The antitumor effect of CAR-T cells targeting transmembrane tumor necrosis factor-alpha combined with PD-1 mAb on breast cancers

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Ba H, *et al. J Immunother Cancer* 2023; 11:e003837. doi: 10.1136/jitc-2021-003837

Supplementary Table 1. Antibodies used in flow cytometry and western blotting

Antibody	Catalog number	Company
Anti-F(ab')$_2$ of IgG-APC	109-136-097	Jackson ImmunoResearch, West Grove, PA, USA
Anti-CD3-percp-cy5.5	45-0037-42	eBioscience, San Diego, CA, USA
Anti-CD3-APC-cy7	561800	BD Pharmingen, San Diego, CA, USA
Anti-CD4-FITC	11-0041-82	eBioscience, San Diego, CA, USA
Anti-CD8-PE	12-0081-82	eBioscience, San Diego, CA, USA
Anti-CD45RO-Pe-cy7	25-0457-42	eBioscience, San Diego, CA, USA
Anti-CD62L-FITC	11-0621-82	eBioscience, San Diego, CA, USA
Anti-CCR7-PE	12-1979-42	eBioscience, San Diego, CA, USA
Anti-PD1-PE	329906	Biolegend, San Diego, CA, USA
Anti-mouse IgG-FITC	ZF-0312	FeiYi, Wuhan, China
Anti-human IgG-FITC	ZF-0308	FeiYi, Wuhan, China
Anti-CD3	A19017	Abclonal, Wuhan, China
Anti-PD-L1	A20344	Abclonal, Wuhan, China
Anti-IκB	A11397	Abclonal, Wuhan, China
Anti-p38	sc-7972	Santa Cruz Biotechnology, CA, USA
Anti-pp38	4511	Cell signaling Technology, MA , USA
Anti-p65	A19653	Abclonal, Wuhan, China
Anti-pp65	3033	Cell signaling Technology, MA, USA
Anti-AKT	10176-2-AP	Proteintech, Wuhan, China
Anti-pAKT	4060	Cell signaling Technology, MA, USA
Anti-actin	AC026	Abclonal, Wuhan, China
Anti-mouse IgG-HRP	7076	Cell signaling Technology, MA, USA
Anti-rabbit IgG-HRP	7074	Cell signaling Technology, MA, USA

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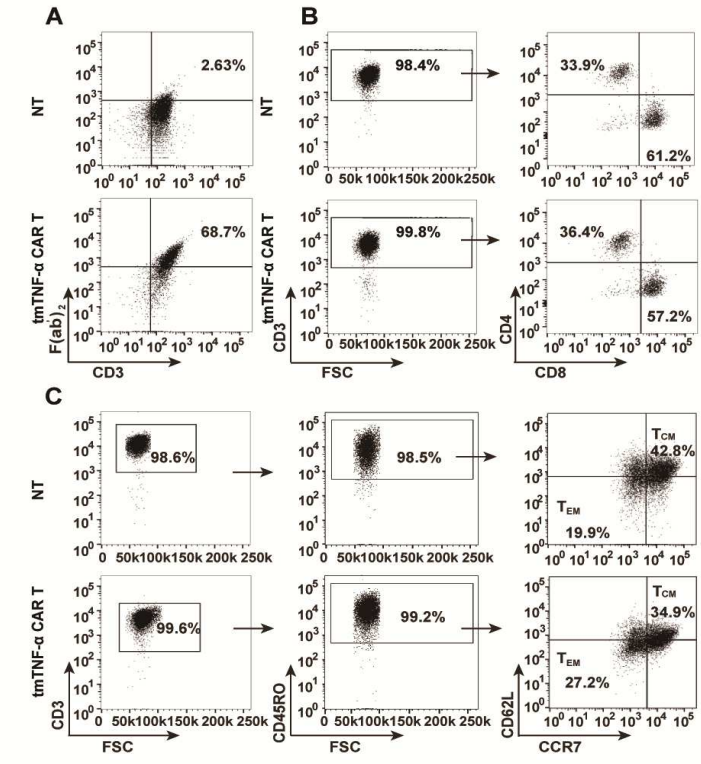
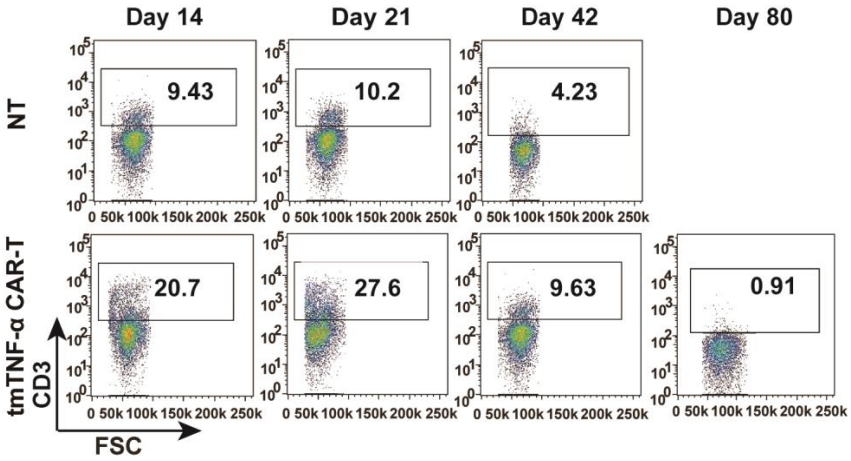
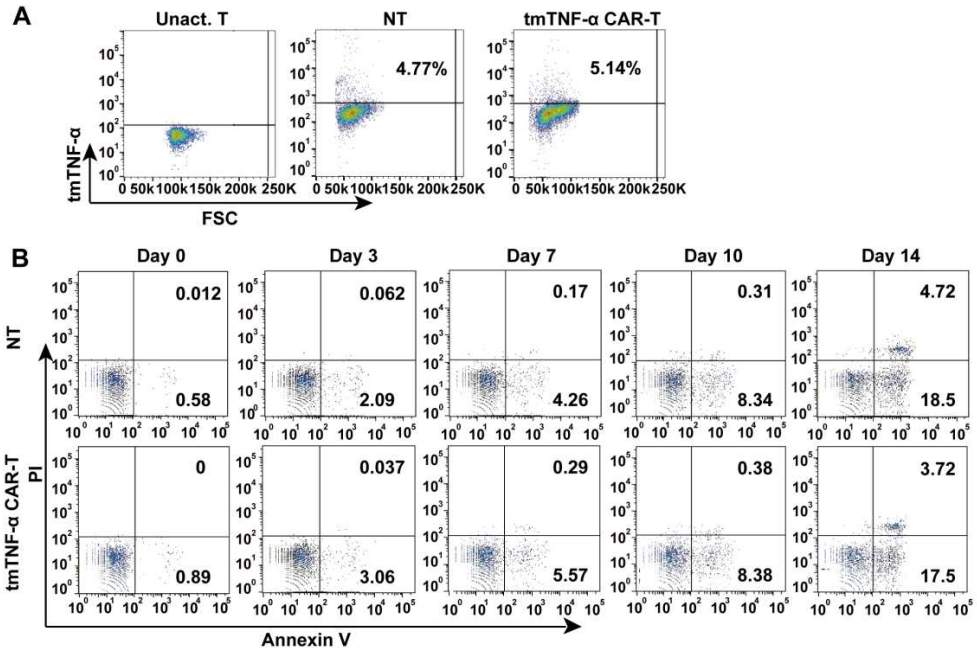
Supplementary Figures



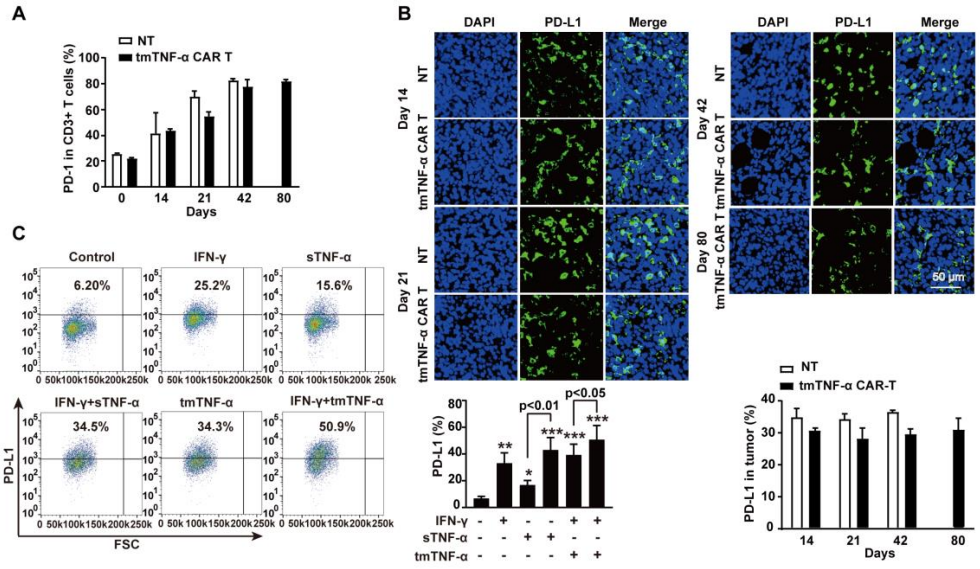
Supplementary Fig. S1. T cell subsets and memory phenotypes of tmTNF-α CAR-T cells. $CD3^+$ T cells isolated from UBC were transduced with tmTNF-α CAR gene-containing lentivirus and further cultured for 14 days in the presence of anti-CD3/anti-CD28 and IL-2. Non-transduced T (NT) cells served as a control. (A) $F(ab')_2$ antibody was used to detect the transduction efficiency of tmTNF-α CAR by flow cytometry and representative cytograms. (B) Representative cytograms of $CD4^+$ and $CD8^+$ T cell subsets. (C) Analysis of the expression of CD45RO, CD62L and CCR7 in CD3-positive T cells by flow cytometry and representative cytograms.



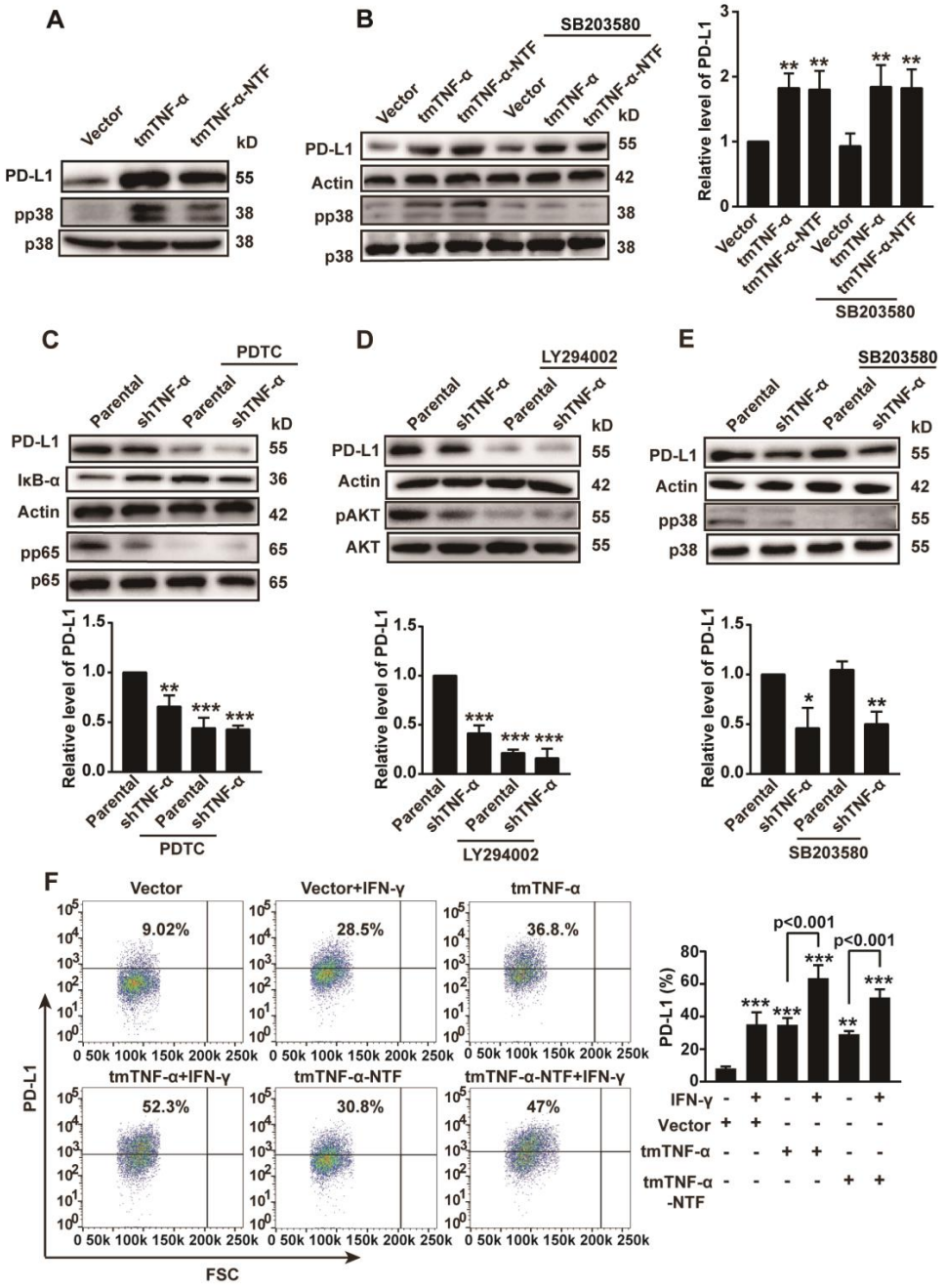
Supplementary Fig. S2. Dynamic changes of CAR-T cells in vivo. NOD/SCID mice were inoculated subcutaneously with 2 x 10^6 MDA-MB-231 cells on the right mamma pat and intravenously administered with 5x10^6 tmTNF-α CAR-T cells or NT cells on Days 7 and 14. Human $CD3^+$ T cells in the periphery blood were analyzed at indicated time points by flow cytometry. Representative cytograms of CD3 T cells in the blood. The data of NT treatment group on Day 80 were missing due to the death of mice.



Supplementary Fig. S3. tmTNF-α expression and apoptosis of NT and CAR-T cells. (A) tmTNF-α expression by unactivated T cells (Unact. T cells), NT and CAR-T cells was detected by flow cytometry and representative cytograms. (B) tmTNF-α CAR-T and NT cells were cultured in medium supplemented with IL-2 (50 IU/ml) in plates coated with anti-CD3/anti-CD28. Apoptosis of NT and CAR-T cells were analyzed at indicated time points by Annexin V/PI and representative cytograms of flow cytometry.

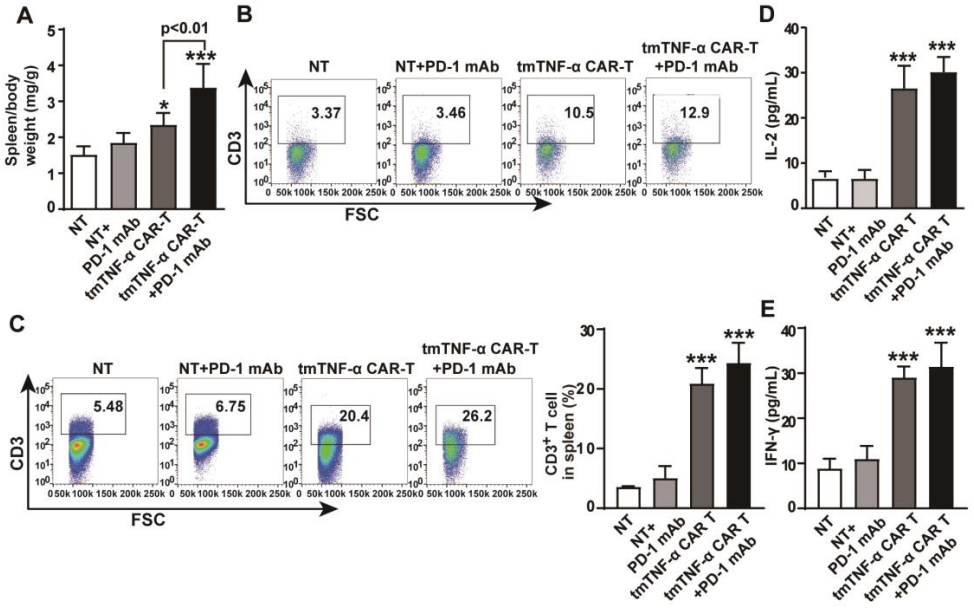


Supplementary Fig. S4. Dynamic changes of PD-1 expression on tmTNF-α CAR-T cells and PD-L1 expression on tumor cells in vivo. NOD/SCID mice were inoculated subcutaneously with 2 x 10^6 MDA-MB-231 cells on the right mamma pat and intravenously administered with 5x10^6 tmTNF-α CAR-T cells or NT cells on Day 7 and 14. (A) The PD-1 expression in CD3 T cells in the tumor tissues was analyzed by flow cytometry (n=3). (B) PD-L1 expression in tumor tissue sections (magnification × 200) determined by indirect immunofluorescence and quantitative data (n=3). The data of NT treatment group on Day 80 were missing due to the death of mice. The data in vivo represent means ± SEM. (C) MDA-MB-468 cells were co-cultured for 24 h with 100 ng/ml sTNF-α or tmTNF-α stably transfected, fixed 293T cells at an E/T ratio of 10:1 in the presence of 0.25 ng/ml IFN-γ. PD-L1 expression was detected by flow cytometry. The data represent means ± SEM of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 versus control.



Supplementary Fig. S5. PD-L1 expression is regulated by tmTNF-α in breast cancer cells. (A, B) After transfection of MDA-MB-468 cells with TNF-α or tmTNF-α-NTF for 24 h, the transfectants were treated with or without a p38 inhibitor, 0.6 M SB203580 for 24 h. Representative images of western blot for PD-L1 expression and p38 phosphorylation and

quantitative data. (C, E) shTNF-α stably transfected or parental MDA-MB-231 cells were treated with 100 μM PDTC (C), 50 μM LY294002 (D) or 0.6 M SB203580 (E) for 24 h. Representative images of western blot for PD-L1 expression, IκBα degradation and the phosphorylation of p65, AKT and p38, and quantitative data. (F) TNF-α or tmTNF-α-NTF transfectants were stimulated for 24 h with 0.25 ng/ml IFN-γ after 48-h transfection, PD-L1 expression was detected by flow cytometry. All quantitative data represent means ± SEM of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 versus parental except F (versus vector).



Supplementary Fig. S6. The effect of PD-1 antibody on the tmTNF-α CAR-T therapy. NOD/SCID mice were inoculated subcutaneously with $2x10^6$ MDA-MB-231 cells on the right mamma pat, and intravenously administered with $5x10^6$ tmTNF-α CAR-T cells or NT cells on Days 7 and 14. 200 μg of PD-1 mAb was intraperitoneally injected every three days, and started on Day 7 prior to infusion of tmTNF-α CAR-T cells. Mice were sacrificed on Day 49 (n=6 per group). (A) Spleen weight/body weight. Representative cytograms of human CD3 T cells in the periphery blood (B) and the spleen (C) analyzed by flow cytometry and quantitative data. (D, E) Serum levels of IL-2 and IFN-γ were detected by ELISA. All quantitative data represent means ± SEM. ***P < 0.001 versus NT.