An oncolytic vaccinia virus expressing anti-CD47 nanobody exerts enhanced antitumor activity by mediating innate and adaptive immune cell infiltration and activation in the lymphoma tumor microenvironment

Mengyuan Li,[1*] Yinyin Zhang,[1*] Lihong Zong,[1*] Minghuan Zhang,[1] Shibing Wang,[2] Wen Lei[1,3] and Wenbin Qian[1,3,4]

[1]Department of Hematology, the Second Affiliated Hospital, College of Medicine, Zhejiang University; [2]Department of Clinical Laboratory, Affiliated Hangzhou First People's Hospital, School of Medicine, Westlake University; [3]Key Laboratory of Cancer Prevention and Intervention, China National Ministry of Education, Zhejiang Provincial Clinical Research Center for Cancer, Cancer Center of Zhejiang University, the Second Affiliated Hospital, College of Medicine, Zhejiang University and [4]Research Center for Life Science and Human Health, Binjiang Institute of Zhejiang University, Hangzhou, China

ML, YZ and LZ contributed equally as first authors.

Correspondence: W. Qian
qianwb@zju.edu.cn

W. Lei
leiwen2017@zju.edu.cn

Received: November 14, 2024.
Accepted: April 3, 2025.
Early view: April 10, 2025.

https://doi.org/10.3324/haematol.2024.286923

Supplementary

Materials and Methods

Cell lines

The cell lines used in our study were purchased from the American Type Culture Collection (ATCC; Manassas, USA), including A20 (Mouse B-cell lymphoma cell), Raji (Human Burkitt's lymphoma cell), Jeko1 (Human mantle lymphoma cell), Pffeifer (Human DLBCL cell), Su-dhl-4 (Human DLBCL cell) and HEK293 (Human embryonic kidney cell). The information on antibodies used in this study is listed in Table S2.

Crispr-cas9 gene knock out

1 μL of 150 pM gRNA was combined with 2 μL of 60 pM TrueCut Cas9 v2 protein (ThemoScientific, Cat#A36498, USA) and PBS was added to reach a total volume of 10 μL. This mixture was incubated at room temperature for 10 minutes to form the Cas9/RNP complex. The complex was then transfected into 10 million Raji cells using a 4D Nucleofector system (Lonza, Germany). The cells were resuspended in 90 μL of SF 4D-Nucleofector™ X Solution (Lonza, Germany) and mixed with the Cas9/RNP complex before being nucleofected with program DS-104.

Oncolysis assay

Cell lines including A20, Raji, Pfeiffer, Jeko1, and Su-DHL-4 cells were resuspended in a cell culture medium and added to a 96-well plate at 1.5×10^4 cells/well, 90 μl/well. Oncolytic vaccine virus (OVV) and OVV-hCD47nb-G1 (volume=10 μl) were added at concentrations of 1, 2, 4, 8, and 16 multiplicity of infection (MOI), and the same volume of PBS was added into the control group. After further cultivation for 72 h, 10 μl CCK8 working solution was added to each well, and the absorbance value was measured after 4 h at a wavelength of 490 nm. The survival percentage was determined using this equation: (experimental group absorbance−background absorbance) / (control group absorbance − background absorbance) × 100%.

Binding and competition assay

The A20, Raji, and Raji-CD47 knock out (KO) cells were maintained in RPMI-1640 (Gibco, Cat#C11875500BT, USA) supplemented with 2% fetal bovine serum (FBS, Gibco, Cat#A5669701, USA), and then co-cultured with the purified mCD47nb, hCD47nb-G1 protein or Hu5F9-G4 at room temperature for 30 minutes. To detect the ability of mCD47nb and hCD47nb-G1 to bind to CD47 antigen on the surface of tumor cells, APC anti-FLag tag antibody (for mCD47nb and hCD47nb-G1) and PE anti-IgG Fc recombinant antibody (for hCD47nb-G1 and Hu5F9-G4, respectively) were used as a secondary antibody. The decreased expression of CD47 because of competition binding was evaluated using an APC anti-human CD47 antibody. The signals were detected by the ACEA Novocyte flow cytometer (SCR_019522, ACEA Biosciences, Inc, San Diego, California, USA).

Enzyme-Linked Immunosorbent Assay (ELISA) assay

Raji cells (2×10^5) were treated with virus at the dose of 0.5, 1, 2, and 4 MOI, respectively. The supernatant was collected at 24, 48, and 72 h after infection. The secreted hCD47nb-G1 proteins in the supernatant were quantified by ELISA assay. The hCD47nb-G1 proteins were purified from the supernatant of virus-infected cells, and then its concentration was determined by NanoDrop (ThemoScientific, USA), which was used to construct a standard curve. In this assay, a recombinant human CD47 protein (ACRO Biosystems Group, Cat#CD7-H5227, China) was used as a coating reagent. HRP anti-Flag antibody (Sigma-Aldrich, Cat#A8592, USA) was the detection antibody.

RBCs aggregation assay and binding activity

The RBCs were separated through centrifugation from the whole blood of 3 healthy donors. The cell suspension was incubated in a U bottom 96-well plate with a series concentration of hCD47nb-G1 or Hu5F9-G4, with PBS as negative control. RBC aggregation was observed after incubation at room

temperature for 2 h. For RBCs binding activity assay, PE anti-human IgG Fc antibody were used as a secondary antibody and then the signals were detected by flow cytometry.

In vitro phagocytosis assay

Given the fact that SIRPα in the murine macrophages could bind to human CD47, we therefore investigated the ability of hCD47nb-G1 inducing phagocytosis using murine bone marrow-derived macrophages (BMDMs) obtained from BALB/c mouse. BMDMs were cultured in RPMI-1640 medium supplemented with 10 ng/ml murine M-CSF (Genescript, Cat# Z02930, China) and 15 % FBS, and then transferred to a 24-well plate at 1 × 10^5 per well and stained with CellTracker Red CMTPX (ThemoScientific, Cat#C34552, USA). Whereas, tumor cells were stained with CellTracker Green CMFDA (ThemoScientific, Cat#C2925, USA). The cells were incubated at 37 ℃ for 15-30 minutes and washed twice with PBS. Finally, tumor cells were co-cultured with BMDMs at 3:1, and then PBS, the supernatant of tumor cells infected with viruses, hCD47nb-G1, Hu5F9-G4, and mCD47nb were added respectively. After further cultivation for 4 hours, flow cytometry and confocal fluorescence microscopy (Carl Zeiss LSM900 with Airyscan, Germany) were used to examine phagocytosis.

Quantitative real-time polymerase chain reaction (qRT-PCR)

To evaluate the effect of mCD47nb or hCD47nb-G1 on transcription change of typical BMDMs cytokine genes, BMDMs and mitomycin treated-A20 or -Raji cells were cocultured at a ratio of 2:1 for 24 h in the presence of 5 ng/μl mCD47nb or hCD47nb-G1, respectively. The total RNA was extracted for measuring the relative transcription of mouse *Il1β*, *Il6*, *Il10*, *Nos2*, *Cxcl2,* and *Mrc1* genes with a couple of corresponding primers for each gene. Additionally, viral replication and spread and infiltration of CD19 CAR-T cells after intratumoral injection were also evaluated by real-time PCR. The Virus Genomic DNA/RNA Extraction Kit (Tiangen, DP315) and RNA-Quick Purification Kit (Yi shan, RN001) were used to isolate RNA and DNA

from approximately 25 mg of tumors and tissues from different murine organs. cDNA was prepared with PrimeScript RT Master Mix (Takara, RR036). Real-time PCR was performed with LightCycler 480 Instrument II (Roche, Germany). As a means of quantifying the viral genomes and hCD47nb-G1 transcripts, we used 40 ng sample cDNA and 100 ng positive control DNA in the presence of SYBR Green I Master (Takara, RR0820). The primers used for qRT-PCR are listed in Table S3.

Western blot

The protein samples were collected from supernatants of 1×10^6 tumor cells infected with OVV-hCD47nb-G1 at a dose of 1 MOI for 24 h. Then, it was combined with a 1 × loading buffer (Beyotime, Cat# P0285, Shanghai, China) and heated for 10 minutes at 100℃, the protein samples were resolved by SDS-PAGE and transferred onto PVDF membranes (Merck Millipore, Cat# K5MA6539B, Germany). After blocking the PVDF membrane in a blocking solution for 1 h, anti-Flag antibody, and secondary antibody were incubated on the membrane. Blots were developed with a super ECL detection reagent (Yeasen, Cat#36208ES60, China). For the CD47/SIRPa interaction blocking assay, BMDMs co-cultured with Raji, Raji-CD47KO, or Raji treated with hCD47nb-G1 (5 ng/μl) at a ratio of 2:1 for 24 h, respectively. The total protein was extracted for Western blot analysis

Evaluation of cytotoxicity and activation of NK cells

Mononuclear cells (MNCs) were isolated from human umbilical cord blood provided by Zhejiang Blood Center Cord Blood Bank (China, Hangzhou), and T-cells were depleted using CD3 microbeads (Miltenyi Biotec，#130-050-101, Germany) according to the manufacturer's instructions. Then $CD3^-$MNCs were stimulated with irradiated feeder cells at a ratio of 1:2 and cultured in AIM-V media (Gibco) supplemented with 2mM L-glutamine (Gibco), 1mM Sodium pyruvate (Gibco), 1mM HEPES (Gibco), 1mM Non-Essential Amino Acids (Gibco), 100 IU/ml penicillin-streptomycin, and 100 IU/ml hIL-2 (Quangang pharmaceutical,

Shandong, China). for 15 days. The cytotoxicity of NK cells was investigated using a Luciferase-based cytotoxicity assay. Brifly, NK cells were cocultured with Raji-luc cells at a ratio of 3:1 in a U bottom plate in the presence of increasing concentrations of purified hCD47nb-G1. Four hours later, 1.5mg/ml of the fluorescent substrate (100 μl) was added after removing the supernatant, and then luciferase activity was detected under Progema microplate reader. The cytotoxicity was calculated using the following formula: (maximum killing group – sample group) / (maximum killing group - minimum killing group) × 100 %.

NK cell degranulation was evaluated by flow cytometry detecting cell surface CD107a after staining with PE anti-human CD107a (LAMP-1) antibody and FITC anti-human CD56 (NCAM) Antibody. Cytokines in the culture consisting of Raji/NK cells were determined using the Human Th1/Th2 cytokine Kit II (BD Biosciences, Cat#551809).

Immunohistochemistry (IHC) analysis

Formalin-fixed paraffin-embedded (FFPE) tissues from 67 DLBCL patients were cut and stained by standard H&E and by IHC with antibodies to CD20, CD3, and CD47. All these FFPE tissues were provided from the Department of Pathology, the Second Affiliated Hospital of Zhejiang University School of Medicine and approved by the Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine in accordance with the Declaration of Helsinki. The sliced images were captured by an optical microscope (Olympus CKX53, Olympus Corp., Japan). CD47 immunostaining was scored by 2 hematopathologists. The antibodies used for animal samples included anti-mouse NKp46 antibody, anti-A27L antibody, anti-Flag tag antibody, and anti-mouse F4/80 antibody. The percentage of positive staining within the whole field of view was quantified using the software ImageJ 1.54g (Wayne Rasband, National Institutes of Health, USA) at 40 × magnification.

SUPPLEMENTAL FIGURE LEGENDS

Supplementary Figure 1 Expression of CD47 on lymphoma cell lines. (A) Flow cytometric assay of CD47 expression on A20 cell. (B) The CD47 expression of Raji, Pffeifer, Su-dhl-4, and Jeko1 cells were examined by flow cytometry.

Supplementary Figure 2 Secreted hCD47nb-G1 by tumor cells infected with oncolytic vaccine virus (OVV)-hCD47nb-G1. (A) The secretion of hCD47nb-G1 was detected by western blot assay. Cells were cultured with OVV or OVV-hCD47nb-G1 at an multiplicity of infection (MOI) of 1 for 24 hours, supernatants were collected and hCD47nb-G1 was detected using an anti-Flag antibody. (B) The amount of hCD47nb-G1 in the supernatants of OVV- hCD47nb-G1 infected Raji cells was determined by Enzyme-Linked Immunosorbent Assay (ELISA) assay.

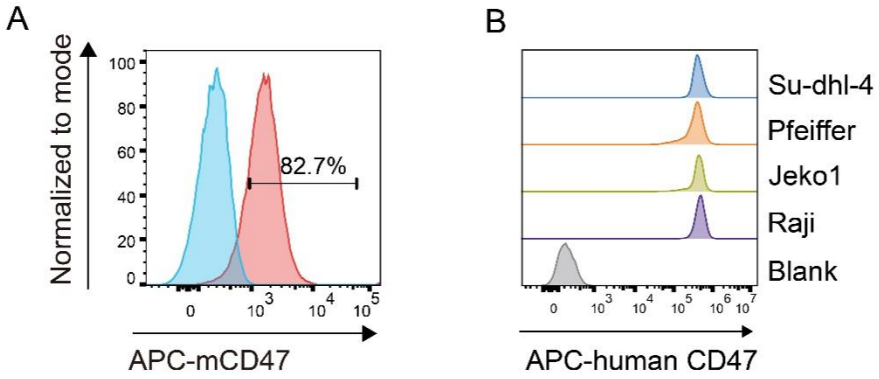
Supplementary Figure 3 The hCD47nb-G1 induced phagocytosis by blocking CD47. (A) Raji-CD47 knock-out (KO) cells were generated using the CRISPR-Cas9 technique. (B) The binding of hCD47nb-G1 (5 ng/μl) to CD47 on Raji wild-type or CD47KO cells was detected by flow cytometry. (C) The competitive binding of hCD47nb-G1 (5 ng/μl) and Hu5F9-G4 (5 ng/μl) to human CD47 antigen on tumor cells. Raji cells were incubated with 5 ng/μl Hu5F9-G4 at room temperature for 30 minutes, washed with phosphate-buffered solution (PBS), then incubated with 5 ng/μl hCD47nb-G1 for another 30 minutes. After a PBS wash, flow cytometry was used to detect the expression of CD47 and binding hCD47nb-G1. (D) The effects of purified hCD47nb-G1 (5 ng/μl), and Hu5F9-G4 (5 ng/μl) on phagocytosis of Raji wild type or Raji-CD47KO cells by BMDMs using flow cytometry. (E) Immunoblots of SIRPα, SHP-1, p-SHP1, STAT3, and p-STAT3 in BMDMs co-cultured with Raji, Raji-CD47KO, or treated with hCD47nb-G1 (5 ng/μl). GAPDH is shown as a loading control. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Comparisons were made using one-way ANOVA with Tukey's multiple comparison tests. All data are shown as mean ± standard deviation (SD).

Supplementary Figure 4 Safety of intratumoral injection therapy with oncolytic vaccine virus (OVV)-hCD47nb-G1. (A) The peripheral blood of mice was collected on day 29, and the levels of white blood cell (WBC), hemoglobin (Hb), and platelet (PLT) levels were checked. Normal reference ranges of mouse WBC, PLT, and Hb were 0.8~6.8 × 10^9/L, 450~1590 × 10^9/L, and 110-143 g/L. (B) At day 14, mice receiving two treatments were euthanized. Bilateral tumors and organs were harvested and viral gene A56R and hCD47nb in tissues were quantified by quantitative reverse transcription polymerase chain reaction (qRT-PCR). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Comparisons were made using one-way ANOVA with Tukey's multiple comparison tests. All data are shown as mean ± standard deviation (SD).

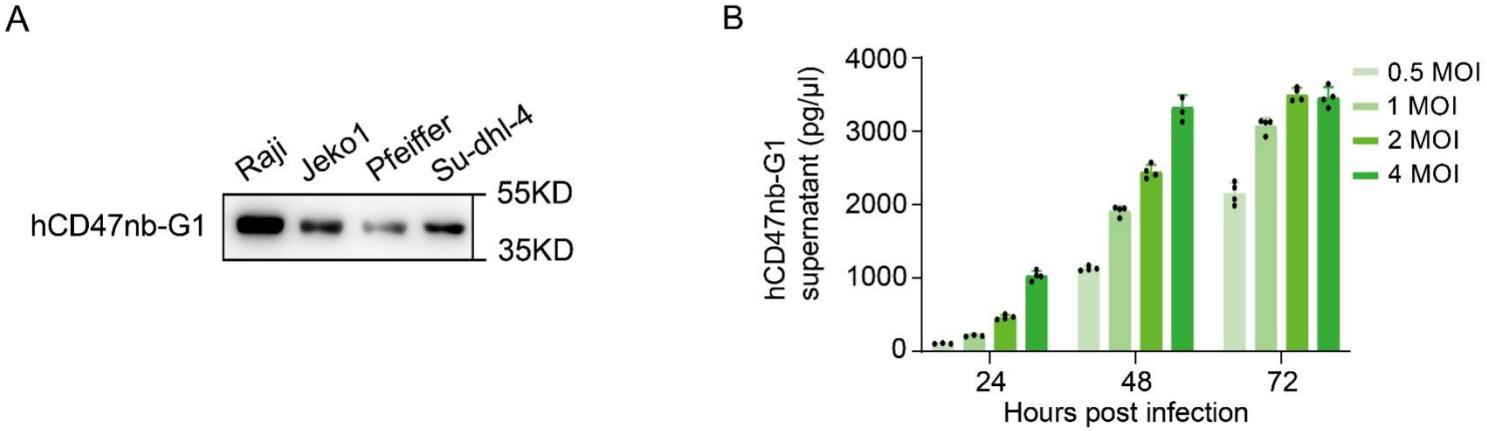
Supplementary Figure 5 Oncolytic vaccine virus (OVV)-hCD47nb-G1 has superior anti-tumor effect compared to Hu5F9-G4 in vivo. (A) Experimental design: NSG mice were injected intravenously (i.v.) with Raji-luc cells. At day 4, mice were randomly assigned to receive phosphate-buffered solution (PBS), Hu5F9-G4 (200 ug/mice, i.p., once a day for one week of treatment), OVV, or OVV-hCD47nb-G1 (1 × 10^7 plaque-forming unit (PFU)/mice, i.p., once) (n = 5). (B) Relative luciferase activity in Raji-luc cells was measured by bioluminescence imaging (BLI). (n = 5). (C) Kaplan-Meier survival curves of mice bearing Raji-luc induced tumor. (D) The peripheral blood of mice was collected on day 14, and the levels of white blood cell (WBC), hemoglobin (Hb), and platelet (PLT) were checked. (E) At day 14, mice were euthanized, and then the organs were harvested and quantitative reverse transcription polymerase chain reaction (qRT-PCR) quantified viral gene A56R in tissues. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Comparisons were made using one-way ANOVA with Tukey's multiple comparisons and Log-rank

(Mantel-Cox) tests. All data are shown as mean ± standard deviation (SD).

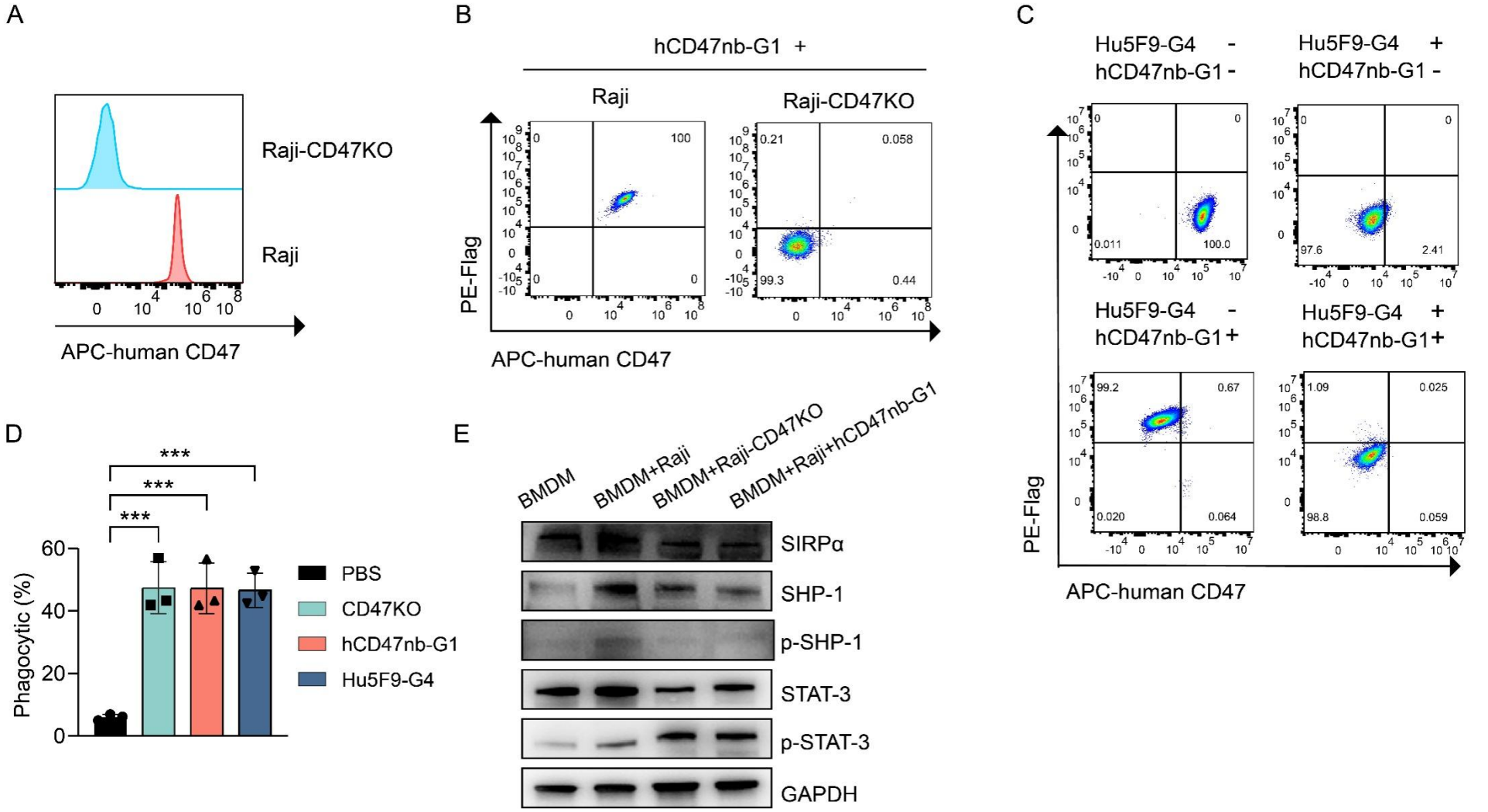
Supplementary figure 1



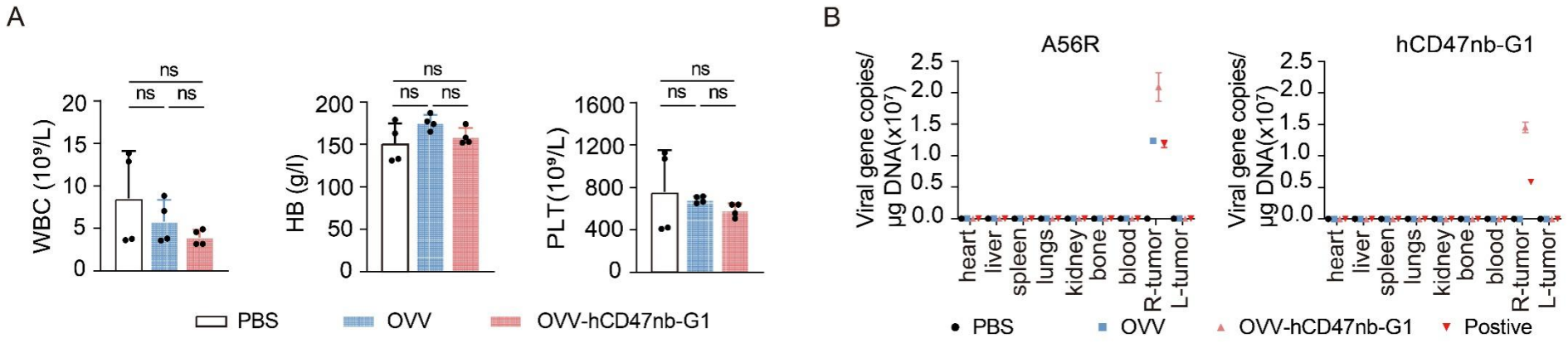
Supplementary figure 2



Supplementary figure 3



Supplementary figure 4



Supplementary figure 5

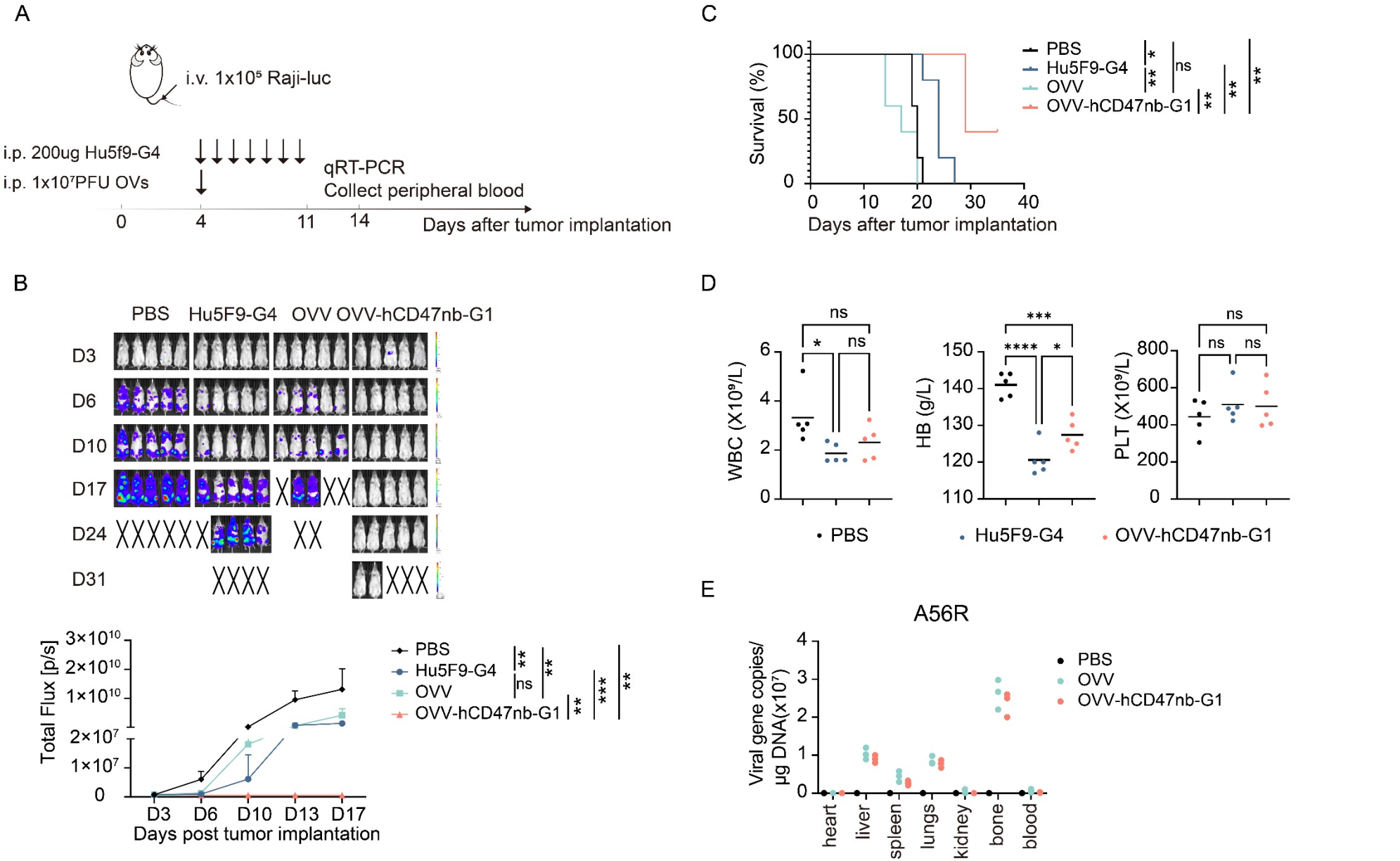


Table S1 Patient characteristics

Characteristic	Primary patients (N=31)	R/R patients (N=36)	*P* value
Median age, y (range)	63 (29~91)	56.5 (24~72)	
Sex, n (%)			0.534
Male	14 (45.2)	19 (52.8)	
Female	17 (54.8)	17 (47.2)	
Origin, n (%)			0.468
GCB	13 (41.9)	12 (33.3)	
non-GCB	18 (58.1)	24 (66.7)	
Extranodal sites, n (%)			0.856
＜1	8 (25.8)	10 (27.8)	
≥1	23 (74.2)	26 (72.2)	
B symptom, n (%)			0.373
A	26 (83.9)	27 (75.0)	
B	5 (16.1)	9 (25.0)	
ECOG, n (%)			0.256
0~1	23 (74.2)	22 (61.1)	
2~4	8 (25.8)	14 (38.9)	
Ann Arbor, n (%)			0.103
I~II	3 (9.7)	9 (25.0)	
III~IV	28 (90.3)	27 (75.0)	
LDH, n (%)			**0.000**
≤248	18 (58.1)	4 (11.1)	
＞248	13 (41.9)	32 (88.9)	
IPI, n (%)			0.953
0~1	5 (16.1)	6 (16.7)	
2~5	26 (83.9)	30 (83.3)	
CD47, n (%)			
0~2	19 (61.3)	22(61.1)	0.128
3~4	12 (38.7)	14(38.9)	

Abbreviation: R/R, relapsed/refractory patients. GCB, germinal center B-cell-like lymphoma. Categorical variables were analyzed using the Pearson Chi-square test.

Table S2. Antibody information

Name	Manufacturer	Cata.number
IHC		
Anti-human CD47	Abcam	Ab218810
Anti-human CD3	ZSGB-BIO	ZA-0503
Anti-human CD20	Abcam	Ab64088
Anti-A27L	Abcam	Ab117453
Anti-Flag tag	Abcam	Ab205606
Anti-mouse F4/80	Abcam	Ab111101
Anti-mouse NKp46	R&D Systems	AF2225-SP

Name	Manufacturer	Cata.number
Western		
Anti-Flag tag	Abcam	Ab205606
GAPDH	Proteintech	60004
SIRP α	Proteintech	14482
SHP-1	Abcam	Ab32559
P-SHP-1	Abcam	Ab51171
STAT3	CST	12640
P-STAT3	CST	9145
HRP Conjugated Goat anti-Rabbit IgG	HuaAn Biotechnology	HA1001
HRP Conjugated Goat anti-mouse IgG	HuaAn Biotechnology	HA1001
FCS		
APC anti-Flag Tag	Biolegend	637308
APC anti-mouse CD3	Biolegend	100236
APC anti-mouse F4/80	Biolegend	123115
APC anti-human CD47	Biolegend	323123
FITC anti-mouse CD3	Biolegend	100203
FITC anti-mouse CD8a	Biolegend	100705
FITC anti-mouse MHCII	Biolegend	107606
FITC anti-human CD56	Biolegend	362545
FITC anti-human CD20	Biolegend	980202
PE anti-mouse CD4	Biolegend	116005
PE anti-mouse CD86	Biolegend	159203
PE anti-human CD107a	Biolegend	301107
PE anti-human IgG Fc	Biolegend	366903
PE/Cyanine7 anti-mouse NKp46	Biolegend	137617
PE/Cyanine7 anti-mouse CD206	Biolegend	141719
PE/Cyanine7 anti-mouse CD62L	Biolegend	104417
Zombie Violet™ Fixable Viability Kit	Biolegend	423113
PerCP anti-mouse CD45	Biolegend	103129
PerCP/Cyanine5.5 anti-mouse CD19	Biolegend	152405
PerCP anti-human CD45	Biolegend	304027
APC/Cyanine7 anti-mouse IFN-γ	Biolegend	505849
APC/Cyanine7 anti-mouse/human CD44	Biolegend	103027
APC/Cyanine7 anti-mouse/human CD11b	Biolegend	101225
APC/Cyanine7 anti- human CD3	Biolegend	300426

Table S3 Primer sequences

GENE	Primer
Il1β-F	GAAATGCCACCTTTTGACAGTG
Il1β-R	TGGATGCTCTCATCAGGACAG
Il6-F	CTGCAAGAGACTTCCATCCAG
Il6-R	AGTGGTATAGACAGGTCTGTTGG

GENE	Primer
Il10-F	CAGTACAGCCGGGAAGACAATAA
Il10-R	CCGCAGCTCTAGGAGCATGT
Nos2-F	TGACGGCAAACATGACTTCAG
Nos2-R	GGTGCCATCGGGCATCT
Ccl2-F	CAGATGCAGTTAACGCCCCA
Ccl2-R	TGAGCTTGGTGACAAAAACTACAG
Mrc1-F	TTCAGCTATTGGACGCGAGG
Mrc1-R	GAATCTGACACCCAGCGGAA
A56-F	TGCTTGGTATAAGGAGCCCAA
A56-R	GTACCGGCATCTCTAGCAGTC
hCD47nb-G1-F	TTCAACTGGTACGTGGACGG
hCD47nb-G1-R	CAGAGCCTTGTTGGACACC
Gapdh-F	TGTGTCCGTCGTGGATCTGA
Gapdh-R	TTGCTGTTGAAGTCGCAGGAG
CD3-F	GGAATCTGGGAAGTAATGCCAA
CD3-R	TCAATGCAGTTCTGACACATTCT
GAPDH-F	CAAGGTCATCCATGACAACTTTG
GAPDH-R	CAAGGTCATCCATGACAACTTTG
CD47-gRNA	TAAGCACTTAAATATAGATC