Supplementary Information

Efficacy and safety of CD19-specific CAR T-cell-based therapy in B-cell acute lymphoblastic leukemia patients with CNSL

SUPPLEMENTARY MATERIALS

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SUPPLEMENTARY METHODS

1. Study design and procedures

This retrospective study design was approved by the institutional ethical review boards of The Affiliated Hospital of Xuzhou Medical University, The First Affiliated Hospital of Zhejiang University, Tianjin First Central Hospital, Tongji Hospital of Tongji University, Tongji Hospital, Tongji Medical College, and Huazhong University of Science and Technology. Clinical trials were registered at www.clinicaltrials.gov as # NCT02782351 and www.chictr.org.cn as # ChiCTR-OPN-16008526.

1.1 Inclusion and exclusion criteria

Eligible patients should be diagnosed as relapsed or refractory B-cell acute lymphoblastic leukemia (B-ALL). B-ALL diagnosis is confirmed according to the World Health Organization classification for tumors of the hematopoietic and lymphoid tissues[1]. Relapsed disease is defined as the reappearance of blasts in blood or bone marrow or in an extramedullary site after a complete remission[2]. Refractory disease is defined as patients experiencing less than a CR after initial induction therapy[2]. Good performance status (ECOG-PS ≤ 2), essentially normal organ function, measurable disease, and a life expectancy of 12 weeks or more were necessary for eligibility. Children candidates could be recruited after the legal guardian had signed the treatment consent form and voluntary consent form. Written informed consent should be obtained from each participant, in compliance with the Declaration of Helsinki.

Patients with uncontrollable infection, active and severe auto-immune disease, acute respiratory/circulatory failure, active graft-versus-host disease (GVHD), any mental or psychological illnesses, and other types of malignant tumors were excluded. Pregnant or lactating women, or female with a pregnancy plan within six months were ineligible.

In addition, patients with CNS-1/2 status before screening were eligible for enrollment. Patients with active CNSL of CNS-3 status before screening, or developing severe cerebral edema or persistent seizure, were excluded until alternative bridging therapies achieved neurologic stabilization and the patients' status returned to baseline[2]. Definition of CNS status for B-ALL is defined according to NCCN guidelines of Acute Lymphoblastic Leukemia,

Version 2.2015. CNSL is defined as CNS-3 status by the most recent relapse or within 30 days before screening.

Table 1

Definition of CNS Status for B-ALL	
	CSF Cell Count and Cytology
CNS-1	No detectable blasts on cytology in a sample of (CSF)
CNS-2	<5/μL WBCs, cytology positive for blasts
CNS-3	⩾5/μL WBCs, cytology positive for blasts or solid mass

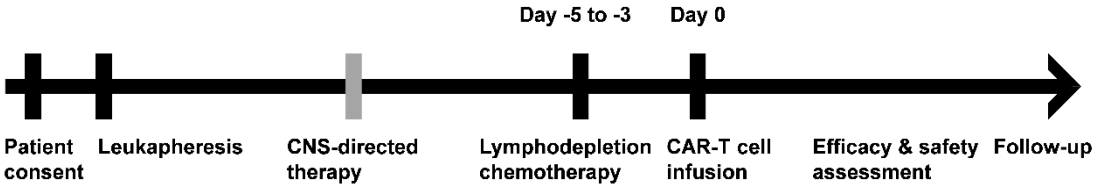
* If the patient has leukemic cells in the peripheral blood and the LP is traumatic and WBC ⩾5/mcL in CSF with blasts, then compare the CSF WBC/red blood cell (RBC) ratio to the blood WBC/RBC ratio. If the CSF ratio is at least two-fold greater than the blood ratio, then the classification is CNS-3; if not, then it is CNS-2.

1.2 Clinical procedures

After providing written informed consent, all eligible patients would undergo leukapheresis. All patients were required to have CNS assessment before screening in the trials. Patients with high disease burden at screening (>20/μL WBCs in CSF, cytology positive for blasts or solid mass) were permitted to have CNS-directed bridging chemotherapy during the manufacturing interval between leukapheresis and CAR T-cell infusion. CNS-directed bridging chemotherapy included IT chemotherapy (methotrexate: 10mg, cytarabine: 50mg, and dexamethasone: 5mg each time), and systemic chemotherapy (high-dose methotrexate, range, 3-5g/m2) at the discretion of the treating physician. After bridging therapy, a CNS re-assessment is required after bridging therapy, and before CAR T-cell infusion. All included patients are given fludarabine (30 mg/m^2/day, day -5 to -3) and cyclophosphamide (750mg/m^2, day -5) for lymphodepletion chemotherapy. Patients are required to receive the last assessment of BM/CNS diseases before infusion, of whom, 27 patients who received bridging chemotherapy after leukapheresis and underwent the last assessment after bridging therapy, and before CAR T-cell infusion. Procedures are demonstrated in Figure 1.

Patterns of infusion included isolated CD19 CAR T-cell therapy and combined CD19 and CD22 CAR T-cell therapy. CAR T cells are infused two days after the end of lymphodepletion chemotherapy. The protocol-specified single intravenous infusion dose range of CD19 CAR T-cell infusion is 1-5× 10^6 cells/kg body weight based on pretreatment disease burden. During the entire infusion process, the patient's vital signs should be closely monitored. 30 to 60 minutes before CAR T-cell infusion, patients are given 325 to 650 mg of acetaminophen to prevent infusion-related reactions.

Figure 1



The response was assessed according to the NCCN Guidelines Version 1.2016. Re-evaluation of the response was performed two weeks, 1 month, 2 months, 3 months, 6 months, and one year after CAR T-cell infusion. All patients were followed up until they died or lost to follow-up. We collected clinical data regarding general information at enrollment, clinical manifestations, laboratory examinations and diagnostic imaging at diagnosis, treatment, and follow-up. In patients with extramedullary disease, the assessment included CSF evaluation, imaging techniques, and physical examination. Patients should be reassessed and given salvage therapy under the condition of disease progression or relapse at any time.

2. CAR T-cell manufacturing

For isolated anti-CD19 CAR T-cell products, lentiviral vectors carrying second generation of CD19 CAR with a single chain variable fragment (scFv) derived from a murine monoclonal antibody against human CD19, a 4-1BB or CD28 co-stimulatory domain, a CD8 hinge, a CD3ζ intracellular domain and a T2A-EGFRt sequence are constructed as previously described[3]. The packaging plasmid required to produce the chimeric antigen receptor lentiviral expression vector are prepared using an endotoxin-free plasmid extraction kit. PBMCs are isolated from peripheral blood of patients and $CD3^+$ T cells are

separated by magnetic beads. After activation, T cells are infected with recombinant anti-CD19 lentivirus, and then CAR T cells are expanded. The expression of EGFRt on the surface of recombinant CAR T cells is detected by flow cytometry as screening markers for CAR T cells and a safety switch for clinical research. To assess the transfection efficiency, T cells are stained with Alexa Fluor 647 labeled protein L or rabbit anti-mouse-F(ab) 2 antibodies (Jackson ImmunoResearch). The apoptosis assay is conducted using an Annexin V Apoptosis Detection Kit (BD Biosciences) according to its instructions. The *in vitro* coculture system is conducted as previously reported for determining the anti-tumor activity of CAR T cells[3].

For combined infusion of anti-CD19 and anti-CD22 CAR T-cell products, lentiviral vectors carrying third generation of CD19 or CD22 CAR with a scFv derived from a murine monoclonal antibody against human CD19 or CD22, tandem 4-1BB&CD28 co-stimulatory domains and a CD3 signaling domain are constructed. The anti-CD19 and anti-CD22 CAR T cells were acquired through the transfection of lentivirus into peripheral $CD3^+$ T cells from PBMCs collected from each patient. Detailed information for lentiviral construction, and cell production of anti-CD19&CD22 CAR T-cell products was described before[4]. Quality-control procedures included assays for transfection efficiency, apoptosis and tumoricidal activity.

3. Adverse events evaluation and management

All adverse events occurring within 30 days after CAR T-cell infusion were recorded. Grading for cytokine release syndrome (CRS) was performed by the treating physician per Lee et al[5], and they were retrospectively regraded in accordance with the recently released American Society for Transplantation and Cellular Therapy (ASTCT) 2019 guidelines. The final analysis of CRS used in the study was based on ASTCT grading system. Neurotoxicity was graded through National Cancer Institute Common Terminology Criteria for Adverse Events v.4.03 system.

Table 2

ASTCT CRS Consensus Grading

CRS parameter	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Fever[1]	Temperature⩾38°C	Temperature⩾38°C	Temperature⩾38°C	Temperature⩾38°C	
			With		
Hypotension	None	Not requiring vasopressors	Requiring a vasopressor with or without vasopressin	Requiring multiple vasopressors (excluding vasopressin)	
			And/or[2]		Death[4]
Hypoxia	None	Requiring low-flow nasal cannula z or blow-by	Requiring high-flow nasal cannula[3], facemask, nonrebreather mask, or Venturi mask	Requiring positive pressure (eg, CPAP, BiPAP, intubation and mechanical ventilation)	

Organ toxicities associated with CRS may be graded according to CTCAE v5.0 but they do not influence CRS grading.
1 Fever is defined as temperature⩾38°C not attributable to any other cause. In patients who have CRS then receive antipyretic or anticytokine therapy such as tocilizumab or steroids, fever is no longer required to grade subsequent CRS severity. In this case, CRS grading is driven by hypotension and/or hypoxia.
2 CRS grade is determined by the more severe event: hypotension or hypoxia not attributable to any other cause. For example, a patient with temperature of 39.5°C, hypotension requiring 1 vasopressor, and hypoxia requiring low-flow nasal cannula is classified as grade 3 CRS.
3 Low-flow nasal cannula is defined as oxygen delivered at⩽6 L/min. Low flow also includes blow-by oxygen delivery, sometimes used in pediatrics. High-flow nasal cannula is defined as oxygen delivered at >6 L/min.
4 Grade 5 CRS is defined as death due to CRS in which another cause is not the principle factor leading to this outcome.

Table 3

CTCAE v.4.03. Neurotoxicity Grading System

Neurotoxicity Domain	Grade 1	Grade 2	Grade 3	Grade 4
Encephalopathy	Mild symptoms	Moderate symptoms; limiting instrumental ADL	Severe symptoms; limiting self-care ADL	Life-threatening consequences; urgent intervention indicated
Seizure	Brief partial seizure and no loss of consciousness	Brief generalized seizure	New-onset seizures (partial or generalized); multiple seizures despite medical intervention	Life-threatening consequences
Dysphasia	Awareness of receptive or expressive characteristics; not impairing ability to communicate	Moderate receptive or expressive characteristics; impairing ability to communicate spontaneously	Severe receptive or expressive characteristics; impairing ability to read, write, communicate intelligibly	
Tremor	Mild symptoms	Moderate symptoms; limiting instrumental ADL	Severe symptoms; limiting self-care ADL	
Headache	Mild pain	Moderate pain; limiting instrumental ADL	Severe pain; limiting self-care ADL	
Confusion	Mild disorientation	Moderate disorientation; limiting instrumental ADL	Severe disorientation; limiting self-care ADL	Life-threatening consequences; urgent intervention indicated
Depressed level of consciousness	Decreased level of alertness	Sedation; slow response to stimuli; limiting instrumental ADL	Difficult to arouse	Life-threatening consequences; coma; urgent intervention indicated
Cerebral edema			New onset; worsening from baseline	Life-threatening consequences; urgent intervention indicated

Table 4

CRS Management Guidelines

Grade 1	• Offer supportive care with antipyretics, IV hydration, and symptomatic management of organ toxicities and constitutional symptoms • May consider empiric broad-spectrum antibiotics and G-CSF if neutropenic. Note: GM-CSF is not recommended • In patients with persistent (＞3 days) or refractory fever, consider managing as per grade 2
Grade 2	• Continue supportive care as per grade 1 and include IV fluid bolus and/or supplemental oxygen as needed • Administer tocilizumab 8 mg/kg IV over 1 hour (not to exceed 800 mg/dose). Repeat every 8 hours if no improvement in signs and symptoms of CRS; limit to a maximum of three doses in a 24 hours, with a maximum of four doses total • In patients with hypotension that persists after two fluid boluses and after one to two doses of tocilizumab, may consider dexamethasone 10 mg IV[1] every 12 hours for one to two doses and then reassess • Manage per grade 3 if no improvement within 24 hours of starting tocilizumab
Grade 3	• Continue supportive care as per grade 2 and include vasopressors as needed • Admit patient to ICU • If echocardiogram was not already performed, obtain ECHO to assess cardiac function and conduct hemodynamic monitoring • Tocilizumab as per grade 2 if maximum dose is not reached within 24 hours plus dexamethasone 10 mg[1] IV every 6 hours and rapidly taper once symptoms improve • If refractory, manage as per grade 4
Grade 4	• Continue supportive care as per grade 3 plus mechanical ventilation as needed • Administer tocilizumab as per grade 2 if maximum is not reached within 24 hours • Initiate high-dose methylprednisolone at a dose of 500 mg IV every 12 hours for 3 days, followed by 250 mg IV every 12 hours for 2 days, 125 mg IV every 12 hours for 2 days, and 60 mg IV every 12 hours until CRS improvement to grade 1 • If not improving, consider methylprednisolone 1,000 mg IV 2 times a day or alternate therapy

G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; ICU, intensive care unit; IV, intravenous.

1 Or equivalent methylprednisolone dose (1 mg/kg).

Table 5

Neurotoxicity Management Guidelines

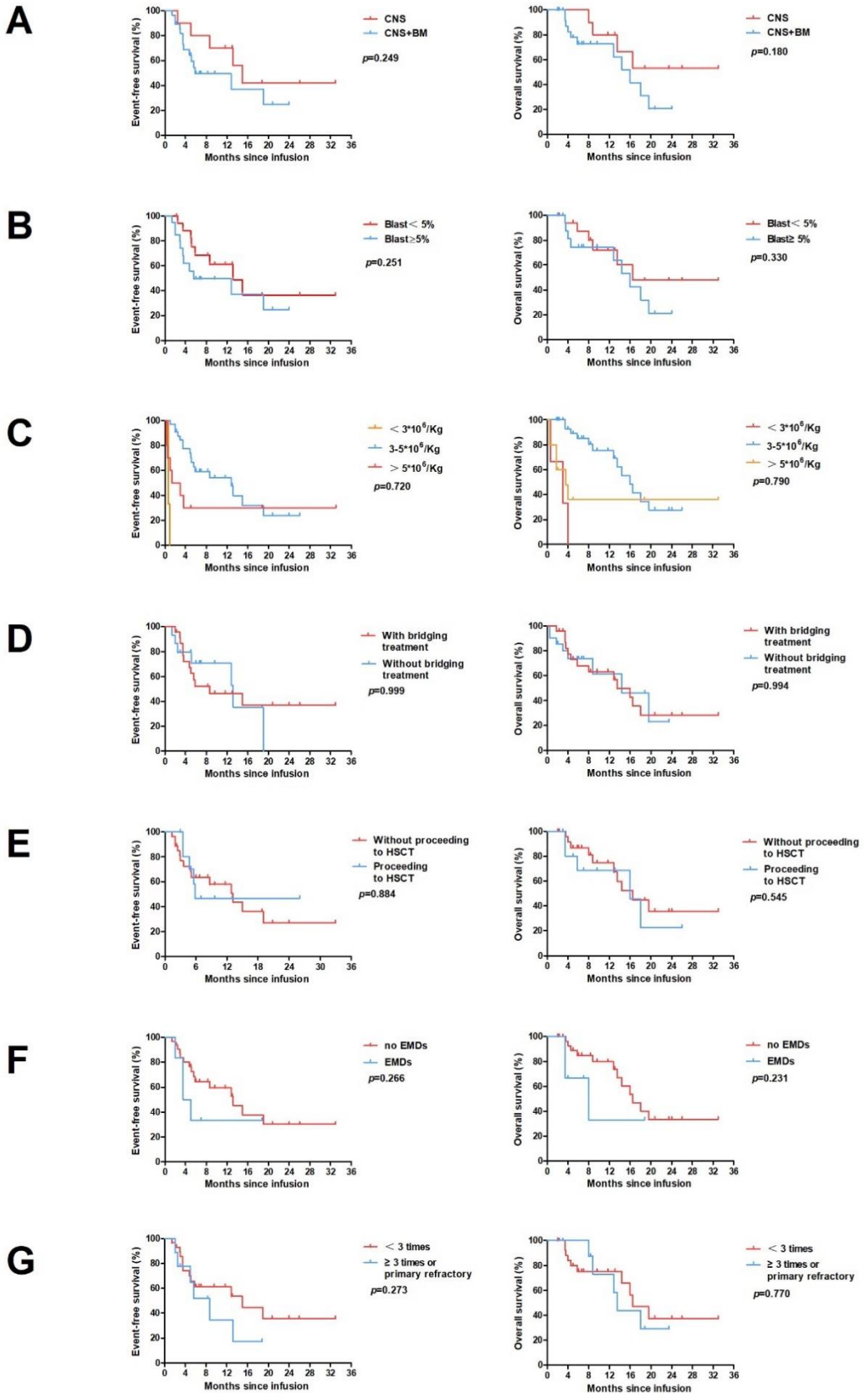
Grade 1	• Supportive care • Closely monitor neurologic status • Consider prophylactic antiepileptic
Grade 2	**Supportive Care and Evaluation** • Continuous cardiac telemetry and pulse oximetry as indicated • Serial neurological examinations to include fundoscopy and Glasgow Coma Score, brain MRI, evaluation of CSF, EEG; consider neurology consult • Administer antiepileptics for patients with seizures **Tocilizumab** • For patients with concurrent CRS, administer tocilizumab 8 mg/kg IV over 1 hour (not to exceed 800 mg); repeat every 4-6 hours as needed if not responsive to IV fluids or increasing supplemental oxygen, for a maximum of 3 doses in 24 hours • Discontinue tocilizumab if patient improves **Corticosteroids** • For patients without concurrent CRS, administer dexamethasone 10 mg[1] IV every 6 hours • For patients with concurrent CRS, if no improvement within 24 hours after starting tocilizumab, administer dexamethasone 10 mg[1] IV every 6 hours
Grade 3	**Supportive Care and Evaluation** • Manage in monitored care or ICU **Tocilizumab** • Per grade 2 • Discontinue tocilizumab if patient improves **Corticosteroids** • Administer dexamethasone 10 mg[1] IV every 6 hours • Taper corticosteroids if patient improves
Grade 4	**Supportive Care and Evaluation** • Per grade 3 • Mechanical ventilation may be required • Administer immunosuppressants if patient does not improve **Tocilizumab** • Per grade 2 **Corticosteroids** • Administer high-dose corticosteroids (eg, methylprednisone 1g/d × 3 days) • Taper corticosteroids if patient improves

CSF, cerebrospinal fluid; EEG, electroencephalogram; ICU, intensive care unit; IV, intravenous; MRI, magnetic resonance imaging.
1 Or equivalent methylprednisolone dose (1 mg/kg).

Organ toxicities associated with CRS were graded according to CTCAE v.4.03, but they do not influence CRS grading according to ASTCT CRS grading system. Management of organ toxicities ranged from supportive care required for management of symptoms to organ-specific advanced management. As management of NEs, levetiracetam was the preferred agent for seizure prophylaxis with optimal dose of 500 to 750 mg twice daily initiated on the day of CAR T-cell infusion. Active seizures are managed with benzodiazepines as acute management, and antiepileptics, such as levetiracetam. For patients who developed severe cerebral edema, intravenous injection of dexamethasone or methylprednisolone was added besides intrathecal injection with dexamethasone. Mannitol (2.5 ml/kg/dose) was used intravenously to prevent and control intracranial hypertension.

SUPPLEMENTARY FIGURES

Figure S1



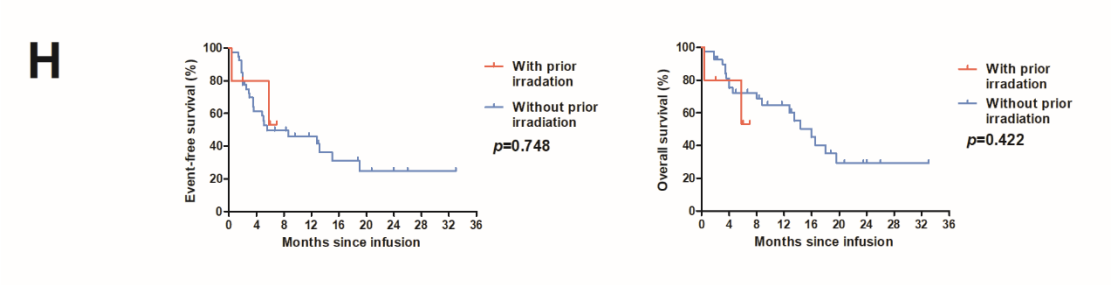


Figure S1. Long-term survival analysis across subgroups in B-ALL patients with CNSL.

Long-term survival analysis showed no significant difference in either EFS or OS across the following subgroups, including (A) disease status (CNS vs. BM+CNS), (B) blast% in BM (＜5% vs. ≥5%), (C) CAR T cell dose (＜3*10^6/Kg vs. 3-5*10^6/Kg vs.＞ 5*10^6/Kg), (D) CNS-directed bridging treatment (N vs. Y), (E) allo-HSCT consolidation (N vs. Y), (F) EMDs (N vs. Y), (G) times of relapse (＜3 times vs. ≥3 times or primary refractory), and (H) prior irradation (N vs. Y).

Figure S2A

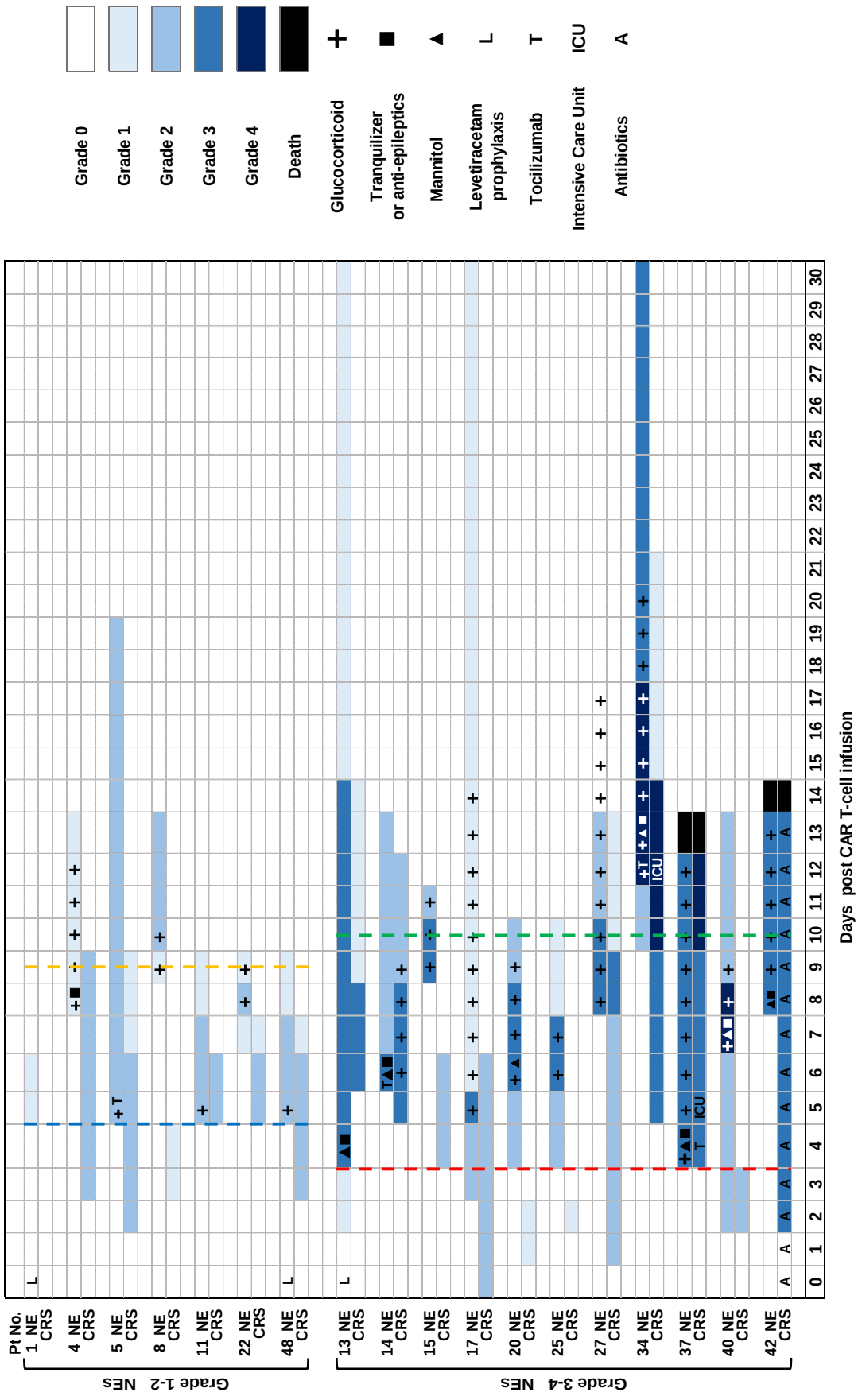

Grade 0
Grade 1
Grade 2
Grade 3
Grade 4
Death
Glucocorticoid
Tranquilizer or anti-epileptics
Mannitol
Levetiracetam prophylaxis
Tocilizumab
Intensive Care Unit
Antibiotics
Pt No. 1 NE CRS
4 NE CRS
5 NE CRS
8 NE CRS
11 NE CRS
22 NE CRS
48 NE CRS
13 NE CRS
14 NE CRS
15 NE CRS
17 NE CRS
20 NE CRS
25 NE CRS
27 NE CRS
34 NE CRS
37 NE CRS
40 NE CRS
42 NE CRS
Grade 1-2 NEs
Grade 3-4 NEs
Days post CAR T-cell infusion

Figure S2B

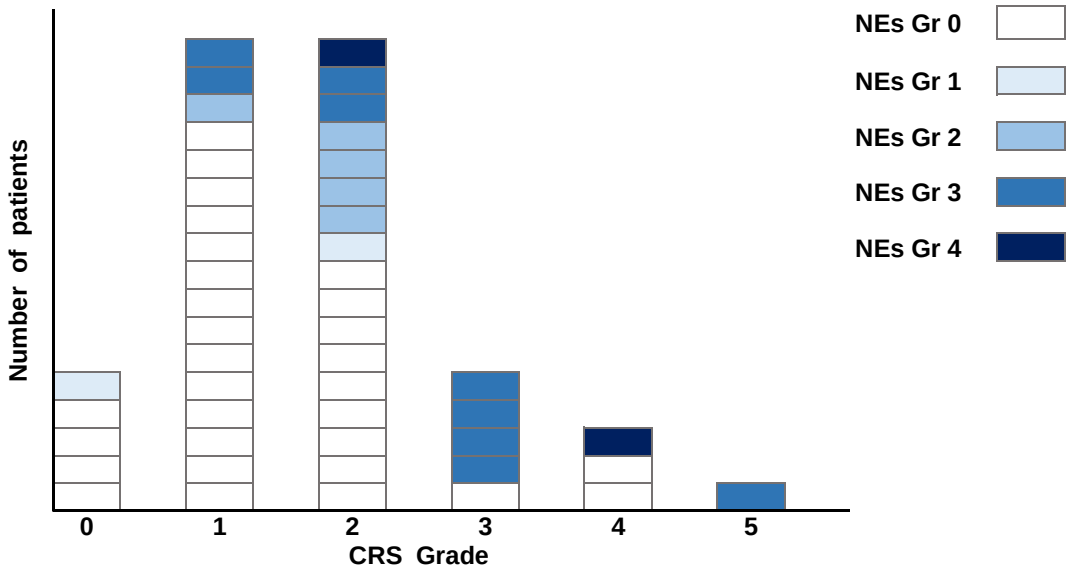


Figure S2. Timeline and management of NEs and CRS.

(A) Colors on the swimmer lane plot indicate the highest grade of any neurologic symptom recorded on each day for patients who developed grade ≥1 NEs through the first 30 days after CAR T-cell infusion. The median onset time of NEs in grade 1-2 (blue dotted line) and grade 3-4 (red dotted line) are indicated. The median duration from the onset of the highest grade of NEs to symptoms improvement in grade 1-2 patients (orange dotted line) and grade 3-4 patients (green dotted line) are indicated. Interventions are indicated as legends. (B) Number of patients with each grade of CRS and NEs. Gr, grade. Spearman analysis showed a significant correlation between the incidences of sCRS and sNEs (r=0.553, *p*=0.024).

Figure S3

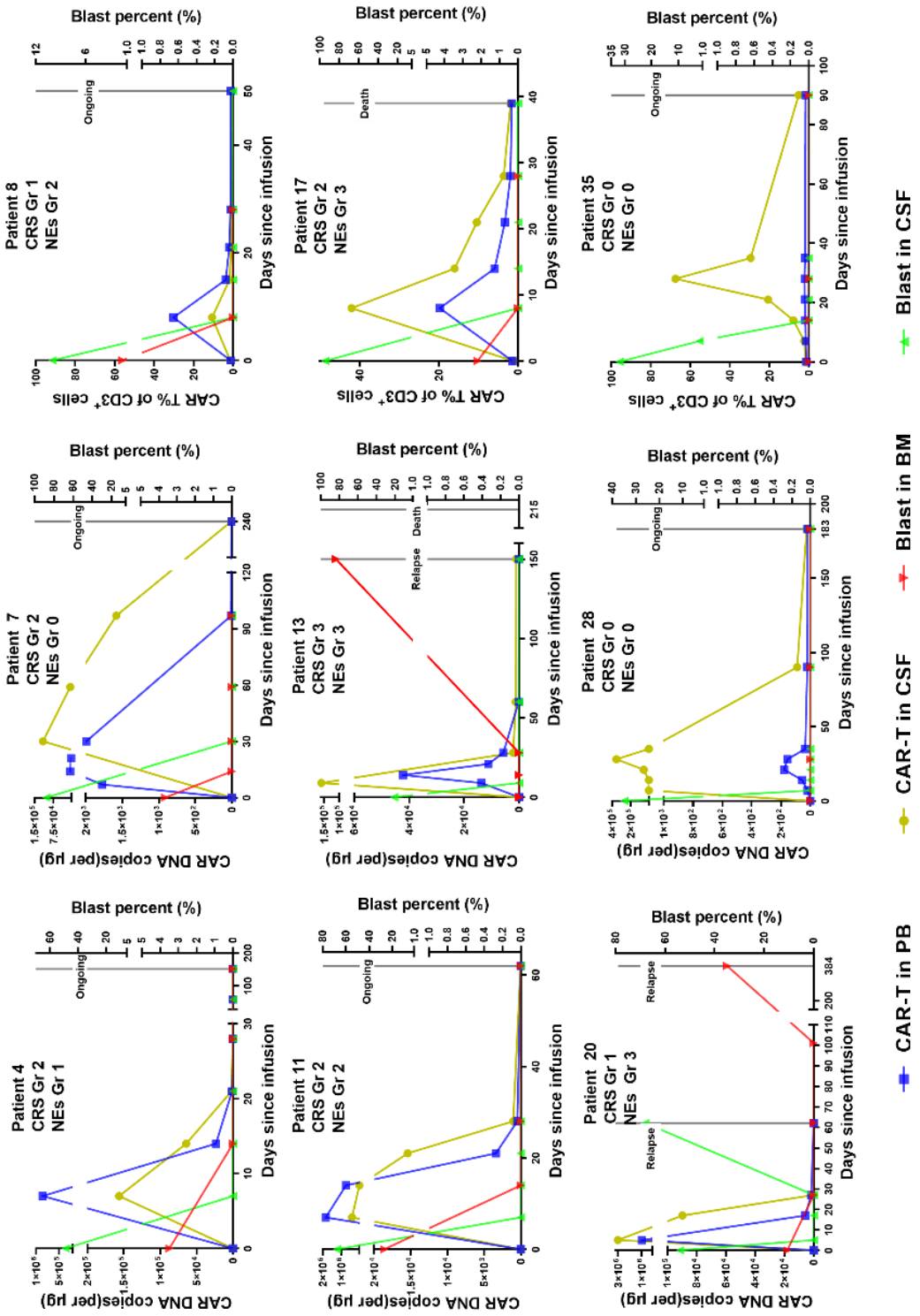


Figure S3. Dynamic changes of CAR T-cell expansion and blasts percentage in both BM and CSF.

Monitoring of CAR T cells in PB and CSF and blasts in BM and CSF in 9 evaluable patients after CAR T-cell infusion. The unconsecutive data was indicated as truncated parts due to unavailability of clinical data during follow-up. Gr, grade.

SUPPLEMENTARY TABLES

Table S1. All grade of neurotoxic events in the first 30 days after CAR T-cell infusion

Pt No.	Grade 1	Grade 2	Grade 3	Grade 4
1	Headache, disturbance in attention, mental status change, encephalopathy			
4	Headache, disturbance in attention, encephalopathy			
5	Cognitive disorder, lethargy	Headache, delirium, tremor, encephalopathy		
8	Headache, dizziness, lethargy	Cognitive disorder, disturbance in attention		
11	Disturbance in attention, headache, lethargy	Encephalopathy memory impairment, cognitive disorder		
22	Cognitive disorder, depressed level of consciousness	Delirium		
48	Depressed level of consciousness			
13		Depressed level of consciousness	Vision disorder	
14	Depressed level of consciousness	Encephalopathy, disturbance in attention, delirium	Cognitive disorder	
15	Depressed level of consciousness		Tremor, delirium, headache	
17	Depressed level of consciousness	Encephalopathy, delirium	Lethargy, disturbance in attention	
20		Delirium	Vision disorder	
25			Vision disorder, cognitive disorder	
27		Encephalopathy, ataxia	Aphasia, memory impairment, delirium	
34		Depressed level of consciousness	Tinnitus, headache	Encephalopathy, seizure
37		Delirium, ataxia	Depressed level of consciousness	Encephalopathy, seizure
40			Depressed level of consciousness	Encephalopathy, motor dysfunction, seizure
42			Depressed level of consciousness	Encephalopathy, seizure

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