GPIbα CAAR T cells function like a Trojan horse to eliminate autoreactive B cells to treat immune thrombocytopenia

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Abstract

Breakthrough treatment for refractory and relapsed immune thrombocytopenia (ITP) patients is urgently needed. Autoantibody-mediated platelet clearance and megakaryocyte dysfunction are important pathogenic mediators of ITP. Glycoprotein (GP) Ibα is a significant autoantigen found in ITP patients and is associated with poor response to standard immunosuppressive treatments. Here, we engineered human T cells to express a chimeric autoantibody receptor (CAAR) with GPIbα constructed into the ligand-binding domain fused to the CD8 transmembrane domain and CD3ζ-4-1BB signaling domains. We performed cytotoxicity assays to assess GPIbα CAAR T-cell selective cytolysis of cells expressing anti-GPIbα B-cell receptors *in vitro*. Furthermore, we demonstrated the potential of GPIbα CAAR T cells to persist and precisely eliminate GPIbα-specific B cells *in vivo*. In summary, we present a proof of concept for CAAR T-cell therapy to eradicate autoimmune B cells while sparing healthy B cells with GPIbα CAAR T cells that function like a Trojan horse. GPIbα CAAR T-cell therapy is a promising treatment for refractory and relapsed ITP patients.

Supplemental Materials

Supplemental Methods

Generation of anti-GPIbα B cells and monoclonal antibodies

The in vivo experiments were approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology (IACUC NO.3284), Wuhan, China. Mouse anti-human GPIbα hybridomas were produced according to the methods created by Koehler and Milstein in 1975.[1] Briefly, BALB/C female mice (6 to 8 weeks) were immunized with four injections of human platelet lysate at a 2 to 3-week interval. Three days after the fourth immunization, mouse splenocytes were fused with Sp2/0 myeloma cells and cultured in a HAT selection medium. Ninety-six well microtiter plates were coated overnight at 4 °C with 100 μl of 1 μg/ml human GPIbα protein(R＆D). Hybridoma supernatants were added and then detected by HRP-conjugated goat anti-mouse IgG. Specific anti-human GPIbα hybridomas were screened by repeated ELISA assay. The filtered hybridoma cells were expanded and cultured, inoculated into the abdominal cavity of mice, and high-efficiency anti-GPIbα monoclonal antibodies were obtained from the ascites and then purified using a protein A/G-Sepharose column.

In vitro cytotoxicity assays and cytokine release determination

Anti-GPIbα hybridomas were labeled with Cell Tracker™ Deep Red dyes (Invitrogen), and control hybridomas were stained with Cell Tracker™ Orange CMRA dyes (Invitrogen) according to the manufacturer's protocols. Donor-matched nontransduced T (NTD-T) cells or GPIbα CAAR-T cells were coincubated with control hybridoma (PE) and the target anti-GPIbα hybridoma cells (APC) at an effector to target (E: T) ratio of 5:1, while the ratio of target hybridoma cells to control hybridoma cells was 3:1. Cytotoxicity was evaluated at 24 hours using flow cytometry based on the changes in the ratio of target hybridoma cells to control hybridoma cells. NTD-T cells or GPIbα CAAR-T cells were incubated with anti-GPIbα hybridomas at varied ratios from 1:1 to10:1. After 16 h post-incubation, the medium supernatants were collected and measured immediately for lactate dehydrogenase (LDH) activities following the manufacturer's instructions. A microplate reader detected the absorbance of each well at OD450 wavelength to determine the LDH content. The killing efficiency was calculated by the following formula: Cytotoxicity = (absorbance of the experimental group - absorbance of target cell natural release group) / (absorbance of target cell highest release group - absorbance of target cell natural release group)× 100%. Proinflammatory cytokines IL-2 and IFN-γ secreted into the cell culture supernatants by the T cells were analyzed using ELISA assay.

Production of GPIbα CAAR-T cells

PsPAX2 packaging plasmids and pMD2.G envelope plasmid were used with polyethyleneimine (PEI) for lentiviral preparation in human embryonic kidney 293 (HEK293T) cells. Primary human T cells isolated from peripheral blood mononuclear cells (PBMCs) were cultured in X-VIVO 15(Lonza) medium supplemented with 100 U/mL IL-

2. T cells were activated by plate-bound anti-CD3(5μg/ml) and anti-CD28 antibodies(5μg/ml) for 2-3 days and were then transduced with GPIbα CAAR lentivirus (multiplicity of infection 25 to 100). GPIbα CAAR expression was detected by flow cytometry with FITC-anti-human CD42b antibody (clone: HIP1).

Anti-GPIbα hybridoma xenograft models

The in vivo experiments were approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology (IACUC NO.3284), Wuhan, China. Luciferase (Luc)/GFP-expressing anti-GPIbα hybridomas were stably generated by retroviral transduction and FACS sorting of GFP-positive cells. 6-8week-old NSG mice (NOD-scid IL2Rγnull; Jackson Laboratories) were intravenously inoculated with anti-GPIbα B cells (10^5 cells per mouse and followed 2 days later by injection with LBD-mutg233k-CAAR-T cells or NTD-T cells (10^7 cells per mouse), monitored for engraftment and therapeutic response by the IVIS® system every few days. After the intraperitoneal injection of D-Luciferin (150 mg/kg), the hybridoma burden of the mouse was measured and quantified by the radiance detected in the region of interest (ROI). Serum samples for antibody quantification were obtained by retro-orbital bleeding, and antibody titers were determined by ELISA against human GPIbα at a dilution ratio of 1:20. Serum anti-GPIbα antibodies were detected with donkey anti-mouse IgG (H+L)-HRP. Control and LBD-mutg233k-CAAR-T cells treated mice were euthanized 21-28 days after hybridoma/T cell injection. T cell persistence and infusion in bone marrow, spleen, and blood samples were assessed by flow cytometry with anti-human CD45(APC)/CD3(BV421) antibodies.

Immunofluorescence imaging analysis

Immunofluorescence imaging analysis was used to evaluate lymphocyte infiltration and persistence in the liver and spleen. Antigen was retrieved from deparaffinized 10 mm sections through microwave treatment, and sections were blocked via incubation with blocking buffer for 30 minutes at room temperature, followed by primary/secondary antibody and DAPI staining. The fluorescent images were recorded by confocal microscopy followed by semiquantitation using Image-Pro Plus software.

Cytometric bead array

The cytometric bead array was used to determine specific platelet autoantibodies of ITP patients. Polystyrene microbeads (PK1-PK5) can excite five different fluorescence intensities (APC) under flow cytometer laser irradiation. The five microbeads (PK1-PK5) were separately coated with five murine anti-human platelet monoclonal antibodies, anti-GPIX (clone: sz-1), anti-granule membrane protein (GMP)140 (clone: sz-51), anti-GPIb (clone: sz-2), anti-GPIIb (clone: sz-22), and anti-GPIIIa (clone: sz-21). Platelets isolated from ITP patients or healthy controls were lysed. Then, platelet lysate was incubated with the coated microbeads and followed by staining with FITC-conjugated goat-anti-human IgG polyclonal antibodies. The five microbeads (PK1-PK5) carrying different fluorescence (APC) intensities represent the five specific platelet autoantibodies. Meanwhile, the fluorescence intensity of FITC is proportional to patients' corresponding platelet autoantibody content. Cutoff values were determined with reference to the FITC

fluorescence intensity of negative controls (mean+3SD).

GPIbα ELISpot assay

This study involving humans was conducted per the Declaration of Helsinki and was approved by the Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (IEC-J (487)). GPIbα CAAR-T-cell-specific cytolysis of anti-GPIbα antibody-producing human B cells was measured using an enzyme-linked immunospot (ELISpot) assay according to the literature [2,3] and the manufacturer's protocol (Mabtech). Briefly, PBMCs isolated from patients and healthy control were stimulated with R828(1μg/ml) and IL-2(10ng/ml) for 2 days and then incubated with GPIbα CAAR-T cells, anti-CD19 CAR-T cells, or NTD-T cells for one day. At the same time, a polyvinylidene difluoride-bottomed 96-well microplate was activated and coated with Recombinant Human CD42b/GPIb alpha Protein (R&D) at a concentration of 10μg/ml or capture mAB MT145(15μg/ml) or BSA(5μg/ml) overnight at 4°C. The plates were washed and blocked with the cell culture medium at room temperature for one hour. Co-incubated cells were resuspended, pipetted into the wells, and cultured overnights. After washing away the cells with PBS, the membranes were incubated with detection mAb (MTG1218-biotin,0.25μg/ml) for 2 hours, followed by washing five times with PBS and incubated with streptavidin HRP diluted at 1:1,000 at room temperature for one hour. Finally, substrate TMB was added, and color development was stopped with deionized water until distinct spots emerged.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 7. Data from at least three individual donors are shown in all figures, and experimental duplicates were always performed. When more than two groups were compared with each other, statistical significance was tested with one-way ANOVA. P values were calculated using the unpaired 2-tailed Student's t test and Mann–Whitney U test. Differences were considered significant at $P<0.05$: NS, P>0.05; *$P < 0.05$; ** $P < 0.01$; ***$P < 0.005$; ****$P < 0.0001$.

Supplementary Table 1. Antibodies used in this study

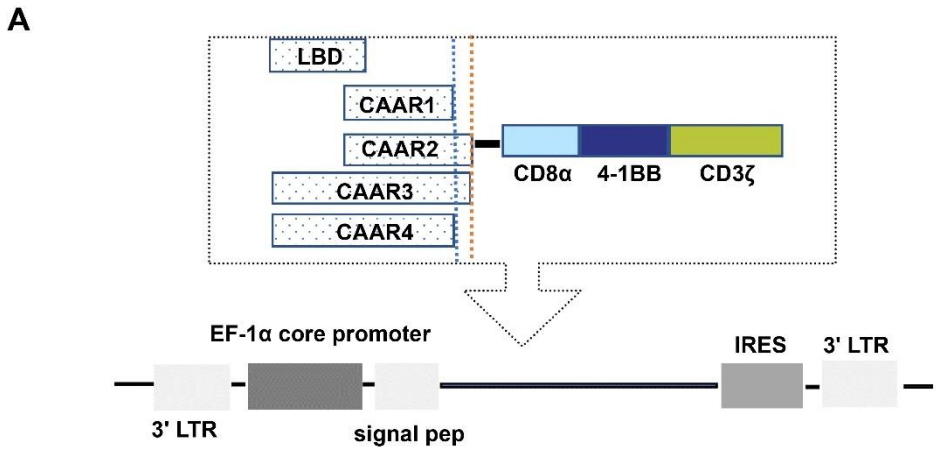
Antibody	Source	Catalogue	Clone	Application
APC Goat anti-mouse IgG (minimal x-reactivity) Antibody	Biolegend	405308	Poly4053	Flow cytometry
APC anti-human IgG Fc Antibody	Biolegend	410712	M1310G05	Flow cytometry
PE anti-human CD4 Antibody	Biolegend	317409	OKT4	Flow cytometry
FITC anti-human CD42b Antibody	Biolegend	303903	HIP1	Flow cytometry
Brilliant Violet 421™ anti-human CD3 Antibody	Biolegend	317343	OKT3	Flow cytometry
APC anti-human CD45 Antibody	Biolegend	304011	HI30	Flow cytometry
Pacific Blue™ anti-human CD8	Biolegend	344717	SK1	Flow

Antibody				cytometry
HRP Goat anti-mouse IgG (minimal x-reactivity) Antibody	Biolegend	405306	Poly4053	ELISA
Anti-CD3 antibody	abcam	ab16669	SP7	mIHC
Recombinant Anti-CD8 alpha antibody	abcam	ab237710	CAL67	ICC/IF
Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488)	abcam	ab150077	Polyclonal	ICC/IF
Goat Anti-Rabbit IgG H&L (Cy3 ®)	abcam	ab6939	Polyclonal	ICC/IF
Goat Anti-Rabbit IgG H&L (Cy5 ®)	abcam	ab6564	Polyclonal	ICC/IF

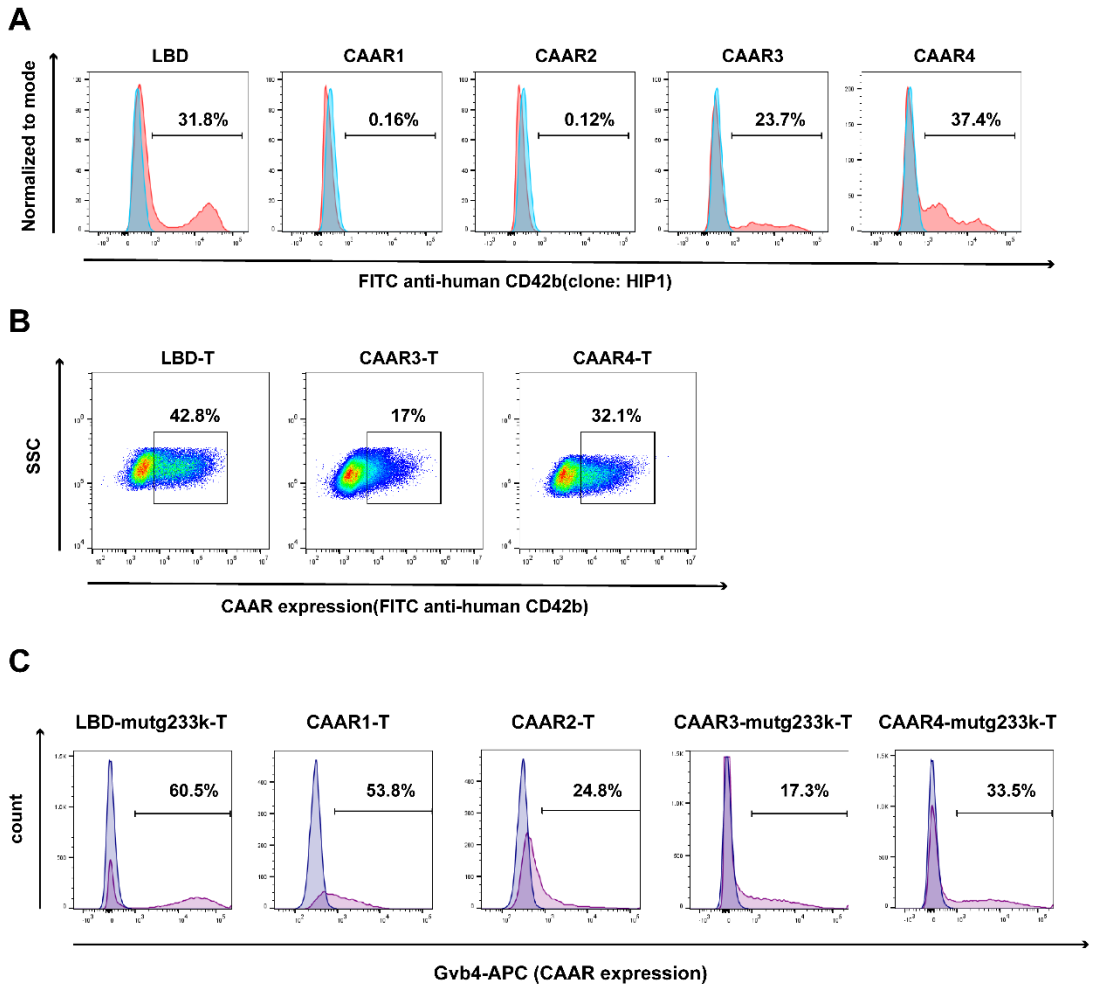
Supplementary Table 2. Kits used in this study

Kit	**Catalogue**	**Source**
Human IgG1 ELISpot BASIC (HRP)	3851-2H	Mabtech
Pan T Cell Isolation Kit, human	130-096-535	Miltenyi Biotec
Zenon Alexa Fluor 647 Mouse IgG1 Labeling Kit	Z25008	Invitrogen™
QuikChange Site-Directed Mutagenesis Kit	200518	Agilent
qPCR Lentivirus Titration Kit	LV900	abm
CellTracker™ Deep Red Dye	C34565	Invitrogen™
Cell Tracker™ Orange CMRA dyes	C34551	Invitrogen™
LDH Cytotoxicity Assay Kit	C0017	Beyotime
IFN gamma Human ELISA Kit	EHIFNG	Invitrogen™
IL-2 Human ELISA Kit	BMS221-2	Invitrogen™
Autoantibodies against Platelet-specific Receptors Detecting Kit（Flow cytometry - fluorescein）	20192400533	Suzhou YuandeWeikang Biological Medicine Co., LTD

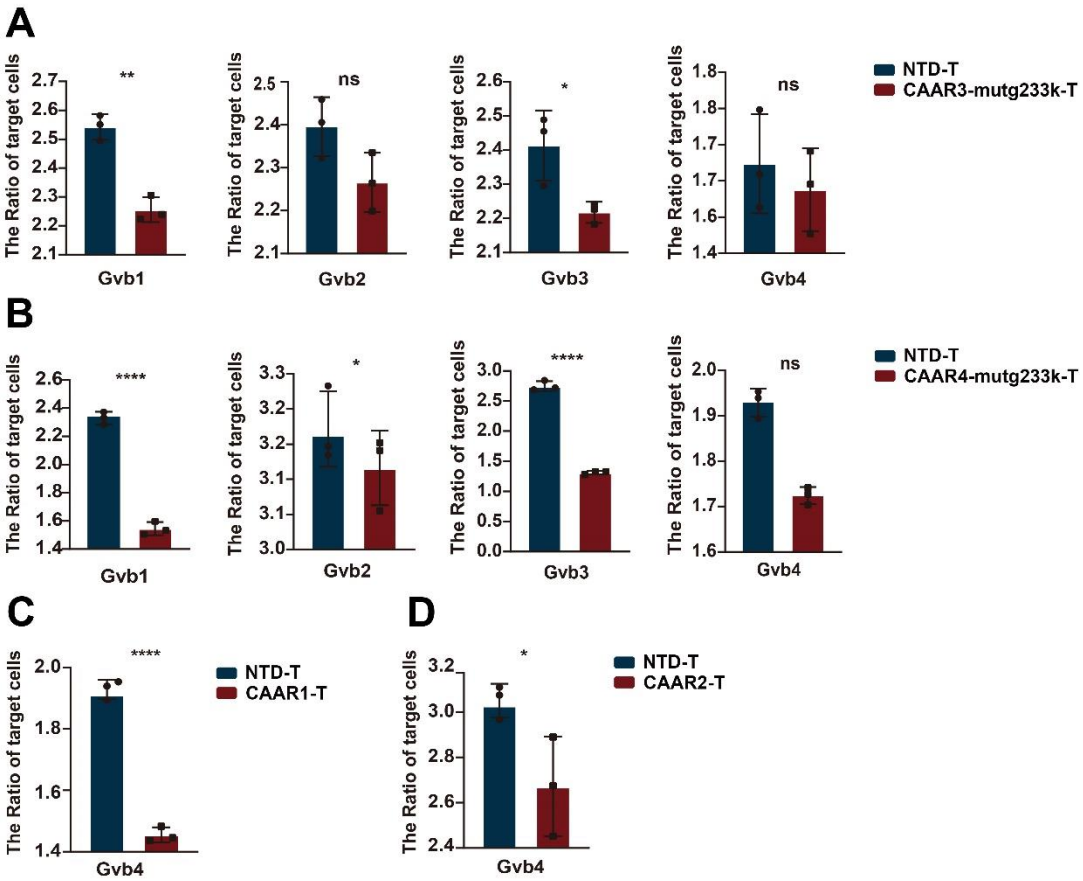
Supplemental Figures and Legends



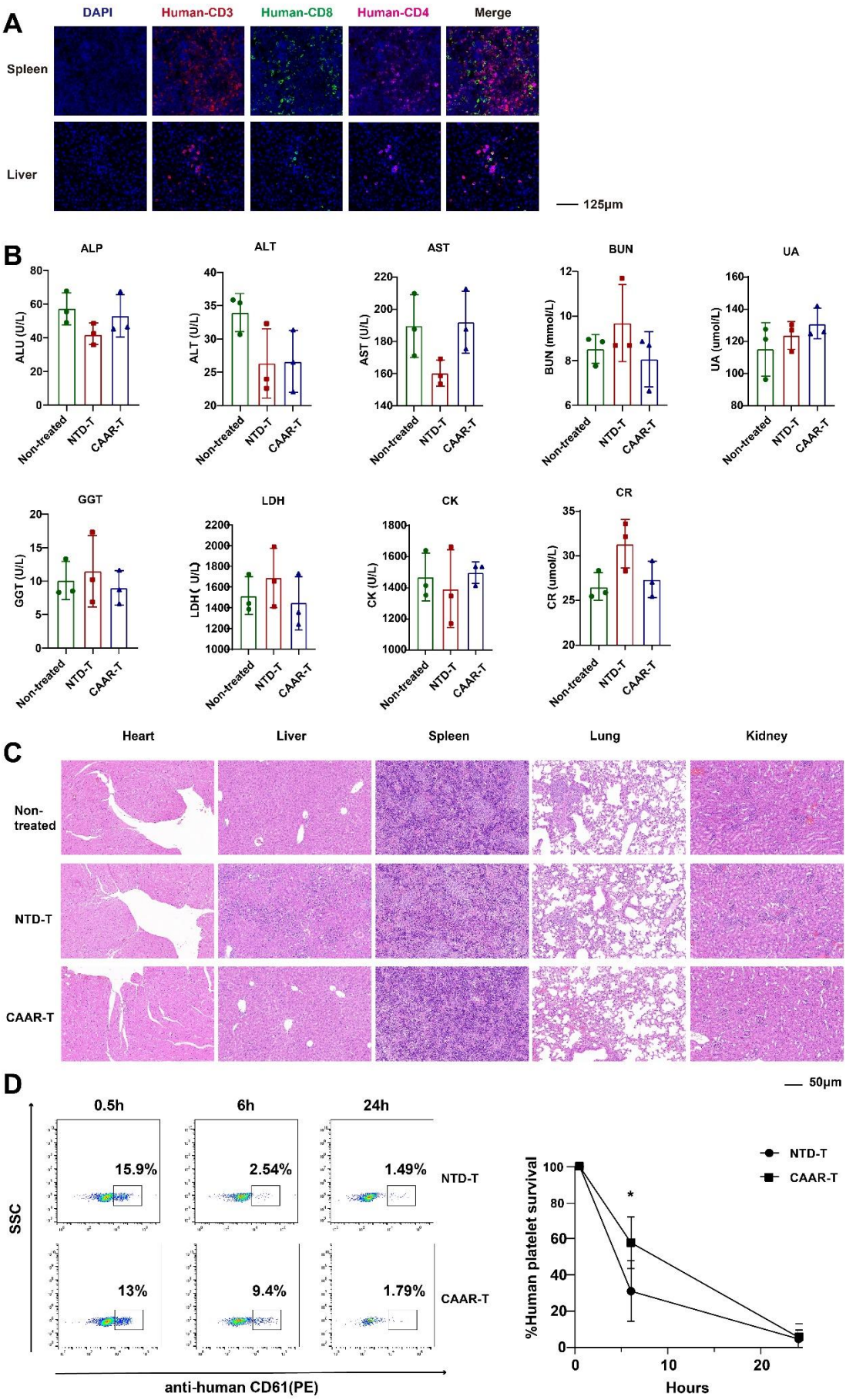
Supplementary figure1. (A) The native GPIbα ectodomain of various lengths was constructed into a second-generation CAR structure containing a CD8α hinge/transmembrane region, 4-1BB costimulatory domain, and intracellular CD3ζ. LTR, long terminal repeats; EF-1, elongation factor-1; IRES, Internal Ribosome Entry Site.



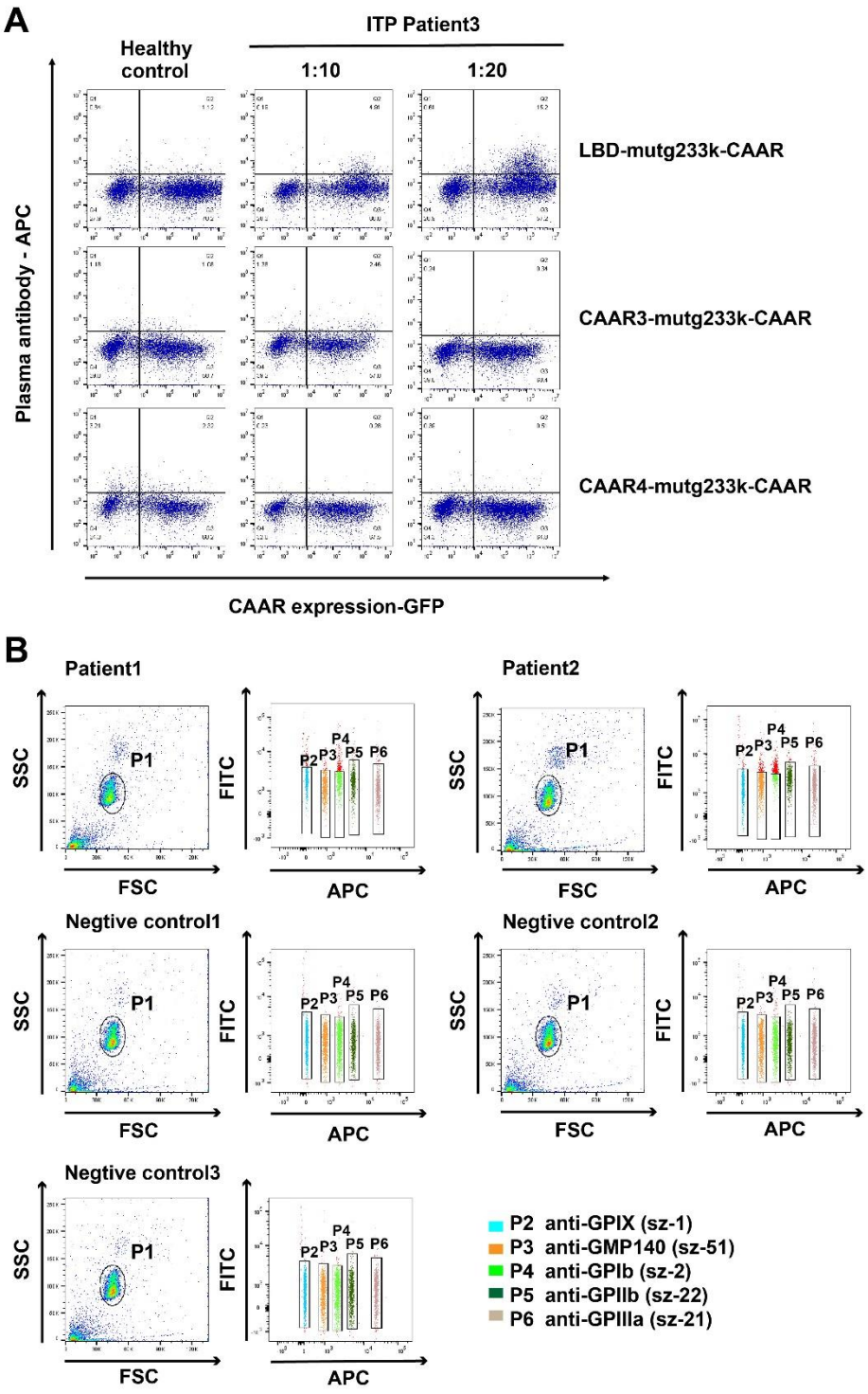
Supplementary figure2. (A) Unmutated GPIbα CAAR plasmids were transfected into HEK293T cells, and CAAR expression was tested with an anti-human CD42b(GPIbα) antibody (clone: HIP1). (B) Primary human T cells were transduced with LBD-CAAR, CAAR3, and CAAR4 lentivirus, and CAAR expression was determined using an anti-human CD42b antibody (clone: HIP1). (C) Primary human T cells were transduced with LBD-mutg233k, CAAR1, CAAR2, CAAR3-mutg233k, and CAAR4-mutg233k lentivirus or NTD-T. The transduction efficiencies of GPIbα CAARs were detected using the APC-conjugated hybridoma antibody Gvb4.



Supplementary figure3. (A) NTD-T or CAA3-mutg233k-T cells were co-incubated with control hybridoma (PE-conjugated) and anti-GPIbα hybridomas (APC-conjugated Gvb1/Gvb2/Gvb3/Gvb4) at an E:T ratio of 5:1. The ratio of the target hybridoma to total control hybridoma cells was 3:1. Cytotoxicity was evaluated at 24 hours using flow cytometry, as indicated by the changes in the ratio of the target hybridoma to the control hybridoma. NTD-T, nontransduced T cells; NS, $P > 0.05$; *$P < 0.05$; ** $P < 0.01$. The same method of detection was also applied in CAA3-mutg233k-T cells (B), CAAR1-T cells (C), and CAAR2-T cells (D). NTD-T, nontransduced T cells; NS, $P > 0.05$; *$P < 0.05$; ****$P < 0.0001$.

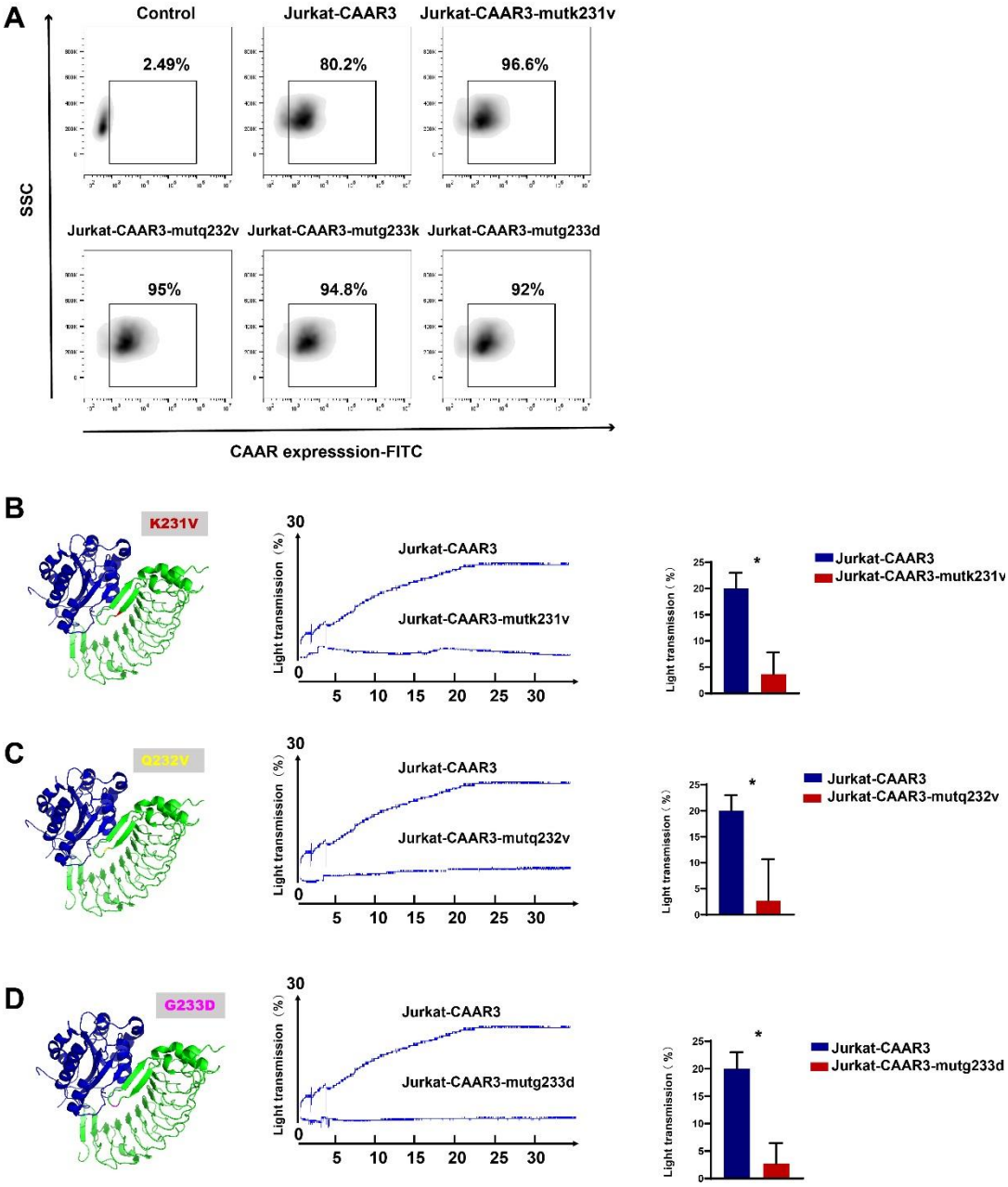

A
DAPI
Human-CD3
Human-CD8
Human-CD4
Merge
Spleen
Liver
125μm
B
ALP
ALT
AST
BUN
UA
GGT
LDH
CK
CR
ALU (U/L)
ALT (U/L)
AST (U/L)
BUN (mmol/L)
UA (umol/L)
GGT (U/L)
LDH(U/L)
CK (U/L)
CR (umol/L)
Non-treated
NTD-T
CAAR-T
C
Heart
Liver
Spleen
Lung
Kidney
Non-treated
NTD-T
CAAR-T
50μm
D
0.5h
6h
24h
15.9%
2.54%
1.49%
NTD-T
13%
9.4%
1.79%
CAAR-T
SSC
anti-human CD61(PE)
%Human platelet survival
Hours
NTD-T
CAAR-T
*

Supplementary figure4. (A) Immunofluorescence imaging analysis of LBD-mutg233k-T-cell penetration and persistence in the liver and spleen of the mice at the end points of each group of experiments. Human T cells were stained with anti-human CD3 (red)/CD8 (green)/CD4(pink) antibody and DAPI (blue; nuclear). Scale bar, 125 μm. (B) Serum biochemical levels of Non-treated, NTD-T, and LBD-mutg233k-T treated mice at sacrifice. ALP, A Lkaline Phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; UA, uric acid; CR, creatinine; GGT, gamma-glutamyl transpeptidase; LDH, lactate dehydrogenase; CK, creatine kinase. NTD-T, nontransduced T cells. (C) Hematoxylin and eosin (H&E) staining of tissues and organs in Non-treated, NTD-T, and LBD-mutg233k-T treated mice to reveal the morphology changes: scale bar, 50 μm. NTD-T, nontransduced T cells. (D) Human platelet-rich plasma (200 μL) at 2×10^{9} resting platelets/mL was injected into the lateral tail vein of the NTD-T-treated and LBD-mutg233k-T-treated mice. Whole blood (20-50 μL) was obtained from the mouse tail vein at various time points (0.5h, 6h, 24h), and human platelet survival was measured by flow cytometry using monoclonal antibody anti-human CD61(clone: VI-PL2). Human platelet percents at 0.5h were taken as the baseline value, and the human platelet survival of the two groups was determined and compared (n=3; *P <0.05).



Supplementary figure5. (A) Plasma from the ITP patient3 and healthy control were diluted at 1:10 and 1:20 and incubated with HEK293T cells expressing LBD-mutg233k, CAAR3-mutg233k, and CAAR4-mutg233k tagged with GFP. The cells were then stained with anti-human IgG antibodies (APC). (B) Platelet-specific antibody detection of ITP patients and negative controls by a cytometric bead array. Cutoff values were determined with reference to negative controls (Mean+3SD). Autoantibodies targeting human platelet GPIX/GPIb/GPIIb/GPIIIa/GMP140 were detected. GMP140, Granule membrane protein

140; SD, Standard Deviation.



Supplementary figure6. (A) Construction and screening of GPIbα mutated Jurkat T cell lines. Jurkat T cells were transduced with CAAR3-mutk231v, CAAR3-mutq232v, and CAAR3-mutg233d lentivirus, and GFP-positive cells were sorted by flow cytometry. (B-D) Ristocetin-induced VWF binding to GPIbα fragments expressed on Jurkat T cells. CAAR3 and mutated CAAR3(K231V/Q232V/G233D) were respectively expressed on the surface of Jurkat T cells, and cell aggregation was measured as an increase in light transmission (%) (n=3; *P <0.05).

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