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Supplemental information

Dual blockade of CD47 and CD24 signaling using a novel bispecific antibody fusion protein enhances macrophage immunotherapy

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Supplemental Material

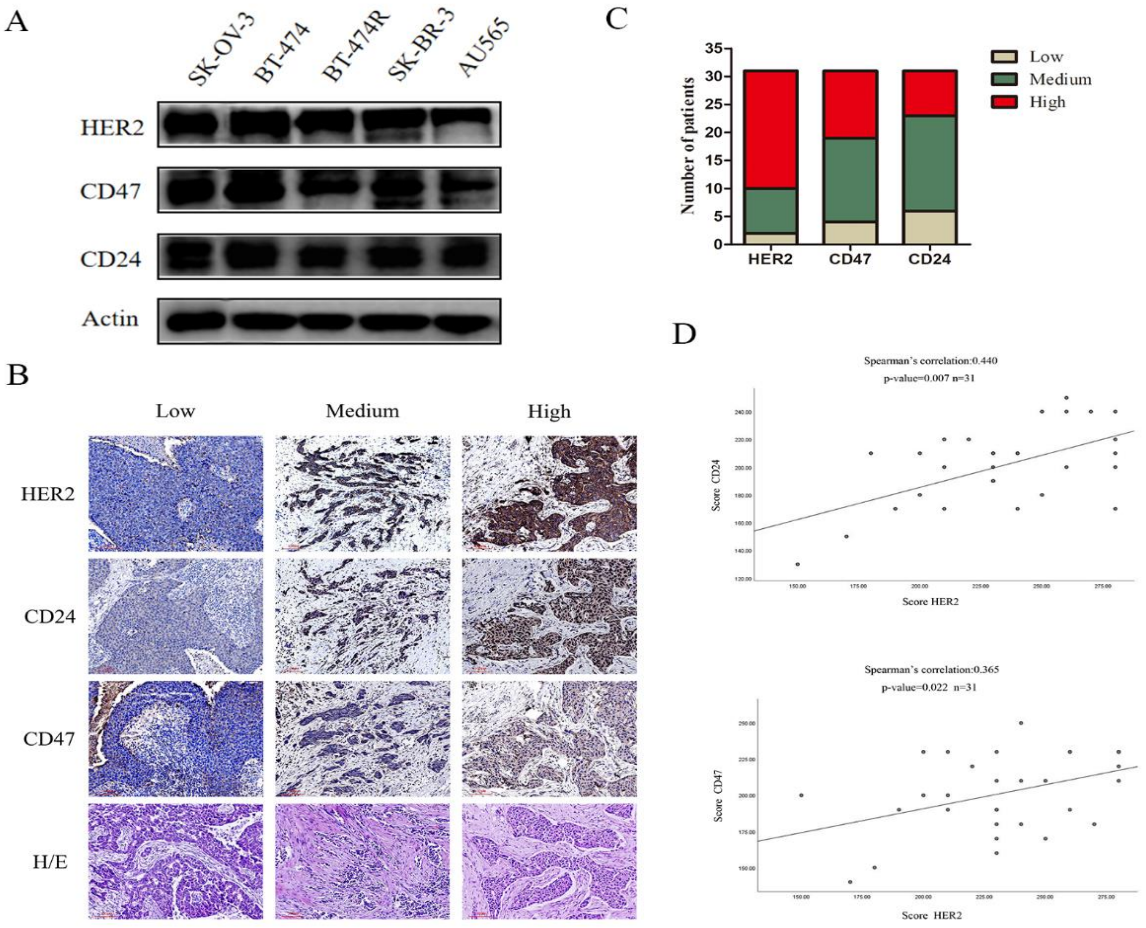

Figure S1. Expression of CD47 and CD24 are linked with HER2 status. (A) Expression of CD47 and CD24 preferring in HER2-expressing cancer cells (SK-OV-3, BT474, BT-474R, SKBR-3 and AU565). (B) Representative IHC of HER2, CD24 and CD47 in tumors from diagnosed HER2-positive breast cancer patients. (C) Numbers of patients with low, medium or high IHC staining of HER2, CD47 and CD24 grouped by HER2-positive breast cancer patients (total tumors n = 31). (D) Correlation between CD47, CD24 and HER2 expression in human breast cancer samples (n=31) was determined and quantified by immunohistochemistry staining.

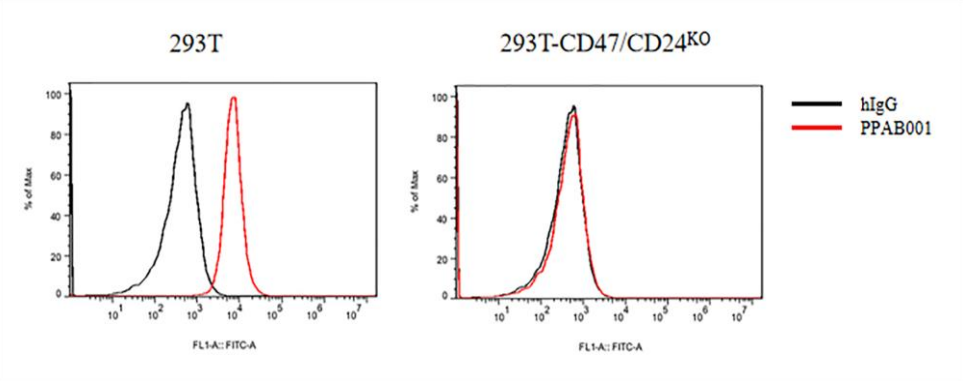

Figure S2. Determination of binding specificity of PPAB001. The binding ability of PPAB001 to 293T cells or CD47 and CD24 knockout 293T cells was detected by flow cytometry.

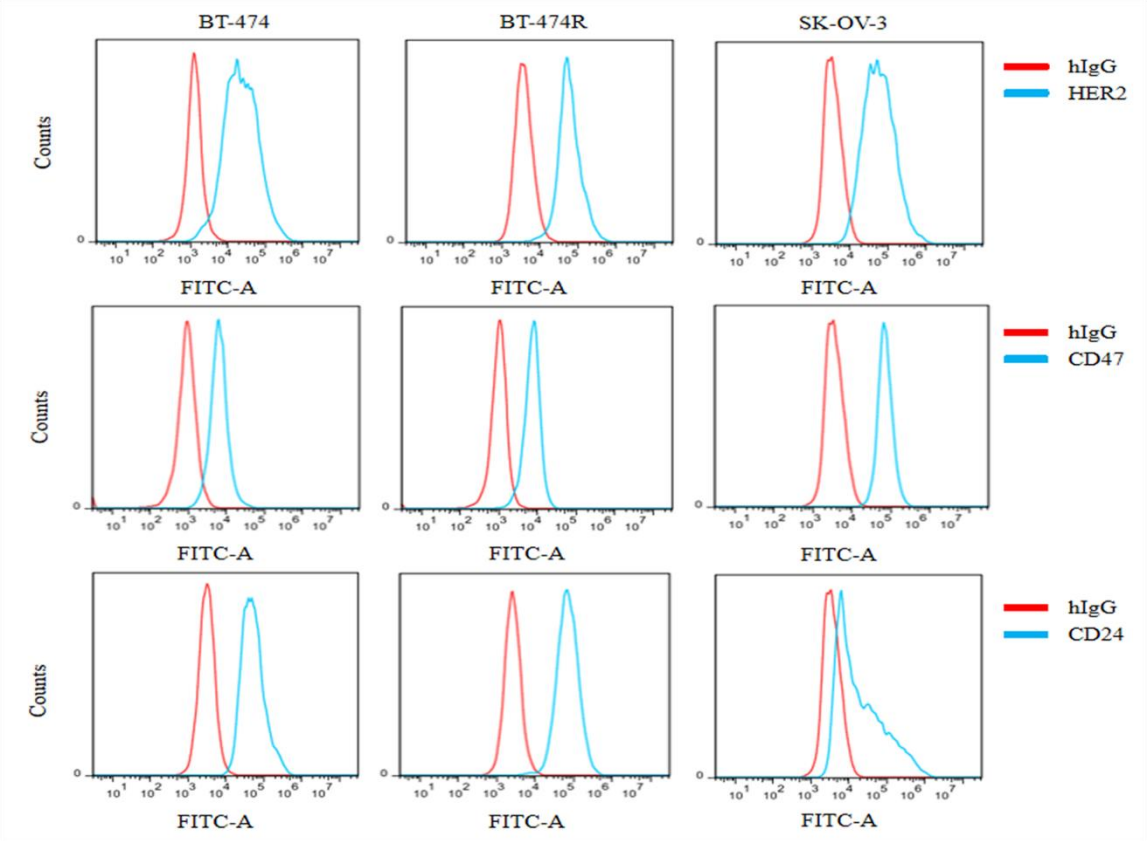

Figure S3. CD47 and CD24 expression in HER2-positive breast cancer. Flow cytometry analysis showing the expression level of CD47 and CD24 on BT-474, BT-474R and SK-OV-3 cells.

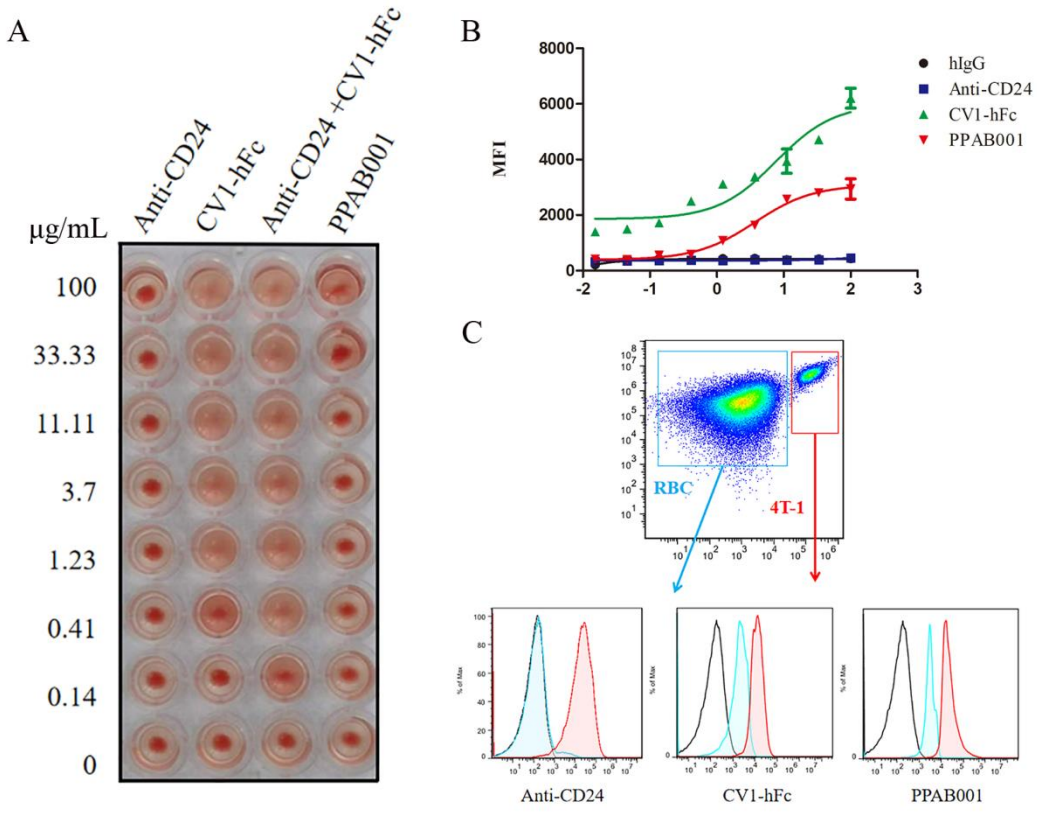

Figure S4. PPAB001 maintains binding to tumor cells, while markedly reducing binding to hRBCs. (A) Human red blood cell (hRBC) agglutination was induced by anti-CD24, CV1-hFc, CV1-hFc plus anti-CD24 or PPAB001. Non-agglutinated RBCs sediment and form a red dot in the bottom of the well. (B) Human red blood cell was incubated with gradient concentrations of hIgG, CV1-hFc, anti-CD24 or PPAB001. Antibody-bound cells were then washed and incubated with fluorescein isothiocyanate (FITC) conjugated goat anti-hIgG secondary antibody, and assessed by flow cytometry. Then, the result was expressed as mean fluorescence intensity (MFI). (C) 4T-1 tumor cells were mixed with a 10-fold excess of mouse blood cells. The cell mixture was incubated with 10 μg/mL of CV1-hFc, anti-CD24 or PPAB001 prior to staining with FITC anti-human IgG secondary antibody and detected by flow cytometry. White, negative control; Blue, RBCs; Red, 4T-1 cells . Data are shown as means ± SD (n = 3).

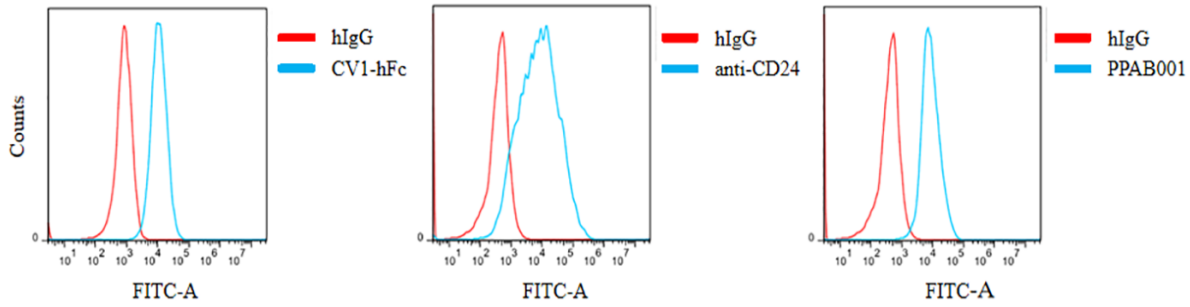

Figure S5. Binding activity analysis of the anti-CD24、CV1-hFc and PPAB001 to mouse 4T-1 cells. 10 μg/mL of each antibody was used in the flow cytometry analyses.

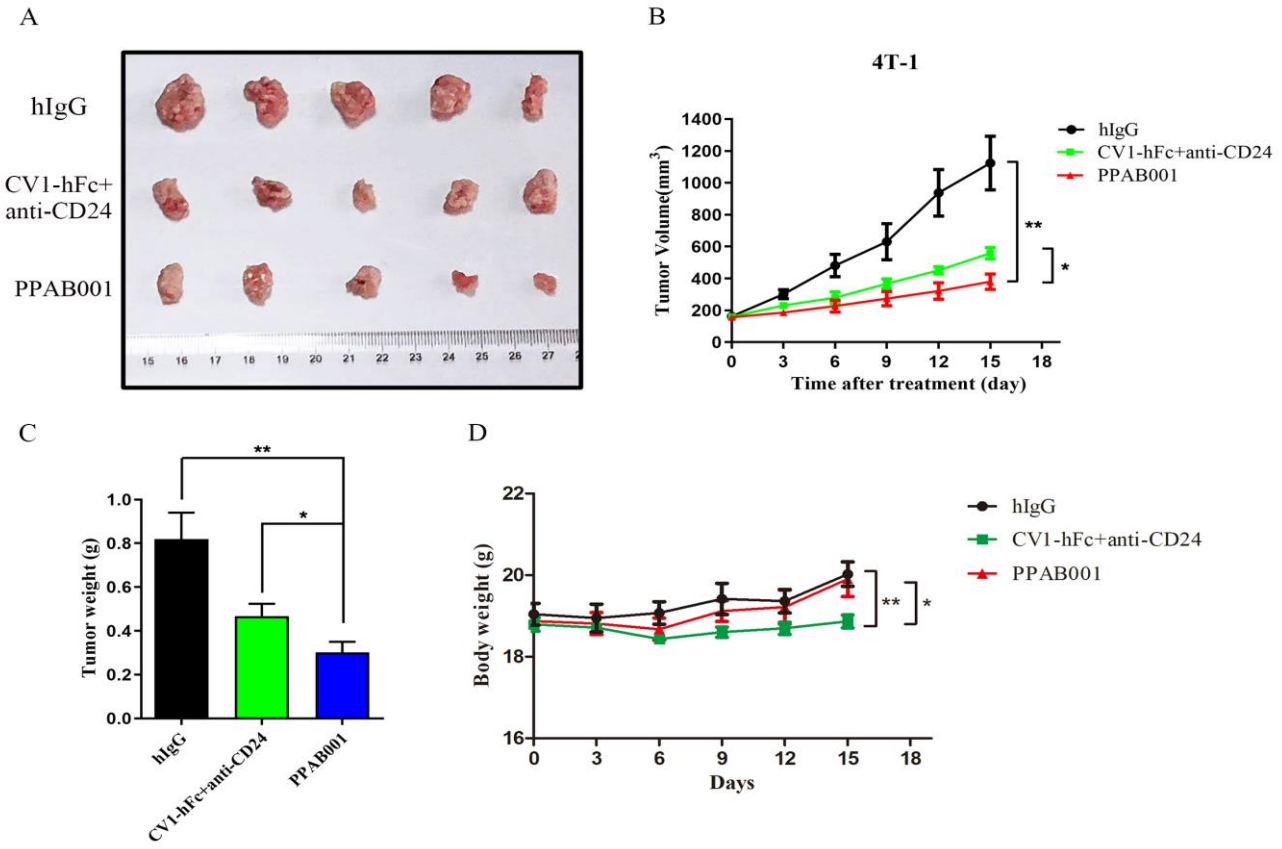

Figure S6. PPAB001 exhibits excellent anti-tumor activity in immunocompetent 4T-1 syngeneic tumor bearing mice. (A) Photos of excised tumors treated with hIgG, PPAB001 or a combination of CV1-hFc and anti-CD24 . (B) C57BL/6J mice were subcutaneously transplanted with 4T-1 cells and treated with hIgG, PPAB001(5 mg/kg), a combination of CV1-hFc with anti-CD24 antibody (2.5 mg/kg for each) (n=5). (C) After xenograft tumors were removed, these tumor masses were weighted.

(D) Body weight of tumor-bearing BALB/c mice on treatment with hIgG, PPAB001 or a combination of CV1-hFc with anti-CD24. Data are shown as means ± SEM (n=5) and statistical significance was determined by a Student's t test. *p < 0.05, **p < 0.01, mean the significant difference compared with hIgG-treated samples.

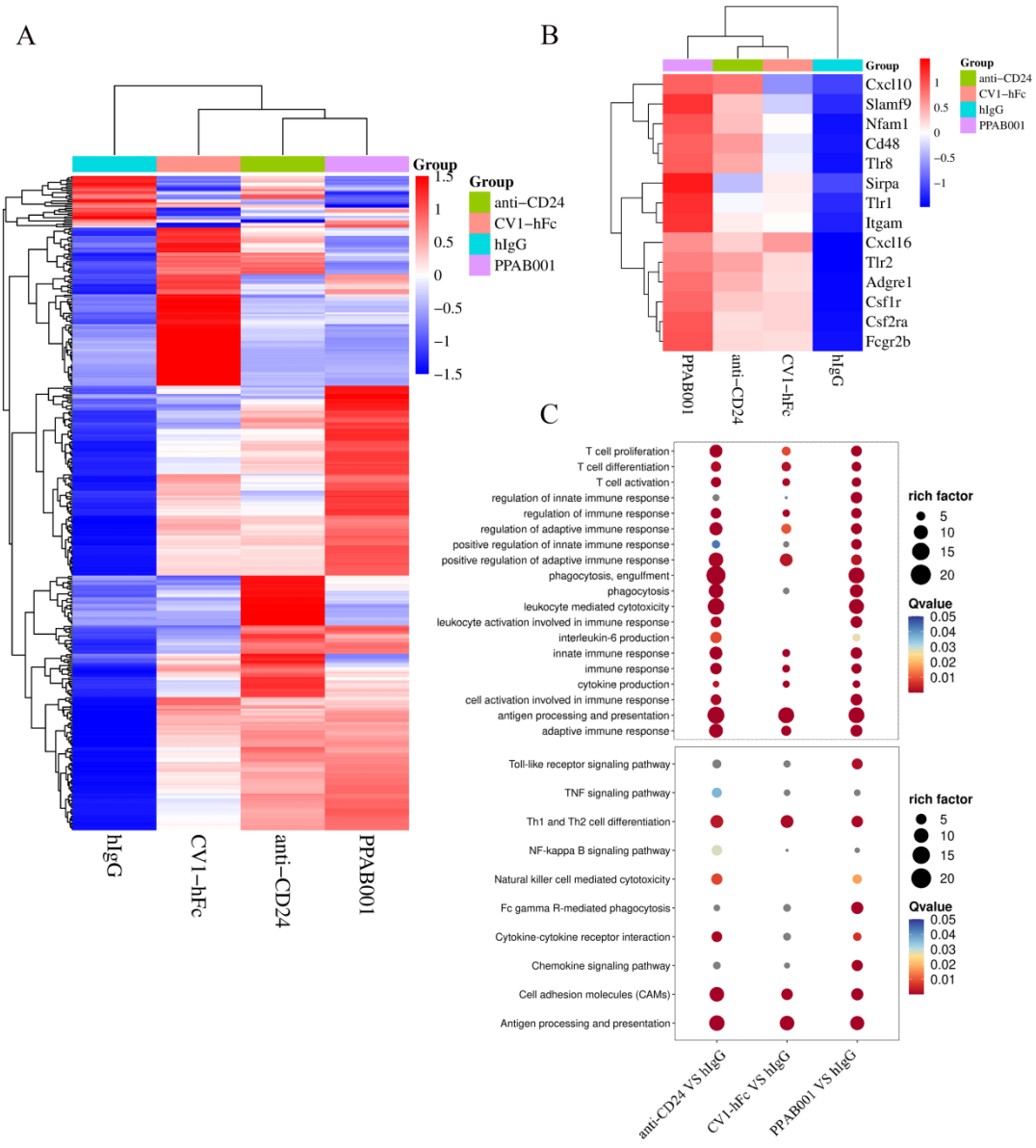

Figure S7. Bulk RNA-seq to evaluate immune landscape in 4T-1 tumors. (A) The heat map showing the average value of differentially expressed gene profiles. (B) The heat maps representing

the expression levels of genes belonging to immune signatures. (C) GO and KEGG enrichment analyses based on the identified DEGs.

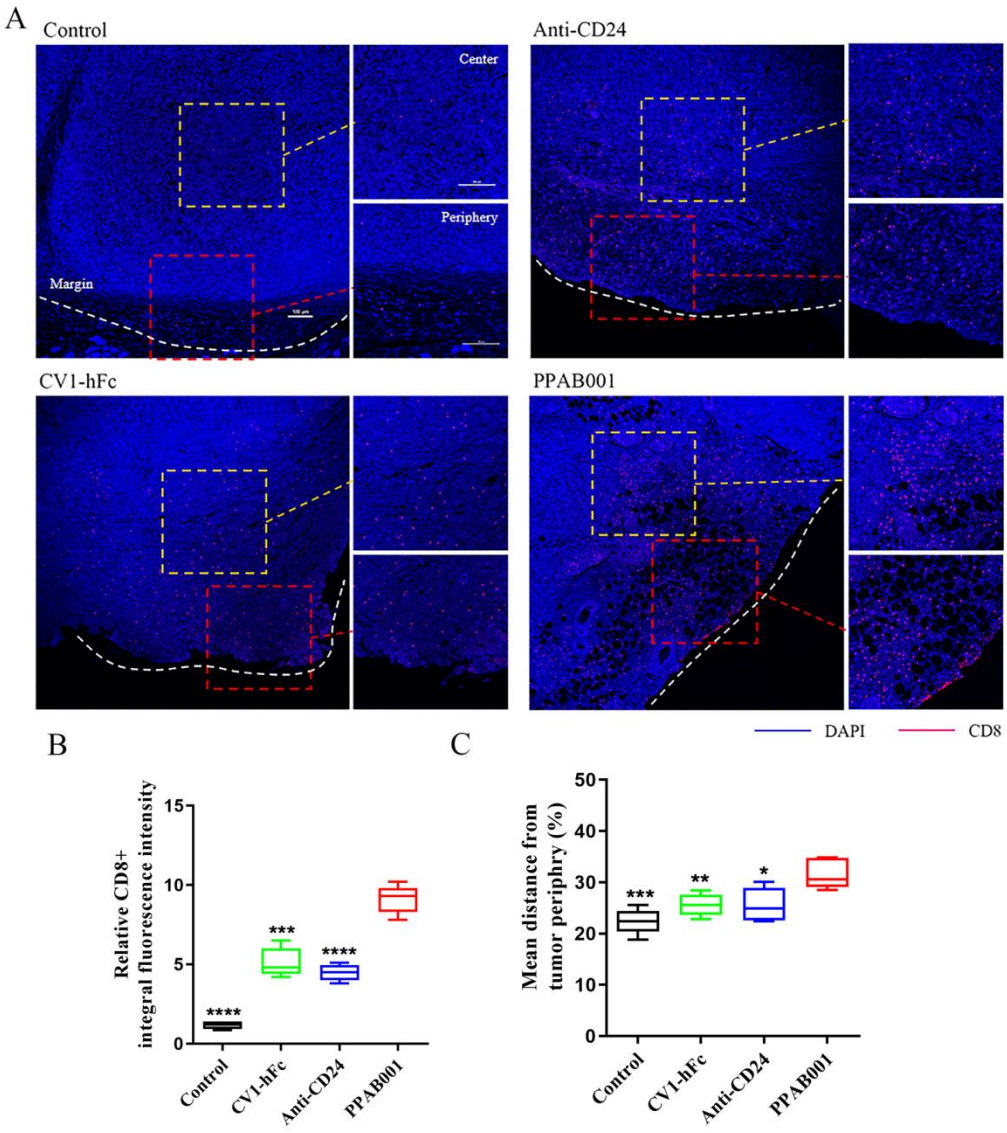

Figure S8. PPAB001 treatment promotes CD8+ T cells infiltration of 4T-1 tumors *in vivo*.

(A) Multiplex IF staining to evaluate the infiltration of CD8+ T cell. The presentative images of infiltrating CD8+ T cells. (B, C) The quantification of CD8+ pixel and infiltration depth. Data are shown as means ± SEM (n=5) and statistical significance was determined by a Student's t test. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 mean the significant difference compared with PPAB001-treated samples.