Supporting Information

Polydopamine/tumor associated antigen nanovaccine with robust prophylactic and therapeutic efficacy for cancer immunotherapy

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Materials:

Dopamine hydrochloride was purchased from Anegi Chemical Reagent Co. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and BSA were purchased from Yuanye Biotechnology Co. The BCA kit was purchased from Biosharp Co. SDS-PAGE gel rapid preparation kit and color pre-stained protein molecular weight standards (10-180 kD) were purchased from Beyotime Biotechnology (Shanghai, China). Lipopolysaccharide (LPS) was purchased from Yuanye Biotechnology Co. Rb a Calreticulin, Ra a HMGB1 and Donkey Anti-Rabbit IgG H&L/FITC were purchased from Proteintech Group, Lnc. CD80/B7-1 Monoclonal antibody, CD86 Polyclonal antibody, Fluorescein (FITC)–conjugated Affinipure Goat Anti-Mouse IgG(H+L) and Fluorescein (FITC)–conjugated Affinipure Goat Anti-Rabbit IgG(H+L) were purchased from Proteintech Group, Lnc. FITC anti-mouse CD3, PE anti-mouse CD4 and APC anti-mouse CD8a were purchased from DAKEWE Biotechnology Co. FITC anti-mouse/human CD11b, PE anti-mouse Ly-6G/Ly-6C (Gr-1), PE anti-mouse/human CD44 and FITC anti-mouse CD62L were purchased from DAKEWE Biotechnology Co. Mouse TNF-α Precoated ELISA Kit, Mouse IFN-γ Precoated ELISA Kit and Mouse IL-6 Precoated ELISA Kit were purchased from DAKEWE Biotechnology Co.

Characterization:

A transmission electron microscope (TEM) image was performed on JEOL JEM-1011 (Japan) with an acceleration voltage of 100 kV. The ultraviolet-visible absorption spectrum was performed on a Shimadzu UV-2450 spectrophotometer. Fluorescence emission spectra were performed on the LS-55 fluorophotometer. Zetasizer Nano-ZS (Malvern Instruments Ltd.) was used to analyze the Zeta potential of the nanoparticles. Endocytosis and immunofluorescence images were taken using the Operetta CLSTM High Content Analysis System (PerkinElmer, USA). The flow cytometry measurement was performed on a Becton Dickinson FACS Aria sorting flow cytometer (Becton-Dickinson, USA).

CRT exposure:

4T1 cells were inoculated into 96-well plates and incubated with Taxol (1 μM) for

24 h. The medium was then aspirated and discarded, and each well was washed three times with 100 μL PBS, following by the addition of 100 μL paraformaldehyde to fix the cells for 20 min. The paraformaldehyde was then aspirated and washed three times with PBS, and 150 μL BSA was added to each well to seal the cells at room temperature for 1 h. The BSA was then aspirated and washed three times with PBS, and 150 μL of primary antibody was added to each well and incubated overnight at 4 °C. Them, the primary antibody was aspirated and collected, and each well was washed three times with PBS for 3-5 min each time, followed by the addition of 150 μL of secondary antibody and incubation in a 4 °C for 1 h. Finally, the secondary antibody was aspirated and collected, and PBS was added three times. Hoechst (150 μL) was added to each well and incubated for 5 min away from light. Finally, the cells were analyzed using the Operetta CLS™ High Content Analysis System.

HMGB1 release:

4T1 cells were inoculated into 96-well plates and incubated with Taxol (1 μM) for 24 h. The medium was then aspirated and discarded, and each well was washed three times with 100 μL PBS, following by the addition of 100 μL paraformaldehyde to fix the cells for 20 min. The paraformaldehyde was then aspirated and discarded, PBS was added three times, then 200 μL Triton X-100 was added to each well and incubated for 5 min to disrupt the cell membrane and increase cell permeability, after which the Triton X-100 was aspirated and discarded, 150 μL BSA was added to each well to seal the cells at room temperature for 1 h. BSA was then aspirated and washed three times with PBS, and 150 μL primary antibody was added to each well and incubated overnight in a 4 °C refrigerator. Then, the primary antibody was aspirated and collected, and each well was washed three times with PBS for 3-5 min each time, followed by the addition of 150 μL of secondary antibody and incubation in a 4 °C refrigerator for 1 h. Finally, the secondary antibody was aspirated and collected, washed three times with PBS, and incubated at 4 °C for 1 h. 150 μL Hoechst was added to each well and incubated for 5 min away from light. Finally, the cells were analyzed using the Operetta CLS™ High Content Analysis System.

In vitro biocompatibility:

DC2.4 cells were inoculated into 96-well plates with different concentrations of PDA, 4T1-Ag and PDAT and incubated for 24 h. Then, 20 μL MTT was added to each well, 4 h later, 150 μL DMSO was added to each well. The absorbance of each well was measured at 490 nm using an enzyme marker.

Cellular endocytosis:

PDA, 4T1-Ag and PDAT were labeled with FITC. DC2.4 cells were inoculated into 96-well plates with PDA_{FITC} (50 μg mL^{-1}), 4T1-Ag_{FITC}, $PDAT_{FITC}$ (50 μg mL^{-1}) and incubated for 6 h. The supernatant was then discarded and the cells were washed three times with PBS. 150 μL paraformaldehyde was added to each well to fix the cells for 10 min, then the paraformaldehyde was aspirated and the cells were washed three times with PBS. Nucleation solution (Hoechst, 150 μL) was added to each well and incubated for 5 min at room temperature. The nucleation solution was then aspirated and washed three times with PBS. Finally, the cells were observed under the Operetta CLS™ High Content Analysis System.

Maturation of DC2.4 cells:

DC2.4 cells were inoculated into a six-well plate, followed by PDA (50 μg mL^{-1}), 4T1-Ag, PDAT (50 μg mL^{-1}), and LPS (50 μg mL^{-1}) and incubated for 12 h. The cells in each well were centrifuged (1500 rpm, 8 min), and the supernatant was collected to determine tumor necrosis factor (TNF-α), interferon-γ (IFN-γ), and interleukin 6 (IL-6) by ELISA. The cells were then stained with PE-CD80 and FITC-CD86 antibodies for 1 h. The cells were blocked with 5% BSA for 0.5 h, washed with PBS, and centrifuged at 1800 rpm for 10 min. The supernatant was discarded and 0.5 mL PBS was added to resuspend the cells. The expression of CD80 and CD86 on DCs was measured by flow cytometry.

Immunofluorescence:

DC2.4 cells were inoculated into 96-well plates and incubated with PDA (50 μg mL^{-1}), 4T1-Ag, PDAT (50 μg mL^{-1}), and LPS (50 μg mL^{-1}) for 12 h. The medium was then aspirated and discarded, and each well was washed three times with 100 μL PBS, followed by the addition of 100 μL paraformaldehyde to fix the cells for 20 min. The paraformaldehyde was then aspirated and washed three times with PBS, and 150 μL

BSA was added to each well to seal the cells at room temperature for 1 h. The BSA was then aspirated and washed three times with PBS, and 150 μL of primary antibody was added to each well and incubated overnight at 4 °C. Then, the primary antibody was aspirated and collected, and each well was washed three times with PBS for 3-5 min each time, followed by the addition of 150 μL secondary antibody and incubation at 4 °C for 1 h. Finally, the secondary antibody was aspirated and collected, PBS was added three times. 150 μL Hoechst was added to each well and incubated for 5 min without light. The coverslip containing the cells in each well was removed and slowly placed on a slide with a drop of glycerol, then the excess glycerol was blotted off with filter paper, and both sides of the coverslip were coated with clear nail polish and observed under the Operetta CLS™ High Content Analysis System.

Animal welfare statement:

All *in vivo* experiments were performed in compliance with the relevant guidelines. (Animal Ethics Standard: Tumor weight shall not exceed 10% of normal body weight, equivalent to a tumor diameter of less than 17 mm for a 25 g mouse.) This work has received approval (No. 2023-0132) for research ethics from Changchun Institute of Applied Chemistry Chinese Academy of Sciences.

In vivo tumor suppression assay in mice：

On day -15, 4T1 cells (20×10^5 cells/100 μL) were injected into the right side of the back of BalB/c mice and the left side of the back of BalB/c mice (10×10^5 cells/100 μL) to establish the tumor model. On day -8, the tumors started to grow. When the tumor volume reached approximately 80 mm^3 (day 1), the mice were injected subcutaneously with the nanovaccine once every 5 days for a total of 5 times. The dose was 5 mg kg^{-1} of nanovaccine per mouse. Body weight and tumor size were monitored every 3 d. After treatment, mice were killed and tumors and major organs were collected.

Animal experiments with nanovaccine prevention models:

The mice were first injected subcutaneously with PBS, PDA, 4T1-Ag or PDAT every five days for a total of five doses (the dose was 5 mg kg^{-1} of nanovaccine per mouse.), and three days after the last dose, 4T1 cells (20×10^5 cells/100 μL) were

injected into the back of BalB/c mice to establish a tumor model. The body weight and tumor volume of mice were monitored.

Detection of immunological indicators:

DC cells, T cells, and myeloid progenitor suppressor cells in lymph nodes and tumors were analyzed immediately after mice sacrifice. Tissues were crushed, rinsed with PBS, centrifuged, and cells were then stained with antibodies. Finally, the cells were washed with PBS and analyzed by flow cytometry.

Cytokines in mouse serum:

After treatment, blood was collected from the eyes of the mice. After centrifugation, the supernatant was collected and the expression of TNF-α, IFN-γ and IL-6 in mouse serum was analyzed by ELISA.

Histological analysis:

To evaluate the safety and anti-tumor effects of the different treatment groups, major organs (heart, liver, spleen, lung and kidney) and tumors were collected, and these tissue sections were stained with hematoxylin-eosin (H&E) for pathological analysis.

Immunofluorescence staining:

At the end of treatment, immunofluorescence staining of tumor tissues was performed to analyze the infiltration of T cells.

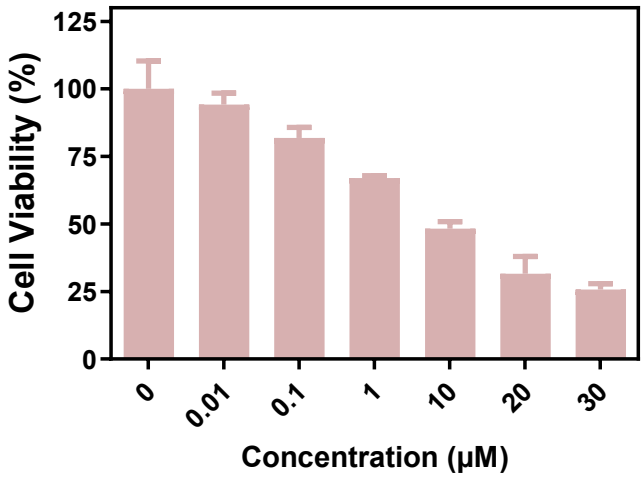

Figure S1. Cell viability of 4T1 cells after incubating with different concentration of Taxol for 48 h. Data are expressed as mean ± SD (significance analysis was performed using a two-tailed t-test. ns: not significant, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, $n = 3$).

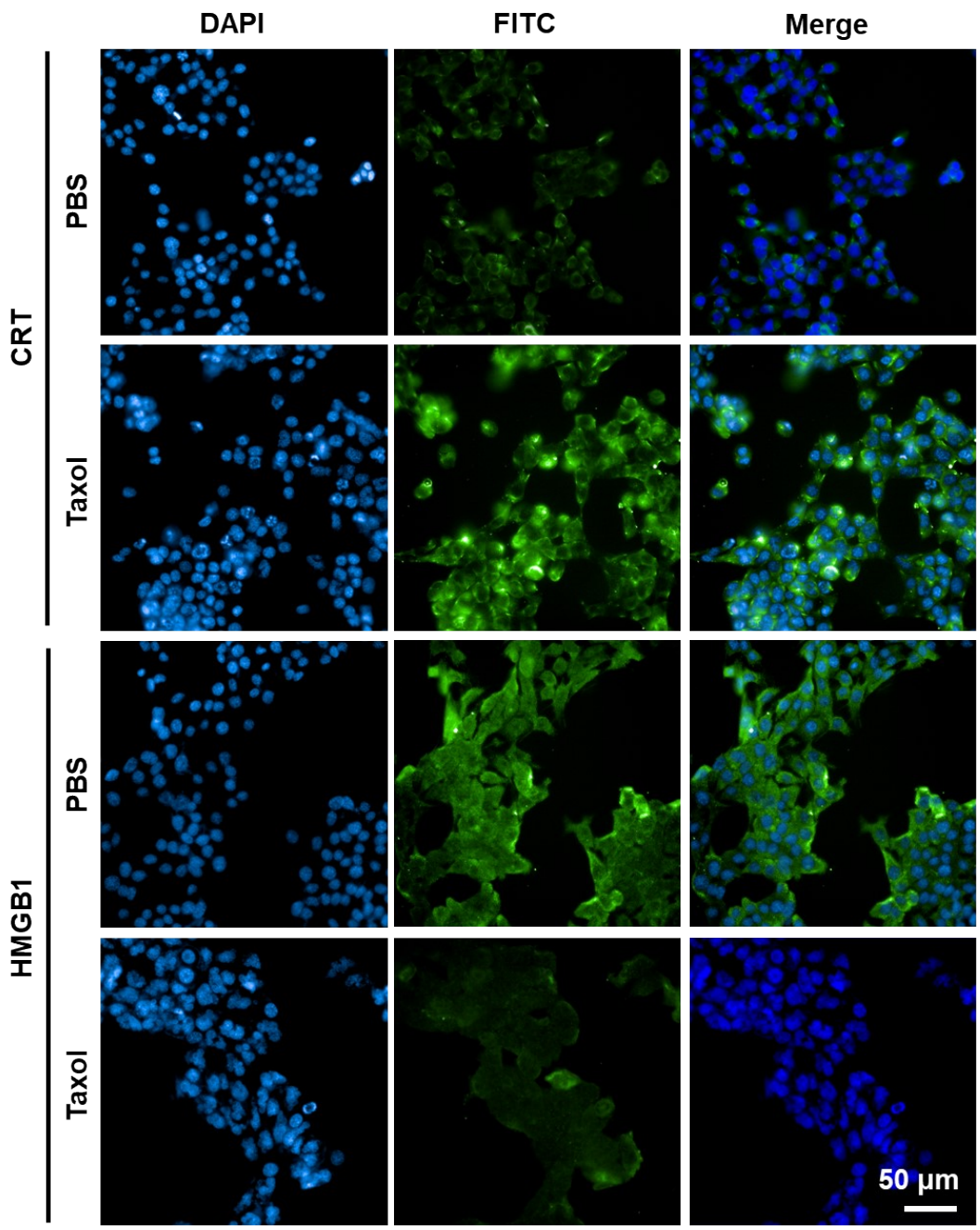

Figure S2. CLS^{TM} High Content images of CRT exposure and HMGB1 release in 4T1 cells.

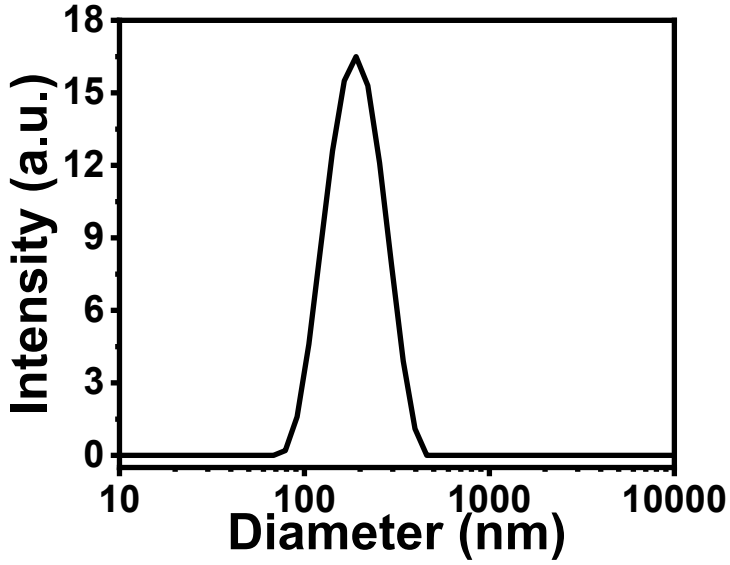

Figure S3. HD of PDA.

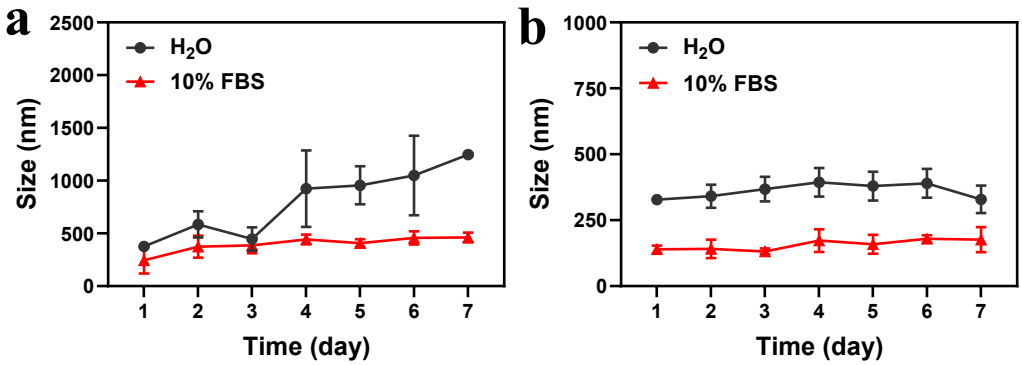

Figure S4. The size change of (a) PDA and (b) PDAT in water and 10% FBS within 7 d. Data are expressed as mean ± SD (significance analysis was performed using a two-tailed t-test. ns: not significant, *p < 0.05, **p < 0.01, and ***p < 0.001, n = 3).

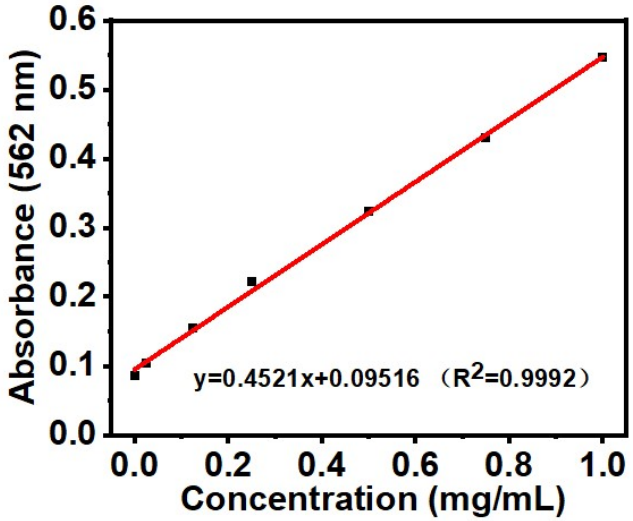

Figure S5. BSA standard curve of BCA protein detection kit.

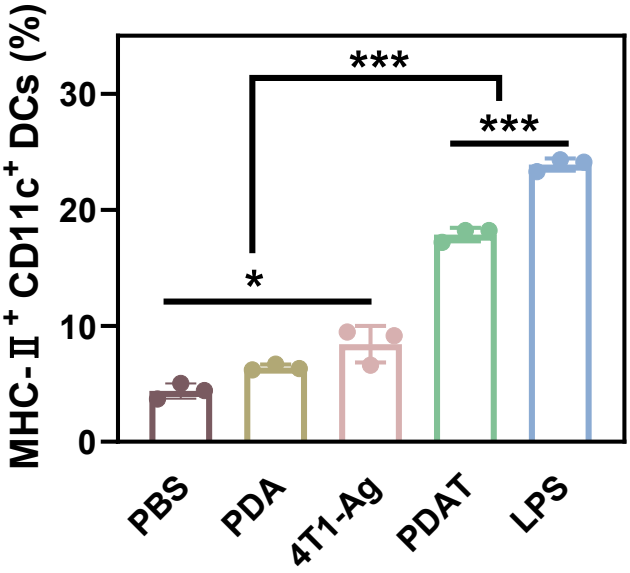

Figure S6. MHC-II expression after 12 h of incubation of PBS, PDA, 4T1-Ag, PDAT and LPS with DC2.4 cells. Data are expressed as mean ± SD (significance analysis was performed using a two-tailed t-test. ns: not significant, *p < 0.05, **p < 0.01, and ***p < 0.001, n = 3).

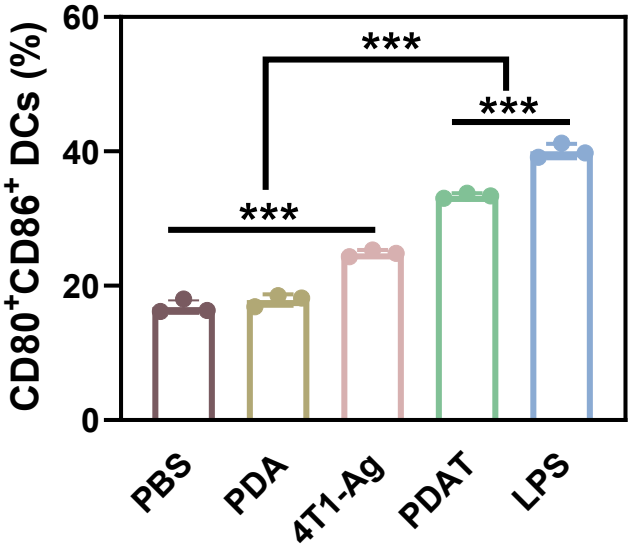

Figure S7. DC maturation after 12 h incubation of PBS, PDA, 4T1-Ag, PDAT and LPS with DC2.4 cells. Data are expressed as mean ± SD (significance analysis was performed using a two-tailed t-test. ns: not significant, *p < 0.05, **p < 0.01, and ***p < 0.001, n = 3).

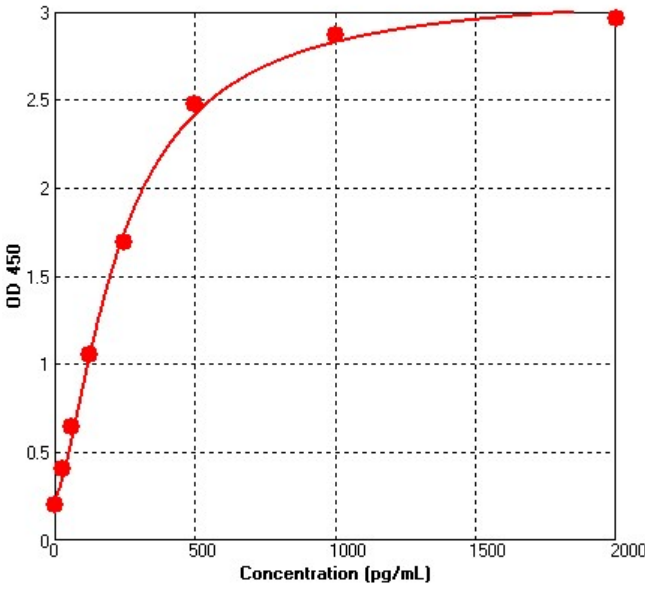

Figure S8. The standard curve of TNF-α in the ELISA test kit: $y=2.88426/[1+(x/233.30036)^{-1.48372}] + 0.24368$ ($R^2=0.9983$)

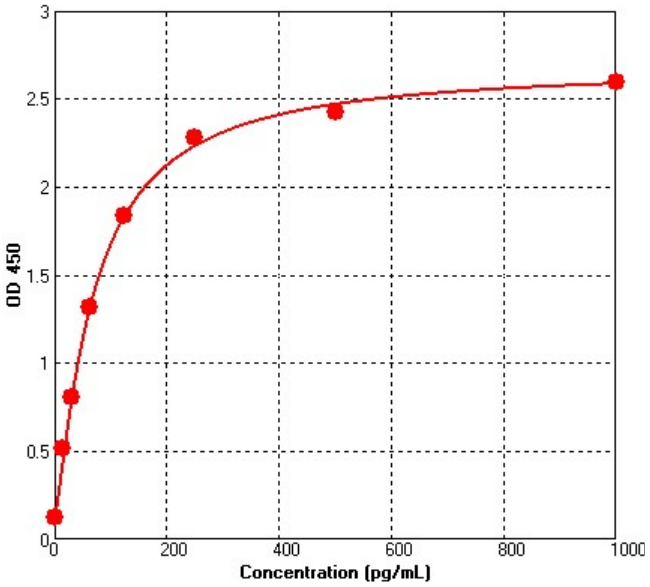

Figure S9. The standard curve of IFN-γ in the ELISA test kit:

$y=2.543/[1+(x/70.05986)^{-1.22338}] + 0.13744$ ($R^2=0.9991$)

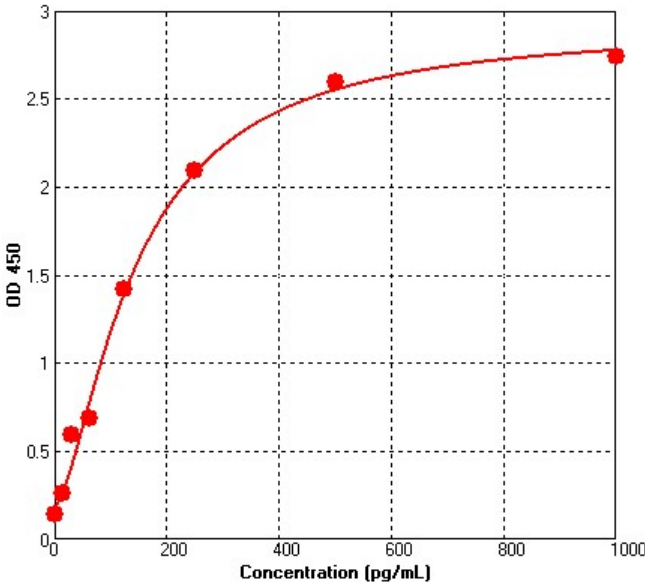

Figure S10. The standard curve of IL-6 in the ELISA test kit:

$y=2.72854/[1+(x/143.76495)-^{1.50499}] + 0.18689$ ($R^2=0.9947$)

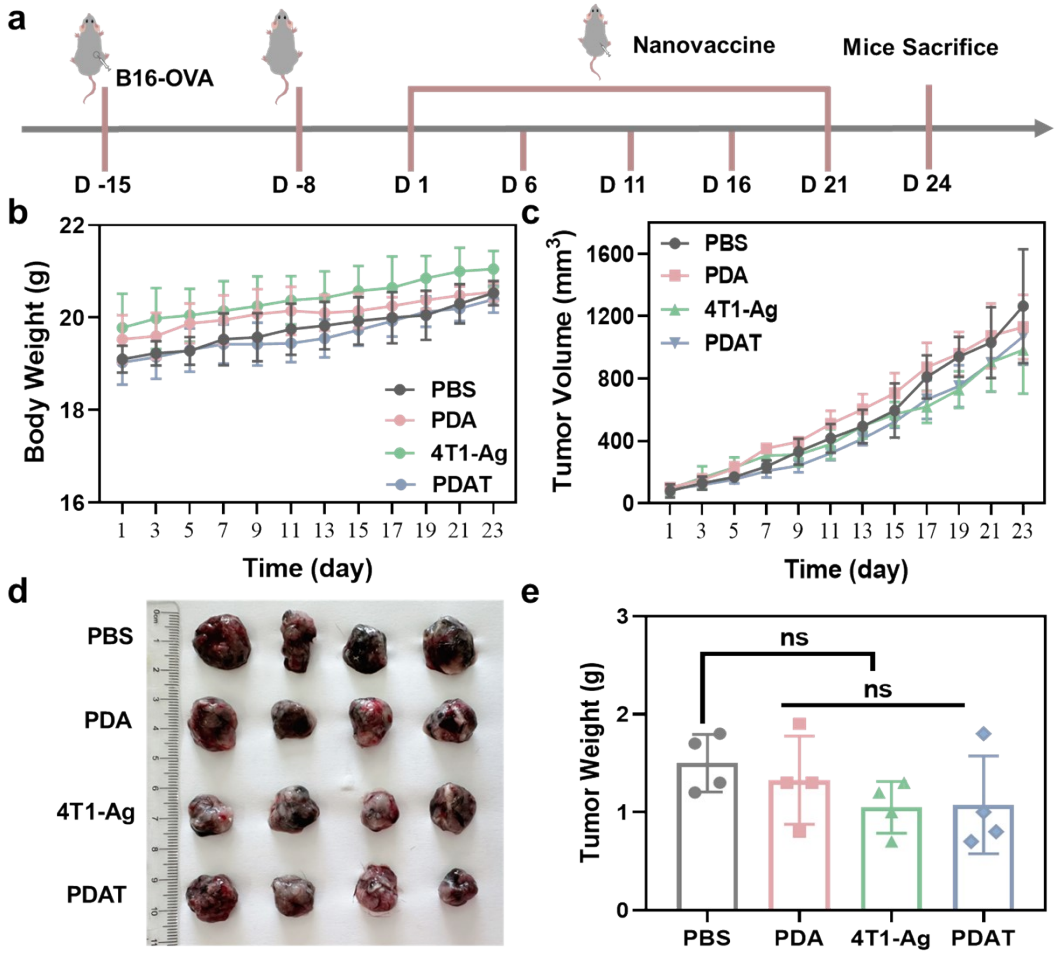

Figure S11. (a) Timeline of treatment of B16-OVA tumors in mice. (b) The curve of mean body weight versus time. (c) The curve of mean tumor volume versus time. (d) Pictures of tumors. (e) Mean weight of tumors. Data are expressed as mean ± SD (significance analysis was performed using a two-tailed t-test. ns: not significant, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, $n = 4$).

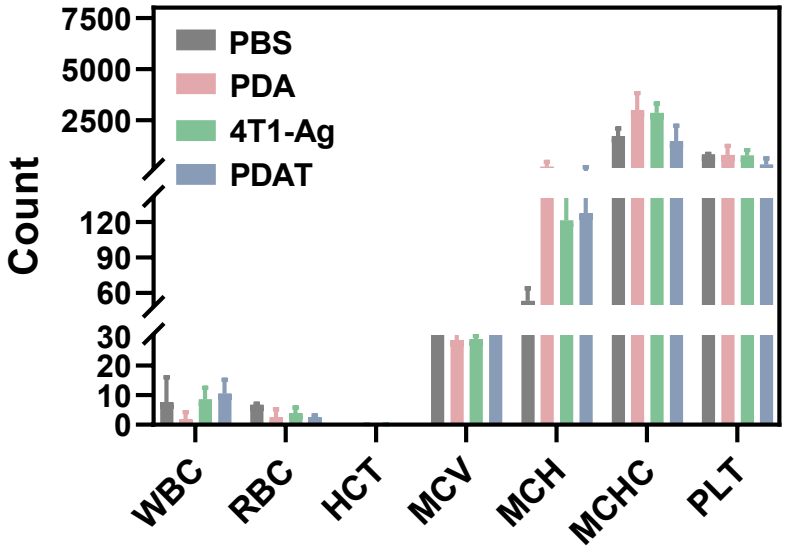

Figure S12. Hematological data of the mice after treatment with PBS, PDA, 4T1-Ag, and PDAT. Data are expressed as mean ± SD (significance analysis was performed using a two-tailed t-test. ns: not significant, *p < 0.05, **p < 0.01, and ***p < 0.001, n = 3).

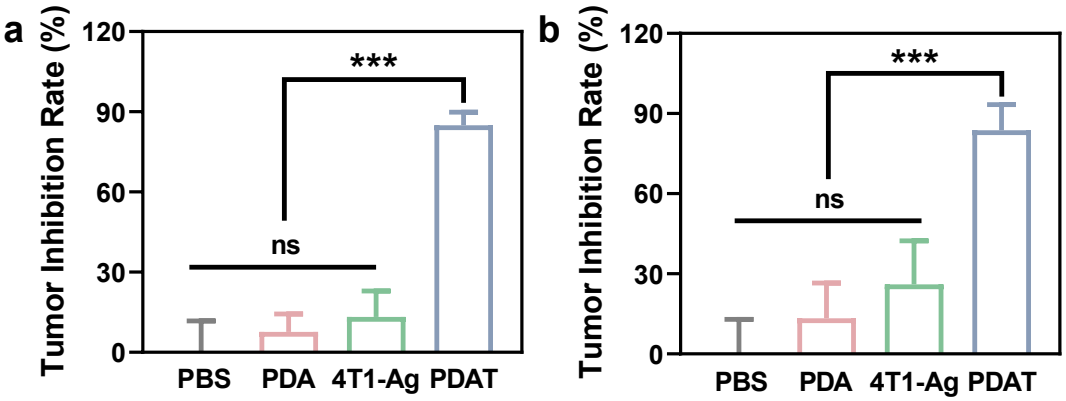

Figure S13. (a) Primary tumor and (b) distant tumor inhibition rates in the treatment groups. Data are expressed as mean ± SD (significance analysis was performed using a two-tailed t-test. ns: not significant, *p < 0.05, **p < 0.01, and ***p < 0.001, n = 5).

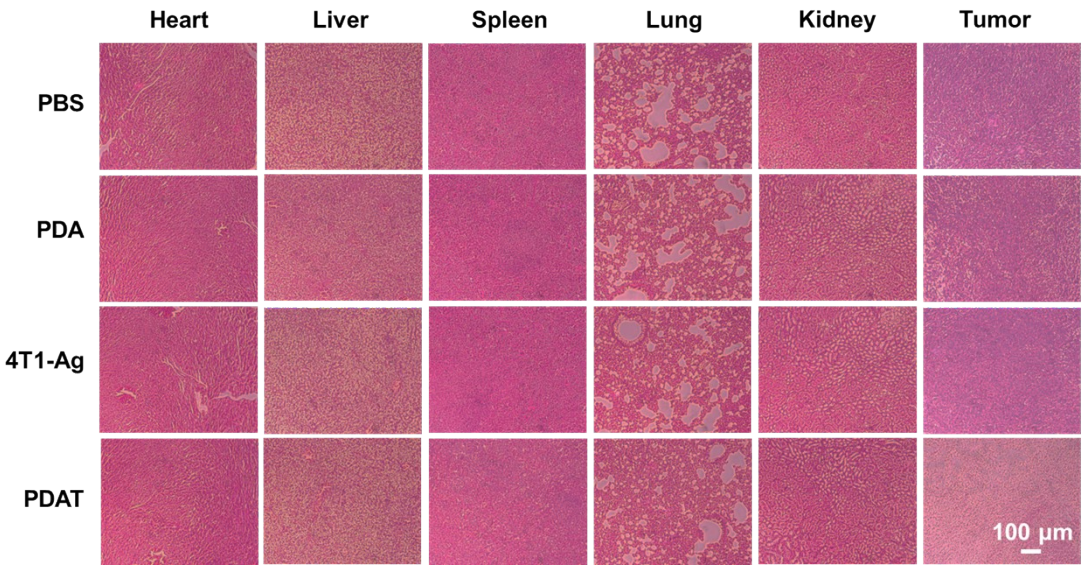

Figure S14. H&E staining of the major organs and tumors of the mice.

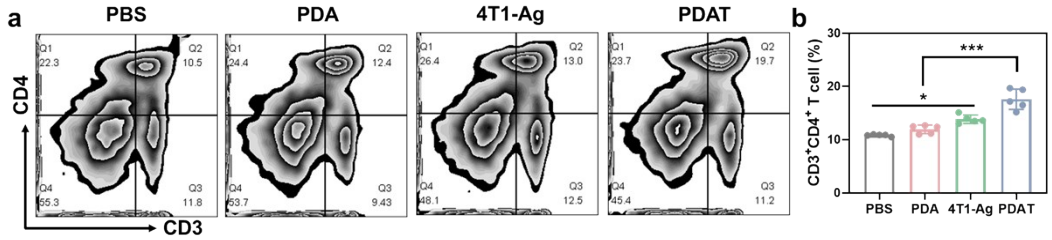

Figure S15. Proportion of $CD3^+$ $CD4^+$ T cells in LNs. Data are expressed as mean ± SD (significance analysis was performed using a two-tailed t-test. ns: not significant, *p < 0.05, **p < 0.01, and ***p < 0.001, n = 5).

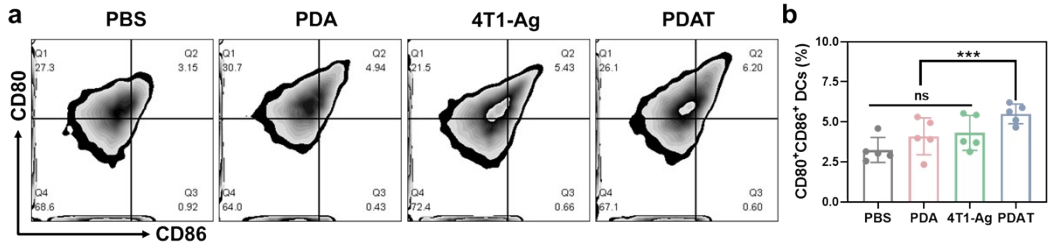

Figure S16. Proportion of mature DC cells in spleens. Data are expressed as mean ± SD (significance analysis was performed using a two-tailed t-test. ns: not significant, *p < 0.05, **p < 0.01, and ***p < 0.001, n = 5).

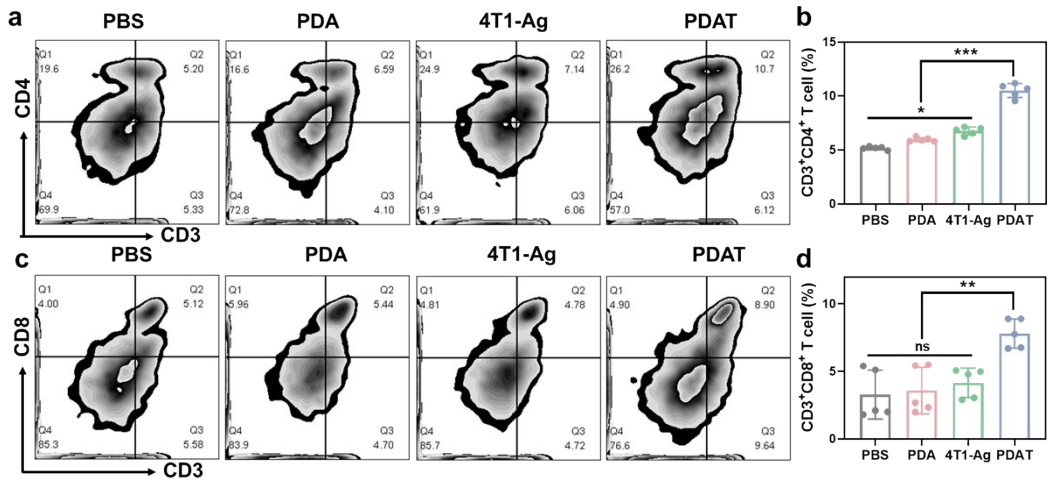

Figure S17. (a, b) Proportion of $CD3^+$ $CD4^+$ T cells in spleens. (c, d) Proportion of $CD3^+$ $CD8^+$ T cells in spleens. Data are expressed as mean ± SD (significance analysis was performed using a two-tailed t-test. ns: not significant, *p < 0.05, **p < 0.01, and ***p < 0.001, n = 5).

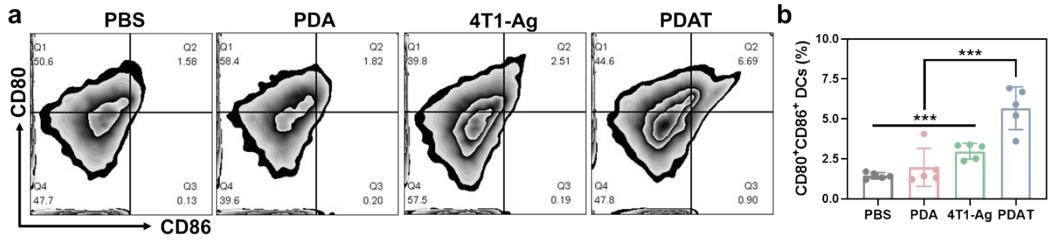

Figure S18. Proportion of mature DC cells in tumors. Data are expressed as mean ± SD (significance analysis was performed using a two-tailed t-test. ns: not significant, *p < 0.05, **p < 0.01, and ***p < 0.001, n = 5).

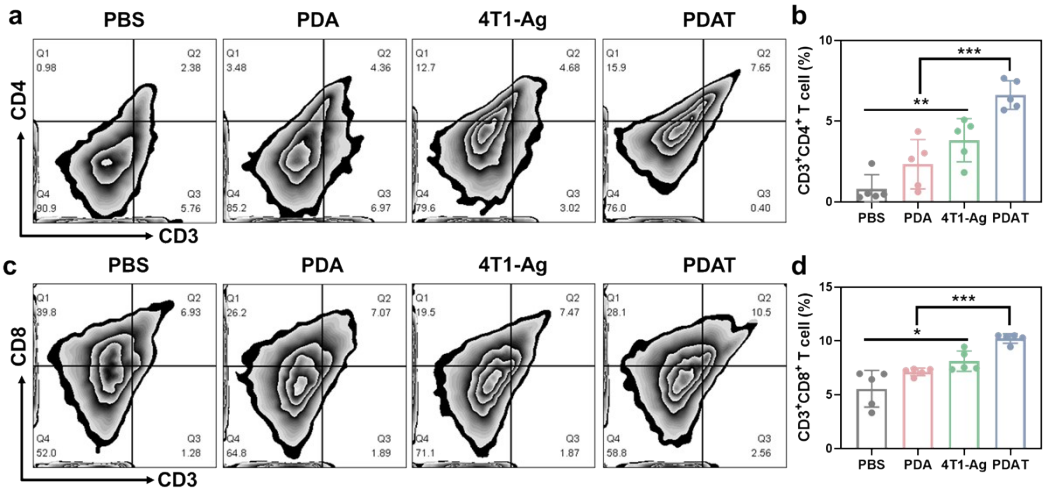

Figure S19. (a, b) Proportion of $CD3^+$ $CD4^+$ T cells in tumor. (c, d) Proportion of $CD3^+$ $CD8^+$ T cells in tumors. Data are expressed as mean ± SD (significance analysis was performed using a two-tailed t-test. ns: not significant, *p < 0.05, **p < 0.01, and ***p < 0.001, n = 5).

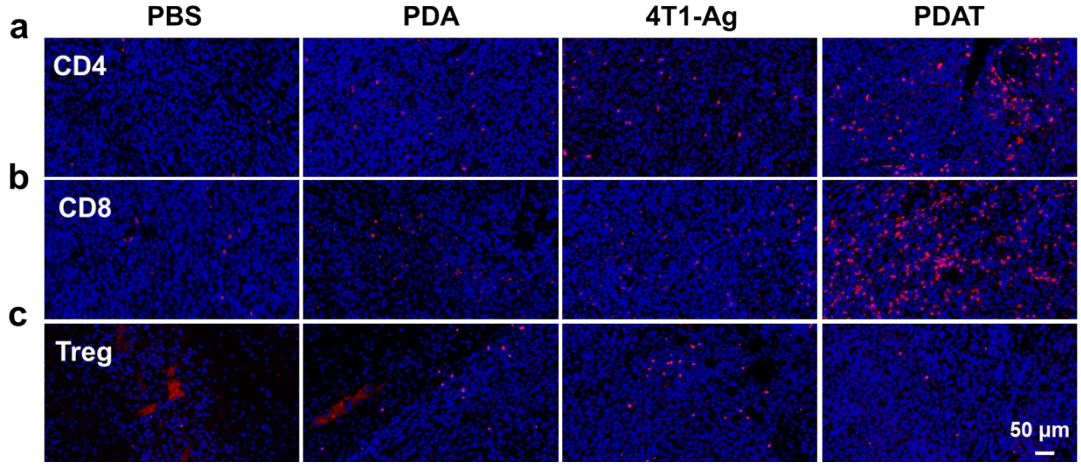

Figure S20. Immunofluorescence analysis of tumor tissues. Infiltration of (a) $CD4^+$ T cells, (b) $CD8^+$ T cells, and (c) Tregs in the tumor tissues.

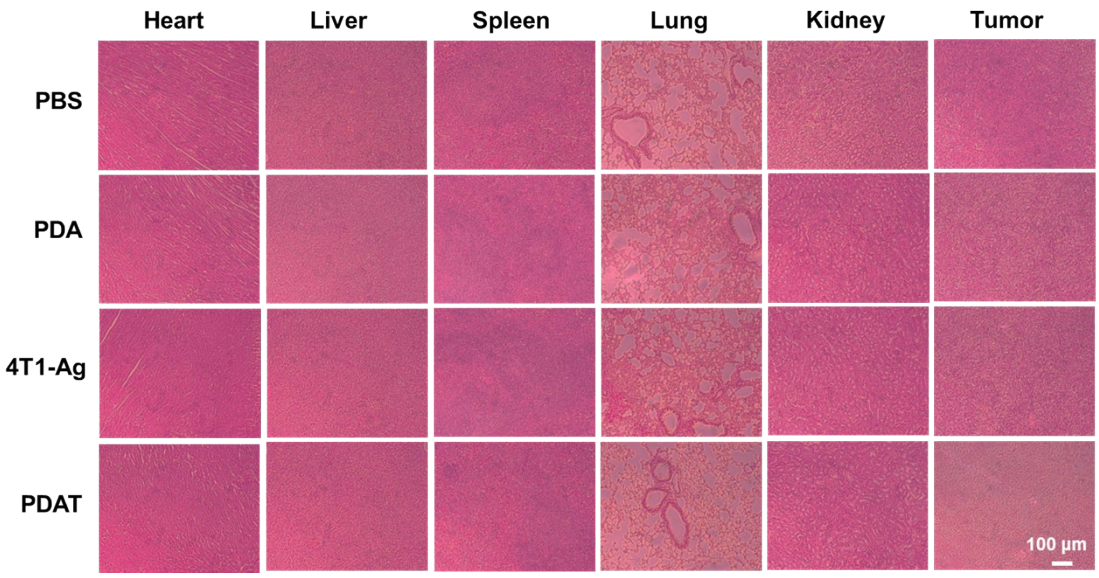

Figure S21. H&E staining of the major organs and tumors of the mice.

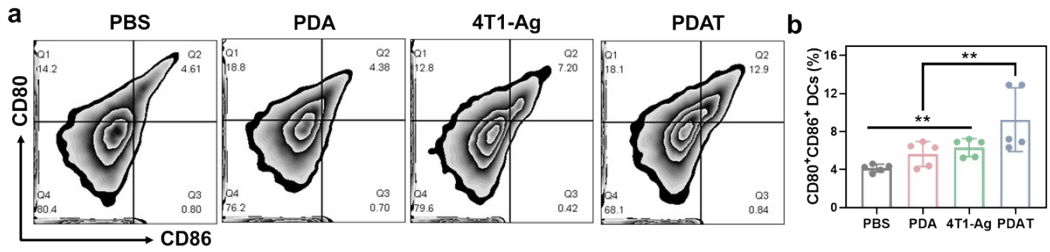

Figure S22. Proportion of mature DCs in the spleens. Data are expressed as mean ± SD (significance analysis was performed using a two-tailed t-test. ns: not significant, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, $n = 5$).

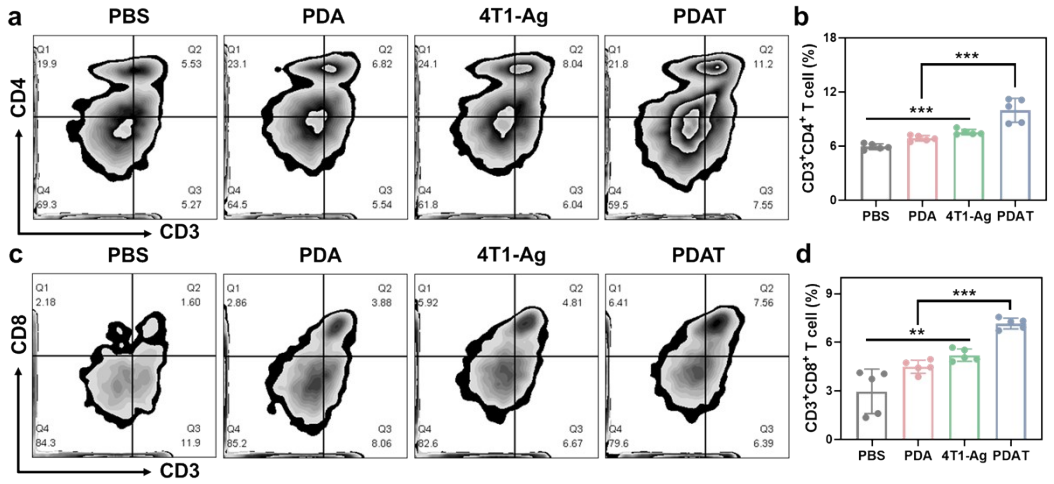

Figure S23. (a, b) Proportion of $CD3^+$ $CD4^+$ T cells in spleens. (c, d) Proportion of $CD3^+$ $CD8^+$ T cells in spleens. Data are expressed as mean ± SD (significance analysis was performed using a two-tailed t-test. ns: not significant, *p < 0.05, **p < 0.01, and ***p < 0.001, n = 5).

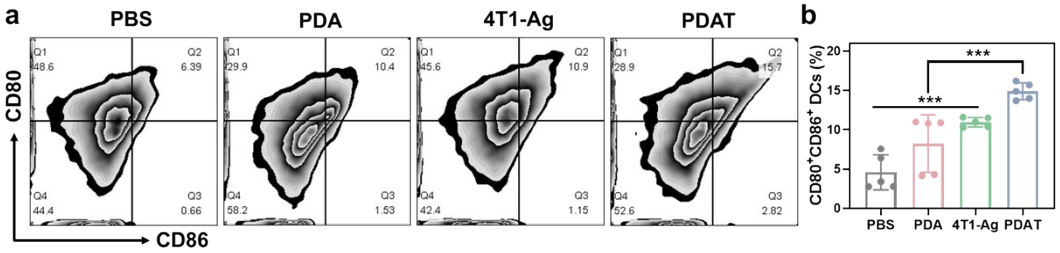

Figure S24. Proportion of mature DCs in tumors. Data are expressed as mean ± SD (significance analysis was performed using a two-tailed t-test. ns: not significant, *p < 0.05, **p < 0.01, and ***p < 0.001, n = 5).

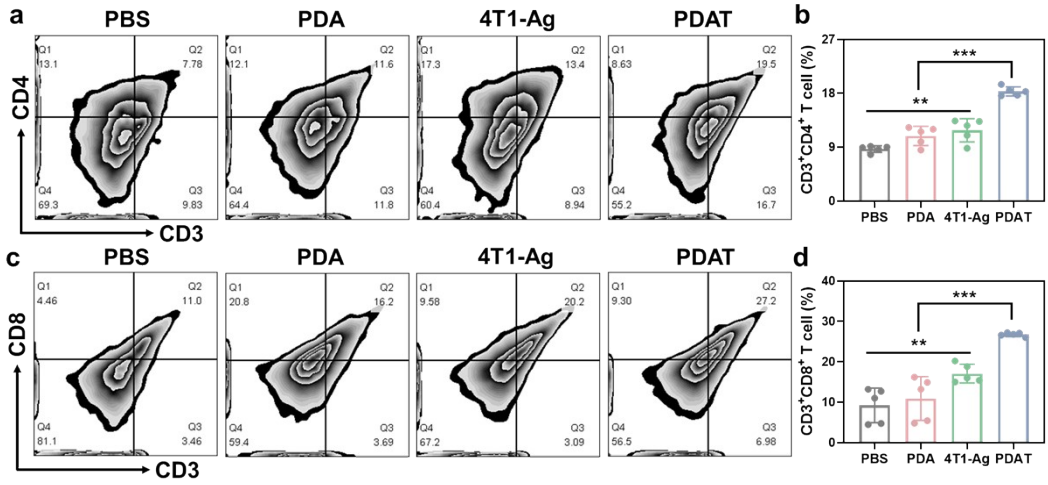

Figure S25. (a, b) Proportion of $CD3^+$ $CD4^+$ T cells in tumors. (c, d) Proportion of $CD3^+$ $CD8^+$ T cells in tumors. Data are expressed as mean ± SD (significance analysis was performed using a two-tailed t-test. ns: not significant, *p < 0.05, **p < 0.01, and ***p < 0.001, n = 5).

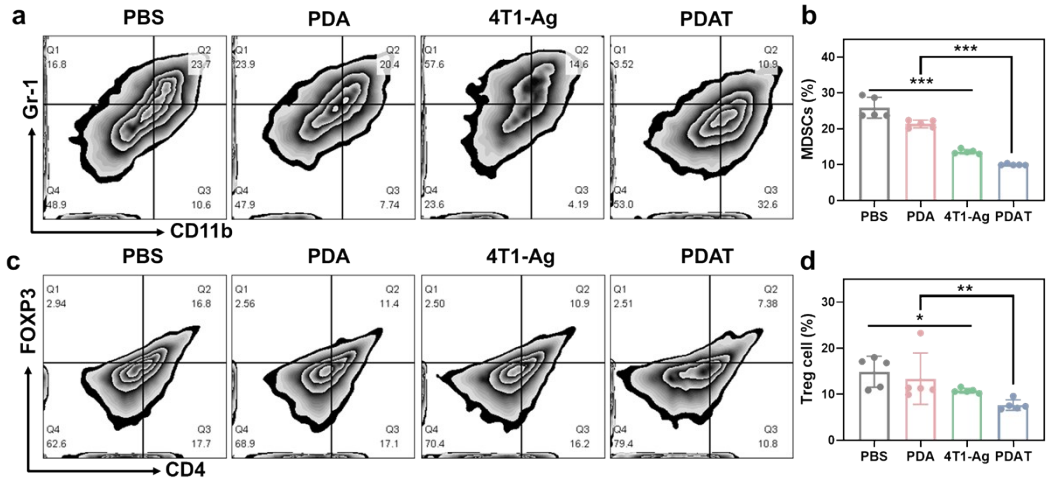

Figure S26. Percentage of (a, b) MDSCs and (c, d) Tregs in tumors. Data are expressed as mean ± SD (significance analysis was performed using a two-tailed t-test. ns: not significant, *p < 0.05, **p < 0.01, and ***p < 0.001, n = 5).