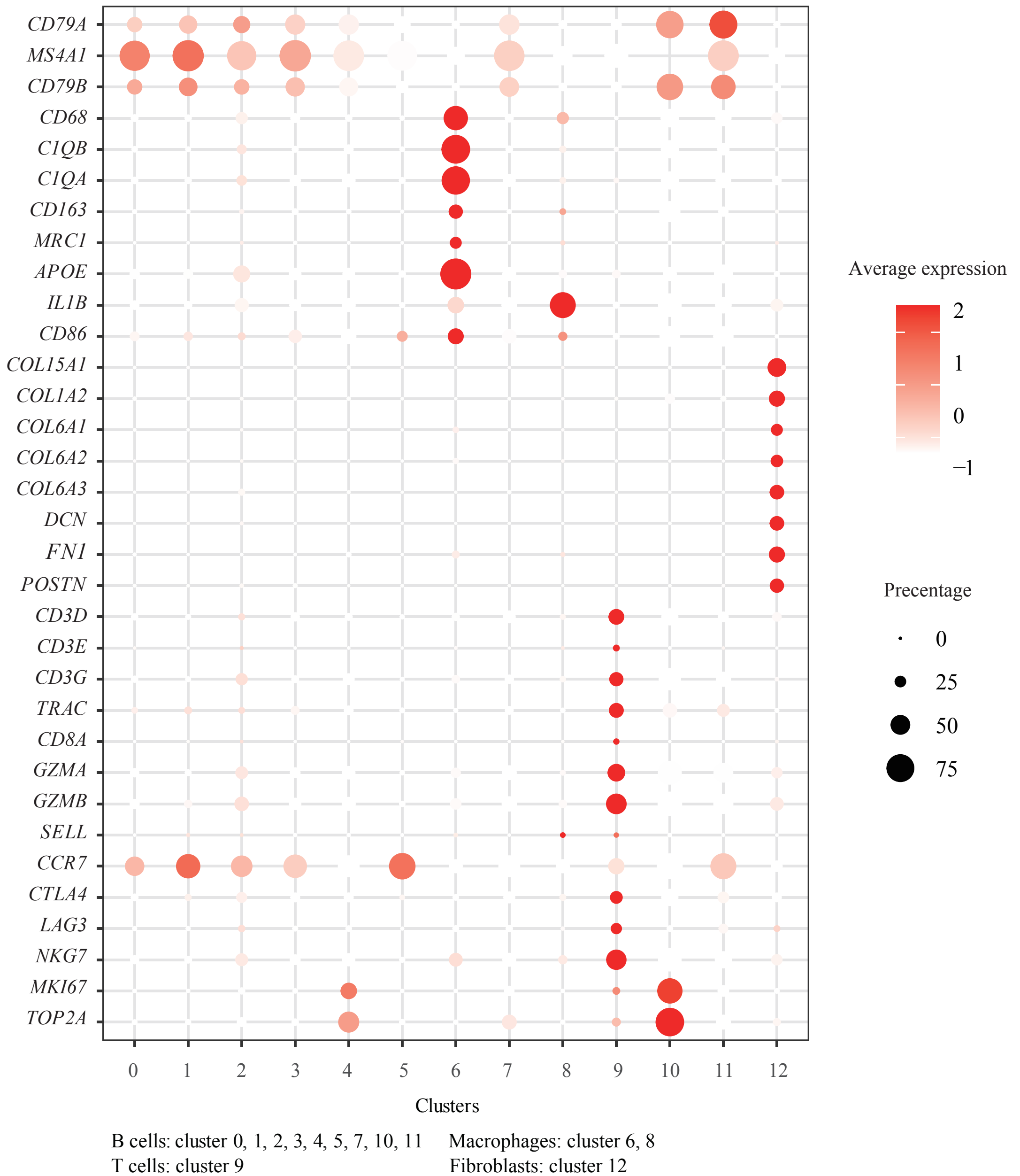



(A) CAR-T cell expansion in peripheral blood were assessed after CD19 CAR-T cell therapy.(B) Area under the curve of CAR-T cells in CR and Non-CR patients after CD28z CAR-T therapy.CAR-T area under the curve is defined as cumulative concentrations of CAR cells per µL of blood during the first 29 days (C) Scatter plot showing the peak value in CR and Non-CR patients in CD28z and 41BBz CAR-T therapy. (D) Scatter plot showing the peak day in CR and Non-CR patients in CD28z and 41BBz CAR-T therapy. (E) Bar plot showing the daily growth rate in CR and Non-CR patients after CD28z and 41BBz CAR-T therapy. (F) CAR-T cell persistence after CD28z and 41BBz CAR-T therapy. CR and Non-CR above were evaluated at day 90 after CAR-T cell therapy.

**Supplementary Figure 3. Gene mutation pattern in patients treated with CD28z CAR-T and 41BBz CAR-T.**

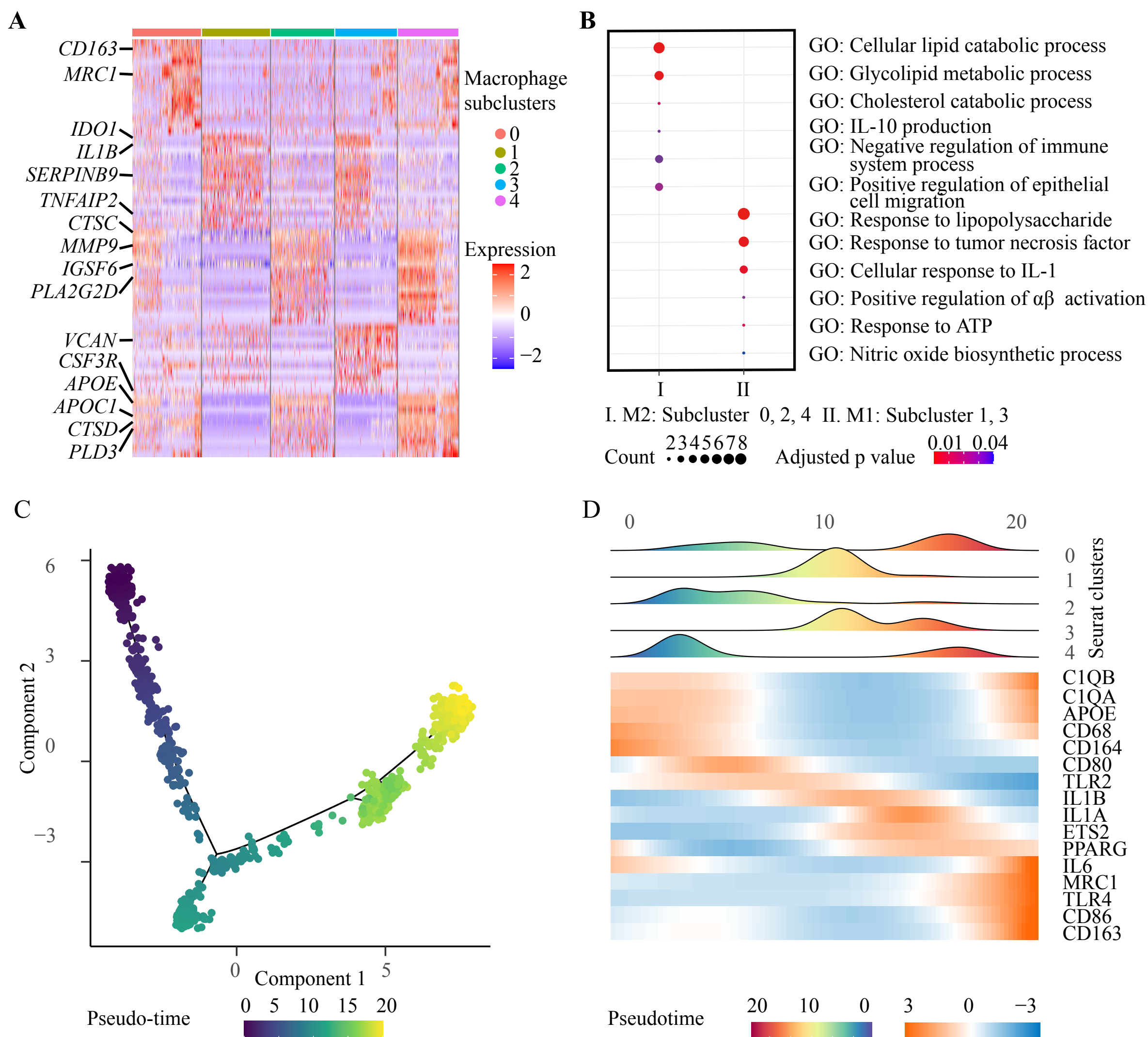


**Supplementary Figure 4. The expression of marker genes of each subclusters**



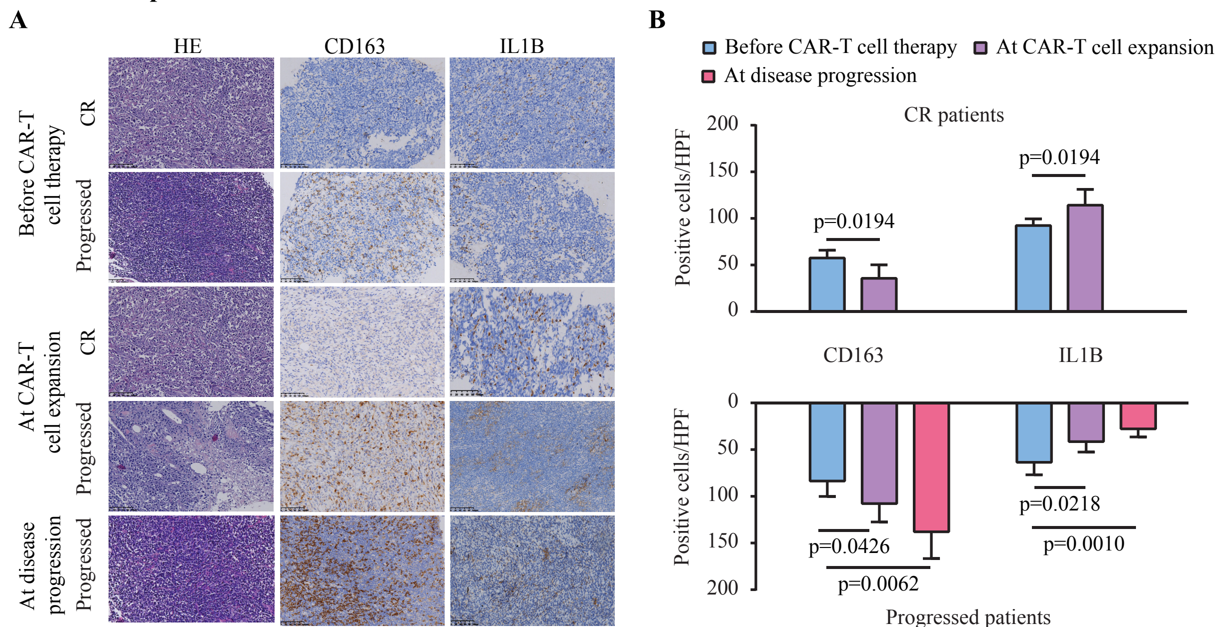

Dot plot showing the gene expression of each cluster. Color bar indicates expression level and dot size indicates percentage.

**Supplementary Figure 5. The proportion and biological function of M1- and M2-subtype macrophages.**




(A) Heatmap showing the gene expression of macrophage subclusters 0,1,2,3 and 4. (B) Dot plot showing gene expression enrichment of each pathway in M1- and M2-subtype macrophages. (C) Pseudo-time trajectory of macrophage subclusters. (D) Ridge distribution of macrophage cell subsets (upper panel) along pseudo-time and heatmap of the expression level of feature genes (lower panel) along the pseudo-time.

**Supplementary Figure 6. Hematoxylin-eosin staining and immunohistochemistry results of CD163 and IL1B expression.**




(A) Hematoxylin-eosin staining and immunohistochemistry results of CD163 and IL1B expression in tumor samples to CR and progressed patients collected before CAR-T cell therapy, at CAR-T cell expansion and at disease progression. (B) CD163 and IL1B expression in tumor samples during stages of CAR-T cell therapy.

**Supplementary Figure 7. The proportion and biological function of T-cell subclusters.**

A

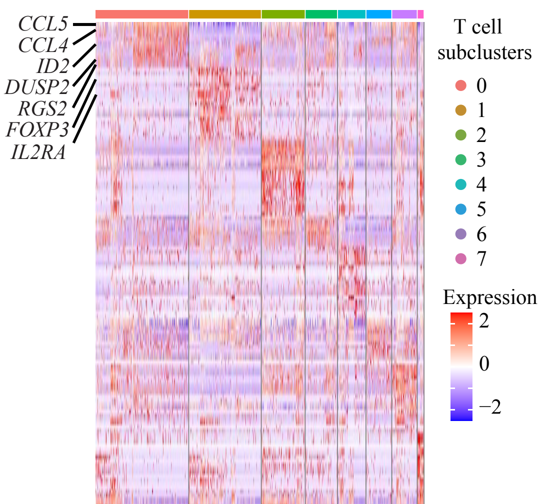
*CD3D*
*CD3E*
*CD3G*
*TRAC*
*CD4*
*IL7R*
*TCF7*
*TBX21*
*GATA3*
*RORC*
*IL2RA*
*FOXP3*
*CD8A*
*CD8B*
*KLRC2*
*KLRK1*
*CD69*
*GZMA*
*GZMB*
*GZMK*
*SELL*
*CCR7*
*CTLA4*
*LAG3*
*HAVCR2*
*PDCD1*
*KLRD1*
*NCAM1*
*GNLY*
*XCL2*
*NKG7*
*MKI67*
*TUBA1B*
*TOP2A*

0 1 2 3 4 5 6 7
T cell subclusters

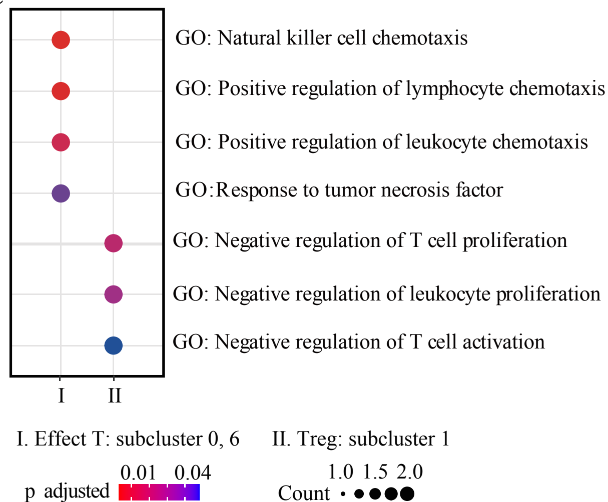
Percentage
0
25
50
75

Average expression
3
2
1
0

B

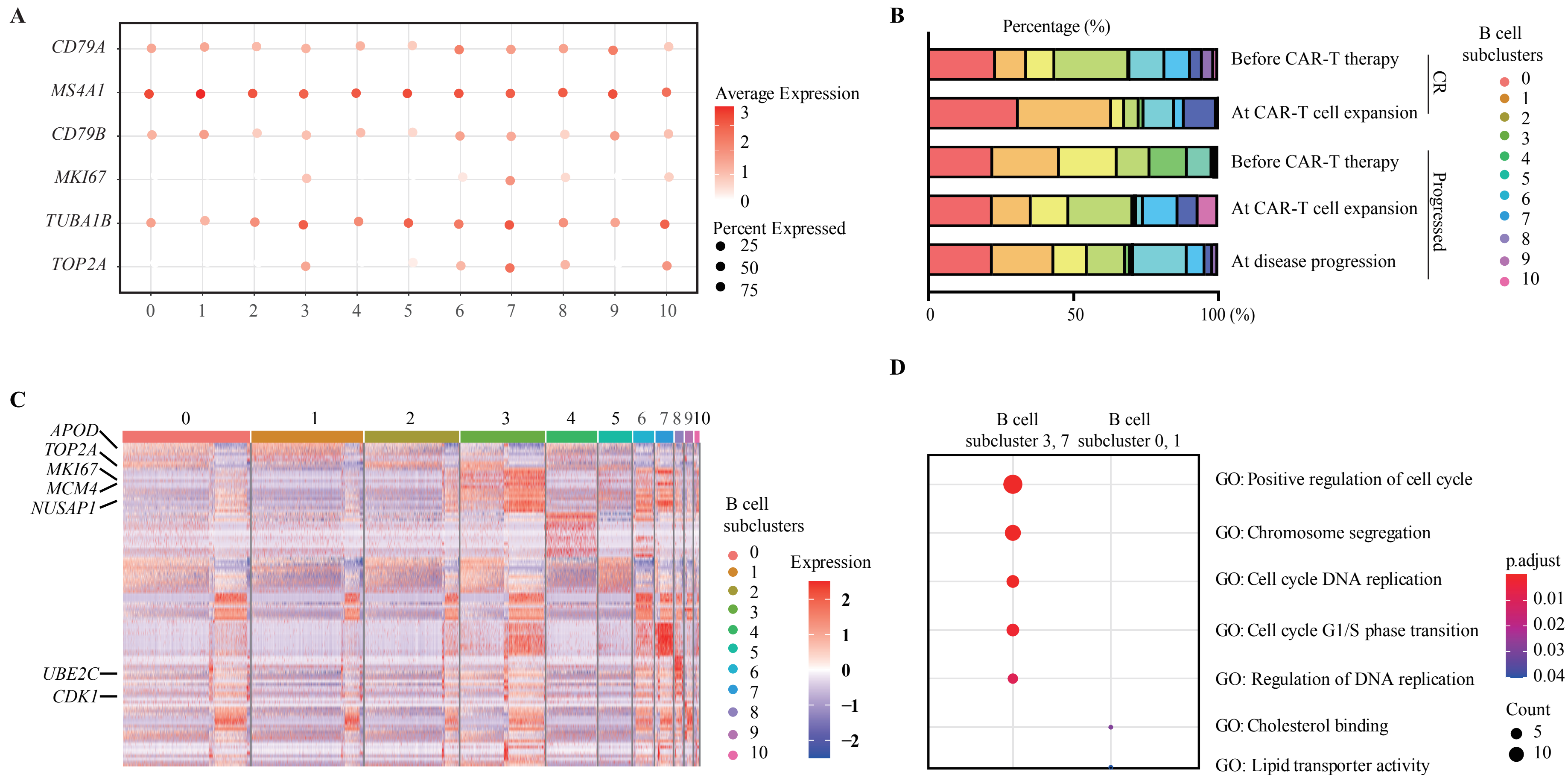



C




(A) Dot plot showing the gene expression of T cell subclusters. (B) Heatmap showing the gene expression of T cell subclusters. (C) Dot plot showing gene expression enrichment of each pathway in effect T cells and Treg cells.

**Supplementary Figure 8. The proportion and biological function of B-cell subclusters.**




(A) Dot plot showing the gene expression of B cell subclusters. (B) The proportion of B cell subclusters. (C)Heatmap showing the gene expression of B cell subclusters. (D) Dot plot showing gene expression enrichment of each pathway in B cell subcluster 3 and 7 (high proliferating population) and B cell subcluster 0 and 1 (low proliferative population). Dot plot showing the gene expression of each B cell subcluster.

## REFERENCES


1 SH S. WHO classificaiton of tumors of haematopoietic and lymphoid tissues. In. World Health Organization classification of tumors, t. e.

2 Yan, Z. X. *et al.* Clinical Efficacy and Tumor Microenvironment Influence in a Dose-Escalation Study of Anti-CD19 Chimeric Antigen Receptor T Cells in Refractory B-Cell Non-Hodgkin's Lymphoma. *Clin Cancer Res* **25**, 6995-7003, doi:10.1158/1078-0432.CCR-19-0101 (2019).

3 Cheson, B. D. *et al.* Recommendations for initial evaluation, staging, and response assessment of Hodgkin and non-Hodgkin lymphoma: the Lugano classification. *J Clin Oncol* **32**, 3059-3068, doi:10.1200/JCO.2013.54.8800 (2014).