Supplementary Information for

Aptamer-assisted tumor localization of bacteria for enhanced biotherapy

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Materials and strains

1-Ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from Adamas-beta (Shanghai, China). Roswell park memorial institute (RPMI) 1640 medium antibiotic/anti-mycotic solution and phosphate buffered saline (1 × PBS) were provided by Sigma-Aldrich (USA). AS1411 (5'-GGT GGT GGT GGT TGT GGT GGT GGT GGT TTT TTT TTT TT-NH_2-3') and TLS11a (ACA GCA TCC CCA TGT GAA CAA TCG CAT TGT GAT TGT TAC GGT TTC CGC CTC ATG GAC GTG CTG TTT-NH_2-3') were synthesized by Tsingke Biological Technology Co., Ltd (Shanghai, China). EcN and *Salmonella typhimurium* were purchased from China general microbiological culture collection center (GMCC, China). 4T1 mammary gland carcinoma cell line was obtained from American type culture collections (ATCC) and H22 hepatocellular carcinoma cell line was purchased from BeNa Culture Collection (BNCC). Both cell lines were cultured in Dulbecco's modified eagle medium (Sigma, USA) supplemented with 10 % (v/v) inactivated foetal bovine serum (FBS) (Sigma, USA) and 1% (v/v) antibiotic/anti-mycotic solution (Sigma, USA) at 37 °C in an incubator with 5% CO_2. Regular mycoplasma evaluations were performed of the cell culture environment to ensure the absence of mycoplasma contamination. Plasmids pBBR1MCS2-Tac-GFP, pMD18-luxCDABE and all other reagents were purchased from domestic suppliers and used as received.

Animals

BALB/c female mice (6~8 weeks, 18-20 g) were provided by Jiesijie laboratory

animal center (Shanghai, China). The protocol of animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University School of Medicine.

Characterization

Photoluminescence (PL) spectra were measured on a FluoroMax-4 spectro-fluorometer. The fluorescence images of cells were characterized by confocal laser scanning microscopy (CLSM, Leica, SP8). Flow cytometric (Beckman CytoFlex) analysis was conducted for quantitative detection of cellular fluorescence. In vivo imaging of mice was performed by in vivo imaging system (Caliper LifeSciences, USA). Morphology observation was performed on a scanning electron microscopy (SEM) (Sirion 200, USA).

Growth of bacteria

EcN carrying pBBR1MCS2-Tac-GFP, or pMD18-luxCDABE were grown at 37 °C overnight in 10 mL liquid LB medium with supplement of 50 μg/ml kanamycin. *Salmonella typhimurium* were grown at 37 °C in tryptic soy broth (TSB) medium. Overnight culture was diluted 1:50 (v/v) to fresh LB liquid medium and grown at 37 °C for 2-3 hours. Bacteria were collected by centrifugation at 6000 ×g for 10 min and resuspended in ice-cold PBS. Bacterial counts were determined by making dilutions of bacterial suspension, culturing them on LB agar plates at 37 °C overnight and counting the colony forming units (CFU).

Preparation of ApCB

Amino-functionalized aptamer was linked on the surface of bacteria by amide condensation. Briefly, 1×10^8 of bacterial cells were dispersed in PBS solution, and 0.1 mL of Amino-functionalized aptamer (0.05 μmol/mL) was added into the bacterial suspension together with 0.55 mg EDC and 0.65 mg NHS at room temperature. After stirring for 3 h, the modified bacteria were separated by centrifugation (10000 rpm, 1 min) and washed with PBS for three times.

Serum stability

Serum stability of ApCB (1×10^9) was evaluated by incubating in 90% phosphate-buffered serum solutions at 37 °C for pre-determined time points (0, 1, 4, 8, 12, 24, and 48 h). At the end of each time point, the fluorescence emission intensity was measured. The samples were placed in quartz cuvettes and excited at 650 nm, while the emission spectra were collected at 670 nm at room temperature.

Cellular uptake

Cells were seeded at a density of 2×10^5 cells per confocal dish in 1 ml RPMI-1640 medium with 10% FBS and antibiotics. 4T1 cells were incubated for 24 h at 37 ℃ in 5% CO_2. Then, the medium was replaced by fresh 1640 medium and treated with equal volume of EcN or ApCB at 37 ℃ for 2 h. Subsequently, after being washed with PBS for three times, the cells were viewed using LSCM. For quantitative analysis of the uptake behavior, the cells were digested and collected for flow

cytometric analysis. To directly observe the binding of bacteria with cancer cells, the co-incubated cells were fixed with 4% glutaraldehyde in 0.5 mL of PBS at 4 ℃ for 40 min. Next, the cells were dehydrated in a series of ethanol/water solution with increasing ethanol content from 30% to 100%. Finally, the associated ethanol in the cells was removed by freeze-drying.

Subcutaneous tumor model

The experiments were performed on female BALB/C mice (6-8 weeks) bred under specific-pathogen-free (SPF) conditions for 6 days. For both 4T1 and H22 tumor models, 1×10^6 cells in 100 μL of serum-free 1640 medium were injected subcutaneously into the right hind leg of each BALB/C mouse. The tumor volume of each mouse was measured by caliper and calculated using formula: $(\text{width})^2 \times \text{length} \times 0.5$.

In vivo binding of bacteria with cancer cells

To develop a 4T1 tumor-bearing mouse model, 1×10^6 cells in 100 μL of serum-free 1640 medium were injected into the right hind leg of each Balb/c nude mouse. The inoculated mice were grouped randomly into three groups including PBS, EcN (1×10^7 CFU) and 5ApCB (1×10^7 CFU) after the size of tumors reached ~200 cm^3. A volume of 100 μL of each above-mentioned solution was administered through the tail vein. For bacterial staining, mice were euthanatized to sample the tumor tissues at day 12 post-injection. The samples were fixed in 4% paraformaldehyde fixative and then transferred to sucrose-containing PBS. After overnight immersion at 4 °C, the samples

were cut into slides and incubated with FITC-labelled anti-*Escherichia coli* antibody in PBS containing 0.1% normal serum for 1-2 hours at room temperature in dark. Cell nuclei were stained by 4',6-diamidino-2-phenylindole (DAPI). After washing with PBS for three times, the samples were mounted for imaging.

Biodistribution

To study the in vivo biodistribution of EcN and 5ApCB, the mice bearing 4T1 tumor were injected via the tail vein with 0.1 mL of normal saline containing 1×10^7 CFU of EcN or 5ApCB. Four mice were used for each group. All the mice were imaged by in vivo imaging system at the indicated time points and then sacrificed for tissue collection. The liver, spleen, lung, heart, kidney, and tumor were sampled and homogenized in a glass homogenizer. Equal weight of each homogenate was diluted serially with LB and 50 μl of each dilution was spread onto LB agar plates with antibiotics before overnight incubation at 37 °C for bacterial counting.

Tumor imaging

To develop a 4T1 tumor-bearing mouse model, 1×10^6 cells in 100 μL of serum-free 1640 medium were injected into the right hind leg of each Balb/c nude mouse. The inoculated mice were grouped randomly after the tumors reaching ~100 cm^3 ($n = 4$ for each group). To examine the accumulation of EcN and 5ApCB in the tumor, the tumor bearing mice were intravenously injected with 1×10^7 CFU of EcN or 5ApCB expressing LuxCDABE. Fluorescence imaging was carried out at 12, 48 and 60 h

post-injection. All the mice were imaged by in vivo imaging system and sacrificed at the predetermined time points for further analysis.

In vivo bacterial-mediated cancer therapy and histological analysis

Tumor-bearing mice were divided into three groups after the size of the tumors reaching ~100 cm^3 (n = 5 for each group), including PBS, VNP (5×10^5 CFU) and 5ApCB or T-5ApCB (5×10^5 CFU). 100 μL of each above-mentioned solution was administered through the tail vein. The size of tumor was measured every other day with a digital caliper. For histology analysis, the tumors were harvested, fixed in 4% paraformaldehyde solution, embedded in paraffin, and then stained with hematoxylin and eosin (H&E). Meanwhile, the tumor tissues of each group were collected for immunofluorescence staining analysis of TUNEL and TNF-α.

Flow cytometric assay for immune responses

Tumor tissues were harvested, treated with 1 mg/mL collagenase I (Gibco) for 1 h and ground using the rubber end of a syringe. Cells were filtered through nylon mesh filters and washed with PBS. Cells were further stained with corresponding fluorochrome-conjugated antibodies. To analyze the T cell subsets in draining tumor, single cell suspensions prepared from these samples were examined by flow cytometry. The following primary antibodies were used: anti-CD3-APC (eBioscience, catalog: 17-0030-82), anti-CD8-PE (eBioscience, catalog: 12-0084-82), anti-IFN-γ-PE-Cy7 (BD eBioscience, catalog: 25-7311-82), and anti-Ki67-FITC (BD

eBioscience, catalog: 11-5698-82). Antibodies were used at a dilution of 1:200. Data analysis was acquired on a CytoFLEX and analyzed using CytExpert (Beckman Coulter, USA) and FlowJo (TreeStar, USA) software.

Statistical analysis

All data were presented as means ± standard deviation (SD). Statistical analysis was performed using Prism 8.0 (GraphPad, USA). Unpaired two tailed Student's *t*-test was used for comparison between two groups. A variance similarity test (F-test) was performed before the *t*-test. Differences were considered statistically significant if $p < 0.05$ ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$).

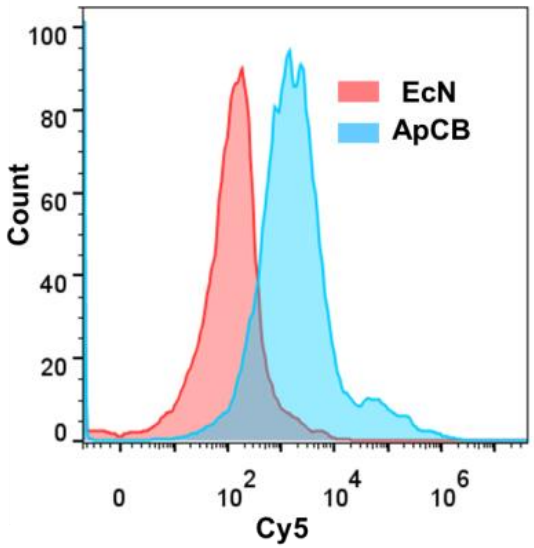

Figure S1. Flow cytometric analysis of EcN and ApCB by tracking the fluorescent intensity of Cy5-labelled AS1411.

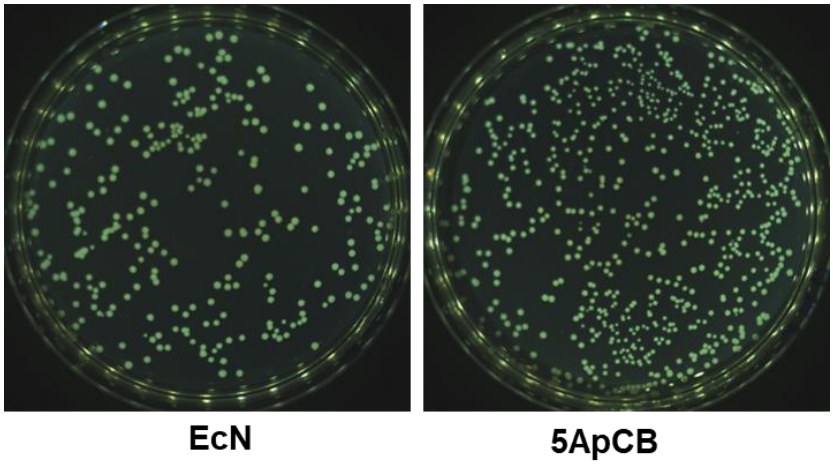

Figure S2. In vivo accumulation of bacteria in tumor site at 12 h post-administration. EcN or 5ApCB (1×10^7 CFU) were injected through the tail vein. Numbers of EcN colonized within the tumor at the indicated time points and spread onto LB agar plates after a proper dilution. Plates were incubated at 37 °C for 24 hours before imaging.

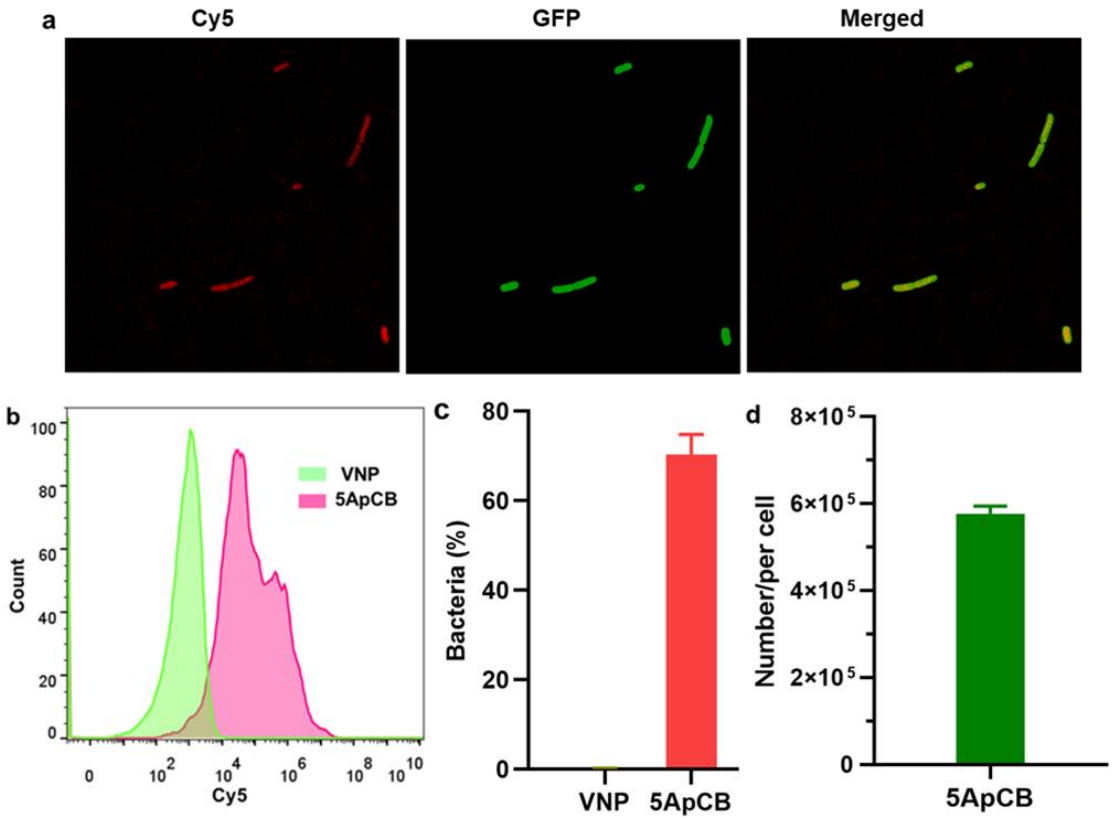

Figure S3. Characterization of aptamer-conjugated VNP. (a) Typical LSCM images of aptamer-conjugated VNP. The red and green channels indicate aptamer conjugated with Cy5 and VNP producing GFP, respectively. Scale bar: 10 μm. (b) Flow cytometric analysis of VNP and VNP conjugated with Cy5-labelled AS1411. (c) Percentage of conjugated VNP under a feed ratio of 5 nmol. (d) Average binding number of aptamers on each VNP quantified by calculating the difference of fluorescent intensity of the aptamer solution after reaction.

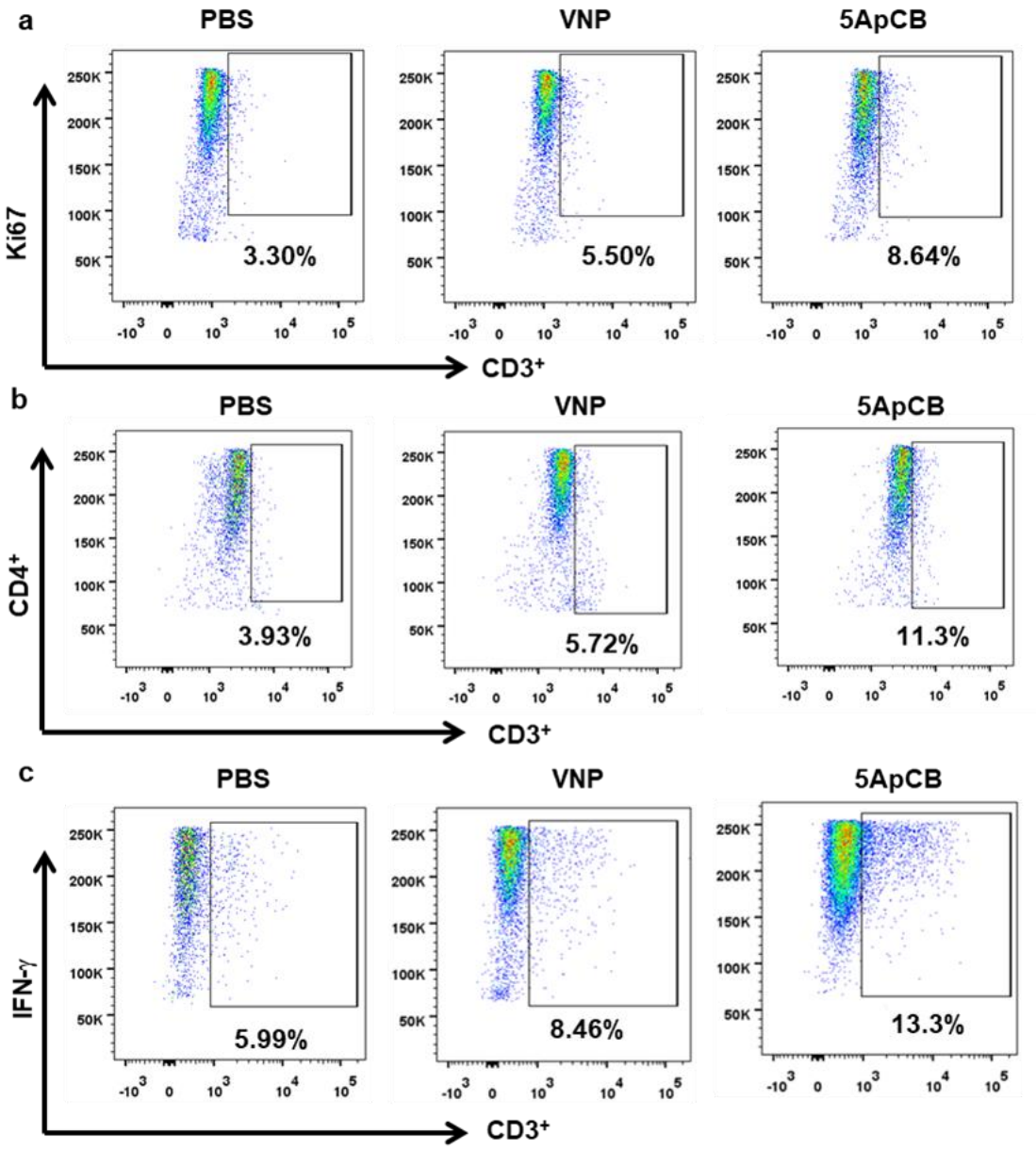

Figure S4. (a) Percentage of Ki67, (b) the population of $CD4^+$ T cells, and (c) Percentage of IFN-γ inside the tumors after different treatment. All cells were gated on $CD3^+$ cells.

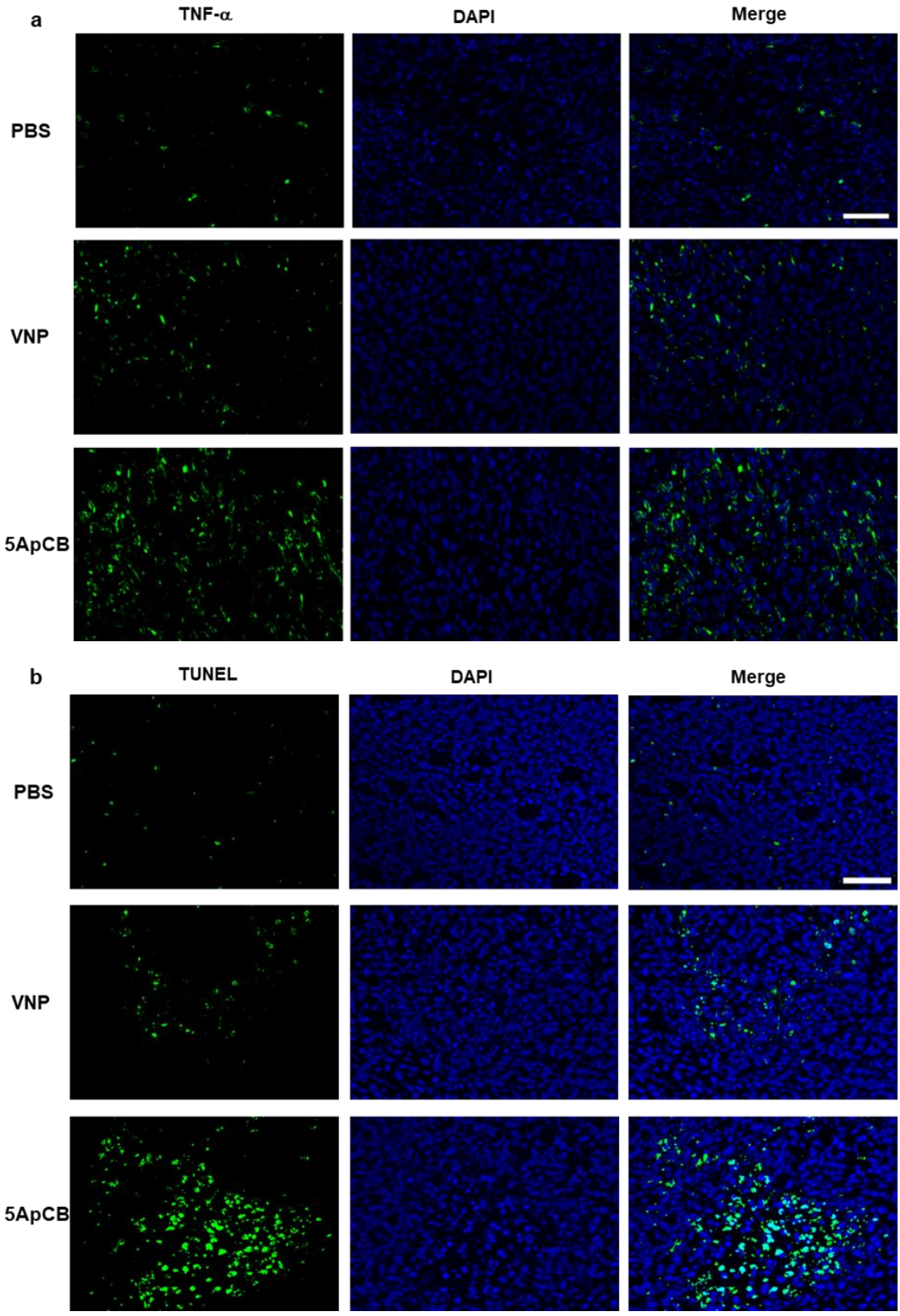

Figure S5. Images of tumor tissues stained with (a) TNF-α and (b) TUNEL after different treatments. Scale bar: 50 μm.