Supplementary information file

TGFβ inhibition via CRISPR promotes the long-term efficacy of CAR-T cells against solid tumors

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The authors declare there is no conflict of interest.

Contents:

Figure S1

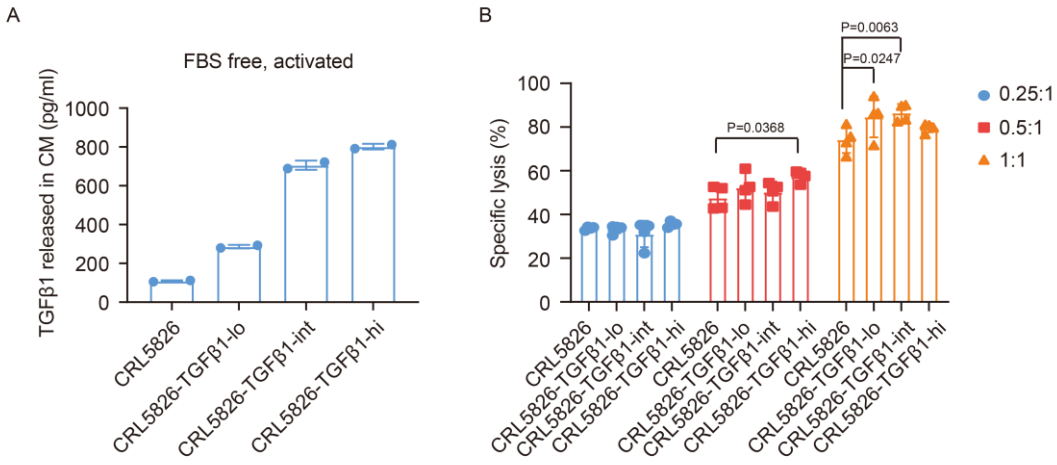
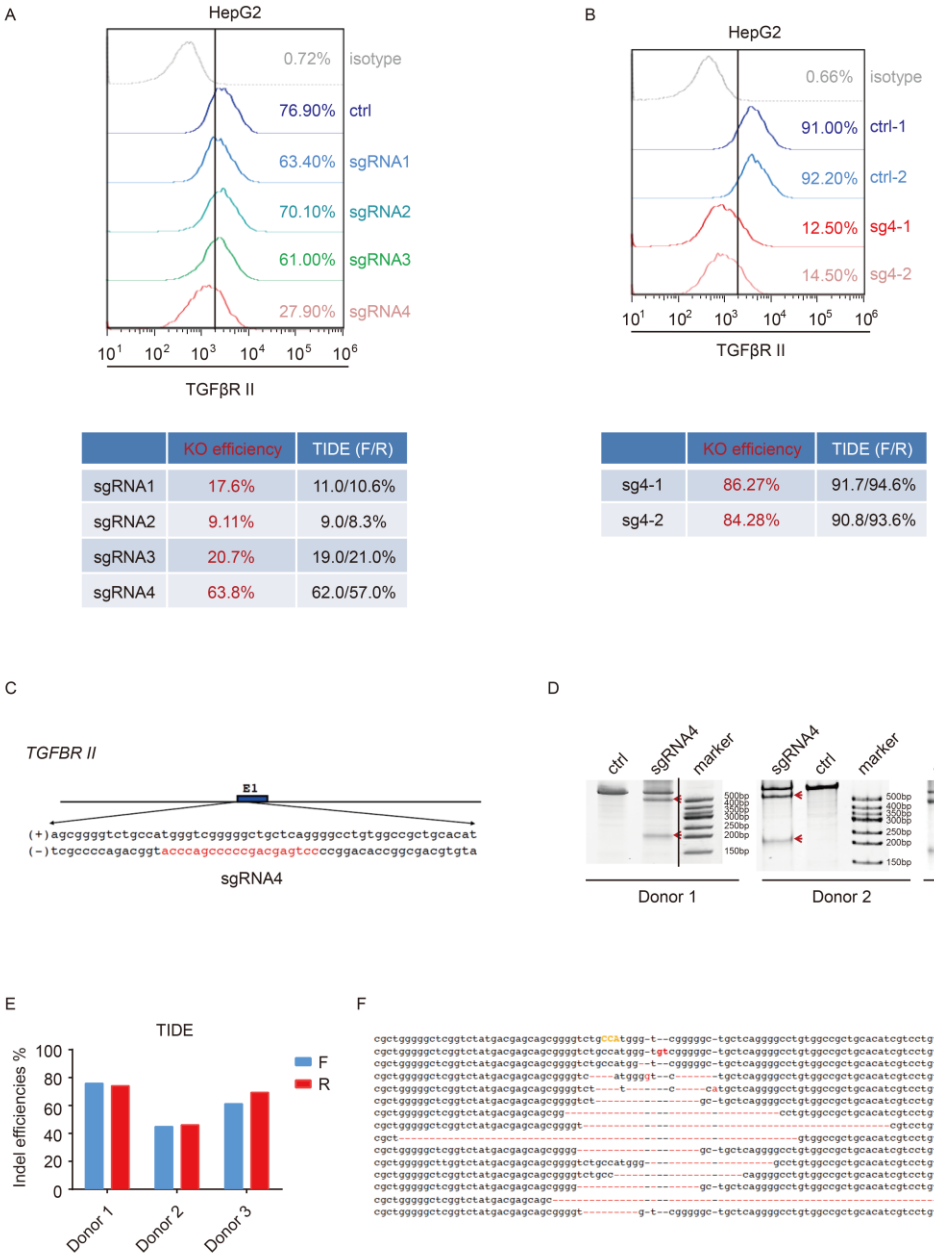


Figure S1: Construction of TGFβ1 over-expression CRL5826 cell line. (A) Concentration of TGFβ 1 released in the culture medium of CRL5826 and TGFβ 1 over-expression CRL5826. (B) Specific lysis of CRL5826 and TGFβ 1 over-expression CRL5826 after co-culture with M28z CAR-T cells at different Effector to Target ratio (E:T). The statistical analysis represented mean ± s.d. of two technical replications per assay in A, and three technical replications per assay in B. Two-way ANOVA and Tukey's multiple comparisons test was used in B. The assays in A were repeated two times, and in B were repeated more than three times.

Figure S2



	KO efficiency	TIDE (F/R)
sgRNA1	17.6%	11.0/10.6%
sgRNA2	9.11%	9.0/8.3%
sgRNA3	20.7%	19.0/21.0%
sgRNA4	63.8%	62.0/57.0%

	KO efficiency	TIDE (F/R)
sg4-1	86.27%	91.7/94.6%
sg4-2	84.28%	90.8/93.6%

Figure S2: Design of sgRNAs targeting *TGFBR2*. (A) Editing efficiencies of sgRNA1-4 on HepG2 cell line were detected by TIDE and FACS. (B) Editing efficiencies of sgRNA4 on HepG2 cell line after optimizing were detected by TIDE and FACS analysis. (C) sgRNA4 targeting *TGFBR2*. (D-F) *TGFBR2* editing efficiencies in CAR-T cells were detected by Surveyor assay (D), TIDE analysis (E) and TA cloning (F).

Figure S3

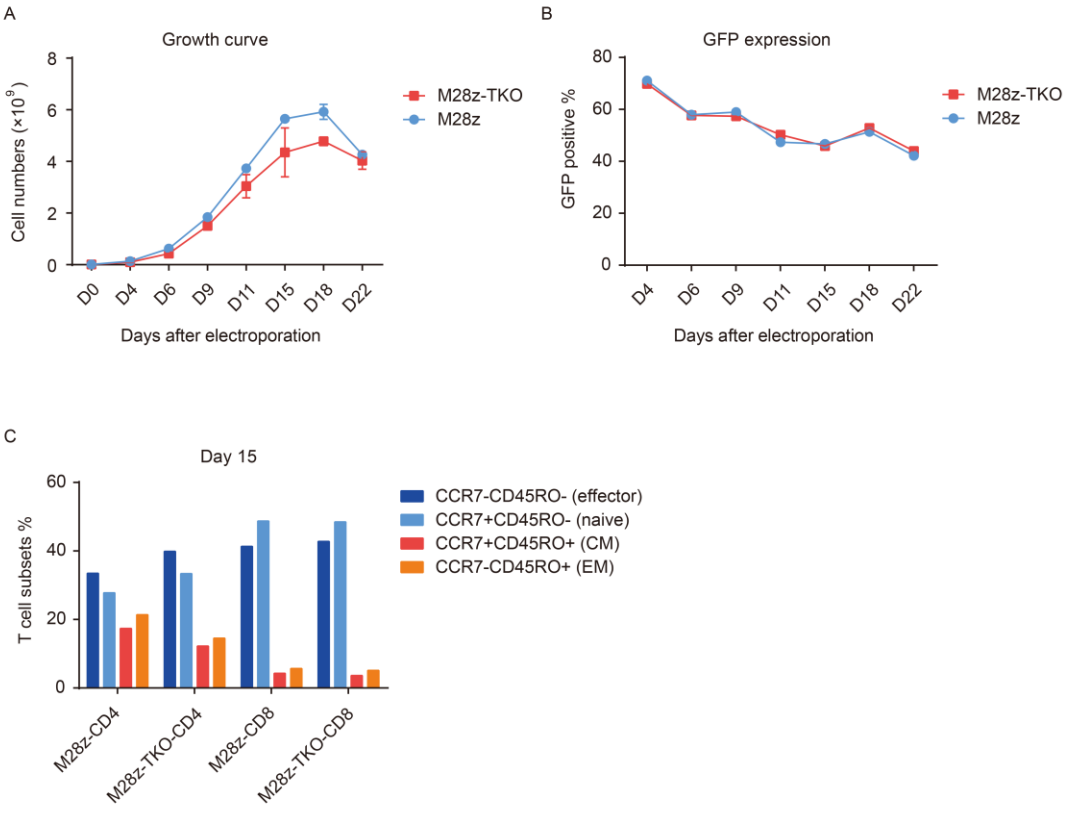


Figure S3: *TGFBR2* editing did not affect the proliferation (A), GFP expression (B) and T cell subsets (C) of CAR-T cells. M28z-TKO, *TGFBR2* KO M28z.

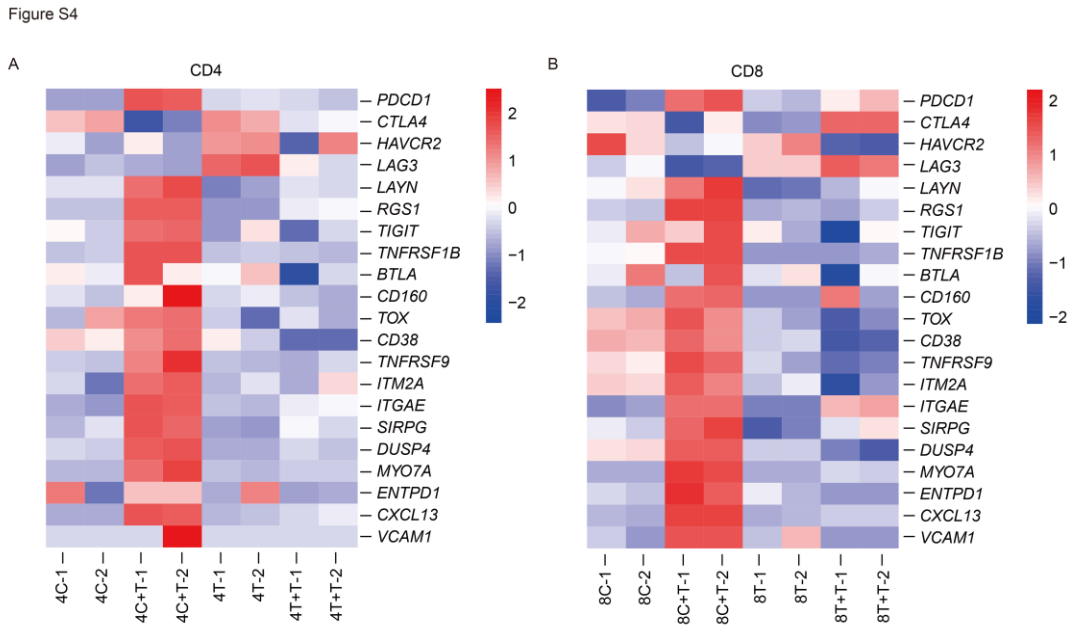


Figure S4: TGFβ1 affects the expression of exhaustion related genes in CD4 (A) and CD8 (B) CAR-T cells. 4C, CD4-ctrl; 4C+T, CD4-ctrl+ TGFβ1; 8C, CD8-ctrl; 8C+T, CD8-ctrl+ TGFβ1; 4T, CD4-TKO; 4T+T, CD4-TKO+ TGFβ1; 8T, CD8-TKO; 8T+T, CD8-TKO+ TGFβ1.

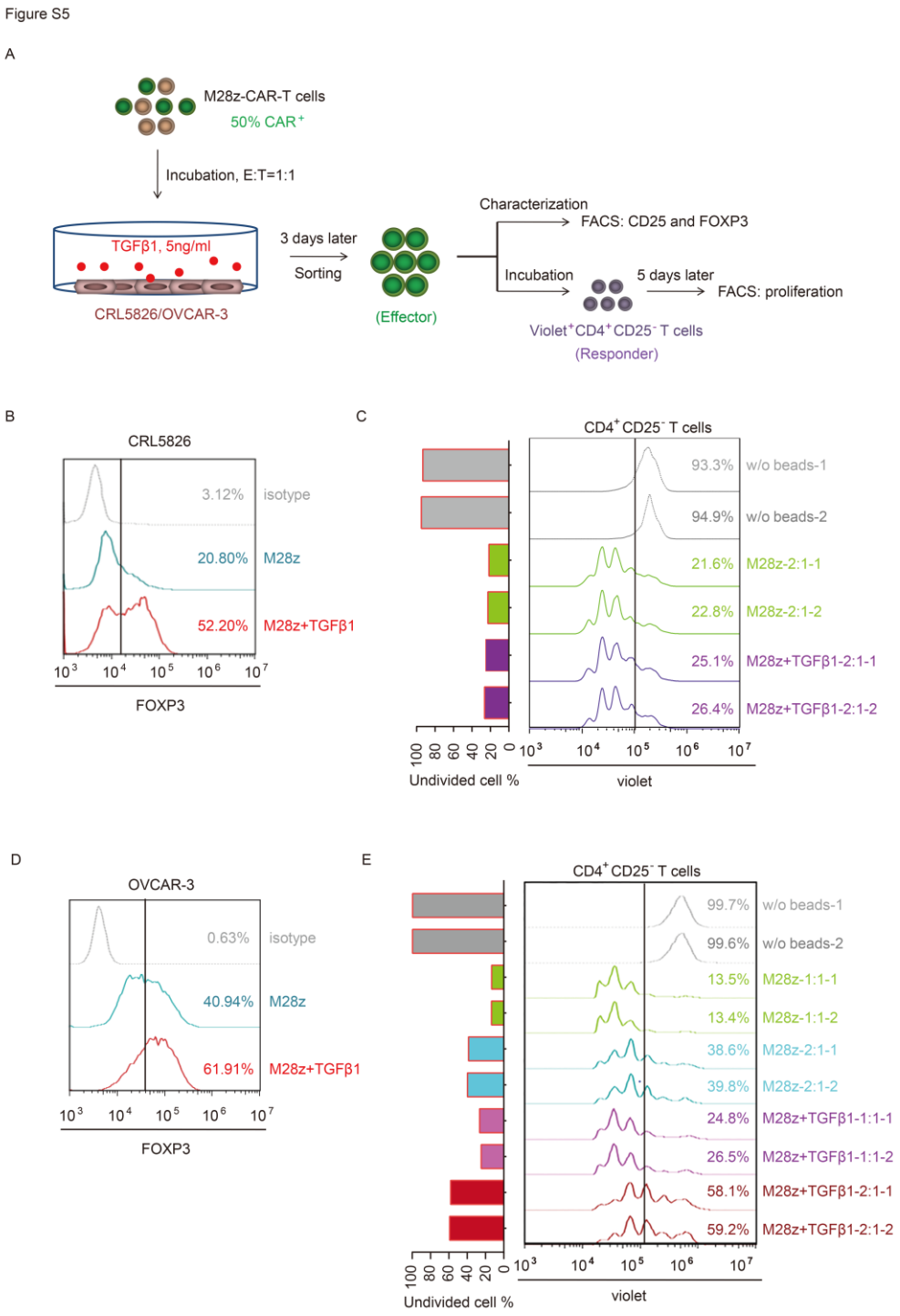


Figure S5: TGFβ1 induced iTreg-like cell conversion of CAR T cells after incubation with tumor cells. (A) Schematic of the proliferation suppression assay. FOXP3 expression in M28z CAR-T cells after incubation with CRL5826 (B) or OVCAR3 (D) in the presence of 5 ng/ml TGFβ1. Proliferation suppression capability of M28z CAR-T cells after incubation with CRL5826 (C) or OVCAR3 (E) in the presence or absence of 5 ng/ml TGFβ1.

Figure S6

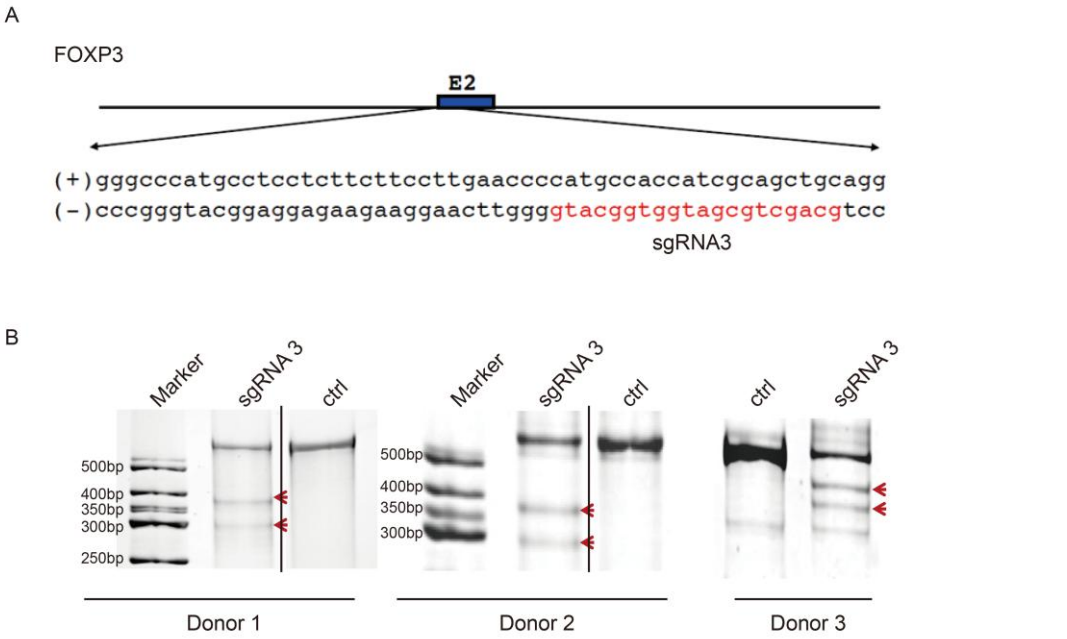


Figure S6: *FOXP3* editing on CAR-T cells. (A) sgRNA3 targeting *FOXP3*. (B-D) *FOXP3* editing efficiencies in CAR-T cells detected by Surveyor assay (B), TIDE analysis (C) and TA clone (D).

Figure S7

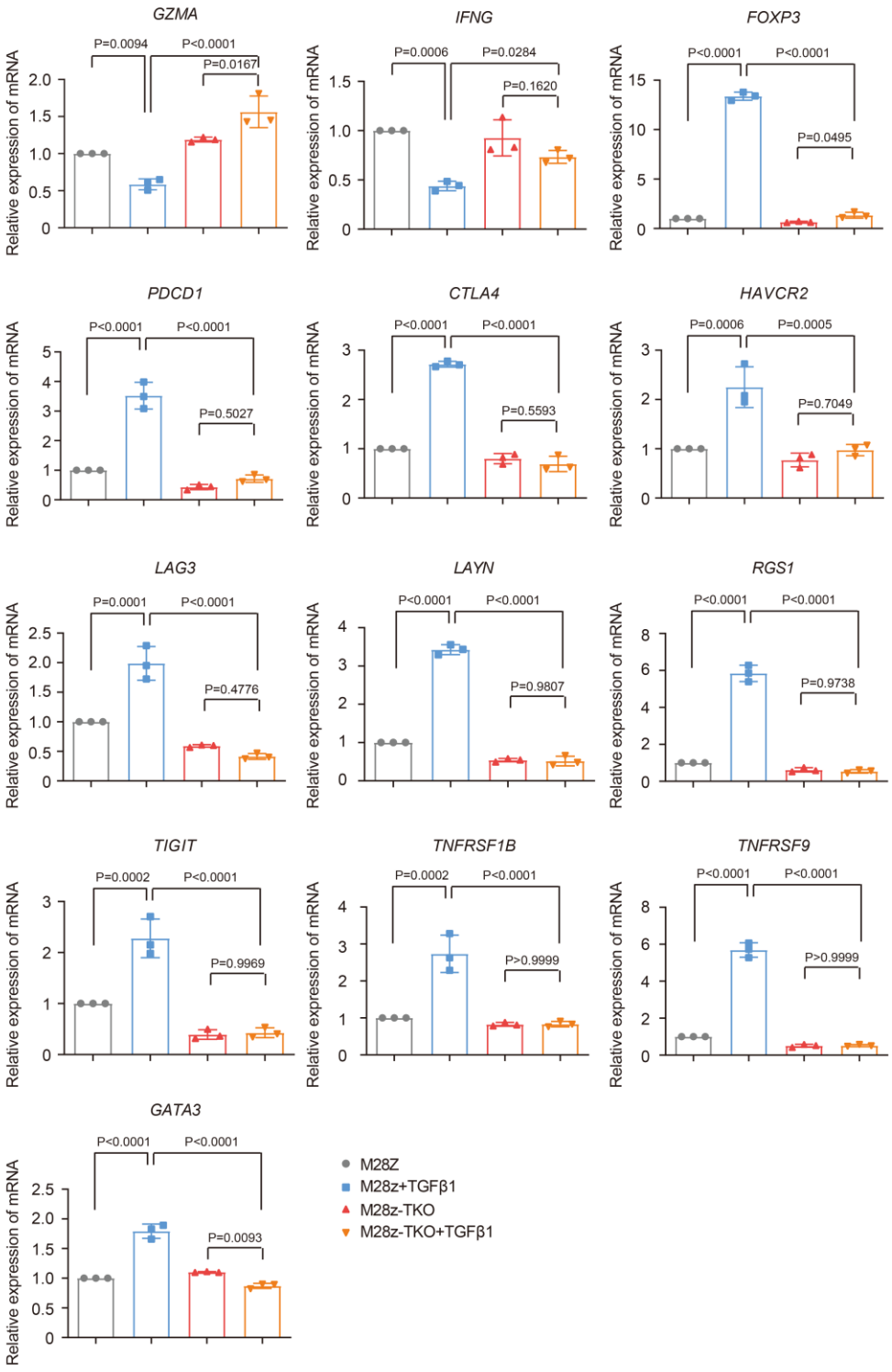


Figure S7: *TGFBR2* KO down-regulated TGFβ1 induced expression of exhaustion related genes. The statistical analysis represented mean ± s.d. of three technical repetitions in one assay. Ordinary one-way ANOVA and Tukey's multiple comparisons test was used. The assays were repeated two times.

Figure S8

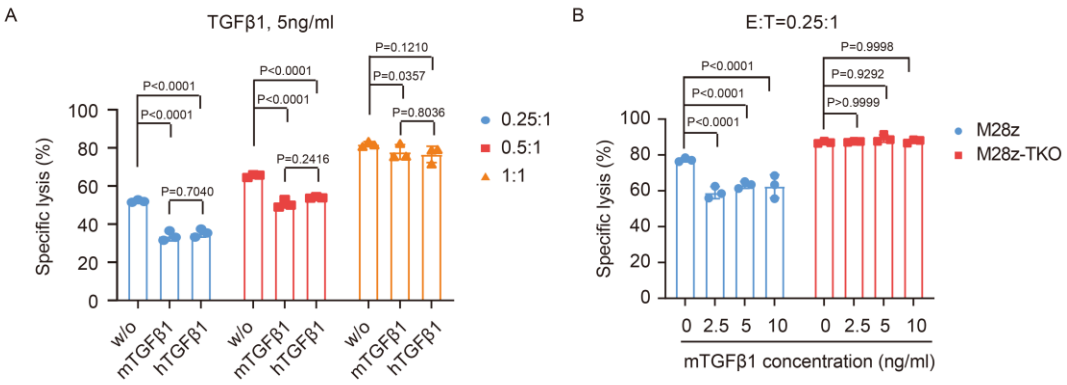


Figure S8: *TGFBR2* KO could totally rescue the inhibitory effects of mTGFβ1.

(A) Specific lysis of CRL5826 after co-culture with M28z CAR-T cells at different E:T ratio in the presence of 5ng/ml human (h) or mouse (m) TGFβ 1. (B) *TGFBR2* KO completely rescues the negative effects of mTGFβ1 on CAR-T cell-mediated tumor lysis. The statistical analysis represented mean ±s.d. of three technical replications per assay in A-B. Two-way ANOVA and Tukey's multiple comparisons test was used in A-B. The assays in A-B were repeated more than three times.

Figure S9

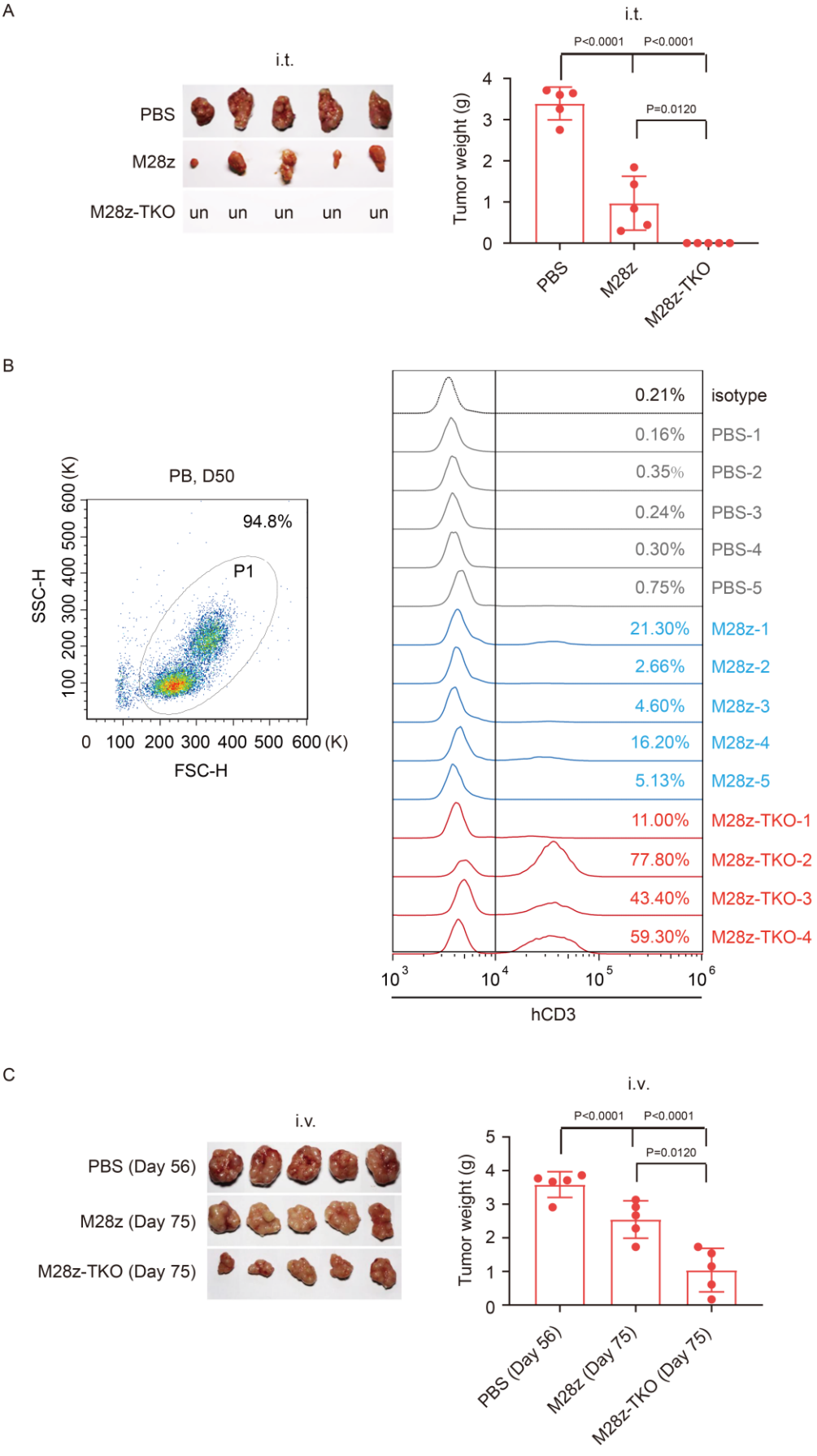

A
i.t.
PBS
M28z
M28z-TKO
un un un un un
i.t.
P<0.0001
P<0.0001
P=0.0120
Tumor weight (g)
0
1
2
3
4
PBS
M28z
M28z-TKO
B
PB, D50
94.8%
P1
SSC-H
100 200 300 400 500 600 (K)
0 100 200 300 400 500 600 (K)
FSC-H
0.21% isotype
0.16% PBS-1
0.35% PBS-2
0.24% PBS-3
0.30% PBS-4
0.75% PBS-5
21.30% M28z-1
2.66% M28z-2
4.60% M28z-3
16.20% M28z-4
5.13% M28z-5
11.00% M28z-TKO-1
77.80% M28z-TKO-2
43.40% M28z-TKO-3
59.30% M28z-TKO-4
10^3 10^4 10^5 10^6
hCD3
C
i.v.
PBS (Day 56)
M28z (Day 75)
M28z-TKO (Day 75)
i.v.
P<0.0001
P<0.0001
P=0.0120
Tumor weight (g)
0
1
2
3
4
5
PBS (Day 56)
M28z (Day 75)
M28z-TKO (Day 75)

Figure S9: Detection of in vivo tumor elimination efficacy of *TGFBR2* KO CAR-T cells in CRL5826 CDX model. Tumor sizes and tumor weights in the end of experiment in CRL5826 CDX model with i.t. (A) and i.v. (C) CAR-T cell injection. (B) The proportion of hCD3 positive cells in mouse peripheral blood 50 days after CAR-T injection. M28z-TKO, *TGFBR2* KO M28z; i.t., intra-tumor; i.v., intravenous; un, undetectable. The statistical analysis represented mean ± s.d., n=5. Ordinary one-way ANOVA and Tukey's multiple comparisons test was used.

Figure S10

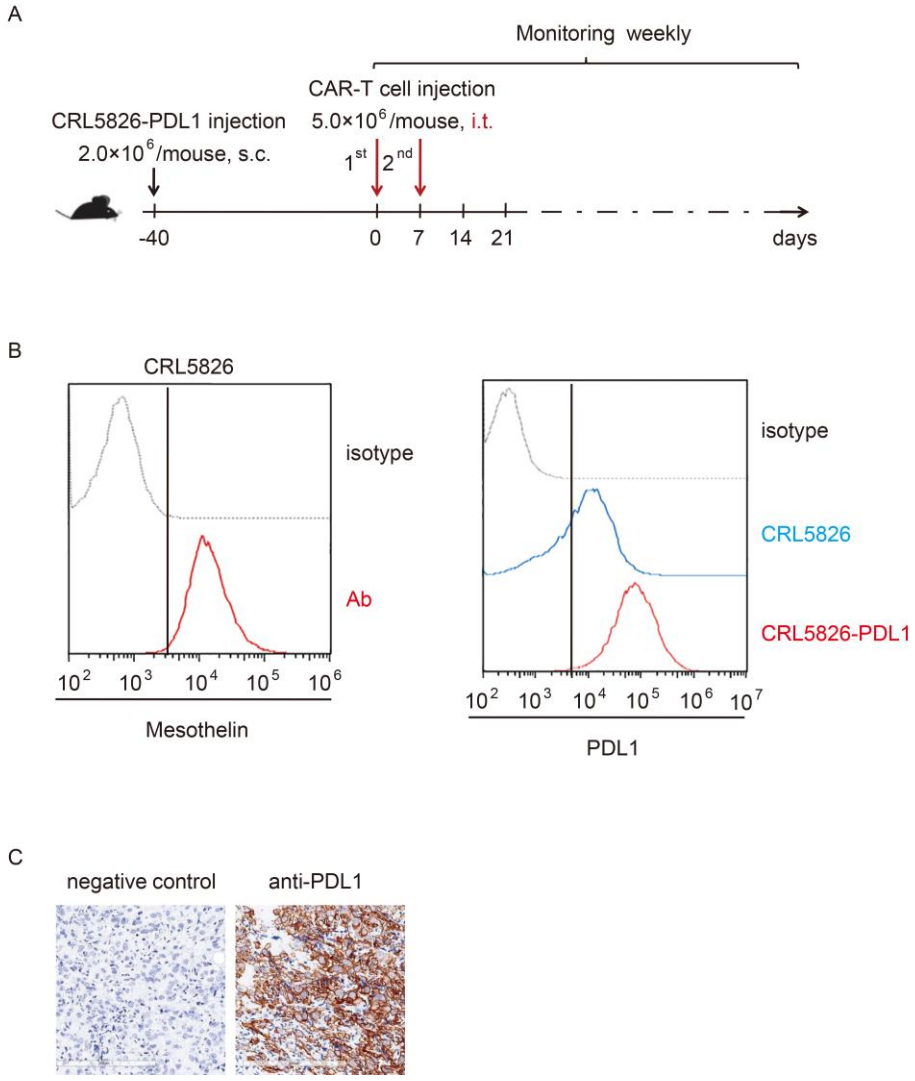


Figure S10: Detection of in vivo tumor elimination efficacy of *TGFBR2* KO CAR-T cells in CRL5826-PDL1 CDX model. (A) Schematic of the *in vivo* experimental design using CRL5826-PDL1 CDX model. (B) Antigen expression on target tumor cells detected by FACS. (C) PDL1 expression in the CDX model.

Figure S11

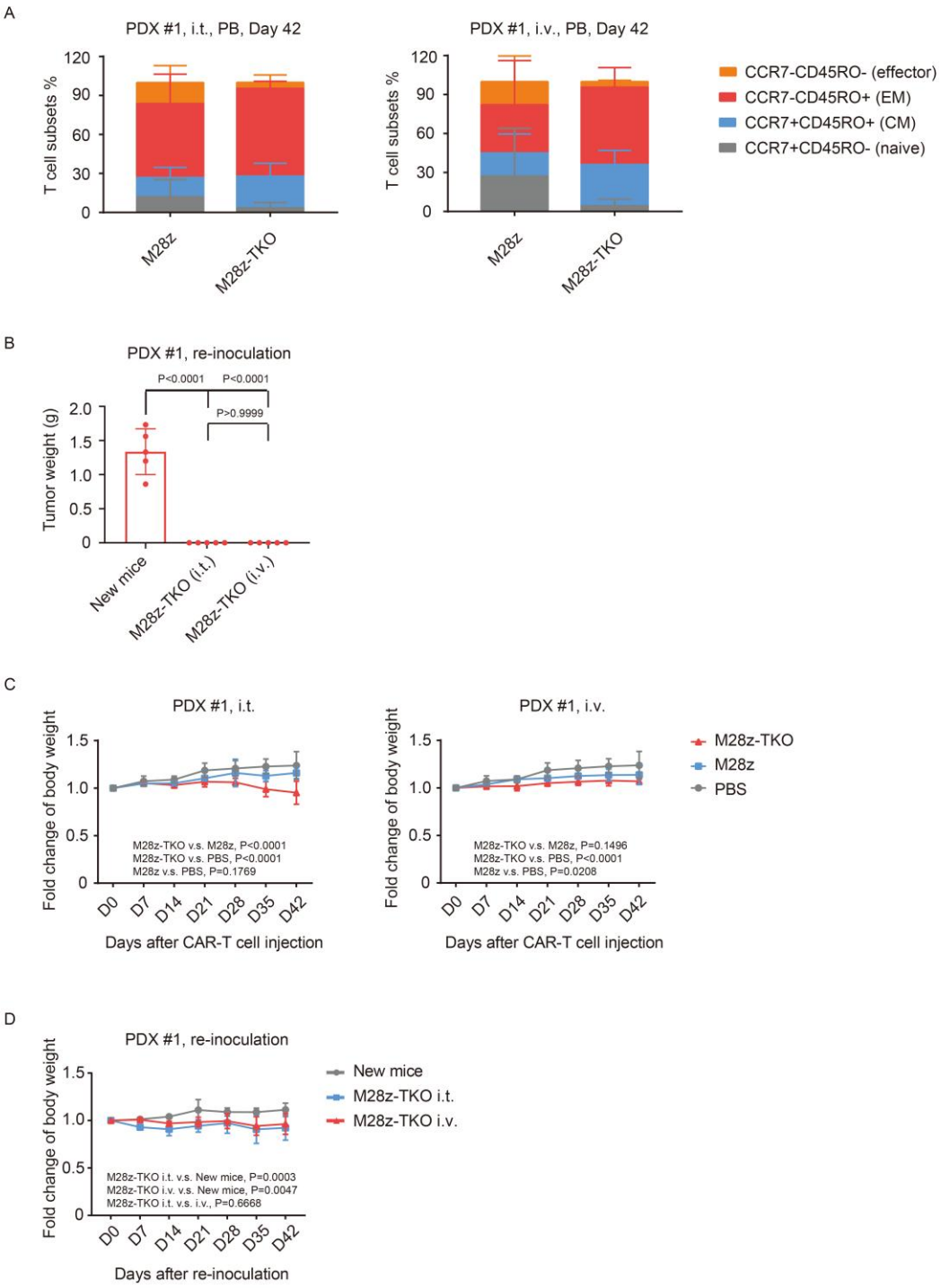


Figure S11: Detection of in vivo tumor elimination efficacy of *TGFBR2* KO CAR-T cells in PDX tumor 1 model. (A) T cell subsets analysis in mouse peripheral blood 42 days after CAR-T cell i.t. or i.v. injection. (B) Tumor weight at the end of PDX tumor 1 re-inoculation experiment. (C-D) Fold change of body weight after

PDX tumor 1 primary inoculation (C) and re-inoculation (D). M28z-TKO, *TGFBR2* KO M28z; i.t., intra-tumor; i.v., intravenous; PB, peripheral blood. The statistical analysis represented mean ± s.d., n=5. Ordinary one-way ANOVA and Tukey's multiple comparisons test was used in B. Two-way ANOVA and Tukey's multiple comparisons test was used in C-D.

Figure S12

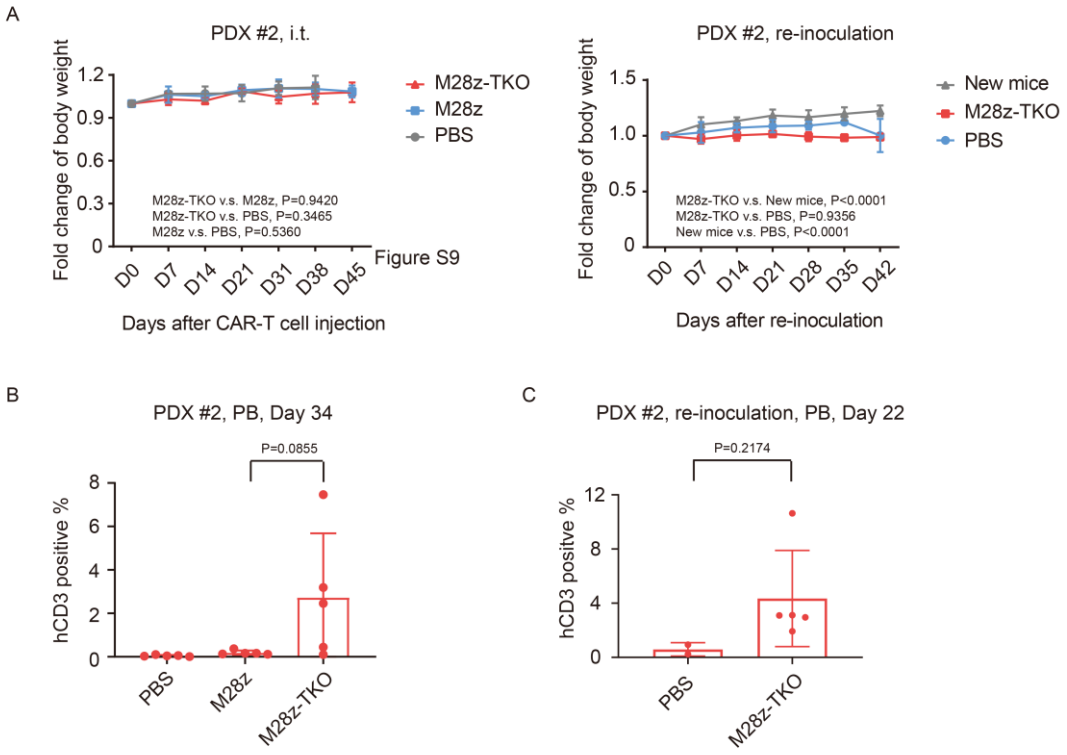


Figure S12: Detection of in vivo tumor elimination efficacy of *TGFBR2* KO CAR-T cells in PDX tumor 2 model. (A) Fold change of body weight after PDX tumor 2 primary inoculation and re-inoculation. (B) The proportion of hCD3 positive cells in peripheral blood 34 days after PDX tumor 2 primary inoculation. (C) The proportion of hCD3 positive cells in peripheral blood 22 days after PDX tumor 2 re-inoculation. M28z-TKO, *TGFBR2* KO M28z; i.t., intra-tumor; PB, peripheral blood. The statistical analysis represented mean ± s.d., n=5 except n=2 in PBS group and n=4 in New mice group in PDX #2 re-inoculation experiment. Two-way ANOVA and Tukey's multiple comparisons test was used in A. Ordinary one-way ANOVA and Tukey's multiple comparisons test was used in B. Unpaired t test was used in C.

Figure S13

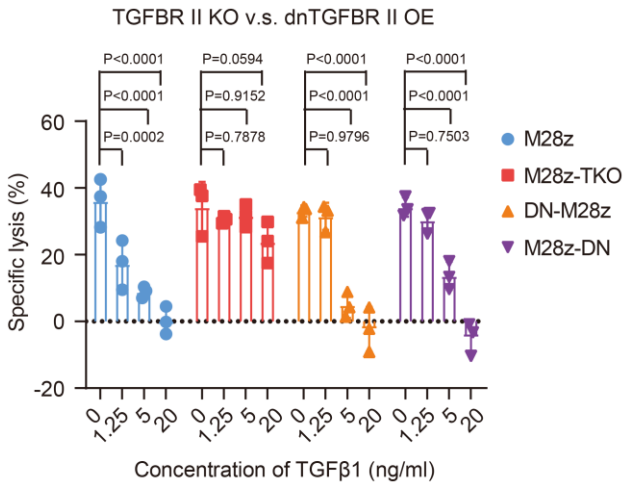


Figure S13: *TGFBR2* KO performed better than dn*TGFBR2* OE in anti-TGFβ 1 negative effect on CAR-T cell specific lysis ability. The statistical analysis represented mean ± s.d. of three technical replications per assay. Two-way ANOVA and Tukey's multiple comparisons test was used. The assays were repeated more than three times.

Table S1: Primer sequences used in this study

Guide sgRNA sequence	
FOXP3 sgRNA	gcagctgcgatggtggcatg
TGFBR2 sgRNA	cctgagcagccccgaccca
PD1 sgRNA	cgactggccagggcgcctgt
***In vitro* transcription primers**	
Forward	taatacgactcactatagNNNNNNNNNNNNNNNNNNNNgtttaagagc tatgctggaaac
Reverse	aaaagcaccgactcggtgcc
Genotyping primers for PCR	
FOXP3-Forward primer	gtgggtatgtacatgtacctgt
FOXP3-Reverse primers	ctaataagttgcagagctggcc
TGFBR2-Forward primer	cacatctggcccgcacatct
TGFBR2-Reverse primer	gggtggctcagaaagagctg
PD1-Forward primer	catctcctctgtctccctgtc
PD1-Reverse primer	gccagggactgagagtgaaag
Genotyping primers for TIDE sequence	
FOXP3-Forward primer	ctagagctggggtgcaacta
FOXP3-Reverse primers	cctcttctcttgtcacatgg
TGFBR2-Forward primer	cacatctggcccgcacatct
TGFBR2-Reverse primer	ggaaactttcctcgtttccgc
PD1-Forward primer	cctgtctctgtctctctctcc
PD1-Reverse primer	tgagagtgaaaggtccctcc
Primers for Q-PCR	
IL2-Forward primer	agaactcaaacctctggaggaag
IL2-Reverse primers	gctgtctcatcagcatattcacac
IFNG-Forward primer	atgtccaacgcaaagcaatac

IFNG-Reverse primer	acctcgaaacagcatctgac
GZMA-Forward primer	ctcactcaataaccagggaagag
GZMA-Reverse primer	gctgggtcatagcatggatag
GZMB-Forward primer	acactcacacacactacaagag
GZMB-Reverse primers	acgcacaactcaatggtact
FOXP3-Forward primer	aggaaggacagcacccttcg
FOXP3-Reverse primer	ctggcagtgcttgaggaagt
PDCD1-Forward primer	aaggcgcagatcaaagagagcc
PDCD1-Reverse primer	caaccaccagggtttggaactg
CTLA4-Forward primer	acgggactctacatctgcaagg
CTLA4-Reverse primer	ggaggaagtcagaatctgggca
TIM3-Forward primer	gactctagcagacagtgggatc
TIM3-Reverse primer	ggtggtaagcatccttggaaagg
LAG3-Forward primer	gcagtgtacttcacagagctgtc
LAG3-Reverse primer	aagccaaaggctccagtcacca
LAYN-Forward primer	acagcctgccaggacctttatg
LAYN-Reverse primer	cgatggctgatggtacatgacc
TIGIT-Forward primer	tggtggtcatctgcacagcagt
TIGIT-Reverse primer	tttctcctgaggtcaccttccac
RGS1-Forward primer	ttgtgcattcagatgctgctaaac
RGS1-Reverse primer	gaggaacctgggataagagtcc
TNFRSF1B-Forward primer	cgttctccaacacgacttcatcc
TNFRSF1B-Reverse primer	acgtgcagactgcatccatgct
TNFRSF9-Forward primer	tcttcctcacgctccgtttctc
TNFRSF9-Reverse primer	tggaaatcggcagctacagcca
GATA3-Forward primer	tgcatgactcactggaggac
GATA3-Reverse primer	tcagggaggacatgtgtctg

GAPDH-Forward primer	atgacatcaagaaggtggtg
GAPDH-Reverse primer	cataccaggaaatgagcttg