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## Supplementary Material

This appendix has been provided by the authors to give readers additional information about their work.

### **Broadly neutralizing antibody-derived CAR-T cells reduce viral reservoir in HIV-1-infected individuals**

Bingfeng Liu, Wanying Zhang, Baijin Xia, Shuliang Jing, Yingying Du, Fan Zou, Rong Li, Lijuan Lu, Shaozhen Chen, Yonghong Li, Qifei Hu, Yingtong Lin, Yiwen Zhang, Zhangping He, Xu Zhang, Xiejie Chen, Tao Peng, Xiaoping Tang, Weiping Cai, Ting Pan, Linghua Li and Hui Zhang

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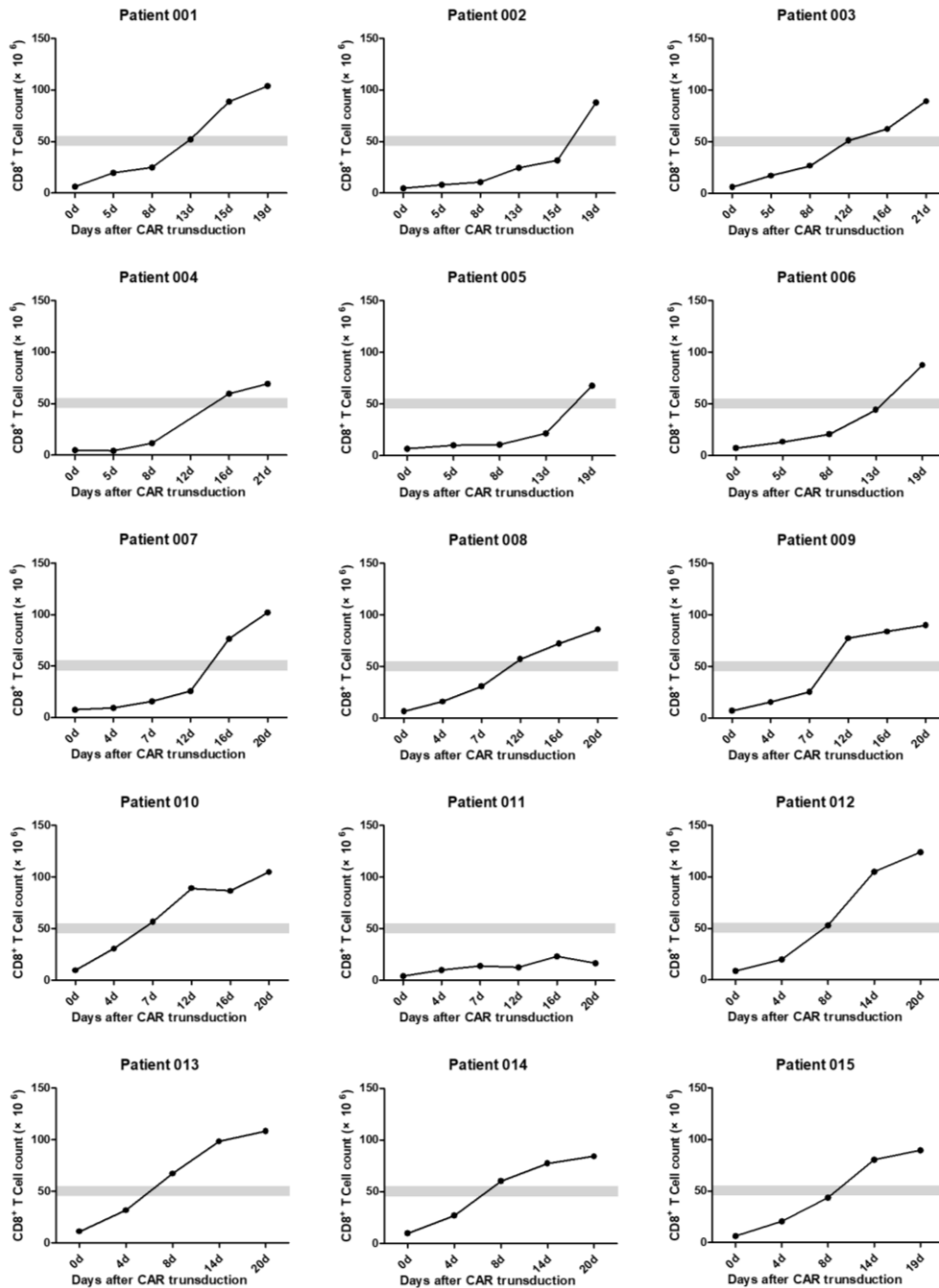
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## Supplemental Methods

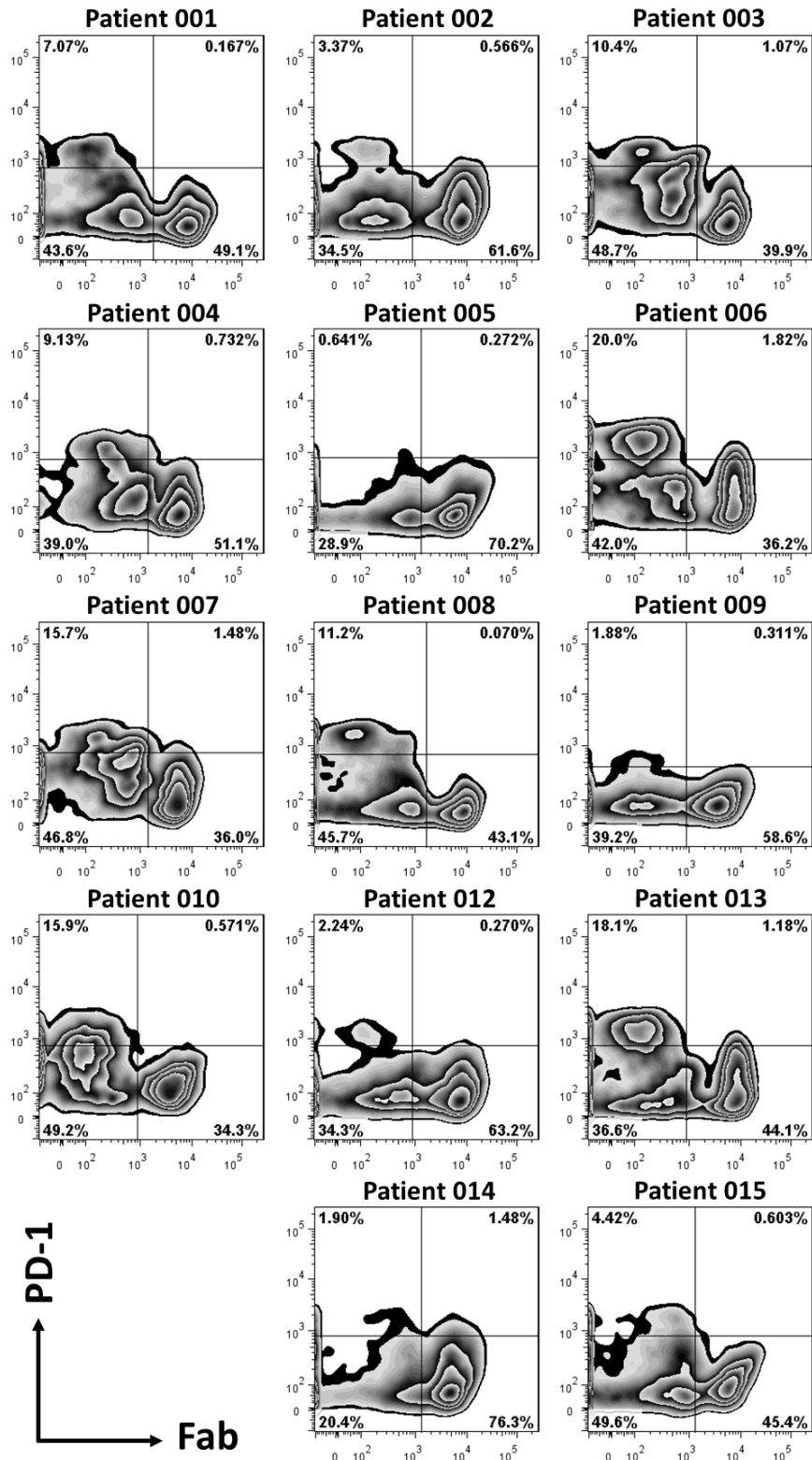
### IFN- $\gamma$ ELISpot

IFN- $\gamma$ -secreting cells were detected by human IFN- $\gamma$  ELISpot assay kit (DKW22-1000-096s; Dakewe) according to the manufacture protocol. The CD8<sup>+</sup> T cells (10<sup>5</sup> per well) from HIV-1-infected participants were incubated with Jurkat-gp160<sub>NL4-3</sub> (Jurkat-derived target cell line expressing HIV-1<sub>NL4-3</sub> Env) at 1: 1 ratio in antibody-precoated plates. Plates were incubated for 24 hours at 37°C and 5% CO<sub>2</sub>. The ELISpot assays were then performed according to the manufacturer's instructions. Spots were counted using an S6 ultra immunoscan reader (Cellular Technology Ltd.). The number of IFN- $\gamma$ -positive T cells was calculated by ImmunoSpot 5.1.34 software (Cellular Technology Ltd.) and converted into the number of spots per million CD8<sup>+</sup> T cells.

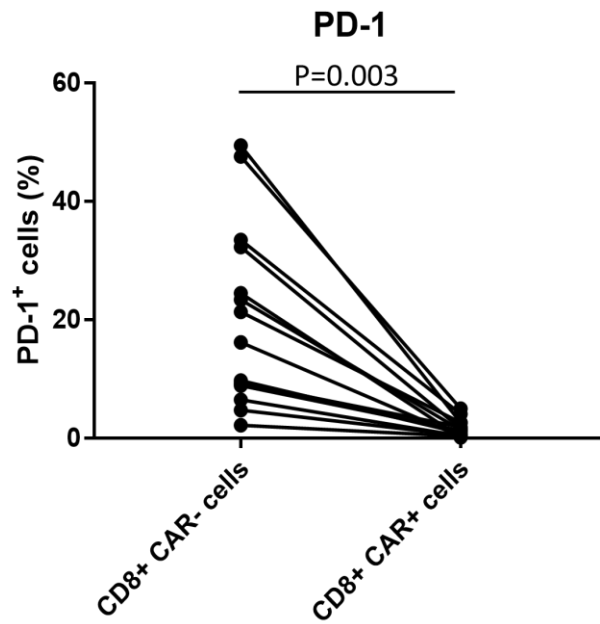
## Supplemental Figures



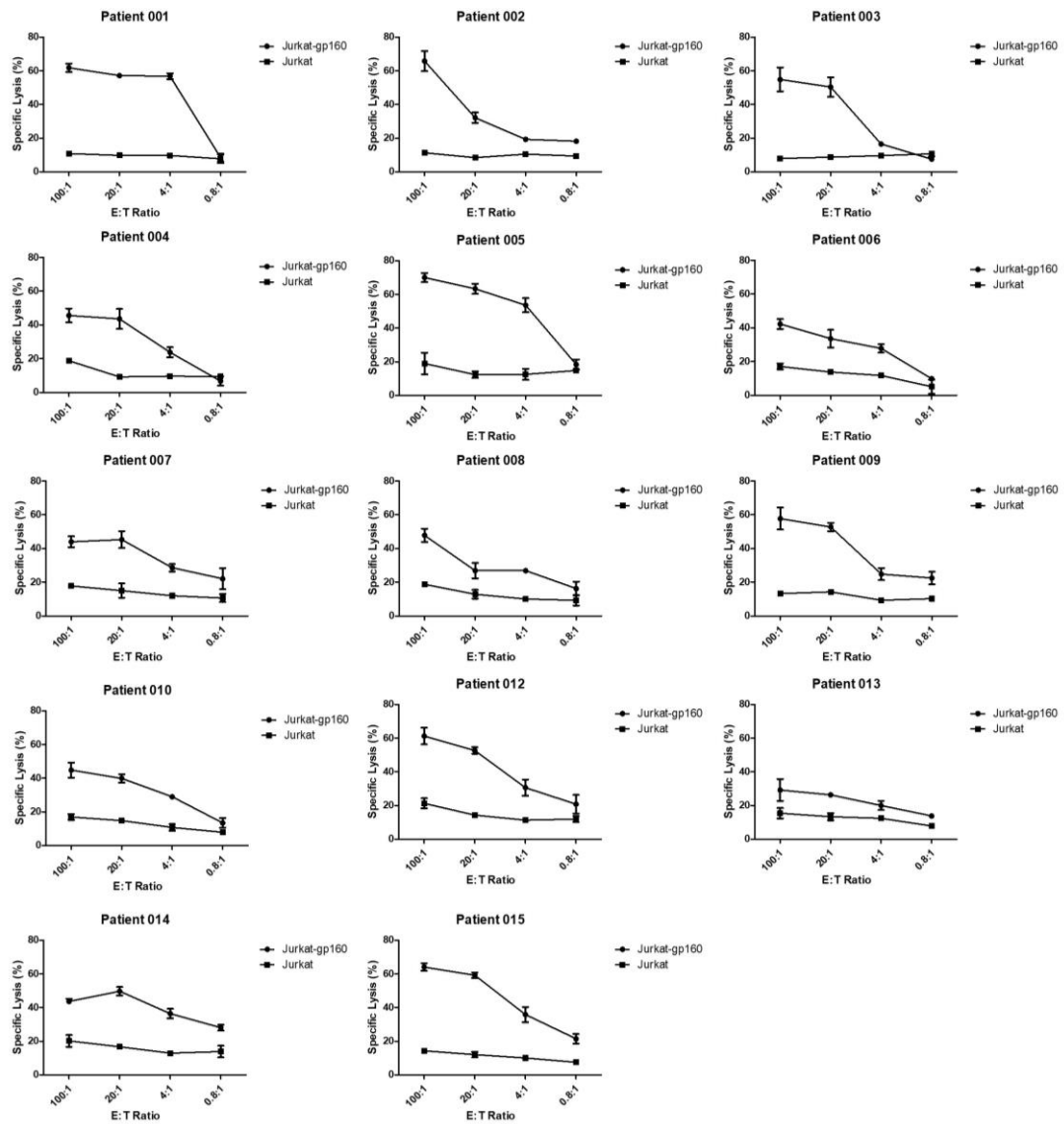
**Supplemental Figure 1. Expansion of CAR-modified T cells.** Panels show growth curves of *ex vivo* expanded CAR-modified CD8<sup>+</sup> T cells for the 15 enrolled patients. CD8<sup>+</sup> T cells were stimulated with anti-CD3/anti-CD28 antibodies and IL-2, transduced by the recombinant lentivirus encoding the bNAb-derived-CAR, and expanded through adding IL-2 every 2 to 3 days. Gray bar, the target cell dose range.



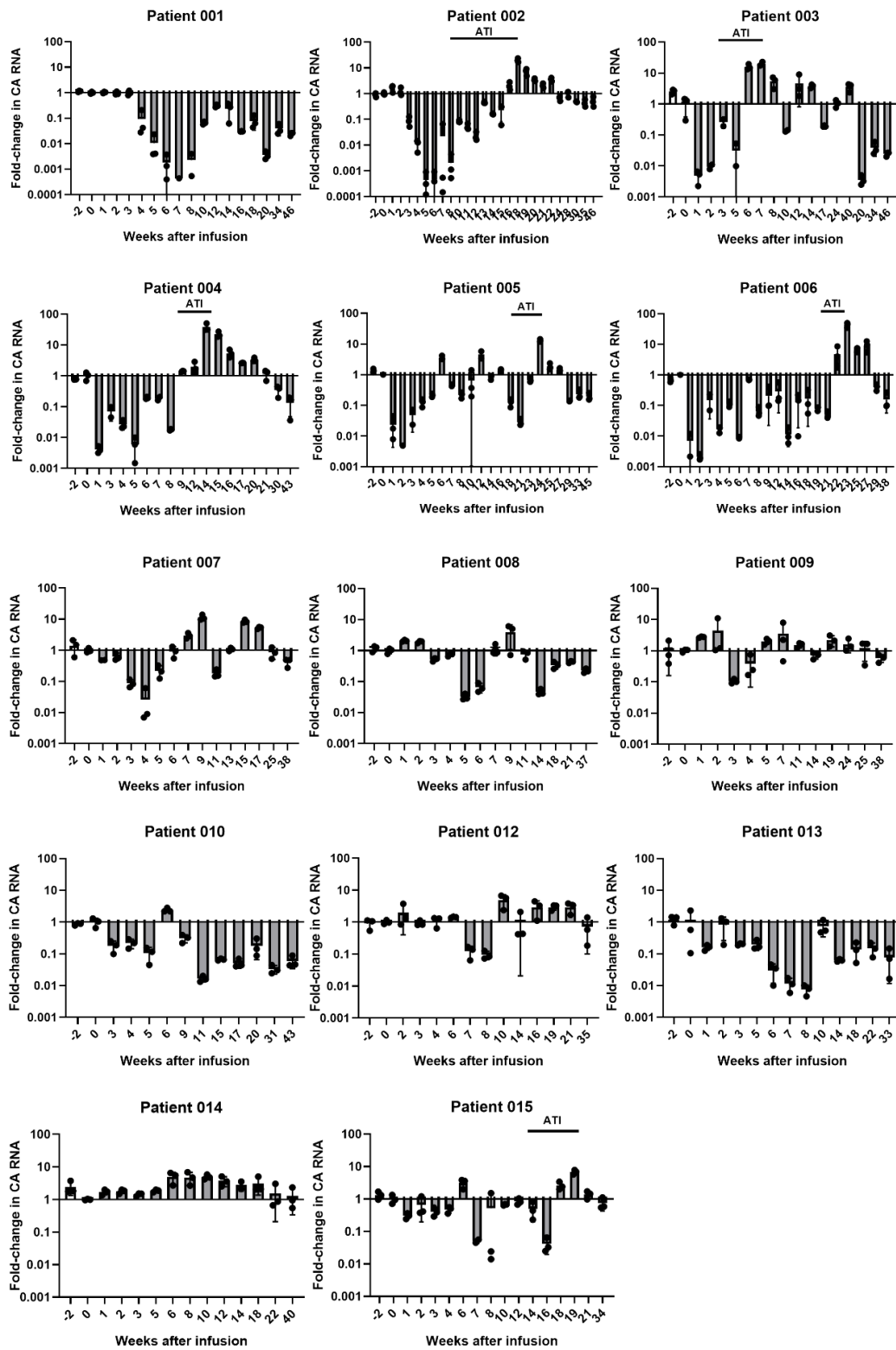
**Supplemental Figure 2. Immunophenotype of infused T cells.** End of production T cells for fourteen patients were analyzed by flow cytometry after being stained with the CD3, CD4, CD8, Fab and PD-1 antibodies. Fab and PD-1 expressions on CAR-transduced T cells are shown after gating on CD3<sup>+</sup> CD8<sup>+</sup> population.



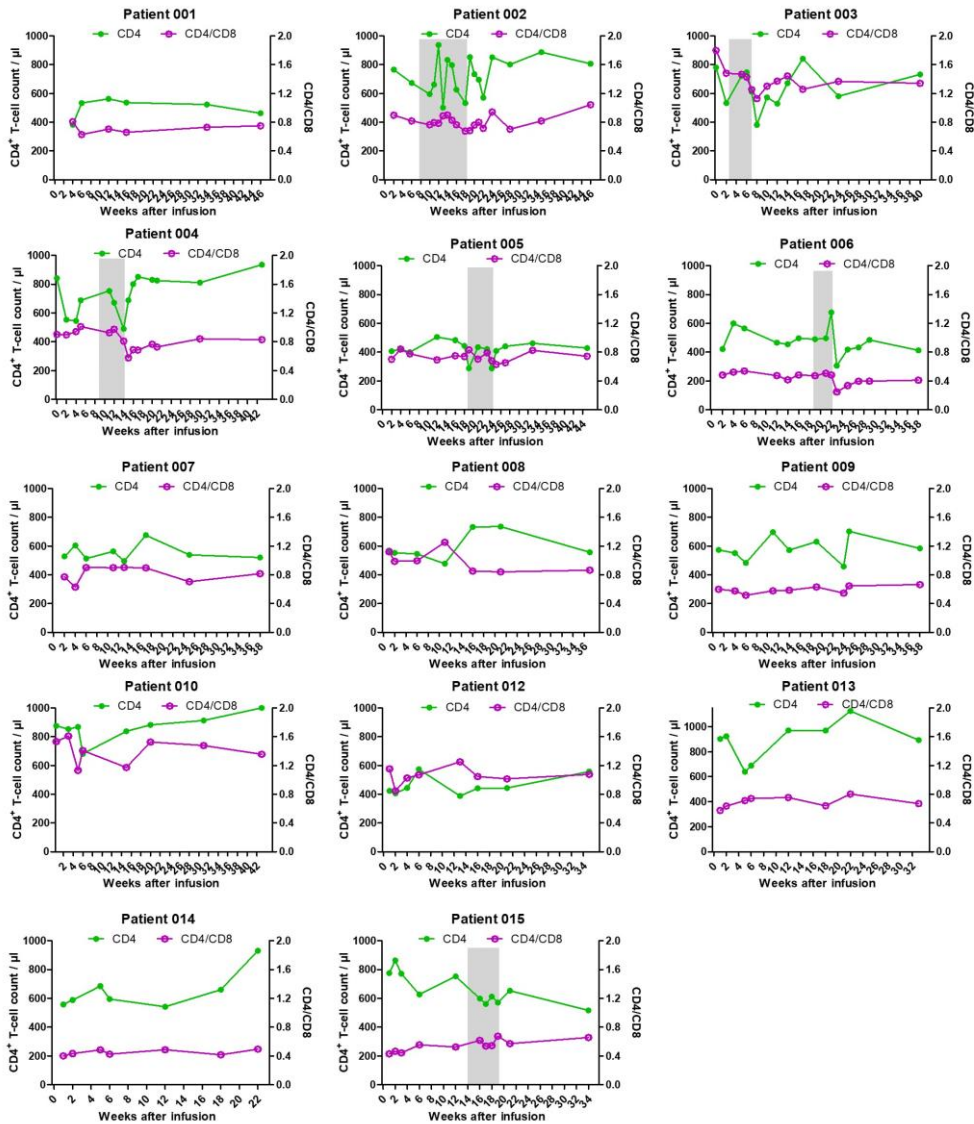
**Supplemental Figure 3. A comparison of PD-1 expression between CD8<sup>+</sup> CAR<sup>-</sup> and CD8<sup>+</sup> CAR<sup>+</sup> subpopulation.** Panel shows the comparison of the PD-1 expression between CD8<sup>+</sup> CAR<sup>-</sup> and CD8<sup>+</sup> CAR<sup>+</sup> subpopulation (n = 14) by flow cytometry. P-values were calculated using the two tailed paired Student's t-test with equal variances.



**Supplemental Figure 4. Cytotoxicity of CAR-T cells.** The bNAb-derived CAR-T cells after *ex vivo* expansion were mixed with Jurkat or Jurkat-gp160<sub>NL4-3</sub> (Jurkat-derived target cell line expressing HIV-1<sub>NL4-3</sub> Env and enhanced green fluorescent protein) as target cells. Twelve hours after co-culture, HIV-1 Env-specific cytotoxicity of bNAb-derived CAR-T cells was assessed by using LDH assay. Data represent the mean of duplicate values, and error bars represent SEM.

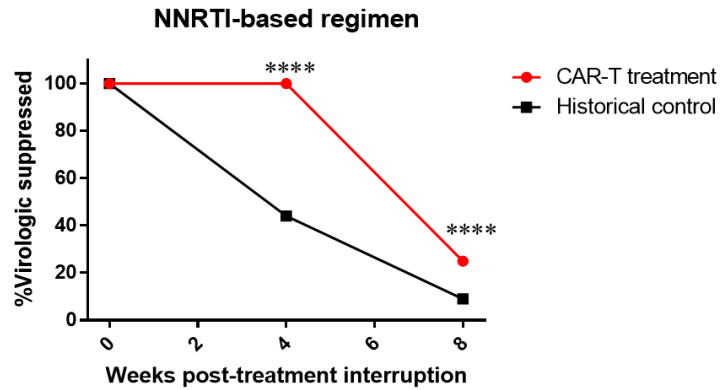


**Supplemental Figure 5.** The Fold-change of CA-RNA levels over baselines (week-0) are shown in fourteen participants before (white bars) and after (grey bars) CAR-T cell administrations. The black line represents the ATI period.



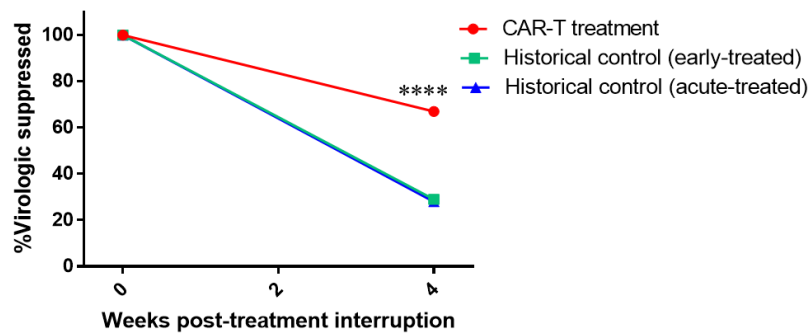
**Supplemental Figure 6. CD4<sup>+</sup> T-cell counts and CD4/CD8 ratios of study participants.** The CD4<sup>+</sup> T-cell counts in plasma (copies/ $\mu$ l) for each of the 14 enrolled patients are shown in green and CD4/CD8 ratios are shown in purple after adoptive transfer. ATI periods are shown by shades of grey.





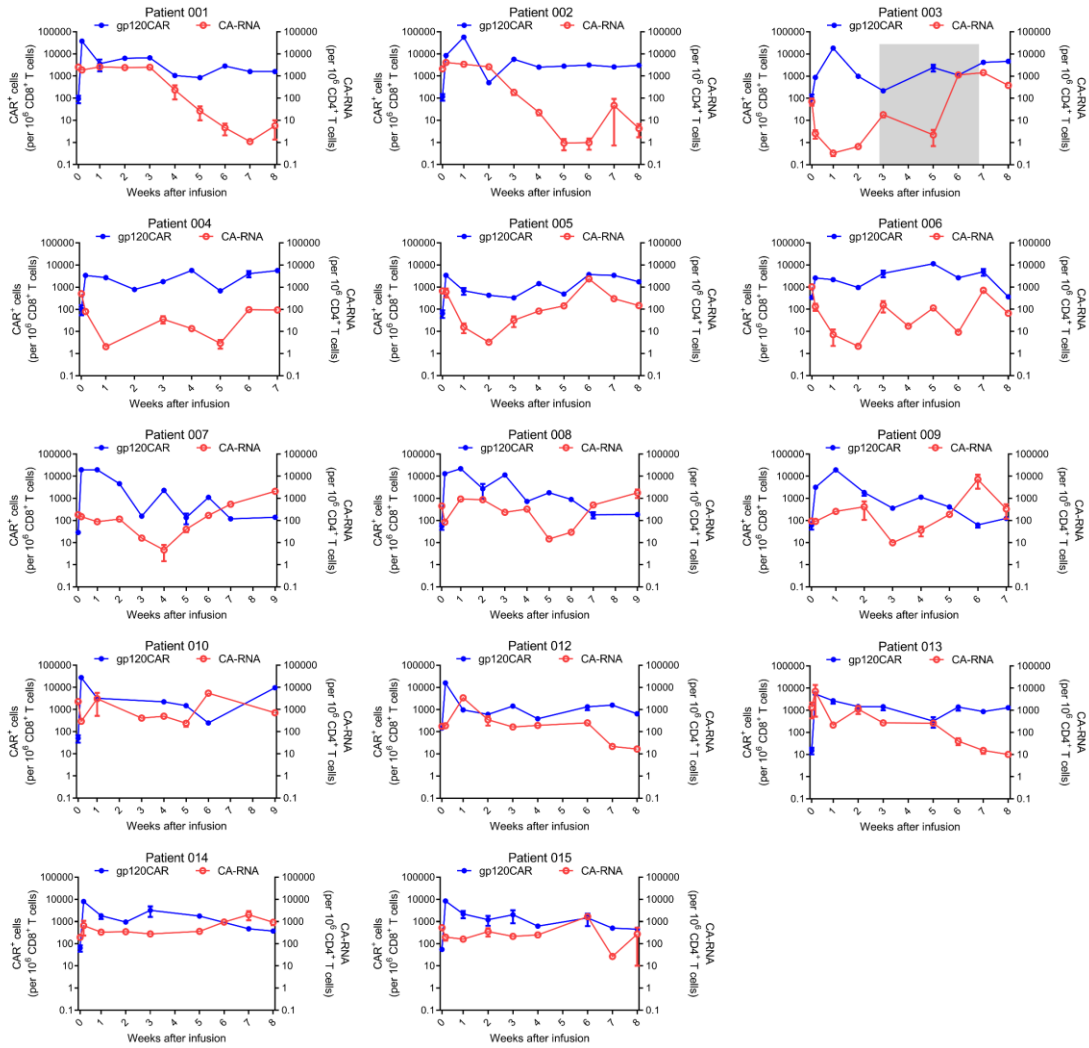
% Suppressed	4 wk	8 wk
CAR T cell treatment	100%	25%
History Control	44%	9%

**Supplemental Figure 7.** Cumulative percentage of virologic suppression within NNRTI-based regime after ATI are shown from CAR-T-receiving participants (n=4) or historical control (n=99) (plasma viral load <200 copies/mL). \*\*\*\*P-value <0.0001 by Fisher's exact testing.

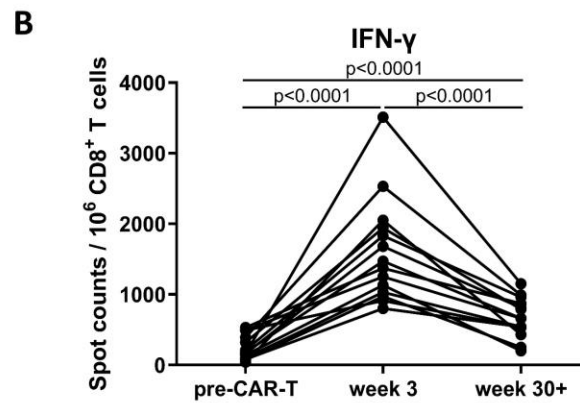
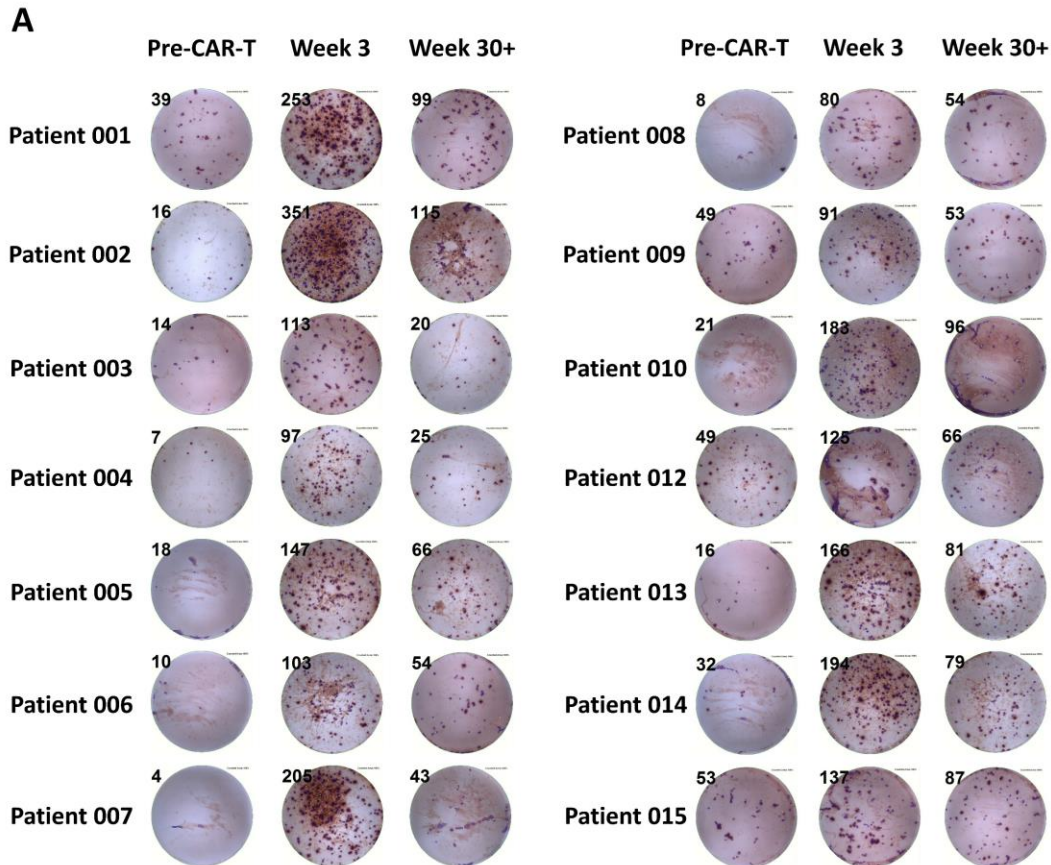


% Suppressed	4 wk
CAR T cell treatment	67%
History Control (early-treated)	29%
History Control (acute-treated)	28%

**Supplemental Figure 8.** Cumulative percentage of virologic suppression after treatment interruption are shown from CAR-T-receiving participants (n=6) or historical controls with treatment during acute- (n=32) or early-infection (n=48) (plasma viral load <200 copies/mL). \*\*\*\*P-value <0.0001 by Fisher's exact testing.

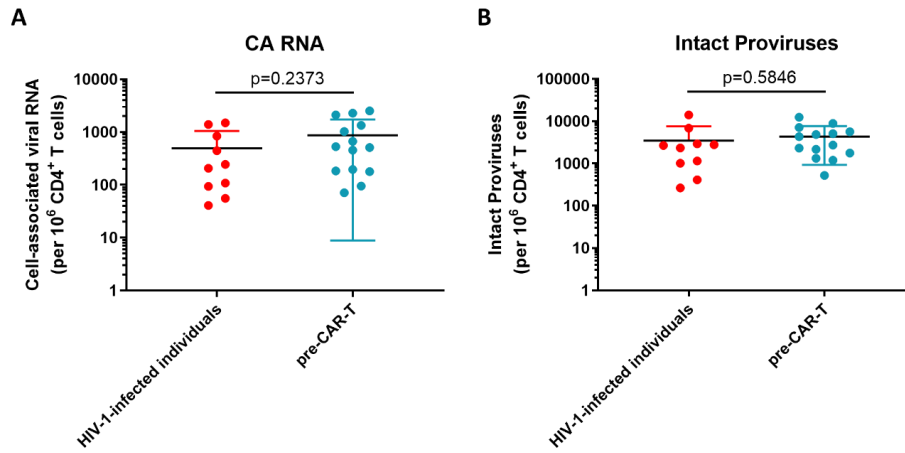


**Supplemental Figure 9. CAR-T cell concentration and CA-RNA before and after adoptive transfer at early time.** Measured CAR<sup>+</sup> cells concentrations (per million CD8<sup>+</sup> T cells) for each of the 14 enrolled patients are shown in blue (log<sub>10</sub> scale on left) and CA-RNA levels (per million CD4<sup>+</sup> T cells) are shown in red (log<sub>10</sub> scale shown on right) after adoptive transfer. ATI periods are shown by shades of grey.

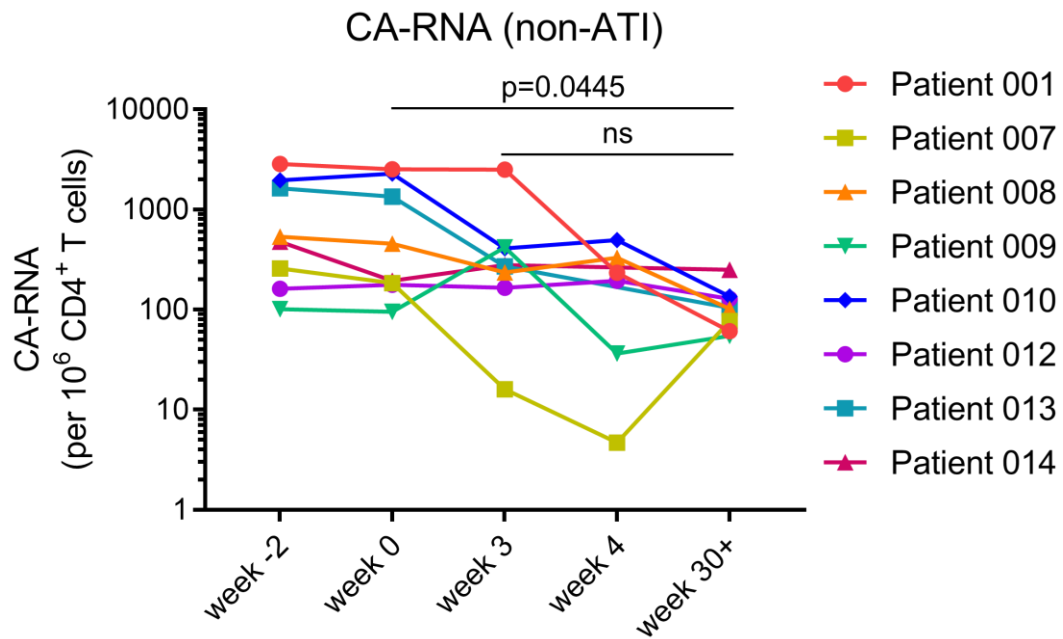


**Supplemental Figure 10. IFN- $\gamma$  ELISpot analysis of fourteen HIV-1-infected participants before and after adoptive transfer to HIV-1 Env-expressing cell line.**

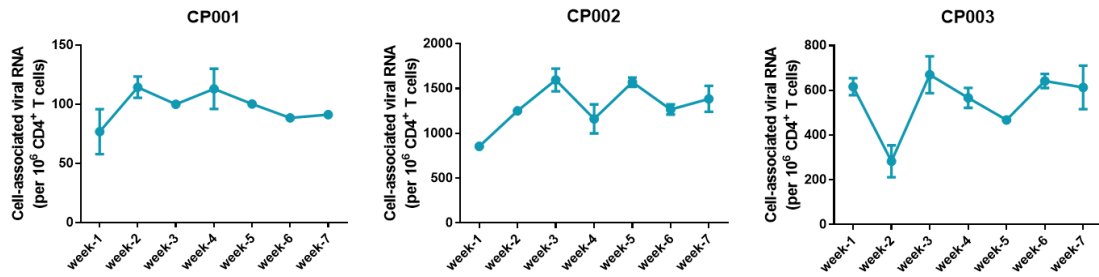
(A) The CD8<sup>+</sup> T cells were plated at  $10^5$  per well and then incubated 24 hours with Jurkat-gp160<sub>NL4-3</sub> (at 1: 1 ratio). (B) The number of IFN- $\gamma$ -positive spots was converted into the number of spots per million CD8<sup>+</sup> T cells. P-values were calculated using the Wilcoxon matched pairs signed-rank test.



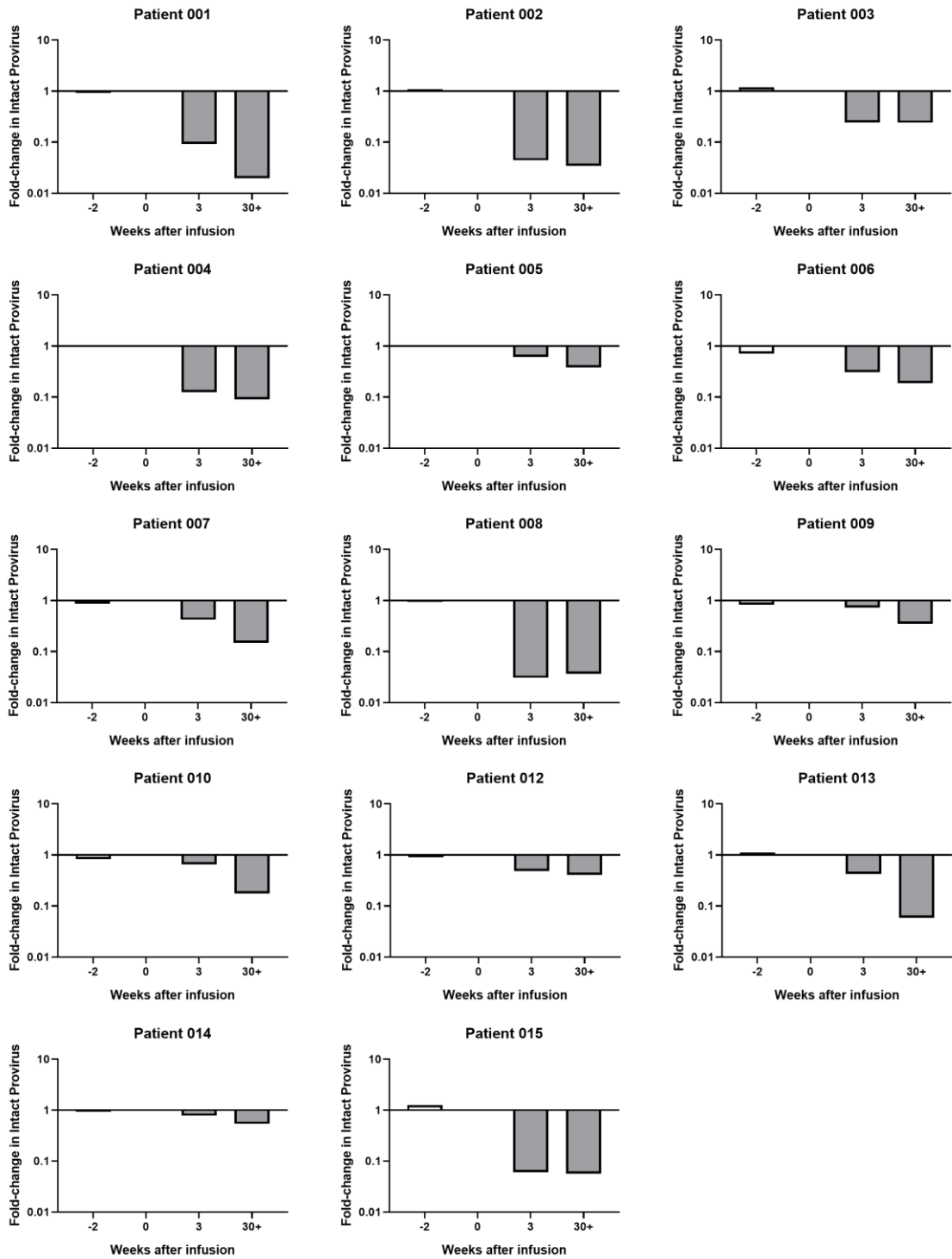
**Supplemental Figure 11.** The panels show the levels of CA-RNA (A) and intact provirus (B) of the samples from ART-receiving HIV-infected volunteers (n=10) or participants in our study (n=14, pre-CAR-T treatment). The samples were collected based on similar criteria. (Clinically stable on cART regimen for at least 12 months with undetectable plasma HIV-1 level; CD4<sup>+</sup> T-cell count  $\geq$  200 cells/ $\mu$ L). P-values were calculated using the two-tailed unpaired Student's t-test with equal variances.



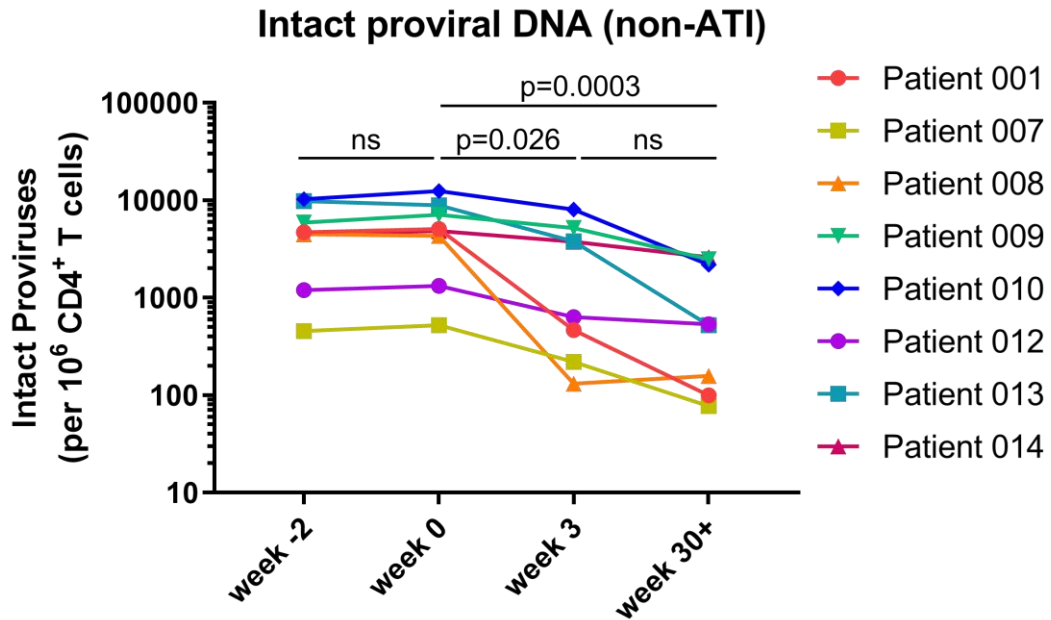
**Supplemental Figure 12. CAR-T treatment decreased the CA-RNA 30 weeks after infusions in non-ATI group.** Panels show the comparison of CA-RNA in non-ATI group before and after administration of CAR-T cells at indicated time-points. Each point represents the mean of triplicate values; P-values were calculated using the Wilcoxon matched pairs signed-rank test.



**Supplemental Figure 13.** The panels show the CA-RNA levels of the samples from three HIV-1-infected individuals who remained on ART and did not receive any immunologic interventions over observation periods. These patients remained clinically stable on ART regimen for at least 12 months with undetectable plasma HIV-1 level; CD4<sup>+</sup> T-cell count  $\geq 350$  cells/ $\mu$ L.

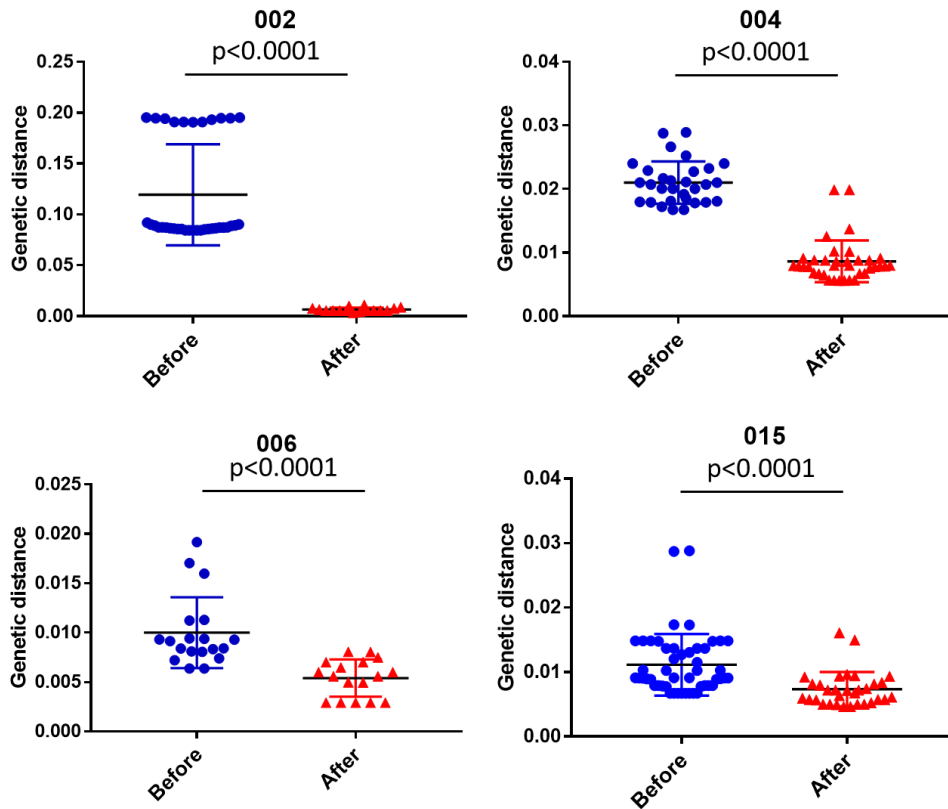


**Supplemental Figure 14.** The Fold-change of intact proviral DNA levels at indicated time points over baselines (week-0) are shown in fourteen participants before (white bars) and after (grey bars) CAR-T cell administration.

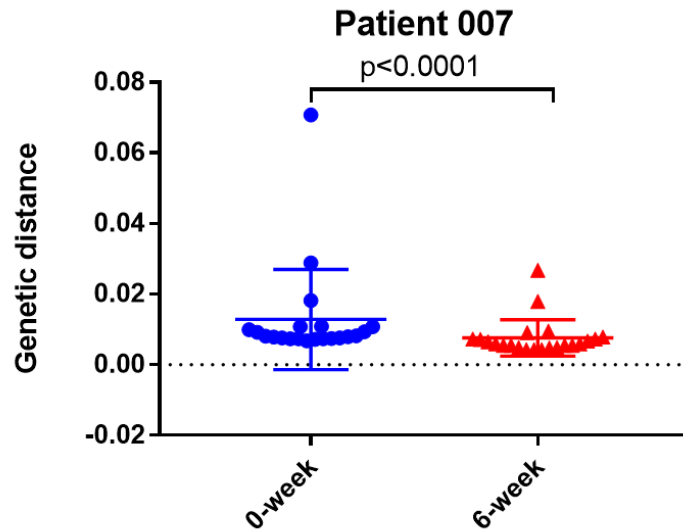


**Supplemental Figure 15. CAR-T treatment decreased the intact HIV-1 proviruses in non-ATI group.** Panels show IPDA results in CD4<sup>+</sup> T cells from 8 non-ATI participants, before and after CAR-T cell administration. P-values were calculated using the Wilcoxon matched pairs signed-rank test.

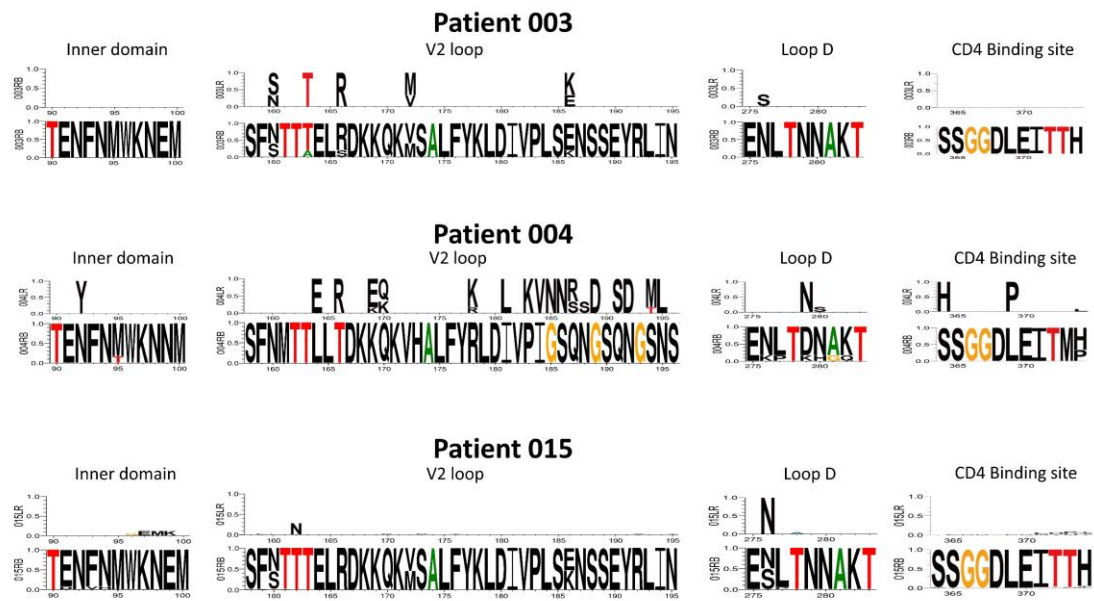




**Supplemental Figure 16. Genetic diversity analysis of Env from the ATI participants.** Single-genome sequencing of induced HIV-1 Env was performed from cell samples of Patient 002, 003, 004, 006 and 015. Genetic diversities were compared between Env sequences from 0-week (pre-CAR-T treatment, blue spot) and cell/plasma samples from the first and second weeks of detectable viremia (red triangle). Each spot or triangle represents the genetic distance between one given clone and the relevant entire population, and the horizontal bar indicates the mean. The two-tailed Mann-Whitney U-test was used to compare the genetic diversities between different groups.



**Supplemental Figure 17. Genetic diversity analysis of Env from the non-ATI participant.** Single-genome sequencing of induced HIV-1 Env was performed from cell samples of Patient 007. Genetic diversities were compared between Env sequences from 0-week (pre-CAR-T treatment, blue spot) and that from 6-week after CAR-T cell infusion (red triangle). Each spot or triangle represents the genetic distance between one given clone and the relevant entire population, and the horizontal bar indicates the mean. The two-tailed Mann-Whitney U-test was used to compare the genetic diversities between different groups.



**Supplemental Figure 18. The combined amino acid changes of Env from pre-CAR-T treatment to rebound in three participants.** To analyze changes in the predicted VRC01 scFv binding regions between pre-CAR-T treatment and rebound virus population in Patient 003, 004 and 015, modified longitudinal logo plots were generated with the program WebLogo. All sequences are compared to the consensus of the rebound viruses and residue numbers are based on the HIV-1 HXB2 sequence. The top line shows amino acid differences in the pre-CAR-T sequences from the rebound consensus.

## Supplemental Tables

**Supplemental Table 1. Characteristics of the participants at baseline.**

Group		non-ATI	ATI
Sex — no. (%)	Male	9 (100)	6 (100)
	Female	0	0
Age (year) — Median (range)		35 (26-47)	30 (26-35)
HIV-1 RNA (Screen)— copies/no. (%)	<20 copies/ml	9 (100)	6(100)
	≥20 copies/ml	0 (0)	0 (0)
Nadir CD4 <sup>+</sup> T-cell count — no. (%)	<200 cells/μl	2 (22.2)	2(33.3)
	200–500 cells/μl	7 (77.8)	4(66.7)
	>500 cells/μl	0 (0)	0 (0)
	Unknown	0 (0)	0 (0)
CD4 <sup>+</sup> T-cell Count (Screen) — no. (%)	<200 cells/μl	0 (0)	0 (0)
	200–500 cells/μl	3 (33.3)	3(50.0)
	>500 cells/μl	6 (66.7)	3(50.0)
	Unknown	0 (0)	0 (0)
Years on ART — Median (range)		4.6 (2.8-7.5)	3.5 (1.8-4.6)
ART regimen — no. (%) **	2 NRTI + 1 NNRTI	7 (77.8)	4(66.7)
	2 NRTI + 1 PI	0 (0)	1 (16.7)
	NRTI + 1 PI	1 (11.1)	0 (0)
	2 NRTI + 1 INSTI	1 (11.1)	1 (16.7)

\*\* NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; INSTI, integrase strand transfer inhibitor.

**Supplemental Table 2. The total cell dose, CAR T cell dose and transduction efficiency for administrations.**

<b>Participant</b>	<b>001</b>	<b>002</b>	<b>003</b>	<b>004</b>	<b>005</b>	<b>006</b>	<b>007</b>	<b>008</b>	<b>009</b>	<b>010</b>	<b>011</b>	<b>012</b>	<b>013</b>	<b>014</b>	<b>015</b>
<b>Total transferred cells (<math>\times 10^6</math>)</b>	80.2	67.2	69.1	50.5	50.5	65.5	81.3	64.6	78.8	86.9	--	100.2	90.7	65.5	70.3
<b>Transduction efficiency (%)</b>	49.3	62.2	41.0	51.8	70.5	38.0	37.5	43.2	58.9	34.9	--	63.5	45.3	77.8	46.0
<b>CAR<sup>+</sup> T cells (<math>\times 10^6</math>)</b>	39.5	41.8	28.3	26.2	35.6	24.9	30.5	27.9	46.4	30.3	--	63.6	41.1	51.0	32.3

**Supplemental Table 3. Complete list of adverse events.**

<b>Study Participant</b>	<b>AE Terminology</b>	<b>Grade*</b>	<b>Resolved</b>	<b>Relationship</b>
<b>001</b>	<b>Fever</b>	<b>1</b>	<b>Yes</b>	<b>Non-Treatment Related Event</b>
<b>007</b>	<b>Fever</b>	<b>1</b>	<b>Yes</b>	<b>Non-Treatment Related Event</b>
<b>010</b>	<b>Fatigue</b>	<b>1</b>	<b>Yes</b>	<b>Non-Treatment Related Event</b>
	<b>Headache</b>	<b>1</b>	<b>Yes</b>	<b>Non-Treatment Related Event</b>

\*All the grade of AEs was evaluated according to the Common Terminology Criteria for Adverse Events (CTCAE) Version 5.0, which was published on November 27 2017.

**Supplemental Table 4. Characteristics of CA RNA, CAR<sup>+</sup> cell count and CD4<sup>+</sup> cell count at the time of ATI.**

Participant	CA RNA (per million CD4 <sup>+</sup> cells)			CAR <sup>+</sup> cell count (per million CD8 <sup>+</sup> cells)	CD4 <sup>+</sup> cell count (copies/ $\mu$ l)
	week 0	pre-ATI	Reduction (%)	pre-ATI	pre-ATI
Patient 002	2115	47	97.8	2590	673
Patient 003	71	1	99.1	979	533
Patient 004	509	9	98.2	1541	689
Patient 005	670	83	87.6	4402	444
Patient 006	1028	168	83.6	3448	497
Patient 015	529	263	50.2	3283	753

**Supplemental Table 5. Characteristics of CA RNA, CAR<sup>+</sup> cell count and CD4<sup>+</sup> cell count in non-ATI participants.**

Participant	CA RNA (per million CD4 <sup>+</sup> cells)			CAR <sup>+</sup> cell count (per million CD8 <sup>+</sup> cells)	CD4 <sup>+</sup> cell count (copies/μl)
	week 0	week 3	Reduction (%)	week 3	week 3-4
Patient 001	2527	2506	0.8	6618	382
Patient 007	184	16	91.3	158	604
Patient 008	454	236	48.0	11435	553
Patient 009	95	10	89.5	362	551
Patient 010	2279	408	82.1	2233	853
Patient 012	178	165	7.3	1430	444
Patient 013	1341	269	79.9	1454	640
Patient 014	195	278	-42.7	3200	685

**Supplemental Table 6. ACTG historical data reported by Li et al (1).**

<b>Characteristic</b>	<b>Total (N=235)</b>	<b>Chronic (N=155)</b>	<b>Acute (N=32)</b>	<b>Early (N=48)</b>
<b>Sex, male, N (%)</b>	213 (91%)	137 (88%)	31 (97%)	45 (94%)
<b>Age, median yrs (Q1, Q3)</b>	41 (35, 46)	42 (38, 50)	36 (29, 43)	37 (28, 42)
<b>Race/ethnicity, N (%)</b>				
<b>White, non-hispanic</b>	166 (71%)	106 (68%)	23 (72%)	37 (77%)
<b>Black, non-hispanic</b>	31 (13%)	25 (16%)	5 (16%)	1 (2%)
<b>Hispanic</b>	32 (14%)	20 (13%)	4 (13%)	8 (17%)
<b>Other</b>	6 (3%)	4 (3%)	0 (0%)	2 (4%)
<b>CD4+ count, median cells/mm<sup>3</sup>(Q1, Q3)</b>	827 (698, 1023)	800 (666, 1004)	852 (736, 1050)	827 (687, 1085)
<b>NNRTI-based ART</b>	99 (42%)	94 (61%)	2 (6%)	3 (6%)
<b>IVDU, ever use, N (%)</b>	17 (7%)	15 (10%)	0 (0%)	2 (4%)
<b>Source study, N (%)</b>				
<b>A5170</b>	96 (41%)	96 (62%)		
<b>A5197</b>	28 (12%)	28 (18%)		
<b>A5068</b>	19 (8%)	19 (12%)		
<b>A5024</b>	12 (5%)	12 (8%)		
<b>ACTG 371</b>	71 (30%)		28 (88%)	43 (90%)
<b>A5187</b>	9 (4%)		4 (13%)	5 (10%)

NNRTi, non-nucleoside reverse transcriptase inhibitor; ART, antiretroviral therapy; IVDU, intravenous drug use; ACTG, AIDS Clinical Trials Group



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**Supplemental Table 7. HLA haplotype of the study participants.**

<b>Participant</b>	<b>HLA-A</b>	<b>HLA-B</b>	<b>HLA-C</b>
<b>001</b>	33:03:01; 24:02:01	58:01:01; 27:07:01	03:02:02; 15:02:01
<b>002</b>	24:02:01; 11:01:01	15:02:01; 15:02:01	08:01:01; 08:01:01
<b>003</b>	02:01:01; 02:01:01	52:01:01; 39:01:01	07:02:01; 12:02:02
<b>004</b>	24:02:01; 02:01:01	13:01:01; 40:01:02	03:04:01; 07:02:01
<b>005</b>	11:01:01; 33:03:01	58:01:01; 27:04:01	12:02:02; 03:02:02
<b>006</b>	24:02:01; 02:06:01	55:02:01; 15:02:01	01:02:01; 08:01:01
<b>007</b>	02:03:01; 11:01:01	35:01:01; 38:02:01	07:02:01; 03:03:01
<b>008</b>	11:01:01; 02:03:01	35:05:01; 15:01:01	04:01:01; 03:03:01
<b>009</b>	02:01:01; 33:03:01	40:06:01; 40:06:01	08:01:01; 08:22:01
<b>010</b>	24:02:01; 30:01:01	40:01:02; 13:02:01	06:02:01; 03:04:01
<b>012</b>	24:02:01; 11:01:01	15:01:01; 46:01:01	01:02:01; 01:02:01
<b>013</b>	11:01:01; 02:07:01	51:02:02; 13:01:01	14:02:01; 03:04:01
<b>014</b>	33:03:01; 24:02:01	51:01:01; 58:01:01	14:02:01; 03:02:02
<b>015</b>	11:01:01; 24:02:01	15:27:01; 38:02:01	04:01:01; 07:02:01

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**Supplemental Table 8. The sequences of primers and probes.**

<b>Primer</b>	<b>Sequence (5'-3')</b>
<b>SK38</b>	ATAATCCACCTATCCCAGTAGGAGAAA
<b>SK39</b>	TTTGGTCCTTGTCTTATGTCCAGAATGC
<b>HIVTotRNA-5F</b>	CTGGCTAACTAGGGAACCCACTGCT
<b>HIVTotRNA-5R</b>	GCTTCAGCAAGCCGAGTCCTGCGTC
<b>qPCR(CD3ζ-flag)-F</b>	GCCTTTACCAGGGTCTCA
<b>qPCR(CD3ζ-flag)-R</b>	ACTTATCGTCGTCATCCTTG
<b>qPCR(VRC-01)-F</b>	ATTTTTTGGCCAGGGGACC
<b>qPCR(VRC-01)-R</b>	AGGATTCTCCTCGACGTCACC
<b>qPCR-Actin-F</b>	CATGTACGTTGCTATCCAGGC
<b>qPCR-Actin-R</b>	CTCCTTAATGTCACGCACGAT
<b>qPCR-GAPDH-F</b>	GGAGCGAGATCCCTCCAAAAT
<b>qPCR-GAPDH-R</b>	GGCTGTTGTCATACTTCTCATGG
<b>ddPCR-Psi-F</b>	CAGGACTCGGCTTGCTGAAG
<b>ddPCR-Psi-R</b>	GCACCCATCTCTCTCCTTCTAGC
<b>ddPCR-Psi probe</b>	FAM-TTTTGGCGTACTCACCAGT-MGB
<b>ddPCR-Env-F</b>	AGTGGTGCAGAGAGAAAAAAGAGC
<b>ddPCR-Env-R</b>	GTCTGGCCTGTACCGTCAGC
<b>ddPCR-Env-intact probe</b>	VIC-CCTTGGGTTCTTGGGA-MGB
<b>ddPCR-Env-hypermut probe</b>	Unlabeled-CCTTAGGTTCTTAGGAGC-MGB
<b>envB5out</b>	TAGAGCCCTGGAAGCATCCAGGAAG
<b>envB3out</b>	TTGCTACTTGTGATTGCTCCATGT
<b>envB5in</b>	CACCTTAGGCATCTCCTAT GGCAGGAAGAAG
<b>envB3in</b>	GTCTCGAGATACTGCTCCCACCC

### Supplemental References

1. Li JZ, Etemad B, Ahmed H, Aga E, Bosch RJ, Mellors JW, et al. The size of the expressed HIV reservoir predicts timing of viral rebound after treatment interruption. *AIDS (London, England)*. 2016;30(3):343-53.

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## Protocol

This trial protocol has been provided by the authors to give readers additional information about their work.

Protocol for: B Liu, W Zhang, et al. Broadly neutralizing antibody-derived CAR-T cells reduce viral reservoir in HIV-1-infected individuals.

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## **PROTOCOL**

This supplement contains the following items:

1. Original protocol, final protocol, summary of changes.
2. Original and current statistical analysis plan.

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## **Original Study Protocol**

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**Protocol ID: GZ8H-QY-mHIVCAR01**

**Broadly Neutralizing Antibody-derived CAR-T Cell  
Reduce Viral Reservoir in HIV-1-infected individuals**

**Investigator:**

Hui Zhang, M.D, Ph.D.

Linghua Li, M.D.

**Research institute:**

Institute of Human Virology, Zhongshan School of Medicine, Sun Yat-sen

University;

Infectious Disease Center, Guangzhou Eighth People's Hospital, Guangzhou Medical

University.

**Founding:**

National Special Research Program of China for Important Infectious Diseases  
(2018ZX10302103 and 2017ZX10202102);

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**Collaborators:**

Qianyang Biomedical Research Institute;

Institute of Pediatrics Guangzhou Women and Children Hospital, Guangzhou Medical

University;

## **Brief summary**

The investigators conducted an open-label trial of the safety and antiviral activity of broad neutralizing antibody (bNAb)-derived Chimeric Antigen Receptor (CAR)-T cell therapy in HIV-1–infected individuals some of whom are undergoing analytical interruption of combination antiretroviral therapy (cART).

## **Objectives**

The primary objective of the study is to assess the safety of single dose of bNAb-derived CAR-T cells administered to persons with viremia suppressed to below detectable levels. Secondary objective is to evaluate the antiviral activity and the *in vivo* persistence of CAR-T products. Key exploratory objectives are the size of HIV-1 reservoir after CAR-T cells administration and the genetic and phenotypic characterization of the rebound viruses. Post hoc analyses of the sequence diversity at the time of viremia rebound and the cytotoxic capacity of CAR-T cells against autologous HIV-1 before and after adoptive transfer are performed.

## **Outcome measures**

### **1. Primary outcome measure**



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- The adverse events (AEs). The AEs of single dose of bNAb-derived CAR-T cells administered to persons with viremia suppressed to below detectable levels. All the grades of AE are evaluated according to the Common Terminology Criteria for Adverse Events (CTCAE).
  - HIV-1 RNA. Level change of HIV-1 RNA in plasma after analytical treatment interruption (ATI).

## **2. Secondary outcome measure**

- HIV-1 RNA. Level change of HIV-1 RNA in plasma before and 6 months after adoptive transfer.
- Persistence of bNAb-derived CAR-T cells *in vivo*. The percentage of bNAb-derived CAR-T cells within peripheral CD8<sup>+</sup> T cells before and after adoptive transfer analyzed by quantitative PCR.
- HIV-1 cell-associated viral RNA. Level change of cell-associated viral RNA in CD4<sup>+</sup> T cells before and after adoptive transfer analyzed by quantitative real-time RT-PCR.

## **3. Other pre-specified outcome measures**

- CD4<sup>+</sup> T cell count. Level change of the CD4<sup>+</sup> T cell number after adoptive transfer.
- The ratio changes of CD4/CD8. The ratio changes of CD4/CD8 in peripheral blood before and after adoptive transfer.
- HIV-1 DNA level. Changes of pro-viral DNA in PBMC before and after adoptive transfer.
- Sequence and phylogenetic analysis of HIV-1 viruses before and after adoptive

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transfer.

- Resistance of rebound viruses after ATI against HIV-1-specific CAR-T cells.

### **Study treatment**

CD8<sup>+</sup> T lymphocytes from PBMCs of HIV-1 infected patients are transduced with bNAb-derived CAR, then the modified CAR-T cells are expanded *in vitro* and adoptively transferred into the autologous patients.

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## 1. Introduction

### 1.1 Study objectives

The primary objective of the study is to assess the safety and antiviral activity of single dose of bNAb-derived CAR-T cells administered to persons with viremia suppressed to below detectable levels. Secondary objective is to evaluate the *in vivo* persistence of CAR-T cells. Key exploratory objectives are the size of HIV-1 reservoir after CAR-T cells administration and the genetic and phenotypic characterization of the rebound viruses. Post hoc analyses of the sequence diversity at the time of viremia rebound and the cytotoxic capacity of CAR-T cells against autologous HIV-1 before and after adoptive transfer are performed.

HIV-1 RNA level and CD4<sup>+</sup> T cell counts will be monitored weekly for two months, then be monitored biweekly for at least six months. The major objectives could be summarized as followings:

- Safety and side-effect profile of single dose of bNAb-derived CAR-T cells administered to persons with viremia suppressed to below detectable levels. All the grades of AEs are evaluated according to the Common Terminology Criteria for Adverse Events (CTCAE) ([https://ctep.cancer.gov/protocolDevelopment/electronic\\_applications/ctc.htm#ctc\\_40](https://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm#ctc_40))
- HIV-1 RNA. Level change of HIV-1 RNA in plasma after analytical treatment interruption (ATI).

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## 1.2 Background and rationale

Combination antiretroviral therapy (cART) significantly suppresses HIV-1 to an undetectable level in the blood, improves immune function, delays progression of the disease, and decreases mortality of HIV-1 patients.(2) However, a certain amount of HIV-1 replication-competent proviruses comprise a latent reservoir, which is quite stable, with a half-life of 44 months, requiring nearly 73.4 years for complete clearance.(3-5) In almost all HIV-1–infected individuals, plasma viral rebound predictably occurs within days after treatment interruption, resulting in the lifelong requirement for cART.(6, 7) To achieve durable suppression of viremia without daily therapy, various strategies have been proposed, including long-acting antiretroviral drugs (LA-ARVs), broadly neutralizing antibodies (bNAbs), and chimeric antigen receptor T (CAR-T) cells.(8) In human clinical trials, viremic individuals who received bNAb therapies showed significant reductions in viremia.(9-11) Moreover, HIV-1–infected individuals who received multiple infusions of VRC01 or 3BNC117, two related bNAbs that target the CD4 binding site on the HIV-1 envelope spike, showed significant viral suppression for 5.6 or 9.9 weeks during analytical treatment interruption (ATI) of cART, respectively.(12, 13) Furthermore, a combination therapy of 3BNC117 and 10-1074 maintains suppression on virus rebound for median of 21 weeks.(14) These findings suggest that the immunotherapy with chimeric antigen receptors (CAR)-modified T-cells, if HIV-1-specific bNAb-derived, may also potentially prevent virus rebound in HIV-1–infected individuals after ATI.

CAR-moiety is typically generated by coupling an antibody-derived, single-chain Fv domain to an intracellular T-cell–receptor zeta chain and costimulatory receptor-signaling domains. The clinical usage of CAR-T cells resulted in complete remission in approximately 83% of lymphocytic leukemia/lymphoma patients.(15, 16) Compared

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to CAR-T cells targeting tumor-associated antigens such as CD19 and CD20 which are also expressed in normal B-lymphocytes, the HIV-1-specific CAR-T cells target HIV-1 envelope (Env) protein which is only expressed on the surface of virus-producing cells.(17-19) At the earlier time, a strategy that fuse the extracellular domain of CD4 with the intracellular domain of the CD3 $\zeta$  chain (CD4 $\zeta$ -CAR) was shown to be safe and feasible in HIV-1-infected individuals. However, the antiviral efficacy was modest and durable control of viral replication in clinical trials was not observed.(20-24) In recent years, the third and fourth generation intracellular CAR moieties have been developed.(16) Moreover, a number of preclinical studies for the bNAb-derived HIV-1-specific CAR-T cells *in vitro* and in animal models have shown the suppression of viral replication or the eradication of virus-producing cells.(25-30) Especially, we previously found that VRC01-derived CAR-T cells effectively eradicate the reactivated viral reservoir isolated from HIV-1-infected individuals receiving cART, and the engineered resistance to triple inhibitory receptors including PD-1, Tim-3, and Lag-3 prevents CAR-T cell exhaustion and improves their maintenance *ex vivo*.(26, 31)

Here, we conduct a phase 1 clinical trial for investigating the effect of single administration of bNAb-derived CAR-T cells upon virus rebound after the discontinuation of suppressive cART.

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## **2. Eligibility assessment and enrollment**

This study can fulfill its objectives only if appropriate patients are enrolled. The following eligibility criteria are designed to select patients for whom protocol treatment is considered appropriate. All relevant medical and non-medical conditions should be taken into consideration when deciding whether this protocol is suitable for a particular patient.

### **2.1 Inclusion criteria**

Patient eligibility should be reviewed and documented by an appropriately qualified member of the investigator's study team before patients are included in the study. Patients must meet all of the following inclusion criteria to be eligible for enrollment into the study.

1. Age between 18 to 60, male or female;
2. HIV-1 infection, documented by any licensed rapid HIV-1 test or HIV-1 enzyme or chemiluminescence immunoassay (E/CIA) test kit at any time prior to study entry and confirmed by a licensed Western blot or a second antibody test by a method other than the initial rapid HIV-1 and/or E/CIA, or by HIV-1 antigen, plasma HIV-1 RNA VL;
3. Clinically stable on cART regimen for at least 12 months with undetectable HIV-1 level ( $< 50$  copies/ml, HIV-1 RNA, Cobas test);
4. Screening CD4<sup>+</sup> T-cell count  $\geq 350$  cells/ $\mu$ L within 14 days prior to study entry.
5. There is no serious damage of liver and kidney function and other indexes are within the basic normal range after comprehensive physical examination (including general examination, routine hematuria examination, blood biochemical examination, chest X-ray, B-ultrasound and ECG, etc.);
6. The following laboratory values obtained within 45 days prior to enrollment: Hgb  $\geq 11$ g/L (Male),  $\geq 10$ g/L (Female); Neutrophil count  $\geq 1 \times 10^9$ /L; Platelet count  $\geq 100 \times 10^9$ /L; Creatinine  $\leq 110 \mu$ mol/L; Aspartate transaminase (AST) and Alanine aminotransferase (ALT)  $\leq 2.5 \times$ ULN;
7. Willingness to have blood samples collected, stored indefinitely, and

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- used for study-related research purposes;
8. Ability and willingness of subject or legal representative to provide informed consent.

## **2.2 Exclusion criteria**

Patients presenting with any of the following will not be included in the study:

1. Ongoing AIDS-related opportunistic infection or any cancer or malignancy;
2. AST and ALT  $> 3 \times$  normal, Creatinine  $> 110 \mu\text{mol/L}$ ;
3. Any uncontrolled active medical disorder including seriously chronic disease, metabolic disorders, neurological and psychiatric disorders that would preclude participation as outlined;
4. History of a pancreatitis;
5. Pregnant or lactation;
6. According to the researcher's judgment, the candidate has a low possibility of inclusion (such as weakness, poor compliance, etc.);
7. History of a severe allergic reaction with generalized urticaria, angioedema, or anaphylaxis in the 2 years prior to enrollment;
8. Acute or chronic hepatitis B or hepatitis C infection;
9. Subject with CMV retinitis or other active CMV infection related diseases;
10. Subject with organ dysfunction;
11. Drug or alcohol abuse or dependence;
12. Currently (Within 3 months) enrolled in another clinical trial or underwent cell therapy;
13. In the opinion of the site investigator, would interfere with adherence to study requirements.

## **2.3 Life style guidelines**

All male and female patients who, in the opinion of the investigator, are biologically capable of having children and are sexually active, must agree to use a highly effective



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method of contraception consistently and correctly for the duration of the active treatment period and for a minimum of 90 days after the last dose of investigational treatment. The investigator, in consultation with the patient, will select the most appropriate method of contraception for the individual patient from the permitted list of contraception methods, and instruct the patient in its consistent and correct use. In addition, the investigator will instruct the patient to call immediately if the selected birth control method is discontinued or if pregnancy is known or suspected.

Highly effective methods of contraception are those that, alone or in combination, result in a failure rate of less than 1‰ per year when used consistently and correctly (i.e., perfect use) and include:

1. Established use of oral, injected or implanted hormonal methods of contraception;
2. Correctly placed intrauterine device (IUD) or intrauterine system (IUS);
3. Male condom or female condom used WITH a spermicide (i.e., foam, gel, film, cream, suppository);
4. Male sterilization with appropriately confirmed absence of sperm in the post-vasectomy ejaculate;
5. Bilateral tubal ligation or bilateral salpingectomy.

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### 3. Study implementation

#### 3.1 Study design

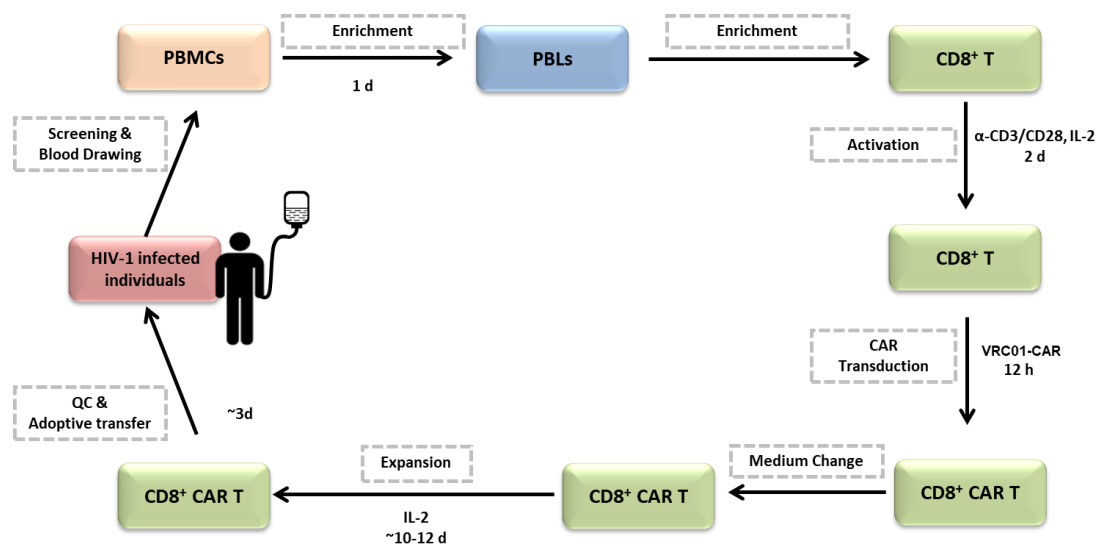
Relevant studies are initiated immediately after the patients are enrolled in the group. After the patients enrolled in the group, the screening will be started when the HIV-1 cannot not be detected. All participants have not undergone any additional immunotherapeutic intervention besides ART and there were no conditioning regimens before CAR-T cell administrations. At the same time, peripheral blood mononuclear cells (PBMCs) are collected from peripheral blood and separated on the basis of CD8 expression. The sorted CD8<sup>+</sup> T cells will be activated and then transduced by bNAb-derived CAR. Then genetic-modified CD8<sup>+</sup> T cells are expanded for 15 to 21 days *ex vivo*. Modified CAR-T cells are administrated into the patient, and the hospital observations will persist for one day. The basic condition of the patients, the immune reaction after bNAb-derived CAR-T cells administration, the HIV-1 viral load in plasma and the size of latent reservoir will be examined every 1-2 weeks after adoptive transfer for at least six months. After the six-month post-treatment, follow-up will be conducted every 1 months. The basic information is shown in the following table.

**Table 1. Basic Information of the Clinical Study**

Item	Content
Study Type	Interventional
Primary Purpose	Treatment
Interventional Study Model	Single Group Assignment
Intervention Description	CD8 <sup>+</sup> T lymphocytes from donor are transduced with bNAb-derived CAR before adoptive transfer into the patient
Number of Arms	1
Masking	None (Open Label)
Enrollment	20

During the study, every single patient will receive single administration of bNAb-

derived CAR-T cells. The patient will undergo a cytokine storm prophylaxis regimen, which will vary according to a specific patient's condition. The patient will be treated with anti-retroviral drugs pre-CAR-T administration and post CAR-T administration. An analytical treatment interruption will be performed when the relative criteria is fulfilled. The transplantation procedure is shown in the following figure.



**Figure 1. Flowchart of the bNAb-derived CAR-T cell therapy**

### 3.2 The CD8<sup>+</sup> T cell sorting

The CD8<sup>+</sup> T cells will be separated from donor mobilized peripheral blood cells using the CliniMACS system. The protocol is as follows:

- 1) Buffer preparation. Human Serum Albumin (HSA) will be added to physiological saline solution for a final concentration of 0.5%;
- 2) Collection of karyocytes. The empty preparation bag will be weighted for following calculation of cell sample. The blood sample will be counted followed by red blood cell lysis. Karyocytes will be counted while 200  $\mu$ l cell suspension will be collected for FACS. The remaining karyocytes will be transported to a preparation bag and weighted;
- 3) Dilution of the karyocytes. The karyocytes will be diluted with two volumes

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- 0.5% HAS containing physiological saline solution and mixed. The preparation bag will be centrifuged at 300 g and the supernatant is removed. The remaining karyocytes suspension will be adjusted to the final weight of 95 g;
- 4) Magnet beads. 7.5 ml CD8 beads will be injected to the karyocytes suspension with a syringe and mixed;
  - 5) Incubation. The mixture of karyocytes suspension and CD8 beads will be incubated at room temperature with an oscillator at 25 rpm for 30 minutes;
  - 6) Cell washing. After diluting the karyocytes suspension with 500 ml buffer, the preparation bag will be centrifuged at 300 g for 15 minutes. 500 ml of the supernatant will be removed. The karyocytes will be washed for two times;
  - 7) Cell preparation. The final volume of the karyocytes suspension will be adjusted to 150 ml. 200  $\mu$ l sample will be collected for FACS;
  - 8) CD8<sup>+</sup> T cell sorting with CliniMACS. Install the LS tube system (162-01) and the preparation bag according to the instruction and perform the separation with DEPLETION 2.1. The sorted positive and negative collection bags will be weighted before and after the separation;
  - 9) Collection of sorted CD8<sup>+</sup> T cells. The collection bag will be weighted and 200  $\mu$ l sample will be collected for FACS.

### **3.3 Activation of CD8<sup>+</sup> T cells**

Cell collection:

Sorted CD8<sup>+</sup> T cells in the collection tubes will be transferred into culture flasks and will be rinsed with physiological saline solution. Cell will be counted and collected by centrifuge at 300 g for 10 minutes.

Culture medium:

CD8<sup>+</sup> T cells will be cultured at  $0.5 \times 10^6$ – $1 \times 10^6$  cells/ml with ImmunoCult-XF T

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Cell Exp Medium (Stem Cell Technologies) supplemented with 5% HAS and Gentamycin Sulfate Injection.

Activation:

CD8<sup>+</sup> T cell culture will be added paramagnetic antibodies (ImmunoCult Human CD3/28 T Cell Activator, Stem Cell Technologies) (25 µL per 1 ml of cell suspension), IL-2 (<sup>125</sup>Ala IL-2, SL Pharm) (150 IU per 1 ml of cell suspension). The culture media will be pre- heated in 37°C incubator before use.

### **3.4 Pseudotyped lentiviral manufacture**

Clinical pseudotyped lentiviral manufacture: The VRC01-CAR lentiviral vectors using design principles previously reported.(26, 31) Pseudotyped lentivirus are produced by transient transfection of HEK293T cells with the lentiviral vector, packaging vector (psPAX2), and envelope vector (pMD.2G). The VRC01-CAR pseudotyped lentivirus is purified via tangential flow filtration (KR2i TFF system, Repligen) and chromatographic purification system (ÄKTA™ pure, GE).

### **3.5 Transduction and expansion**

Two days after initial CD8<sup>+</sup> T cell activation, transduction of pseudotyped lentivirus encoding VRC01-CAR is performed in retronectin-coated cell-culture bags and containing culture medium, and 150 IU/ml IL-2. After 12 hours, transduced CD8<sup>+</sup> T cells are washed, counted and resuspended at  $0.5 \times 10^6$ – $1 \times 10^6$  cells/ml in fresh culture medium with 150 IU/ml IL-2. Then genetic-modified CD8<sup>+</sup> T cells are expanded with IL-2–dependent growth for 15 to 21 days *ex vivo*. The target cell dose range is at least  $5 \times 10^7$  CD3<sup>+</sup> CD8<sup>+</sup> cells.

Release criteria for the expanded CD8<sup>+</sup> T cells are as follows:

- 1) Cell viability  $\geq$  90%;
- 2) CD3<sup>+</sup> CD8<sup>+</sup> T cells  $\geq$  95%;
- 3) Endotoxins  $\leq$  5 EU/ml;

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- 4) Mycoplasma: negative;
  - 5) Bacterial culture: negative;
  - 6) Fungal culture: negative;
  - 7) Transduction efficiency (percentage CAR<sup>+</sup> of viable CD3<sup>+</sup> CD8<sup>+</sup> T cells): ≥ 30-60%;
  - 8) The gp120-specific cytotoxicity of more than 40% lysis at 100:1 E:T ratio.

### **3.6 CAR-T cell suspension infusion**

The expanded CAR-T cells will be prepared under the good manufacturing practice (GCP). Release criteria for the expanded CD8<sup>+</sup> T cells are as follows: sterility by Chinese Pharmacopeia (2015), negative fungal, and mycoplasma testing, negative Gram stain, endotoxin less than 5 U/kg, more than 90% viability by Trypan Blue exclusion, CD3 and CD8 staining by flow cytometry, IL-2–dependent growth, and gp120-specific cytotoxicity of more than 40% lysis at 100:1 E:T ratio.

These cells will be infused into the patient on day 0 (/and day 1, if necessary) of transplantation, with or without co-infusion of thawed, selected negative cells. The cell infusion procedures are as followings:

- Obtain blood pressure, temperature, pulse, respirations and pulse oximetry at the start of the infusion, every 10 minutes and at the completion of each product;
- Glucose gluconate injection is intravenously injected before cells infusion;
- Begin infusion slowly for the first 20 ml. If the patient tolerates the first 20 ml, increase the rate to infuse over 10-15 minutes;
- Infuse cell suspension at the speed about 50 ml per hour unless otherwise ordered. It is imperative that the cells are infused slowly due to the potential immune reaction;
- Cell suspension will be rinsed with 100 ml normal physiological saline solution at the end of infusion by back priming to assure as many cells as possible are infused into the patient;

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- Assess the patient frequently for possible reactions, (i.e. shortness of breath, dyspnea, tachycardia, chest pain, rales, erythema, rash, hives, flank pain, temperature increase, nausea, vomiting, rigors, bronchial spasms and respiratory arrest);
  - If reaction suspected, slow infusion, medicate as needed per medication orders and notify the physician immediately.

#### **4. Analytical treatment interruption (ATI)**

Analytical treatment interruption (ATI) of cART is favorable to evaluate the anti-virus effect of the CAR-T cell therapy. Considering the potential risks for ATI, the ATI will be performed at least four weeks after CAR-T cell administration. To prevent the risk of efavirenz monotherapy and the emergence of resistant strains after stopping ART, the individuals whose regimens contained efavirenz stop taking it one week before discontinuing the other agents. The individuals with integrase inhibitor-based or protease inhibitor-based regimes discontinue three ART agents simultaneously. When all the ART agents are discontinued, the time of plasma viral suppression begins to measure. The ATI is performed when the following criteria are achieved.

##### **4.1 Criteria for initiate treatment interruption**

- A written informed consent is provided by the patient;
- No adverse events at the time of interruption;
- The CD4<sup>+</sup> T cell counts maintain in a normal range (> 400 cells/ $\mu$ l peripheral blood);
- The plasma viral load is under the detectable level (< 20 copies per milliliter);
- The cell-associated viral RNA is reduced at least 50%;
- *In vivo* CAR-T cells can be persistently detectable (> 100 copies per million CD8<sup>+</sup> T cells).

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## **4.2 Criteria for reinstate anti-viral treatment**

- The plasma viral load exceeding 200 copies per milliliter;
- The CD4<sup>+</sup> T cells decrease under 350 cells/ $\mu$ l;
- Any AIDS-related opportunistic infections are observed;
- Occurrence of severe CAR-T-related adverse events;
- Request for reinitiating anti-retroviral therapy by the patient.



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## **5. Quality control**

### **5.1 Quality control and quality assurance**

During study conduct, the Emergency Response Group (ERG), led by Dr. Linghua Li, the PI of this study and Dr. Xilong Deng, the chair of Emergency Department of the Guangzhou 8<sup>th</sup> People's Hospital, will conduct periodic monitoring visits to ensure that the protocol and Good Clinical Practices (GCPs) are being followed. The monitors may review source documents to confirm that the data recorded on CRFs is accurate. The investigator and institution will allow the ERG monitors or its agents and appropriate regulatory authorities direct access to source documents to perform this verification.

It is important that the investigation and their relevant personnel are available during the monitoring visits and possible audits or inspections and that sufficient time is devoted to the process.

### **5.2 Safety meeting**

The ERG will review and monitor toxicity and accrual data from this study. The committee is composed of clinical specialists with experience in virology and who are the principal investigators in the study. Information that raises any questions about participant safety will be addressed with the study team. The ERG will meet quarterly and/or more often if required to review toxicity and accrual data. Information to be provided to the ERG may include: all grade 2 or higher unexpected adverse events that have been reported; any response information; audit results, and a summary provided by the study team. Other information (e.g. scans, laboratory values) will be provided upon request.

### **5.3 Monitoring**

Involvement in this study as a participating investigator implies acceptance of potential

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audits or inspections, including source data verification, by representatives designated by the Protocol Chair. The purpose of these audits or inspections is to examine study-related activities and documents to determine whether these activities are conducted, and data are recorded, analyzed, and accurately reported in accordance with the protocol, institutional policy, and any applicable regulatory requirements. Monitoring will begin at the time of participant registration and will continue during protocol performance and completion.

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## 6. Data collection and evaluation

### 6.1 Schedule

Except if noted otherwise, the same study procedures must be matched for participants. AE/SAE will be reported throughout the study, as described in the specific section of the protocol. Three periods are involved in this section of “Data Collection and Evaluation”, including pre-screening, study treatment period and long-term follow-up period.

- 1) Pre-screening procedures for baseline data will occur within 30 days before pre-treatment unless otherwise noted. Informed consent document (ICD), an Institutional Review Board (IRB) or Independent Ethics Committee (IEC) approved ICD, must be signed and dated before any study-specific procedures are done. Results from laboratory tests or assessments, performed as a standard of care, prior to the date of informed consent but within the allowed timeframe for screening procedures, can be used for determining the patient's eligibility and will be entered in the CRF. Use of pre-consent results to support inclusion/exclusion criteria must be clearly documented in the patient's source documents. The following will be collected at screening:
  - Sign informed consent form;
  - Review study eligibility (inclusion and exclusion criteria);
  - Locally obtained characterization of viral infection;
  - Disease history and prior treatment;
  - Focused physical examination;
  - Vital signs;
  - Laboratory evaluation;
  - Other focused items.
- 2) The “study treatment period” is defined as the period starting with the CAR-T cells administration up to and including the end-of-treatment visit. The protocol of

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this period involves parts of section “Study Implementation” and “Data Collection and Evaluation”.

- 3) For long term follow-up visit, disease assessments will be conducted for all patients for 1 years or longer. Visits for disease assessment are planned to start approximately one weeks after the last disease assessment then continuing until the patient completes 1 years (relative to the day of administration), has documented undetectable *in vivo* bNAb-derived CAR-T cells, whichever occurs first. The data collected at follow-up include persistence of infused CAR-T cells, HIV-1 RNA viral load in plasma, HIV-1 cell-associated viral RNA, HIV-1 DNA measurement, sequencing and genetic diversity analysis of HIV-1 Env and the other laboratory measurement.

## **6.2 Adoptive transfer**

HIV-1-specific CAR-T therapy involves the intravenous infusion of bNAb-derived CD8<sup>+</sup> CAR-T cells to reestablish anti-viral immunosurveillance in patients with continuous cART regimen for at least 12 months. The CD8<sup>+</sup> T cells sources are mobilized peripheral blood from the donor.

Adoptive transfer, patient care and follow-up were performed at Guangzhou Eighth People’s Hospital. The patient provided informed consent in accordance with the Declaration of Helsinki. During the study, every single patient will undergo a single administration of bNAb-derived CAR-T cells. The patient will receive a graft containing CD8<sup>+</sup> T cells with transduction of bNAb-derived CAR (CliniMACS sorting system). The patient will be treated with anti-retroviral drugs pre-treatment and post treatment. The adoptive transfer will be evaluated by the *in vivo* persistence of the CAR-T products.

## **6.3 Safety measures**

Safety endpoints include adverse events (graded according to the Common Terminology Criteria for Adverse Events (CTCAE) Version 5.0), clinical examination

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(including blood pressure and pulse), laboratory tests (hematology, chemistry, coagulation and urinalysis) and electrocardiograms (ECGs) run in triplicate. Additional procedures or samples may be undertaken as medically required of the discretion of the investigator. In addition to the local laboratory tests, additional blood samples may be taken, or additional tests may be conducted in existing samples, for analysis at either central or local laboratories in order to provide the best possible guidance on improving the medical management of study patients on emerging safety findings during the conduct of the study. Generally, the *in vivo* persistence of CAR-T cells and HIV-1 related assessments (HIV-1 RNA/DNA, Reservoir diversity, sequence and phylogenetic analysis, viral outgrowth assay and cytotoxicity determination) will be conducted by the collaborator, Sun Yat-sen University, or the other institutes.

#### **6.4 Quantitative PCR to detect CAR-modified T cells**

Patient PBMCs collected at baseline and at serial time points after CAR-T-cell infusions were collected and separated by Ficoll centrifugation and then cryopreserved. Batched cells were thawed and primary human CD8<sup>+</sup> T cells were obtained from PBMCs by positive magnetic selection through Anti-Human CD8 Magnetic Particles - DM, BD-IMag<sup>TM</sup>. The genomic DNA was harvested using an AllPure Total DNA/RNA Micro Kit (Magen). The CAR transgene was detected by performing quantitative PCR as previously described, using either a primer amplifying the fragment spanning the junction of the CD3 $\zeta$  domain and adjacent Flag domain (forward primer: 5'-GCCTTTACCAGGGTCTCA-3', reverse primer: 5'-ACTTATCGTCGTCATCCTTG-3'), or a primer amplifying the fragment of VRC01 scFv (forward primer: 5'-ATTTTTTGGCCAGGGGACC-3', reverse primer: 5'-AGGATTCTCCTCGACGTCACC-3'). Quantitative real-time PCR was performed in triplicate using SYBR Premix ExTaq II Kit (Takara), in a C1000 Touch Thermal Cycler (BIO-RAD CFX96<sup>TM</sup> Real-Time System).(26) Copy numbers per microgram of genomic DNA, generated from a standard curve of 10-fold serial dilutions of purified plasmid, were used to calculate the percentage of CAR<sup>+</sup> cells among CD8<sup>+</sup> T cells,

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assuming 1 copy/cell.

### **6.5 Quantitative real-time RT-PCR analysis**

Primary human CD4<sup>+</sup> T cells were obtained from PBMCs by negative magnetic selection through Human CD4<sup>+</sup> T Lymphocyte Enrichment Set-DM, BD-IMag<sup>TM</sup>. Total RNA was isolated with Trizol reagent (Life Technologies) and then subjected to cDNA synthesis using PrimeScript RT reagent Kit (Takara). All primers were annealed at 37°C and RT was processed at 42°C. Quantitative PCR was performed with SYBR Premix ExTaq II Kit (Takara) by following the manufacturer's instructions. The expressions of HIV-1 unspliced RNAs were determined by real-time qRT-PCR with the primer pair SK38 (5'-ATAATCCACCTATCCCAGTAGGAGAAA-3') and SK39 (5'-TTTGGTCCTTGTCTTATGTCCAGAATGC-3'). An *in vitro*-synthesized HIV-1 RNA, after quantification, was used as the external control for measuring cell associated viral RNA.(32) Quantification was normalized to the housekeeping gene GAPDH or  $\beta$ -actin.

### **6.6 Assessment of HIV-1 proviral DNA**

Sorted CD4<sup>+</sup> T cell populations were subjected to DNA extraction using commercial kits purchased from Magen (catalog number R5111-02). Genomic DNA was collected for quantitative PCR using Gag primer pair GagF (5'-ACATCAAGCAGCCATGCAAA-3'), GagR (5'-TCTGGCCTGGTGCAATAGG-3') and probe (5'-VIC-CTATCCCATTCTGCAGCTTCCTCATTGATG-TAMRA-3'). qPCR was performed using the following program: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 94°C for 15s and 60°C for 1 min.

### **6.7 Viral outgrowth**

Recovery and amplification of replication-competent viruses were described previously.(33) Briefly,  $1 \times 10^6$  CD4<sup>+</sup> T cells from HIV-1-infected individuals were stimulated by  $1 \times 10^7$  irradiated allogeneic PBMC from uninfected donors and the 1

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$\mu\text{g/ml}$  PHA-M at day 1, and typically, three additions of  $5 \times 10^6$  activated  $\text{CD4}^+$  lymphoblasts from uninfected donors as target cells were added for HIV-1 outgrowth at day 2, day 7 and day 14, respectively. The cells were cultured in RPMI-1640 media + IL-2 (10 ng/ml, recombinant human, R&D Systems) all the time. After 14 days co-culture, the recovered viruses were harvested and tested for HIV-1 p24 protein.

### **6.8 *In vitro* HIV-1 infection and drug withdrawal model**

*In vitro* HIV-1 infection model was previously described with minor modifications.(26) Briefly, the PBMCs from healthy donors were stimulated by adding 1 mg/ml PHA and 10 ng/ml IL-2 to the conditioned RPMI1640 media with 10% heat-inactivated fetal bovine serum and antibiotics for two days before isolation of  $\text{CD4}^+$  T cells.  $\text{CD4}^+$  T cells were infected with an outgrown HIV-1 from patients (p24 titer of 1 ng/ml). Three hours after infection, the culture media was changed by centrifugation. The infected  $\text{CD4}^+$  T cells were cultured in basal media + IL-2 (10 ng/ml, recombinant human, R&D Systems) and further incubated at  $37^\circ\text{C}$  in a humidified incubator with 5%  $\text{CO}_2$ . Six days after HIV-1 infection, azidothymidine (Zidovudine, Sigma-Aldrich) and lopinavir (Sigma-Aldrich) were added into the  $\text{CD4}^+$  T cell culture both at  $50 \mu\text{M}$  to inhibit virus production and prevent further infection events. The cells were then cultured in the presence of low-concentration of IL-2 (1 ng/ml). Anti-HIV-1 drugs were withdrawn when the viral production was significantly decreased to the marginal level for p24 detection (about 6-8 day after drugs adding). Then,  $0.5 \times 10^6$   $\text{CD4}^+$  T cells were mixed with autologous CAR-T cells or control  $\text{CD8}^+$  T cells at 1: 1 ratio in the conditioned media plus IL-2 (10 ng/ml) at 1 ml in 24-well plate. Every two days the cultures were tested for HIV-1 p24 antigen with the HIV-1 p24 Antigen Assay kit by following the manufacturer's instructions.

### **6.9 Genetic diversity analysis of activated HIV-1 viruses**

HIV-1 RNA extraction and single-genome amplification was performed as previously described.(34, 35) In brief, HIV-1 RNA was extracted from cell and plasma samples

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followed by first-strand cDNA synthesis using HiScript II 1st Strand cDNA Synthesis KIT (Vazyme). cDNA synthesis for plasma-derived HIV-1 RNA was performed using the antisense primer envB3out 5'-TTGCTACTTGTGATTGCTCCATGT-3'. gp160 was amplified using envB5out 5'-TAGAGCCCTGGAAGCATCCAGGAAG-3' and envB3out 5'-TTGCTACTTGTGATTGCTCCATGT-3' in the first round and in the second round with nested primers envB5in 5'-CACCTTAGGCATCTCCTATGGCAGGAAGAAG-3' and envB3in 5'-GTCTCGAGATACTGCTCCCACCC-3'. PCRs were performed using Phanta Max Super-Fidelity DNA Polymerase (Vazyme) and run at 94 °C for 2 min; 35 cycles of 94 °C for 15 s, 55 °C for 30 s and 68 °C for 4 min; and 68 °C for 15 min. Second-round PCR was performed with 1 µl of the PCR product from the first round as template and Phanta Max Super-Fidelity DNA Polymerase (Vazyme) at 94 °C for 2 min; 45 cycles of 94 °C for 15 s, 55 °C for 30 s and 68 °C for 4 min; and 68 °C for 15 min. cDNA synthesis for cell and plasma RNA was performed using the antisense primer R3B6R 5'-TGAAGCACTCAAGGCAAGCTTTATTGAGGC-3'. The env 3' half-genome was amplified in a single PCR using B3F3 primer 5'-TGGAAGGTGAAGGGGCAGTAGTAATAC-3' and R3B6R primer 5'-TGAAGCACTCAAGGCAAGCTTTATTGAGGC-3'. PCR was performed using Phanta Max Super-Fidelity DNA Polymerase (Vazyme) and run at 94 °C for 2 min; 45 cycles of 94 °C for 15 s, 55 °C for 30 s and 68 °C for 5 min; and 68 °C for 15 min. Amplicons were run on precast 1% agarose gels (BIOWESTE) and the PCR products were proceeded to deoxyadenosine (A)-tailing at the 3'-end of the PCR products utilizing Ex Taq DNA polymerase (Takara) without thermal cycling as follows: 95°C, 5 min; 72°C, 30 min. The A-tailed PCR products were TA-ligated into pMD-18 T vector (Takara).

### **6.10 Sequence and phylogenetic analysis**

Nucleotide alignments of intact *env* sequences were translation-aligned using MAGE 7. Sequences with premature stop codons and frameshift mutations that fell in the gp120



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surface glycoprotein region were excluded from all analyses. The sequences from each group were aligned using MUSCLE. The average genetic distance between one give clone and the relevant entire population were calculated by MEGA 7 and represented as genetic diversity index. The phylogenetic bootstrap trees were generated for each sample using **maximum likelihood** method with 1000 bootstrap replications implemented in MEGA seven to depict the global landscape of HIV-1 diversity. Logograms were generated using the Weblogo 3.0 tool. To analyze changes between reservoir and rebound viruses, env sequences were aligned at the amino acid level to a HXB2 reference using BioEdit.

### **6.11 Cytotoxicity determination**

The specific killing activity of pre-stimulated CD8<sup>+</sup> T cells towards Jurkat or HEK293T cells expressing HIV-1 envelope glycoprotein at indicated ratios was measured after co-culture for 8 hours by lactate dehydrogenase assay using the CytoTox 96 nonradioactive cytotoxicity kit (G1781, Promega, as described previously.(26, 31) The manufacturer's instructions were followed. Absorbance values of wells containing effector cells alone and target cells alone were combined and subtracted as background from the values of the co-cultures. Wells containing target cells alone were mixed with a lysis reagent for 30 min at 37°C and the resulting luminescence was set as 100% lysis. Cytotoxicity was calculated by using the following formula: %Cytotoxicity = (Experimental – Effector spontaneous – Target spontaneous) / (Target maximum – Target spontaneous) × 100%.

### **6.12 Statistical considerations**

This is an open-label, monocenter, single arm clinical study. The investigators performed this study to evaluate the safety and feasibility of administration with autologous bNAb-derived CAR-T cells for chronic HIV-1-infected patients. Patients will be treated with cART to achieve undetectable HIV-1 in peripheral blood before adoptive transfer. CD8<sup>+</sup> T cells from donors will be infused into the autologous patients

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after transduction with bNAbs-derived CAR.

The primary endpoint, the persistence of bNAbs-derived CAR-T cells, will be evaluated by qPCR at various test points according to the data collection plan, as summarized by simple descriptive summary statistics. In addition, all laboratory correlative studies will also be reported descriptively. Laboratory studies will be performed to characterize the safety and feasibility of bNAbs-derived CAR-T cells administration. The other endpoints will be analyzed according to the primary endpoint statistical methods.

### **6.13 IFN- $\gamma$ ELISpot**

IFN- $\gamma$ -secreting cells were detected by human IFN- $\gamma$  ELISpot assay kit (DKW22-1000-096s; Dakewe) according to the manufacture protocol. CD8<sup>+</sup> T cells ( $10^5$  per well) from HIV-1-infected participants were incubated with Jurkat-gp160<sub>NL4-3</sub> (Jurkat-derived target cell line expressing HIV-1<sub>NL4-3</sub> Env) at 1: 1 ratio in antibody-precoated plates. Plates were incubated for 24 hours at 37°C and 5% CO<sub>2</sub>. The ELISpot assays were then performed according to the manufacturer's instructions. Spots were counted using an S6 ultra immunoscan reader (Cellular Technology Ltd.). The number of IFN- $\gamma$ -positive T cells was calculated by ImmunoSpot 5.1.34 software (Cellular Technology Ltd.) and converted into the number of spots per million CD8<sup>+</sup> T cells.

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## **7. Adverse event reporting**

### **7.1 Adverse event**

An adverse event (AE) is any undesirable sign, symptom or medical condition or experience that develops or worsens in severity after starting study treatment or any procedure specified in the protocol, even if the event is not considered to be related to the study. All observed or volunteered AE regardless of treatment group or suspected causal relationship to the investigational treatment will be reported as described in the following sections.

For all AEs, the investigator must pursue and obtain information adequate both to determine the outcome of the AE and to assess whether it meets the criteria for classification as a serious adverse event requiring immediate notification to its designated representative. For all AEs, sufficient information should be obtained by the investigator to determine the causality of the AE. The investigator is required to assess causality. Follow-up by the investigator may be required until the event or its sequelae resolve or stabilize at a level acceptable to the investigator.

As part of ongoing safety reviews conducted by the investigator, any non-serious adverse event that is determined by the ERG to be serious will be reported by the ERG as an SAE. To assist in the determination of case seriousness further information may be requested from the investigator to provide clarity and understanding of the event in the context of the clinical trial.

### **7.2 Serious adverse event**

A serious adverse event (SAE) is any untoward medical occurrence that:

- Results in death;
- Is life-threatening (immediate risk of death);
- Requires inpatient hospitalization or prolongation of existing hospitalization;
- Results in persistent or significant disability/incapacity (substantial

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disruption of the ability to conduct normal life functions);

- Results in congenital anomaly/birth defect;
- Progression of the malignancy under study (including signs and symptoms of progression) should not be reported as an SAE unless the outcome is fatal within the safety reporting period.

Hospitalization due to signs and symptoms of disease progression should not be reported as an SAE. If the treatment has a fatal outcome during the study or within the safety reporting period, then the event leading to death must be recorded as an AE and as an SAE with Common Terminology Criteria for Adverse Events (CTCAE, Version 5.0) Grade 5.

Medical and scientific judgment is exercised in determining whether an event is an important medical event. An important medical event may not be immediately life-threatening and/or result in death or hospitalization. However, if it is determined that the event may jeopardize the patient or may require intervention to prevent one of the other AE outcomes, the important medical event should be reported as serious.

Examples of such events are intensive treatment for or development of acute immune response after treated-cells infusion, and the gene therapy induced tumorigenesis.

All suspected serious adverse events related to the study and requiring additional medical intervention should be followed according to the institution's standard of care, preferably in consultation with appropriate specialists.

### **7.3 Expected/unexpected adverse events**

Expected adverse events are those that have been previously identified as resulting from administration of the autologous CAR-T cells, anti-retroviral therapy of HIV-1 infection.

For the purposes of this study, an adverse event is considered unexpected when it varies in nature, intensity or frequency from information provided by the ERG or when it is

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not included in the informed consent document as a potential risk.

#### **7.4 Causality assessment**

The investigator's assessment of causality must be provided for all AEs (serious and non-serious); the investigator must record the causal relationship in the CRF, as appropriate, and report such an assessment in accordance with the serious adverse reporting requirements if applicable. An investigator's causality assessment is the determination of whether there exists a reasonable possibility that the investigational treatment caused or contributed to an AE. Generally, the facts (evidence) or arguments to suggest a causal relationship should be provided. If the investigator does not know whether or not the investigational treatment caused purposes, then the event will be handled as “related to investigational treatment” for reporting investigator's causality assessment is “unknown but not related to investigational treatment”, this should be clearly documented on study records.

In addition, if the investigator determines an SAE is associated with study procedures, the investigator must record this causal relationship in the source documents and CRF, as appropriate, and report such an assessment in accordance with the SAE reporting requirements, if applicable.

Attribution is the relationship between an adverse event or SAE and the study treatment. Attribution will be assigned as follows:

- Definite – The AE is clearly related to the study treatment;
- Probable – The AE is likely related to the study treatment;
- Possible – The AE may be related to the study treatment;
- Unlikely - The AE is doubtfully related to the study treatment;
- Unrelated - The AE is clearly NOT related to the study treatment.

#### **7.5 Recording and reporting**

Participating investigators will assess the occurrence of AE and SAE at all participant evaluation time points during the study. All grade 3 or higher AE and SAE, whether

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reported by the participant, discovered during questioning, directly observed, or detected by physical examination, laboratory test or other means, will be recorded in the participant's medical record and on the appropriate study-specific case report forms. The descriptions and grading scales found in the revised Common Terminology Criteria for Adverse Events (CTCAE) version 5.0 will be utilized for AE reporting. All appropriate treatment areas should have access to a copy of the CTCAE version 5.0.

The study must be conducted in compliance with local safety reporting requirements, and reporting requirements. Investigators must report to the ERG any AE, whether or not they are considered related to the investigational interventions.

All adverse events, both serious and non-serious, and deaths that are encountered from initiation of study intervention, throughout the study, and within 30 days of the last study intervention should be followed to their resolution, or until the participating investigator assesses them as stable, or the participating investigator determines the event to be irreversible, or the participant is lost to follow-up. The presence and resolution of AE /SAE (with dates) should be documented on the appropriate case report form and recorded in the medical record to facilitate source data verification. For some SAE, the study sponsor or designee may follow-up by telephone and/or monitoring visit to obtain additional case details. Participants should be instructed to report any serious post-study event(s) that might reasonably be related to participation in this study.

#### **7.6 General toxicity management considerations**

**Replication-competent lentivirus (RCL)** may be generated during the CAR-T manufacturing phase or subsequently after introduction of vector transduced cells into the patient. However, an RCL resulting from the production phase is highly unlikely since elements are incorporated in the design of the vector system that minimize vector recombination and generation of RCL. Furthermore, the vector used to transduce the product undergoes sensitive assays for detection of RCL before it can be released to a

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subject.

**Uncontrolled T cell proliferation or malignancy:** In pre-clinical studies, bNAb-derived CAR-T cells have only proliferated in response to HIV-1 gp120.(26) In the context of this protocol it is highly unlikely that the T cells will proliferate in response to signals from the normal tissues.

If any subject develops excessive CAR-T cell accumulation or malignancy, ganciclovir will be administered to eradicate the infused CAR-T cells because the vector contains a herpes simplex virus-1 thymidine kinase (TK) as the suicide gene, furthermore, corticosteroids can also be utilize to eradicate the uncontrolled proliferating CAR-T cells.

**Infusion reaction:** Acetaminophen and diphenhydramine hydrochloride may be repeated every 6 hours as needed. A course of non-steroidal anti-inflammatory medication may be prescribed if the patient continues to have fever not relieved by acetaminophen. It is recommended that patients not receive corticosteroids at any time, except in the case of a life-threatening emergency, since this may have an adverse effect on infused CAR-T cells.

**Febrile reaction.** In the event of febrile reaction, an evaluation for infection should be initiated, and patients managed appropriately with antibiotics, fluids and other supportive care as medically indicated and determined by the treating physician. In the event that the patient develops sepsis or systemic bacteremia following CAR-T cell infusion, appropriate cultures and medical management should be initiated. If a contaminated CAR-T cell product is suspected, the product can be retested for sterility using archived samples. Consideration of a cytokine release syndrome (see below) should be given.

**Cytokine Release Syndrome (CRS):** CRS has been observed in lymphocytic

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leukemia/lymphoma patients after treatment with anti-CD19/20-CAR-T cells. Patients with clinical responses exhibited some level of CRS that ranged from mild to severe consisting of fevers, hypotension, capillary leak, hypoxia or other symptoms. Cytokine production is caused by the activation, expansion and cytolytic function of T cells. Subsequent to this experience, ganciclovir will be administered to eradicate the infused CAR-T cells because the vector contains a herpes simplex virus-1 thymidine kinase (TK) as the suicide gene, selective tocilizumab (an anti-IL6-receptor antibody) therapy can also be utilized with effective toxicity management and successful ongoing bNAb-derived CAR-T cell expansion in patients.



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## **8. Data handling and record keeping**

### **8.1 Case report forms/electronic data record**

As used in this protocol the term CRF should be understood to refer to either a paper form or an electronic data record or both, depending on the data collection method used in this study. A CRF is required and should be completed for each included patient. The completed original CRFs are the sole property of investigation team and should not be made available in any form or third parties, except for authorized representatives of the team or appropriate regulatory authorities, without written permission from the team.

The PI has ultimate responsibility for the collection and reporting of all clinical, safety and laboratory data entered on the CRFs and any other data collection forms (source documents) and ensuring that they are accurate, authentic/original, attributable, complete, consistent, legible, timely (contemporaneous), enduring and available when required. The CRFs must be signed by the PI or by an authorized staff member to attest that the data contained on the CRFs is true. Any corrections to entries made in the CRFs, source documents must be dated, initialed and explained (if necessary) and should not obscure the original entry.

In most cases, the source documents are the hospital's or the physician's patient chart. In these cases, data collected on the CRFs must match the data in those charts.

In some cases, the CRF, or part of the CRF, may also serve as source documents. In these cases, a document should be available at the investigator's site as well as at the Emergency Response Group and clearly identify those data that will be recorded in the CRF, and for which the CRF will stand as the source document.

### **8.2 Record retention**

To enable evaluations and/or audits from regulatory authorities, the investigator agrees to keep records including the identity of all participating patients, all original signed informed consent documents, copies of all CRFs, safety reporting forms, source

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documents, and detailed records of treatment disposition, and adequate documentation of relevant correspondence. The records should be retained by the investigator according to International Conference on Harmonization (ICH) (<https://www.ich.org/page/search-index-ich-guidelines>) and local regulations.

If the investigator becomes unable for any reason to continue to retain study records for the required period (e.g., retirement, relocation), the ERG should be prospectively notified. The study records must be transferred to a designee acceptable to the ERG, such as another investigator, another institution, or to an independent third party arranged by the ERG. Investigator records must be kept for a minimum of 10 years after completion or discontinuation of the study or for longer if required by applicable local regulations. The investigator must obtain the written permission before disposing of any records, even if retention requirements have been met.

## **9. Ethics**

The study will be conducted in accordance with ethical principles founded in the Declaration of Helsinki. The Institutional Review Board (IRB) & Independent Ethics Committee (IEC) will review all appropriate study documentation in order to safeguard the rights, safety, and well-being of the subjects. The study will only be conducted at sites where IRB/IEC approval has been obtained. The Protocol, Investigator's Brochure, ICF, advertisements (if applicable), written information given to the subjects, safety updates, annual progress reports, and any revisions to these documents will be provided to the IRB/IEC.

### **9.1 IRB & IEC**

It is the responsibility of the investigators to have approval of the study protocol, protocol amendments, informed consent documents, and other relevant documents, eg, recruitment advertisements, if applicable, from the IRB/IEC. All correspondences with the IRB/IEC should be retained in the Investigator Files. Copies of IRB/IEC approvals should be forwarded to the Emergency Response Group. The only circumstance in

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which an amendment may be initiated prior to IRB/IEC approval is where the change is necessary to eliminate apparent immediate hazards to the patients. In that event, the investigator must notify the IRB/IEC and the Emergency Response Group in writing immediately after the implementation.

### **9.2 Ethical conduct of the study**

The study will be conducted in accordance with legal and regulatory requirements, as well as the general principles set forth in the International Ethical Guidelines for Biomedical Research Involving Human Patients (Council for International Organizations of Medical Sciences 2002), Guidelines for GCP (ICH 1996), and the Declaration of Helsinki (World Medical Association 1996 & 2008). In addition, the study will be conducted in accordance with the protocol, the ICH guideline on GCP, and applicable local regulatory requirements and laws.

### **9.3 Patient information and consent**

All parties will ensure protection of patient personal data and will not include patient names on any forms, reports, publications, or in any other disclosures, except where required by laws.

Patient names, address and other identifiable data will be replaced by a numerical code consisting of a numbering system. In case of data transfer, the ERG will maintain high standards of confidentiality and protection of patient personal data. The informed consent document must be in compliance with ICH GCP, local regulatory requirements, and legal requirements.

The investigator must ensure that each study patient, or his/her legal representative, is fully informed about the nature and objectives of the study and possible risks associated with participation. The investigator, or a person designated by the investigator, will obtain written informed consent from each patient or the patient's legal representative before any study-specific activity is performed. The investigator will retain the original of each patient's signed consent document.

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#### **9.4 Patient recruitment**

Advertisements approved by ethics committees and investigator databases may be used as recruitment procedures.

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## **Current Study Protocol**



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**Protocol ID: GZ8H-QY-mHIVCAR01**

**Broadly Neutralizing Antibody-derived CAR-T Cell  
Reduce Viral Reservoir in HIV-1-infected individuals**

**Investigator:**

Hui Zhang, M.D, Ph.D.

Linghua Li, M.D.

**Research institute:**

Institute of Human Virology, Zhongshan School of Medicine, Sun Yat-sen

University;

Infectious Disease Center, Guangzhou Eighth People's Hospital, Guangzhou Medical

University.

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**Collaborators:**

Qianyang Biomedical Research Institute;

Institute of Pediatrics Guangzhou Women and Children Hospital, Guangzhou Medical

University;

## **Brief summary**

The investigators conducted an open-label trial of the safety and antiviral activity of broad neutralizing antibody (bNAb)-derived Chimeric Antigen Receptor (CAR)-T cell therapy in HIV-1–infected individuals some of whom are undergoing analytical interruption of combination antiretroviral therapy (cART).

## **Objectives**

The primary objective of the study is to assess the safety of single dose of bNAb-derived CAR-T cells administered to persons with viremia suppressed to below detectable levels. Secondary objective is to evaluate the antiviral activity and the *in vivo* persistence of CAR-T products. Key exploratory objectives are the size of HIV-1 reservoir after CAR-T cells administration and the genetic and phenotypic characterization of the rebound viruses. Post hoc analyses of the sequence diversity at the time of viremia rebound and the cytotoxic capacity of CAR-T cells against autologous HIV-1 before and after adoptive transfer are performed.

## **Outcome measures**

### **1. Primary outcome measure**

- 
- The adverse events (AEs). The AEs of single dose of bNAb-derived CAR-T cells administered to persons with viremia suppressed to below detectable levels. All the grades of AE are evaluated according to the Common Terminology Criteria for Adverse Events (CTCAE).
  - HIV-1 RNA. Level change of HIV-1 RNA in plasma after analytical treatment interruption (ATI).

## **2. Secondary outcome measure**

- HIV-1 RNA. Level change of HIV-1 RNA in plasma before and 6 months after adoptive transfer.
- Persistence of bNAb-derived CAR-T cells *in vivo*. The percentage of bNAb-derived CAR-T cells within peripheral CD8<sup>+</sup> T cells before and after adoptive transfer analyzed by quantitative PCR.
- HIV-1 cell-associated viral RNA. Level change of cell-associated viral RNA in CD4<sup>+</sup> T cells before and after adoptive transfer by analyzed quantitative real-time RT-PCR.

## **3. Other pre-specified outcome measures**

- CD4<sup>+</sup> T cell count. Level change of the CD4<sup>+</sup> T cell number after adoptive transfer.
- The ratio changes of CD4/CD8. The ratio changes of CD4/CD8 in peripheral blood before and after adoptive transfer.
- HIV-1 DNA level. Changes of pro-viral DNA in PBMC before and after adoptive transfer.
- Sequence and phylogenetic analysis of HIV-1 viruses before and after adoptive

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transfer.

- Resistance of rebound viruses after ATI against HIV-1-specific CAR-T cells.

### **Study treatment**

CD8<sup>+</sup> T lymphocytes from PBMCs of HIV-1 infected patients are transduced with bNAb-derived CAR, then the modified CAR-T cells are expanded *in vitro* and adoptively transferred into the autologous patients.

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## 1. Introduction

### 1.1 Study objectives

The primary objective of the study is to assess the safety and antiviral activity of single dose of bNAb-derived CAR-T cells administered to persons with viremia suppressed to below detectable levels. Secondary objective is to evaluate the *in vivo* persistence of CAR-T cells. Key exploratory objectives are the size of HIV-1 reservoir after CAR-T cells administration and the genetic and phenotypic characterization of the rebound viruses. Post hoc analyses of the sequence diversity at the time of viremia rebound and the cytotoxic capacity of CAR-T cells against autologous HIV-1 before and after adoptive transfer are performed.

HIV-1 RNA level and CD4<sup>+</sup> T cell counts will be monitored weekly for two months, then be monitored biweekly for at least six months. The major objectives could be summarized as followings:

- Safety and side-effect profile of single dose of bNAb-derived CAR-T cells administered to persons with viremia suppressed to below detectable levels. All the grades of AEs are evaluated according to the Common Terminology Criteria for Adverse Events (CTCAE) ([https://ctep.cancer.gov/protocolDevelopment/electronic\\_applications/ctc.htm#ctc\\_40](https://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm#ctc_40))
- HIV-1 RNA. Level change of HIV-1 RNA in plasma after analytical treatment interruption (ATI).

### 1.2 Background and rationale

Combination antiretroviral therapy (cART) significantly suppresses HIV-1 to an undetectable level in the blood, improves immune function, delays progression of the disease, and decreases mortality of HIV-1 patients.(2) However, a certain amount of

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HIV-1 replication-competent proviruses comprise a latent reservoir, which is quite stable, with a half-life of 44 months, requiring nearly 73.4 years for complete clearance.(3-5) In almost all HIV-1–infected individuals, plasma viral rebound predictably occurs within days after treatment interruption, resulting in the lifelong requirement for cART.(6, 7) To achieve durable suppression of viremia without daily therapy, various strategies have been proposed, including long-acting antiretroviral drugs (LA-ARVs), broadly neutralizing antibodies (bNAbs), and chimeric antigen receptor T (CAR-T) cells.(8) In human clinical trials, viremic individuals who received bNAb therapies showed significant reductions in viremia.(9-11) Moreover, HIV-1–infected individuals who received multiple infusions of VRC01 or 3BNC117, two related bNAbs that target the CD4 binding site on the HIV-1 envelope spike, showed significant viral suppression for 5.6 or 9.9 weeks during analytical treatment interruption (ATI) of cART, respectively.(12, 13) Furthermore, a combination therapy of 3BNC117 and 10-1074 maintains suppression on virus rebound for median of 21 weeks.(14) These findings suggest that the immunotherapy with chimeric antigen receptors (CAR)-modified T-cells, if HIV-1-specific bNAb-derived, may also potentially prevent virus rebound in HIV-1–infected individuals after ATI.

CAR-moiety is typically generated by coupling an antibody-derived, single-chain Fv domain to an intracellular T-cell–receptor zeta chain and costimulatory receptor-signaling domains. The clinical usage of CAR-T cells resulted in complete remission in approximately 83% of lymphocytic leukemia/lymphoma patients.(15, 16) Compared to CAR-T cells targeting tumor-associated antigens such as CD19 and CD20 which are also expressed in normal B-lymphocytes, the HIV-1–specific CAR-T cells target HIV-1 envelope (Env) protein which is only expressed on the surface of virus-producing cells.(17-19) At the earlier time, a strategy that fuse the extracellular domain of CD4 with the intracellular domain of the CD3 $\zeta$  chain (CD4 $\zeta$ -CAR) was shown to be safe and feasible in HIV-1–infected individuals. However, the antiviral efficacy was modest and durable control of viral replication in clinical trials was not observed.(20-24) In recent



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years, the third and fourth generation intracellular CAR moieties have been developed.(16) Moreover, a number of preclinical studies for the bNAb-derived HIV-1–specific CAR-T cells *in vitro* and in animal models have shown the suppression of viral replication or the eradication of virus-producing cells.(25-30) Especially, we previously found that VRC01-derived CAR-T cells effectively eradicate the reactivated viral reservoir isolated from HIV-1–infected individuals receiving cART, and the engineered resistance to triple inhibitory receptors including PD-1, Tim-3, and Lag-3 prevents CAR-T cell exhaustion and improves their maintenance *ex vivo*.(26, 31)

Here, we conduct a phase 1 clinical trial for investigating the effect of single administration of bNAb-derived CAR-T cells upon virus rebound after the discontinuation of suppressive cART.

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## **2. Eligibility assessment and enrollment**

This study can fulfill its objectives only if appropriate patients are enrolled. The following eligibility criteria are designed to select patients for whom protocol treatment is considered appropriate. All relevant medical and non-medical conditions should be taken into consideration when deciding whether this protocol is suitable for a particular patient.

### **2.1 Inclusion criteria**

Patient eligibility should be reviewed and documented by an appropriately qualified member of the investigator's study team before patients are included in the study. Patients must meet all of the following inclusion criteria to be eligible for enrollment into the study.

1. Age between 18 to 60, male or female;
2. HIV-1 infection, documented by any licensed rapid HIV-1 test or HIV-1 enzyme or chemiluminescence immunoassay (E/CIA) test kit at any time prior to study entry and confirmed by a licensed Western blot or a second antibody test by a method other than the initial rapid HIV-1 and/or E/CIA, or by HIV-1 antigen, plasma HIV-1 RNA VL;
3. Clinically stable on cART regimen for at least 12 months with undetectable HIV-1 level ( $< 50$  copies/ml, HIV-1 RNA, Cobas test);
4. Screening CD4<sup>+</sup> T-cell count  $\geq 350$  cells/ $\mu$ L within 14 days prior to study entry.
5. There is no serious damage of liver and kidney function and other indexes are within the basic normal range after comprehensive physical examination (including general examination, routine hematuria examination, blood biochemical examination, chest X-ray, B-ultrasound and ECG, etc.);
6. The following laboratory values obtained within 45 days prior to enrollment: Hgb  $\geq 11$ g/L (Male),  $\geq 10$ g/L (Female); Neutrophil count  $\geq 1 \times 10^9$ /L; Platelet count  $\geq 100 \times 10^9$ /L; Creatinine  $\leq 110$   $\mu$ mol/L; Aspartate transaminase (AST) and Alanine aminotransferase (ALT)  $\leq 2.5 \times$ ULN;

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7. Willingness to have blood samples collected, stored indefinitely, and used for study-related research purposes;
  8. Ability and willingness of subject or legal representative to provide informed consent.

## **2.2 Exclusion criteria**

Patients presenting with any of the following will not be included in the study:

1. Ongoing AIDS-related opportunistic infection or any cancer or malignancy;
2. AST and ALT  $> 3 \times$  normal, Creatinine  $> 110 \mu\text{mol/L}$ ;
3. Any uncontrolled active medical disorder including seriously chronic disease, metabolic disorders, neurological and psychiatric disorders that would preclude participation as outlined;
4. History of a pancreatitis;
5. Pregnant or lactation;
6. According to the researcher's judgment, the candidate has a low possibility of inclusion (such as weakness, poor compliance, etc.);
7. History of a severe allergic reaction with generalized urticaria, angioedema, or anaphylaxis in the 2 years prior to enrollment;
8. Acute or chronic hepatitis B or hepatitis C infection;
9. Subject with CMV retinitis or other active CMV infection related diseases;
10. Subject with organ dysfunction;
11. Drug or alcohol abuse or dependence;
12. Currently (Within 3 months) enrolled in another clinical trial or underwent cell therapy;
13. In the opinion of the site investigator, would interfere with adherence to study requirements.

## **2.3 Life style guidelines**

All male and female patients who, in the opinion of the investigator, are biologically capable of having children and are sexually active, must agree to use a highly effective

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method of contraception consistently and correctly for the duration of the active treatment period and for a minimum of 90 days after the last dose of investigational treatment. The investigator, in consultation with the patient, will select the most appropriate method of contraception for the individual patient from the permitted list of contraception methods, and instruct the patient in its consistent and correct use. In addition, the investigator will instruct the patient to call immediately if the selected birth control method is discontinued or if pregnancy is known or suspected.

Highly effective methods of contraception are those that, alone or in combination, result in a failure rate of less than 1‰ per year when used consistently and correctly (i.e., perfect use) and include:

1. Established use of oral, injected or implanted hormonal methods of contraception;
2. Correctly placed intrauterine device (IUD) or intrauterine system (IUS);
3. Male condom or female condom used WITH a spermicide (i.e., foam, gel, film, cream, suppository);
4. Male sterilization with appropriately confirmed absence of sperm in the post-vasectomy ejaculate;
5. Bilateral tubal ligation or bilateral salpingectomy.

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### 3. Study implementation

#### 3.1 Study design

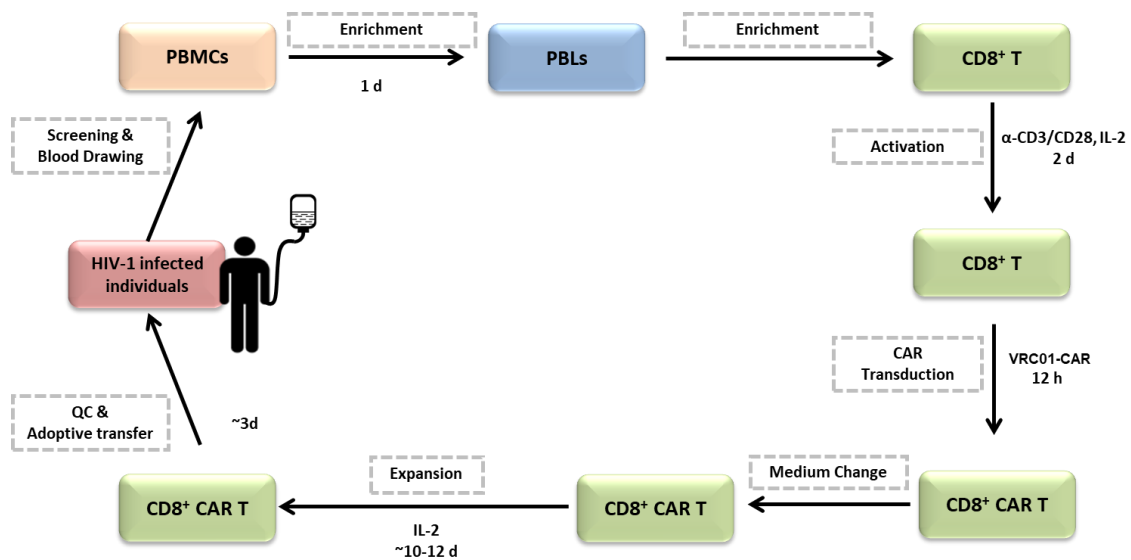
Relevant studies are initiated immediately after the patients are enrolled in the group. After the patients enrolled in the group, the screening will be started when the HIV-1 cannot not be detected. All participants have not undergone any additional immunotherapeutic intervention besides ART and there were no conditioning regimens before CAR-T cell administrations. At the same time, peripheral blood mononuclear cells (PBMCs) are collected from peripheral blood and separated on the basis of CD8 expression. The sorted CD8<sup>+</sup> T cells will be activated and then transduced by bNAb-derived CAR. Then genetic-modified CD8<sup>+</sup> T cells are expanded for 15 to 21 days *ex vivo*. Modified CAR-T cells are administrated into the patient, and the hospital observations will persist for one day. The basic condition of the patients, the immune reaction after bNAb-derived CAR-T cells administration, the HIV-1 viral load in plasma and the size of latent reservoir will be examined every 1-2 weeks after adoptive transfer for at least six months. After the six-month post-treatment, follow-up will be conducted every 1 months. The basic information is shown in the following table.

**Table 1. Basic Information of the Clinical Study**

Item	Content
Study Type	Interventional
Primary Purpose	Treatment
Interventional Study Model	Single Group Assignment
Intervention Description	CD8 <sup>+</sup> T lymphocytes from donor are transduced with bNAb-derived CAR before adoptive transfer into the patient
Number of Arms	1
Masking	None (Open Label)
Enrollment	20

During the study, every single patient will receive single administration of bNAb-

derived CAR-T cells. The patient will undergo a cytokine storm prophylaxis regimen, which will vary according to a specific patient's condition. The patient will be treated with anti-retroviral drugs pre-CAR-T administration and post CAR-T administration. An analytical treatment interruption will be performed when the relative criteria is fulfilled. The transplantation procedure is shown in the following figure.



**Figure 1. Flowchart of the bNAb-derived CAR-T cell therapy**

### 3.2 The CD8<sup>+</sup> T cell sorting

The CD8<sup>+</sup> T cells will be separated from donor mobilized peripheral blood cells using the CliniMACS system. The protocol is as follows:

- 1) Buffer preparation. Human Serum Albumin (HSA) will be added to physiological saline solution for a final concentration of 0.5%;
- 2) Collection of karyocytes. The empty preparation bag will be weighted for following calculation of cell sample. The blood sample will be counted followed by red blood cell lysis. Karyocytes will be counted while 200  $\mu$ l cell suspension will be collected for FACS. The remaining karyocytes will be transported to a preparation bag and weighted;
- 3) Dilution of the karyocytes. The karyocytes will be diluted with two volumes

- 
- 0.5% HAS containing physiological saline solution and mixed. The preparation bag will be centrifuged at 300 g and the supernatant is removed. The remaining karyocytes suspension will be adjusted to the final weight of 95 g;
- 4) Magnet beads. 7.5 ml CD8 beads will be injected to the karyocytes suspension with a syringe and mixed;
  - 5) Incubation. The mixture of karyocytes suspension and CD8 beads will be incubated at room temperature with an oscillator at 25 rpm for 30 minutes;
  - 6) Cell washing. After diluting the karyocytes suspension with 500 ml buffer, the preparation bag will be centrifuged at 300 g for 15 minutes. 500 ml of the supernatant will be removed. The karyocytes will be washed for two times;
  - 7) Cell preparation. The final volume of the karyocytes suspension will be adjusted to 150 ml. 200  $\mu$ l sample will be collected for FACS;
  - 8) CD8<sup>+</sup> T cell sorting with CliniMACS. Install the LS tube system (162-01) and the preparation bag according to the instruction and perform the separation with DEPLETION 2.1. The sorted positive and negative collection bags will be weighted before and after the separation;
  - 9) Collection of sorted CD8<sup>+</sup> T cells. The collection bag will be weighted and 200  $\mu$ l sample will be collected for FACS.

### **3.3 Activation of CD8<sup>+</sup> T cells**

Cell collection:

Sorted CD8<sup>+</sup> T cells in the collection tubes will be transferred into culture flasks and will be rinsed with physiological saline solution. Cell will be counted and collected by centrifuge at 300 g for 10 minutes.

Culture medium:

CD8<sup>+</sup> T cells will be cultured at  $0.5 \times 10^6$ – $1 \times 10^6$  cells/ml with ImmunoCult-XF T

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Cell Exp Medium (Stem Cell Technologies) supplemented with 5% HAS and Gentamycin Sulfate Injection.

Activation:

CD8<sup>+</sup> T cell culture will be added paramagnetic antibodies (ImmunoCult Human CD3/28 T Cell Activator, Stem Cell Technologies) (25 µL per 1 ml of cell suspension), IL-2 (<sup>125</sup>Ala IL-2, SL Pharm) (150 IU per 1 ml of cell suspension). The culture media will be pre- heated in 37°C incubator before use.

### **3.4 Pseudotyped lentiviral manufacture**

Clinical pseudotyped lentiviral manufacture: The VRC01-CAR lentiviral vectors using design principles previously reported.(26, 31) Pseudotyped lentivirus are produced by transient transfection of HEK293T cells with the lentiviral vector, packaging vector (psPAX2), and envelope vector (pMD.2G). The VRC01-CAR pseudotyped lentivirus is purified via tangential flow filtration (KR2i TFF system, Repligen) and chromatographic purification system (ÄKTA™ pure, GE).

### **3.5 Transduction and expansion**

Two days after initial CD8<sup>+</sup> T cell activation, transduction of pseudotyped lentivirus encoding VRC01-CAR is performed in retronectin-coated cell-culture bags and containing culture medium, and 150 IU/ml IL-2. After 12 hours, transduced CD8<sup>+</sup> T cells are washed, counted and resuspended at  $0.5 \times 10^6$ – $1 \times 10^6$  cells/ml in fresh culture medium with 150 IU/ml IL-2. Then genetic-modified CD8<sup>+</sup> T cells are expanded with IL-2–dependent growth for 15 to 21 days *ex vivo*. The target cell dose range is at least  $5 \times 10^7$  CD3<sup>+</sup> CD8<sup>+</sup> cells.

Release criteria for the expanded CD8<sup>+</sup> T cells are as follows:

- 1) Cell viability  $\geq$  90%;
- 2) CD3<sup>+</sup> CD8<sup>+</sup> T cells  $\geq$  95%;
- 3) Endotoxins  $\leq$  5 EU/ml;



- 
- 4) Mycoplasma: negative;
  - 5) Bacterial culture: negative;
  - 6) Fungal culture: negative;
  - 7) Transduction efficiency (percentage CAR<sup>+</sup> of viable CD3<sup>+</sup> CD8<sup>+</sup> T cells): ≥ 30-60%;
  - 8) The gp120-specific cytotoxicity of more than 40% lysis at 100:1 E:T ratio.

### **3.6 CAR-T cell suspension infusion**

The expanded CAR-T cells will be prepared under the good manufacturing practice (GCP). Release criteria for the expanded CD8<sup>+</sup> T cells are as follows: sterility by Chinese Pharmacopeia (2015), negative fungal, and mycoplasma testing, negative Gram stain, endotoxin less than 5 U/kg, more than 90% viability by Trypan Blue exclusion, CD3 and CD8 staining by flow cytometry, IL-2–dependent growth, and gp120-specific cytotoxicity of more than 40% lysis at 100:1 E:T ratio.

These cells will be infused into the patient on day 0 (/and day 1, if necessary) of transplantation, with or without co-infusion of thawed, selected negative cells. The cell infusion procedures are as followings:

- Obtain blood pressure, temperature, pulse, respirations and pulse oximetry at the start of the infusion, every 10 minutes and at the completion of each product;
- Glucose gluconate injection is intravenously injected before cells infusion;
- Begin infusion slowly for the first 20 ml. If the patient tolerates the first 20 ml, increase the rate to infuse over 10-15 minutes;
- Infuse cell suspension at the speed about 50 ml per hour unless otherwise ordered. It is imperative that the cells are infused slowly due to the potential immune reaction;
- Cell suspension will be rinsed with 100 ml normal physiological saline solution at the end of infusion by back priming to assure as many cells as possible are infused into the patient;

- 
- Assess the patient frequently for possible reactions, (i.e. shortness of breath, dyspnea, tachycardia, chest pain, rales, erythema, rash, hives, flank pain, temperature increase, nausea, vomiting, rigors, bronchial spasms and respiratory arrest);
  - If reaction suspected, slow infusion, medicate as needed per medication orders and notify the physician immediately.

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## **4. Analytical treatment interruption (ATI)**

Analytical treatment interruption (ATI) of cART is favorable to evaluate the anti-virus effect of the CAR-T cell therapy. Considering the potential risks for ATI, the ATI will be performed at least four weeks after CAR-T cell administration. To prevent the risk of efavirenz monotherapy and the emergence of resistant strains after stopping ART, the individuals whose regimens contained efavirenz stop taking it one week before discontinuing the other agents. The individuals with integrase inhibitor-based or protease inhibitor-based regimes discontinue three ART agents simultaneously. When all the ART agents are discontinued, the time of plasma viral suppression begins to measure. The ATI is performed when the following criteria are achieved.

### **4.1 Criteria for initiate treatment interruption**

- A written informed consent is provided by the patient;
- No adverse events at the time of interruption;
- The CD4<sup>+</sup> T cell counts maintain in a normal range (> 400 cells/μl peripheral blood);
- The plasma viral load is under the detectable level (< 20 copies per milliliter);
- The cell-associated viral RNA is reduced at least 50%;
- *In vivo* CAR-T cells can be persistently detectable (> 100 copies per million CD8<sup>+</sup> T cells).

### **4.2 Criteria for reinitiate anti-viral treatment**

- The plasma viral load exceeding 200 copies per milliliter;
- The CD4<sup>+</sup> T cells decrease under 350 cells/μl;
- Any AIDS-related opportunistic infections are observed;
- Occurrence of severe CAR-T–related adverse events;
- Request for reinitiating anti-retroviral therapy by the patient.

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## **5. Quality control**

### **5.1 Quality control and quality assurance**

During study conduct, the Emergency Response Group (ERG), led by Dr. Linghua Li, the PI of this study and Dr. Xilong Deng, the chair of Emergency Department of the Guangzhou 8<sup>th</sup> People's Hospital, will conduct periodic monitoring visits to ensure that the protocol and Good Clinical Practices (GCPs) are being followed. The monitors may review source documents to confirm that the data recorded on CRFs is accurate. The investigator and institution will allow the ERG monitors or its agents and appropriate regulatory authorities direct access to source documents to perform this verification.

It is important that the investigation and their relevant personnel are available during the monitoring visits and possible audits or inspections and that sufficient time is devoted to the process.

### **5.2 Safety meeting**

The ERG will review and monitor toxicity and accrual data from this study. The committee is composed of clinical specialists with experience in virology and who are the principal investigators in the study. Information that raises any questions about participant safety will be addressed with the study team. The ERG will meet quarterly and/or more often if required to review toxicity and accrual data. Information to be provided to the ERG may include: all grade 2 or higher unexpected adverse events that have been reported; any response information; audit results, and a summary provided by the study team. Other information (e.g. scans, laboratory values) will be provided upon request.

### **5.3 Monitoring**

Involvement in this study as a participating investigator implies acceptance of potential

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audits or inspections, including source data verification, by representatives designated by the Protocol Chair. The purpose of these audits or inspections is to examine study-related activities and documents to determine whether these activities are conducted, and data are recorded, analyzed, and accurately reported in accordance with the protocol, institutional policy, and any applicable regulatory requirements. Monitoring will begin at the time of participant registration and will continue during protocol performance and completion.

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## 6. Data collection and evaluation

### 6.1 Schedule

Except if noted otherwise, the same study procedures must be matched for participants. AE/SAE will be reported throughout the study, as described in the specific section of the protocol. Three periods are involved in this section of “Data Collection and Evaluation”, including pre-screening, study treatment period and long-term follow-up period.

- 1) Pre-screening procedures for baseline data will occur within 30 days before pre-treatment unless otherwise noted. Informed consent document (ICD), an Institutional Review Board (IRB) or Independent Ethics Committee (IEC) approved ICD, must be signed and dated before any study-specific procedures are done. Results from laboratory tests or assessments, performed as a standard of care, prior to the date of informed consent but within the allowed timeframe for screening procedures, can be used for determining the patient's eligibility and will be entered in the CRF. Use of pre-consent results to support inclusion/exclusion criteria must be clearly documented in the patient's source documents. The following will be collected at screening:
  - Sign informed consent form;
  - Review study eligibility (inclusion and exclusion criteria);
  - Locally obtained characterization of viral infection;
  - Disease history and prior treatment;
  - Focused physical examination;
  - Vital signs;
  - Laboratory evaluation;
  - Other focused items.
  
- 2) The “study treatment period” is defined as the period starting with the CAR-T cells administration up to and including the end-of-treatment visit. The protocol of

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this period involves parts of section “Study Implementation” and “Data Collection and Evaluation”.

- 3) For long term follow-up visit, disease assessments will be conducted for all patients for 1 years or longer. Visits for disease assessment are planned to start approximately one weeks after the last disease assessment then continuing until the patient completes 1 years (relative to the day of administration), has documented undetectable *in vivo* bNAb-derived CAR-T cells, whichever occurs first. The data collected at follow-up include persistence of infused CAR-T cells, HIV-1 RNA viral load in plasma, HIV-1 cell-associated viral RNA, HIV-1 DNA measurement, sequencing and genetic diversity analysis of HIV-1 Env and the other laboratory measurement.

## **6.2 Adoptive transfer**

HIV-1-specific CAR-T therapy involves the intravenous infusion of bNAb-derived CD8<sup>+</sup> CAR-T cells to reestablish anti-viral immunosurveillance in patients with continuous cART regimen for at least 12 months. The CD8<sup>+</sup> T cells sources are mobilized peripheral blood from the donor.

Adoptive transfer, patient care and follow-up were performed at Guangzhou Eighth People’s Hospital. The patient provided informed consent in accordance with the Declaration of Helsinki. During the study, every single patient will undergo a single administration of bNAb-derived CAR-T cells. The patient will receive a graft containing CD8<sup>+</sup> T cells with transduction of bNAb-derived CAR (CliniMACS sorting system). The patient will be treated with anti-retroviral drugs pre-treatment and post treatment. The adoptive transfer will be evaluated by the *in vivo* persistence of the CAR-T products.

## **6.3 Safety measures**

Safety endpoints include adverse events (graded according to the Common Terminology Criteria for Adverse Events (CTCAE) Version 5.0), clinical examination

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(including blood pressure and pulse), laboratory tests (hematology, chemistry, coagulation and urinalysis) and electrocardiograms (ECGs) run in triplicate. Additional procedures or samples may be undertaken as medically required of the discretion of the investigator. In addition to the local laboratory tests, additional blood samples may be taken, or additional tests may be conducted in existing samples, for analysis at either central or local laboratories in order to provide the best possible guidance on improving the medical management of study patients on emerging safety findings during the conduct of the study. Generally, the *in vivo* persistence of CAR-T cells and HIV-1 related assessments (HIV-1 RNA/DNA, Reservoir diversity, sequence and phylogenetic analysis, viral outgrowth assay and cytotoxicity determination) will be conducted by the collaborator, Sun Yat-sen University, or the other institutes.

#### **6.4 Quantitative PCR to detect CAR-modified T cells**

Patient PBMCs collected at baseline and at serial time points after CAR-T-cell infusions were collected and separated by Ficoll centrifugation and then cryopreserved. Batched cells were thawed and primary human CD8<sup>+</sup> T cells were obtained from PBMCs by positive magnetic selection through Anti-Human CD8 Magnetic Particles - DM, BD-IMag<sup>TM</sup>. The genomic DNA was harvested using an AllPure Total DNA/RNA Micro Kit (Magen). The CAR transgene was detected by performing quantitative PCR as previously described, using either a primer amplifying the fragment spanning the junction of the CD3 $\zeta$  domain and adjacent Flag domain (forward primer: 5'-GCCTTTACCAGGGTCTCA-3', reverse primer: 5'-ACTTATCGTCGTCATCCTTG-3'), or a primer amplifying the fragment of VRC01 scFv (forward primer: 5'-ATTTTTTGGCCAGGGGACC-3', reverse primer: 5'-AGGATTCTCCTCGACGTCACC-3'). Quantitative real-time PCR was performed in triplicate using SYBR Premix ExTaq II Kit (Takara), in a C1000 Touch Thermal Cycler (BIO-RAD CFX96<sup>TM</sup> Real-Time System).(26) Copy numbers per microgram of genomic DNA, generated from a standard curve of 10-fold serial dilutions of purified plasmid, were used to calculate the percentage of CAR<sup>+</sup> cells among CD8<sup>+</sup> T cells,



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assuming 1 copy/cell.

### **6.5 Quantitative real-time RT-PCR analysis**

Primary human CD4<sup>+</sup> T cells were obtained from PBMCs by negative magnetic selection through Human CD4<sup>+</sup> T Lymphocyte Enrichment Set-DM, BD-IMag™. Total RNA was isolated with Trizol reagent (Life Technologies) and then subjected to cDNA synthesis using PrimeScript RT reagent Kit (Takara). All primers were annealed at 37°C and RT was processed at 42°C. Quantitative PCR was performed with SYBR Premix ExTaq II Kit (Takara) by following the manufacturer's instructions. The expressions of HIV-1 unspliced RNAs were determined by real-time qRT-PCR with the primer pair SK38 (5'-ATAATCCACCTATCCCAGTAGGAGAAA-3') and SK39 (5'-TTTGGTCCTTGTCTTATGTCCAGAATGC-3'). An *in vitro*-synthesized HIV-1 RNA, after quantification, was used as the external control for measuring cell associated viral RNA.(32) Quantification was normalized to the housekeeping gene GAPDH or β-actin.

### **6.6 Intact proviral DNA assay (IPDA)**

The procedures for IPDA described previously were followed with minor modifications.(36, 37) In general, the IPDA is performed on DNA from  $2 \times 10^6$  CD4<sup>+</sup> T cells. Genomic DNA is extracted using the QIAamp DNA Mini Kit (Qiagen) with precautions to avoid excess DNA fragmentation. Quantification of intact, 5'deleted, and 3'deleted and/or hypermutated proviruses was carried out using primer/probe combinations optimized for subtype B HIV-1. The primer/ probe mix consists of oligonucleotides for two independent hydrolysis probe reactions that interrogate conserved regions of the HIV-1 genome to discriminate intact from defective proviruses (Supplementary Table 2). HIV-1 reaction A targets the packaging signal ( $\psi$ ) that is a frequent site of small deletions and is included in many large deletions in the proviral genome. The  $\psi$  amplicon is positioned at HXB2 coordinates 692–797. This reaction uses forward and reverse primers, as well as a 5'6-FAM-labeled hydrolysis probe.

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Successful amplification of HIV-1 reaction A produced FAM fluorescence in droplets containing  $\psi$ , detectable in channel 1 of the droplet reader. HIV-1 reaction B targets the RRE of the proviral genome, with the amplicon positioned at HXB2 coordinates 7736–7851. This reaction used forward and reverse primers, as well as two hydrolysis probes: a 5'VIC labeled probe specific for wild-type proviral sequences, and a 5'unlabelled probe specific for APOBEC3G/H hypermutated proviral sequences (Supplementary file 2). Successful amplification of HIV-1 reaction B produced a VIC fluorescence in droplets containing a wild-type form of RRE, detectable in channel 2 of the droplet reader, whereas droplets containing a hypermutated form of RRE were not fluorescent. Droplets containing HIV-1 proviruses were scored as follows. Droplets positive for FAM fluorescence only, which arises from amplification, was scored as containing 30 defective proviruses, with the defect attributable to either APOBEC3G mediated hypermutation or 3' deletion. Droplets positive for VIC fluorescence only, which arises from wild-type RRE amplification, was scored as containing 5' defective proviruses, with the defect attributable to 5' deletion. Droplets positive for both FAM and VIC fluorescence was scored as containing intact proviruses. Double-negative droplets contained no proviruses or rare proviruses with defects affecting both amplicons.

### **6.7 Viral outgrowth**

Recovery and amplification of replication-competent viruses were described previously.(33) Briefly,  $1 \times 10^6$  CD4<sup>+</sup> T cells from HIV-1-infected individuals were stimulated by  $1 \times 10^7$  irradiated allogeneic PBMC from uninfected donors and the 1  $\mu$ g/ml PHA-M at day 1, and typically, three additions of  $5 \times 10^6$  activated CD4<sup>+</sup> lymphoblasts from uninfected donors as target cells were added for HIV-1 outgrowth at day 2, day 7 and day 14, respectively. The cells were cultured in RPMI-1640 media + IL-2 (10 ng/ml, recombinant human, R&D Systems) all the time. After 14 days co-culture, the recovered viruses were harvested and tested for HIV-1 p24 protein.

### **6.8 *In vitro* HIV-1 infection and drug withdrawal model**

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*In vitro* HIV-1 infection model was previously described with minor modifications.(26) Briefly, the PBMCs from healthy donors were stimulated by adding 1 mg/ml PHA and 10 ng/ml IL-2 to the conditioned RPMI1640 media with 10% heat-inactivated fetal bovine serum and antibiotics for two days before isolation of CD4<sup>+</sup> T cells. CD4<sup>+</sup> T cells were infected with an outgrown HIV-1 from patients (p24 titer of 1 ng/ml). Three hours after infection, the culture media was changed by centrifugation. The infected CD4<sup>+</sup> T cells were cultured in basal media + IL-2 (10 ng/ml, recombinant human, R&D Systems) and further incubated at 37°C in a humidified incubator with 5% CO<sub>2</sub>. Six days after HIV-1 infection, azidothymidine (Zidovudine, Sigma-Aldrich) and lopinavir (Sigma-Aldrich) were added into the CD4<sup>+</sup> T cell culture both at 50 μM to inhibit virus production and prevent further infection events. The cells were then cultured in the presence of low-concentration of IL-2 (1 ng/ml). Anti-HIV-1 drugs were withdrawn when the viral production was significantly decreased to the marginal level for p24 detection (about 6-8 day after drugs adding). Then, 0.5 ×10<sup>6</sup> CD4<sup>+</sup> T cells were mixed with autologous CAR-T cells or control CD8<sup>+</sup> T cells at 1: 1 ratio in the conditioned media plus IL-2 (10 ng/ml) at 1 ml in 24-well plate. Every two days the cultures were tested for HIV-1 p24 antigen with the HIV-1 p24 Antigen Assay kit by following the manufacturer's instructions.

### **6.9 Genetic diversity analysis of activated HIV-1 viruses**

HIV-1 RNA extraction and single-genome amplification was performed as previously described.(34, 35) In brief, HIV-1 RNA was extracted from cell and plasma samples followed by first-strand cDNA synthesis using HiScript II 1st Strand cDNA Synthesis KIT (Vazyme). cDNA synthesis for plasma-derived HIV-1 RNA was performed using the antisense primer envB3out 5'-TTGCTACTTGTGATTGCTCCATGT-3'. gp160 was amplified using envB5out 5'-TAGAGCCCTGGAAGCATCCAGGAAG-3' and envB3out 5'-TTGCTACTTGTGATTGCTCCATGT-3' in the first round and in the second round with nested primers envB5in 5'-CACCTTAGGCATCTCCTATGGCAGGAAGAAG-3' and envB3in 5'-GTCTCGAGATACTGCTCCCACCC-3'.

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PCRs were performed using Phanta Max Super-Fidelity DNA Polymerase (Vazyme) and run at 94 °C for 2 min; 35 cycles of 94 °C for 15 s, 55 °C for 30 s and 68 °C for 4 min; and 68 °C for 15 min. Second-round PCR was performed with 1 µl of the PCR product from the first round as template and Phanta Max Super-Fidelity DNA Polymerase (Vazyme) at 94 °C for 2 min; 45 cycles of 94 °C for 15 s, 55 °C for 30 s and 68 °C for 4 min; and 68 °C for 15 min. cDNA synthesis for cell and plasma RNA was performed using the antisense primer R3B6R 5'-TGAAGCACTCAAGGCAAGCTTTATTGAGGC-3'. The *env* 3' half-genome was amplified in a single PCR using B3F3 primer 5'-TGGAAAGGTGAAGGGGCAGTAGTAATAC-3' and R3B6R primer 5'-TGAAGCACTCAAGGCAAGCTTTATTGAGGC-3'. PCR was performed using Phanta Max Super-Fidelity DNA Polymerase (Vazyme) and run at 94 °C for 2 min; 45 cycles of 94 °C for 15 s, 55 °C for 30 s and 68 °C for 5 min; and 68 °C for 15 min. Amplicons were run on precast 1% agarose gels (BIOWESTE) and the PCR products were proceeded to deoxyadenosine (A)-tailing at the 3'-end of the PCR products utilizing Ex Taq DNA polymerase (Takara) without thermal cycling as follows: 95°C, 5 min; 72°C, 30 min. The A-tailed PCR products were TA-ligated into pMD-18 T vector (Takara).

### **6.10 Sequence and phylogenetic analysis**

Nucleotide alignments of intact *env* sequences were translation-aligned using MAGE 7. Sequences with premature stop codons and frameshift mutations that fell in the gp120 surface glycoprotein region were excluded from all analyses. The sequences from each group were aligned using MUSCLE. The average genetic distance between one give clone and the relevant entire population were calculated by MEGA 7 and represented as genetic diversity index. The phylogenetic bootstrap trees were generated for each sample using **maximum likelihood** method with 1000 bootstrap replications implemented in MEGA seven to depict the global landscape of HIV-1 diversity. Logograms were generated using the Weblogo 3.0 tool. To analyze changes between

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reservoir and rebound viruses, env sequences were aligned at the amino acid level to a HXB2 reference using BioEdit.

### **6.11 Cytotoxicity determination**

The specific killing activity of pre-stimulated CD8<sup>+</sup> T cells towards Jurkat or HEK293T cells expressing HIV-1 envelope glycoprotein at indicated ratios was measured after co-culture for 8 hours by lactate dehydrogenase assay using the CytoTox 96 nonradioactive cytotoxicity kit (G1781, Promega, as described previously.(26, 31) The manufacturer's instructions were followed. Absorbance values of wells containing effector cells alone and target cells alone were combined and subtracted as background from the values of the co-cultures. Wells containing target cells alone were mixed with a lysis reagent for 30 min at 37°C and the resulting luminescence was set as 100% lysis. Cytotoxicity was calculated by using the following formula: %Cytotoxicity = (Experimental – Effector spontaneous – Target spontaneous) / (Target maximum – Target spontaneous) × 100%.

### **6.12 Statistical considerations**

This is an open-label, monocenter, single arm clinical study. The investigators performed this study to evaluate the safety and feasibility of administration with autologous bNAb-derived CAR-T cells for chronic HIV-1-infected patients. Patients will be treated with cART to achieve undetectable HIV-1 in peripheral blood before adoptive transfer. CD8<sup>+</sup> T cells from donors will be infused into the autologous patients after transduction with bNAb-derived CAR.

The primary endpoint, the persistence of bNAb-derived CAR-T cells, will be evaluated by qPCR at various test points according to the data collection plan, as summarized by simple descriptive summary statistics. In addition, all laboratory correlative studies will also be reported descriptively. Laboratory studies will be performed to characterize the safety and feasibility of bNAb-derived CAR-T cells administration. The other endpoints will be analyzed according to the primary endpoint

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statistical methods.

### **6.13 IFN- $\gamma$ ELISpot**

IFN- $\gamma$ -secreting cells were detected by human IFN- $\gamma$  ELISpot assay kit (DKW22-1000-096s; Dakewe) according to the manufacture protocol. CD8<sup>+</sup> T cells (10<sup>5</sup> per well) from HIV-1-infected participants were incubated with Jurkat-gp160<sub>NL4-3</sub> (Jurkat-derived target cell line expressing HIV-1<sub>NL4-3</sub> Env) at 1: 1 ratio in antibody-precoated plates. Plates were incubated for 24 hours at 37°C and 5% CO<sub>2</sub>. The ELISpot assays were then performed according to the manufacturer's instructions. Spots were counted using an S6 ultra immunoscan reader (Cellular Technology Ltd.). The number of IFN- $\gamma$ -positive T cells was calculated by ImmunoSpot 5.1.34 software (Cellular Technology Ltd.) and converted into the number of spots per million CD8<sup>+</sup> T cells.

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## **7. Adverse event reporting**

### **7.1 Adverse event**

An adverse event (AE) is any undesirable sign, symptom or medical condition or experience that develops or worsens in severity after starting study treatment or any procedure specified in the protocol, even if the event is not considered to be related to the study. All observed or volunteered AE regardless of treatment group or suspected causal relationship to the investigational treatment will be reported as described in the following sections.

For all AEs, the investigator must pursue and obtain information adequate both to determine the outcome of the AE and to assess whether it meets the criteria for classification as a serious adverse event requiring immediate notification to its designated representative. For all AEs, sufficient information should be obtained by the investigator to determine the causality of the AE. The investigator is required to assess causality. Follow-up by the investigator may be required until the event or its sequelae resolve or stabilize at a level acceptable to the investigator.

As part of ongoing safety reviews conducted by the investigator, any non-serious adverse event that is determined by the ERG to be serious will be reported by the ERG as an SAE. To assist in the determination of case seriousness further information may be requested from the investigator to provide clarity and understanding of the event in the context of the clinical trial.

### **7.2 Serious adverse event**

A serious adverse event (SAE) is any untoward medical occurrence that:

- Results in death;
- Is life-threatening (immediate risk of death);
- Requires inpatient hospitalization or prolongation of existing hospitalization;
- Results in persistent or significant disability/incapacity (substantial

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disruption of the ability to conduct normal life functions);

- Results in congenital anomaly/birth defect;
- Progression of the malignancy under study (including signs and symptoms of progression) should not be reported as an SAE unless the outcome is fatal within the safety reporting period.

Hospitalization due to signs and symptoms of disease progression should not be reported as an SAE. If the treatment has a fatal outcome during the study or within the safety reporting period, then the event leading to death must be recorded as an AE and as an SAE with Common Terminology Criteria for Adverse Events (CTCAE, Version 5.0) Grade 5.

Medical and scientific judgment is exercised in determining whether an event is an important medical event. An important medical event may not be immediately life-threatening and/or result in death or hospitalization. However, if it is determined that the event may jeopardize the patient or may require intervention to prevent one of the other AE outcomes, the important medical event should be reported as serious.

Examples of such events are intensive treatment for or development of acute immune response after treated-cells infusion, and the gene therapy induced tumorigenesis.

All suspected serious adverse events related to the study and requiring additional medical intervention should be followed according to the institution's standard of care, preferably in consultation with appropriate specialists.

### **7.3 Expected/unexpected adverse events**

Expected adverse events are those that have been previously identified as resulting from administration of the autologous CAR-T cells, anti-retroviral therapy of HIV-1 infection.

For the purposes of this study, an adverse event is considered unexpected when it varies in nature, intensity or frequency from information provided by the ERG or when it is



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not included in the informed consent document as a potential risk.

#### **7.4 Causality assessment**

The investigator's assessment of causality must be provided for all AEs (serious and non-serious); the investigator must record the causal relationship in the CRF, as appropriate, and report such an assessment in accordance with the serious adverse reporting requirements if applicable. An investigator's causality assessment is the determination of whether there exists a reasonable possibility that the investigational treatment caused or contributed to an AE. Generally, the facts (evidence) or arguments to suggest a causal relationship should be provided. If the investigator does not know whether or not the investigational treatment caused purposes, then the event will be handled as “related to investigational treatment” for reporting investigator's causality assessment is “unknown but not related to investigational treatment”, this should be clearly documented on study records.

In addition, if the investigator determines an SAE is associated with study procedures, the investigator must record this causal relationship in the source documents and CRF, as appropriate, and report such an assessment in accordance with the SAE reporting requirements, if applicable.

Attribution is the relationship between an adverse event or SAE and the study treatment. Attribution will be assigned as follows:

- Definite – The AE is clearly related to the study treatment;
- Probable – The AE is likely related to the study treatment;
- Possible – The AE may be related to the study treatment;
- Unlikely - The AE is doubtfully related to the study treatment;
- Unrelated - The AE is clearly NOT related to the study treatment.

#### **7.5 Recording and reporting**

Participating investigators will assess the occurrence of AE and SAE at all participant evaluation time points during the study. All grade 3 or higher AE and SAE, whether

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reported by the participant, discovered during questioning, directly observed, or detected by physical examination, laboratory test or other means, will be recorded in the participant's medical record and on the appropriate study-specific case report forms. The descriptions and grading scales found in the revised Common Terminology Criteria for Adverse Events (CTCAE) version 5.0 will be utilized for AE reporting. All appropriate treatment areas should have access to a copy of the CTCAE version 5.0.

The study must be conducted in compliance with local safety reporting requirements, and reporting requirements. Investigators must report to the ERG any AE, whether or not they are considered related to the investigational interventions.

All adverse events, both serious and non-serious, and deaths that are encountered from initiation of study intervention, throughout the study, and within 30 days of the last study intervention should be followed to their resolution, or until the participating investigator assesses them as stable, or the participating investigator determines the event to be irreversible, or the participant is lost to follow-up. The presence and resolution of AE /SAE (with dates) should be documented on the appropriate case report form and recorded in the medical record to facilitate source data verification. For some SAE, the study sponsor or designee may follow-up by telephone and/or monitoring visit to obtain additional case details. Participants should be instructed to report any serious post-study event(s) that might reasonably be related to participation in this study.

## **7.6 General toxicity management considerations**

**Replication-competent lentivirus (RCL)** may be generated during the CAR-T manufacturing phase or subsequently after introduction of vector transduced cells into the patient. However, an RCL resulting from the production phase is highly unlikely since elements are incorporated in the design of the vector system that minimize vector recombination and generation of RCL. Furthermore, the vector used to transduce the product undergoes sensitive assays for detection of RCL before it can be released to a

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subject.

**Uncontrolled T cell proliferation or malignancy:** In pre-clinical studies, bNAb-derived CAR-T cells have only proliferated in response to HIV-1 gp120.(26) In the context of this protocol it is highly unlikely that the T cells will proliferate in response to signals from the normal tissues.

If any subject develops excessive CAR-T cell accumulation or malignancy, ganciclovir or tafasitamab (an anti-CD19 antibody) will be administered to eradicate the infused CAR-T cells because the vector contains a herpes simplex virus-1 thymidine kinase (TK) and a truncated CD19 gene as the suicide genes, furthermore, corticosteroids can also be utilize to eradicate the uncontrolled proliferating CAR-T cells.

**Infusion reaction:** Acetaminophen and diphenhydramine hydrochloride may be repeated every 6 hours as needed. A course of non-steroidal anti-inflammatory medication may be prescribed if the patient continues to have fever not relieved by acetaminophen. It is recommended that patients not receive corticosteroids at any time, except in the case of a life-threatening emergency, since this may have an adverse effect on infused CAR-T cells.

**Febrile reaction.** In the event of febrile reaction, an evaluation for infection should be initiated, and patients managed appropriately with antibiotics, fluids and other supportive care as medically indicated and determined by the treating physician. In the event that the patient develops sepsis or systemic bacteremia following CAR-T cell infusion, appropriate cultures and medical management should be initiated. If a contaminated CAR-T cell product is suspected, the product can be retested for sterility using archived samples. Consideration of a cytokine release syndrome (see below) should be given.

**Cytokine Release Syndrome (CRS):** CRS has been observed in lymphocytic

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leukemia/lymphoma patients after treatment with anti-CD19/20-CAR-T cells. Patients with clinical responses exhibited some level of CRS that ranged from mild to severe consisting of fevers, hypotension, capillary leak, hypoxia or other symptoms. Cytokine production is caused by the activation, expansion and cytolytic function of T cells. Subsequent to this experience, ganciclovir or tafasitamab (an anti-CD19 antibody) will be administered to eradicate the infused CAR-T cells because the vector contains a herpes simplex virus-1 thymidine kinase (TK) and a truncated CD19 gene as the suicide genes, selective tocilizumab (an anti-IL6-receptor antibody) therapy can also be utilized with effective toxicity management and successful ongoing bNAb-derived CAR-T cell expansion in patients.

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## **8. Data handling and record keeping**

### **8.1 Case report forms/electronic data record**

As used in this protocol the term CRF should be understood to refer to either a paper form or an electronic data record or both, depending on the data collection method used in this study. A CRF is required and should be completed for each included patient. The completed original CRFs are the sole property of investigation team and should not be made available in any form or third parties, except for authorized representatives of the team or appropriate regulatory authorities, without written permission from the team.

The PI has ultimate responsibility for the collection and reporting of all clinical, safety and laboratory data entered on the CRFs and any other data collection forms (source documents) and ensuring that they are accurate, authentic/original, attributable, complete, consistent, legible, timely (contemporaneous), enduring and available when required. The CRFs must be signed by the PI or by an authorized staff member to attest that the data contained on the CRFs is true. Any corrections to entries made in the CRFs, source documents must be dated, initialed and explained (if necessary) and should not obscure the original entry.

In most cases, the source documents are the hospital's or the physician's patient chart. In these cases, data collected on the CRFs must match the data in those charts.

In some cases, the CRF, or part of the CRF, may also serve as source documents. In these cases, a document should be available at the investigator's site as well as at the Emergency Response Group and clearly identify those data that will be recorded in the CRF, and for which the CRF will stand as the source document.

### **8.2 Record retention**

To enable evaluations and/or audits from regulatory authorities, the investigator agrees to keep records including the identity of all participating patients, all original signed informed consent documents, copies of all CRFs, safety reporting forms, source

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documents, and detailed records of treatment disposition, and adequate documentation of relevant correspondence. The records should be retained by the investigator according to International Conference on Harmonization (ICH) (<https://www.ich.org/page/search-index-ich-guidelines>) and local regulations.

If the investigator becomes unable for any reason to continue to retain study records for the required period (e.g., retirement, relocation), the ERG should be prospectively notified. The study records must be transferred to a designee acceptable to the ERG, such as another investigator, another institution, or to an independent third party arranged by the ERG. Investigator records must be kept for a minimum of 10 years after completion or discontinuation of the study or for longer if required by applicable local regulations. The investigator must obtain the written permission before disposing of any records, even if retention requirements have been met.

## **9. Ethics**

The study will be conducted in accordance with ethical principles founded in the Declaration of Helsinki. The Institutional Review Board (IRB) & Independent Ethics Committee (IEC) will review all appropriate study documentation in order to safeguard the rights, safety, and well-being of the subjects. The study will only be conducted at sites where IRB/IEC approval has been obtained. The Protocol, Investigator's Brochure, ICF, advertisements (if applicable), written information given to the subjects, safety updates, annual progress reports, and any revisions to these documents will be provided to the IRB/IEC.

### **9.1 IRB & IEC**

It is the responsibility of the investigators to have approval of the study protocol, protocol amendments, informed consent documents, and other relevant documents, eg, recruitment advertisements, if applicable, from the IRB/IEC. All correspondences with the IRB/IEC should be retained in the Investigator Files. Copies of IRB/IEC approvals should be forwarded to the Emergency Response Group. The only circumstance in

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which an amendment may be initiated prior to IRB/IEC approval is where the change is necessary to eliminate apparent immediate hazards to the patients. In that event, the investigator must notify the IRB/IEC and the Emergency Response Group in writing immediately after the implementation.

### **9.2 Ethical conduct of the study**

The study will be conducted in accordance with legal and regulatory requirements, as well as the general principles set forth in the International Ethical Guidelines for Biomedical Research Involving Human Patients (Council for International Organizations of Medical Sciences 2002), Guidelines for GCP (ICH 1996), and the Declaration of Helsinki (World Medical Association 1996 & 2008). In addition, the study will be conducted in accordance with the protocol, the ICH guideline on GCP, and applicable local regulatory requirements and laws.

### **9.3 Patient information and consent**

All parties will ensure protection of patient personal data and will not include patient names on any forms, reports, publications, or in any other disclosures, except where required by laws.

Patient names, address and other identifiable data will be replaced by a numerical code consisting of a numbering system. In case of data transfer, the ERG will maintain high standards of confidentiality and protection of patient personal data. The informed consent document must be in compliance with ICH GCP, local regulatory requirements, and legal requirements.

The investigator must ensure that each study patient, or his/her legal representative, is fully informed about the nature and objectives of the study and possible risks associated with participation. The investigator, or a person designated by the investigator, will obtain written informed consent from each patient or the patient's legal representative before any study-specific activity is performed. The investigator will retain the original of each patient's signed consent document.

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#### **9.4 Patient recruitment**

Advertisements approved by ethics committees and investigator databases may be used as recruitment procedures.



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## 10. References

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## Summary of Protocol Changes

Section of Current protocol	Rationale	Current Version
6.6	<p>Added the indexes to monitor the HIV-1 latent reservoir during and after the ATI through a newly developed intact proviral DNA assay (IPDA) based on droplet digital PCR</p>	<p><b>Intact proviral DNA assay (IPDA)</b></p> <p>The procedures for IPDA described previously were followed with minor modifications. In general, the IPDA is performed on DNA from <math>2 \times 10^6</math> CD4<sup>+</sup> T cells. Genomic DNA is extracted using the QIAamp DNA Mini Kit (Qiagen) with precautions to avoid excess DNA fragmentation. Quantification of intact, 5' deleted, and 3' deleted and/or hypermutated proviruses was carried out using primer/probe combinations optimized for subtype B HIV-1. The primer/ probe mix consists of oligonucleotides for two independent hydrolysis probe reactions that interrogate conserved regions of the HIV-1 genome to discriminate intact from defective proviruses (Supplementary Table 2). HIV-1 reaction A targets the packaging signal (<math>\psi</math>) that is a frequent site of small deletions and is included in many large deletions in the proviral genome. The <math>\psi</math> amplicon is positioned at HXB2 coordinates 692–797. This reaction uses forward and reverse primers, as well as a 5'-FAM-labeled hydrolysis probe. Successful amplification of HIV-1 reaction A produced FAM fluorescence in droplets containing <math>\psi</math>, detectable in channel 1 of the droplet reader. HIV-1 reaction B targets the RRE of the proviral genome, with the amplicon positioned at HXB2 coordinates 7736–7851. This reaction used forward and reverse primers, as well as two hydrolysis probes: a 5'-VIC labeled probe specific for wild-type proviral sequences, and a 5'-unlabelled probe specific for APOBEC3G/H hypermutated proviral sequences (Supplementary file 2). Successful amplification of HIV-1 reaction B produced a VIC fluorescence in droplets containing a wild-type form of RRE, detectable in channel 2 of the droplet reader, whereas droplets containing a hypermutated form of RRE were not fluorescent.</p> <p>Droplets containing HIV-1 proviruses were scored as follows. Droplets positive for FAM fluorescence only, which arises from amplification, was scored as containing 30 defective proviruses, with the defect attributable to either APOBEC3G mediated hypermutation or 3' deletion. Droplets positive for VIC fluorescence only, which arises from wild-type RRE amplification, was scored as containing 5' defective proviruses, with the defect attributable to 5' deletion. Droplets positive for both FAM and VIC fluorescence was scored as containing intact proviruses. Double-negative droplets contained no proviruses or rare proviruses with defects affecting both amplicons.</p>

7.6	Improved the safety of HIV-1-specific CAR-T cells which may persistently survive <i>in vivo</i> for a long time, the engineered CAR-T cells carry another suicide gene as a safety switch to eliminate potential adverse effects	<p><b>Uncontrolled T cell proliferation or malignancy:</b> In pre-clinical studies, bNAb-derived CAR-T cells have only proliferated in response to HIV-1 gp120. In the context of this protocol it is highly unlikely that the T cells will proliferate in response to signals from the normal tissues.</p> <p>If any subject develops excessive CAR-T cell accumulation or malignancy, ganciclovir or tafasitamab (an anti-CD19 antibody) will be administered to eradicate the infused CAR-T cells because the vector contains a herpes simplex virus-1 thymidine kinase (TK) and a truncated CD19 gene as the suicide genes, furthermore, corticosteroids can also be utilized to eradicate the uncontrolled proliferating CAR-T cells.</p>
7.6	Improved the safety of HIV-1-specific CAR-T cells which may persistently survive <i>in vivo</i> for a long time, the engineered CAR-T cells carry another suicide gene as a safety switch to eliminate potential adverse effects	<p><b>Cytokine Release Syndrome (CRS):</b> CRS has been observed in lymphocytic leukemia/lymphoma patients after treatment with anti-CD19/20-CAR-T cells. Patients with clinical responses exhibited some level of CRS that ranged from mild to severe consisting of fevers, hypotension, capillary leak, hypoxia or other symptoms. Cytokine production is caused by the activation, expansion and cytolytic function of T cells.</p> <p>Subsequent to this experience, ganciclovir or tafasitamab (an anti-CD19 antibody) will be administered to eradicate the infused CAR-T cells because the vector contains a herpes simplex virus-1 thymidine kinase (TK) and a truncated CD19 gene as the suicide genes, selective tocilizumab (an anti-IL6-receptor antibody) therapy can also be utilized with effective toxicity management and successful ongoing bNAb-derived CAR-T cell expansion in patients.</p>

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## Original and Current Statistical Analysis Plan

### Statistical analysis plan

This is an open-label trial of the safety, *in vivo* persistence of CAR-T cells, and antiviral activity of bNAb-derived CAR-T cell therapy in HIV-1–infected individuals some of whom are undergoing analytical interruption of cART. The investigators performed this study to evaluate the safety and feasibility of administration with bNAb-derived CAR-T cells for chronic HIV-1–infected patients. Patients will be infused with combined antiviral therapy (cART) to achieve undetectable HIV-1 virus in peripheral blood before CAR-T cell administrations. Autologous CD8<sup>+</sup> CAR-T cells from donors will be infused into the patients after modified with HIV-1 gp120-specific CAR moiety. The cART interruption was performed when the relative treatment criteria are achieved.

The primary endpoint, level change of HIV-1 RNA in plasma and persistence of bNAb-derived CAR-T cells *in vivo*, will be evaluated at various test points according to the data collection plan, as summarized by simple descriptive summary statistics. In addition, all laboratory and imaging correlative studies will also be reported descriptively and graphically. Laboratory studies will be performed to characterize the safety and feasibility of administration with bNAb-derived CAR-T cells. The other endpoints will be analyzed according to the primary endpoint statistical methods.