Supporting Information

Aggregable Nanoparticles-Enabled Chemotherapy and Autophagy Inhibition Combined with Anti-PD-L1 Antibody for Improved Glioma Treatment

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ABBREVIATIONS

AK peptide: Alanine-Alanine-Asparagine-Cysteine-Lysine, AANCK

AK-PEG-SH: AK-polyethylene glycol-thiol

ATP: adenosine triphosphate

AuNPs: gold nanoparticles

BBB: blood-brain barrier

CM-PEG-SH: carboxyl polyethylene glycol thiol

CABT-PEG-SH: CABT- polyethylene glycol-thiol

CABT: 2-Cyano-6-Aminobenzothiazole

CNS: central nervous system

CTLA-4: cytotoxic T lymphocyte-associated antigen 4

DCC: dicyclohexylcarbodiimide

D&H-A-AK: AK modified AuNPs co-loaded with DOX and HCQ

D&H-A-CABT: CABT modified AuNPs co-loaded with DOX and HCQ

D&H-A-P: PEG modified AuNPs co-loaded with DOX and HCQ

D-A-AK: AK modified AuNPs co-loaded with DOX

D-A-CABT: CABT modified AuNPs co-loaded with DOX

DMEM: Dulbecco's Modified Eagle Medium

DLS: dynamic laser scattering

DOX: doxorubicin

EPR: enhanced permeability and retention

FBS: fetal bovine serum

GAPDH: glyceraldehyde-3-phosphate dedydrogenase

GSC: glioma stem cell

H-A-AK: AK modified AuNPs co-loaded with HCQ

H-A-CABT: CABT modified AuNPs co-loaded with HCQ

HCQ: hydroxychloroquine

H&E: hematoxylin-eosin

HEPES: N-(2-hydroxyethyl)piperazine-N′-ethanesulfonic acid

IFN-γ: interferon-γ

IHC: immunohistochemistry

IL-10: interleukin-10

KDR: kinase domain insert receptor

LC3: microtube-associated protein 1 light chain 3

MHC: major histocompatibility complex

MMP-2: matrix metalloproteinase-2

MMP-9: matrix metalloproteinase-9

mPEG-SH: methoxy-polyethylene glycol thiol

mTOR: mammalian target of rapamycin

NF-κB: nuclear factor kappa B

NHS: N-hydroxysuccinimide

NSET: nanosurface energy transfer

PAS: periodic acid-Schiff

PDI: polydispersity index

PD-1: programmed death 1

PD-L1: programmed death-ligand 1

PI3K/AKT: phosphatidylinositol 3-kinase/a serine/threonine protein kinase

ROS: reactive oxygen species

TCR: T cell receptor

Tem: T memory cells

TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling

VE-Cadherin: vascular endothelial cadherin

VEGF: vascular endothelial growth factor

VM: vasculogenic mimicry

EXPERIMENTAL SECTION

Materials: Chloroauric acid was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Doxorubicin hydrochloride was achieved from Beijing Huafeng United Technology Co., Ltd (Beijing, China). Hydroxychloroquine was purchased from TCI Development Co., Ltd (Shanghai, China). Methoxy-polyethylene glycol thiol (mPEG-SH, MW = 5000) and carboxyl polyethylene glycol thiol (CM-PEG-SH, MW = 5000) were purchased from Layson Bio Inc (Arab, USA). AK peptide (sequence: Ala-Ala-Asn-Cys-Asp) was custom-made from PHTD Peptide Co., Ltd (Zhengzhou, China). 2-Cyano-6-Aminobenzothiazole (CABT) was obtained from Shanghai Chemical Pharm-Intermediate Tech. Co., Ltd (Shanghai, China). Legumain was obtained from Abcam Ltd (Hong kong, China). Lysotracker Red (DND-99) was purchased from Life Technologies (Shanghai, China). eRFP-LC3 plasmid was purchased from Changsha Yingrun Biological Technology Co. Ltd (Changsha, China). Lipofectamine 2000 was ordered from Thermo Fisher Scientific (Waltham, USA). Annexin V-FITC/PI apoptosis detection kit was purchased from BD Biosciences (San Jose, USA). Matrigel basement membrane matrix was achieved from Corning (New York, USA). Mouse IFN-γ ELISA Kit II was purchased from BD Biosciences (San Jose, USA). Red blood cell lysis solution was obtained from Beijing Labgic Technology Co., Ltd. Collagen IV was achieved from Thermo Fisher Scientific (Waltham, USA). Ficoll-paque PREMIUM sterile solution was purchased from GE Healthcare Life Sciences (Beijing, China). Dulbecco's Modified Eagle Medium (DMEM), Improved Minimal Essential Medium (Opti-MEM) and fetal bovine serum

(FBS) were purchased from Gibco (Grand Island, USA). Penicillin-streptomycin and trypsin were purchased from Solarbio life science (Beijing, China). Plastic cell culture dishes and plates were obtained from Wuxi NEST Biotechnology Co., Ltd (Wuxi, China). All chemical reagents were analytical grade or better.

Antibody: Rabbit anti-LC3 antibody, Rabbit anti-GAPDH antibody, Rabbit anti-MMP2 antibody and rabbit anti-MMP9 antibody were achieved from Abclonal Technology (Wuhan, China). Rabbit anti-VE Cadherin antibody was purchased from Abcam Ltd (Hong Kong, China). Anti-PD-L1 monoclonal antibody (Atezolizumab) for glioma treatment was purchased from Selleckchem (USA). Anti-CD11c (Clone N418, Cat: 117306, BioLegend), Anti-CD83 (Clone: Michel-19, Cat: 121508, BioLegend), Anti-CD86 (Clone: GL-1, Cat: 105008, BioLegend), Anti-CD3 (Clone: 17A2, Cat: 565643, BD Pharmingen), Anti-CD4 (Clone: RM4-5, Cat: 553047, BD Pharmingen), Anti-CD8 (Clone: 53-6.7, Cat: 553032, BD Pharmingen), Anti-Foxp3 (Cat: A12051, RRID: AB 2758962, Abconal Technology), Anti-CD44 (Clone: IM7, Cat: 553133, BD Pharmingen), Anti-CD62L (Clone: MEL-14, Cat: 553151, BD Pharmingen)

Cell line: C6 (murine glioma) cells were consistently cultured in our laboratory. Dulbecco's Modified Eagle Medium (DMEM) contains 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Subculture were maintained in humidified incubator with 5% CO_2 supply at 37 ℃ and all experiments were performed during the logarithmic phase of cell growth.

Animal: Male BALB/C mice were obtained from Chengdu Experimental Animal

Center (Chengdu, China) and were maintained under standard housing conditions. All animal experiments were carried out in strict accordance with the guidelines evaluated and were approved by the ethics committee of Sichuan University.

Synthesis of Citrate-stabilized AuNPs: AuNPs were synthesized according to the sodium citrate reduction described previously.[1] Briefly, 2.5 mL $HAuCl_4$ solution (2.2 mg/mL) was diluted in ultrapure water to a final volume of 50 mL and heated to boiling under vigorous stirring. 2 mL sodium citrate solution was added rapidly into the boiling solution. The solution was kept boiling until the color of solution turned to wine red, followed by continuous stirring under room temperature until the AuNPs solution was harvested.

Synthesis of pH-sensitive DOX: The pH-sensitive DOX was synthesized following the procedures described in our previous research.[1]

Synthesis of pH-sensitive HCQ: The pH-sensitive HCQ was synthezed following a procedure elucidated in Figure S1, the detail procedures can be divided into five steps:

(a) HCQ · H_2SO4 (1.01 g) was dissolved in 10 mL of saturated sodium bicarbonate solution, 50 mL dichloromethane was added into solution. After violent shaking and static stratification, organic phase was collected and evaporated in vacuo. This step was repeated for three times and the HCQ was faint yellow oil product.

(b) HCQ (201.3 mg, 0.6 mmol, 1 eq) was dissolved in 50 mL tetrahydrofuran (THF) and succinic anhydride (SA, 66.1 mg, 0.66 mmol, 1.1 eq) was introduced into solution. The solution was constantly stirring under 80 ℃ over 12 h. After the completion of

the reaction (monitored by TLC), the mixture was evaporated in vacuo to yield compound 1. The reaction was monitored by thin-layer chromatography (TLC) and directly input into next step reaction without purification.

(c) Dicyclohexylcarbodiimide (DCC, 248.1 mg, 1.2 mmol, 2 eq) and N-hydroxysuccinimide (NHS, 138.5 mg, 1.2 mmol, 2 eq) were further added into the solution in step 1 after completion, the solution was constantly stirring for over 12 h. After the completion of the reaction (monitored by TLC), the solution was filter with diatomaceous earth to remove the insoluble by-products (DCC crystal), then the organic solution was evaporated in vacuo to yield yellow oil (Compound 2).

(d) Compound 3 was synthesized according to the procedures described in our previous study.[2] 102.5 mg compound 2 (0.192 mmol, 1 eq) was dissolved in 5 mL methanol and added with 29.1 mg compound 3 (0.199 mmol, 1 eq). The mixture was reacted at room temperature with constant stirring. After the completion of the reaction (monitored by TLC), the mixture was evaporated in vacuo. However the residue did not undergo a further purification owing to the instability of hydrazone bond. (Compound 4).

(e) Compound 4 obtained in step 4 was dissolved in dissolved in 10 mL of anhydrous methanol was added 55.3 mg potassium carbonate (0.4 mmol, 2eq). The mixture was stirred at room temperature for 4-30 h. After the completion of the reaction (monitored by TLC), the solvent was evaporated in the vacuo to yield the final compound 5, pH-sensitive HCQ. However the residue did not undergo a further purification owing to the activity of thiol and the instability of hydrazone bond.

Synthesis of SH-PEG-AK and SH-PEG-CABT: The legumain-responsive functional fragments, SH-PEG-AK and SH-PEG-CABT, were synthesized following the procedures described in our previous research.[3]

Preparation of D&H-A-A&C: The D&H-A-A&C were composed of D&H-A-AK and D&H-A-CABT. To obtain D&H-A-AK, 1 mL AuNPs solution was pre-incubated with 5 μL SH-PEG-AK (1 mg/mL) for 1 h under 37 ℃, 150 rpm/min. Then, 5 μL pH-sensitive DOX methanol solution (1 mg/mL) and 30 μL pH-sensitive HCQ methanol solution (1 mg/mL) were simultaneously added into the solution. The solution was further incubated for another 8 h at same condition. Similarly, to obtain D&H-A-CABT, 1 mL AuNPs solution was pre-incubated with 5 μL SH-PEG-CABT (1 mg/mL) for 1 h under 37 ℃, 150 rpm/min. Then, 5 μL pH-sensitive DOX methanol solution (1 mg/mL) and 30 μL pH-sensitive HCQ methanol solution (1 mg/mL) were simultaneously added into the solution. The solution was further incubated for another 8 h at same condition.

Preparation of D&H-A-P: To obtain D&H-A-P, 1 mL AuNPs solution was pre-incubated with 5 μL mPEG-SH (1 mg/mL) for 1 h under 37 ℃, 150 rpm/min. Then, 5 μL pH-sensitive DOX methanol solution (1 mg/mL) and 30 μL pH-sensitive HCQ methanol solution (1 mg/mL) were simultaneously added into the solution.

Preparation of H-A-A&C: The H-A-A&C were composed of H-A-AK and H-A-CABT. To obtain H-A-AK, 1 mL AuNPs solution was pre-incubated with 5 μL SH-PEG-AK (1 mg/mL) for 1 h under 37 ℃, 150 rpm/min. Then, 30 μL pH-sensitive HCQ methanol solution (1 mg/mL) were simultaneously added into the solution. The

solution was further incubated for another 8 h at same condition. Similarly, to obtain H-A-CABT, 1 mL AuNPs solution was pre-incubated with 5 μL SH-PEG-CABT (1 mg/mL) for 1 h under 37 ℃, 150 rpm/min. Then, 30 μL pH-sensitive HCQ methanol solution (1 mg/mL) were simultaneously added into the solution. The solution was further incubated for another 8 h at same condition.

Preparation of D-A-A&C: The D-A-A&C were composed of D-A-AK and D-A-CABT. To obtain D-A-AK, 1 mL AuNPs solution was pre-incubated with 5 μL SH-PEG-AK (1 mg/mL) for 1 h under 37 ℃, 150 rpm/min. Then, 5 μL pH-sensitive DOX methanol solution (1 mg/mL) were simultaneously added into the solution. The solution was further incubated for another 8 h at same condition. Similarly, to obtain D-A-CABT, 1 mL AuNPs solution was pre-incubated with 5 μL SH-PEG-CABT (1 mg/mL) for 1 h under 37 ℃, 150 rpm/min. Then, 5 μL pH-sensitive DOX methanol solution (1 mg/mL) were simultaneously added into the solution. The solution was further incubated for another 8 h at same condition.

In vitro DOX release: D&H-A-A&C, D&H-A-PEG and D-A-A&C were re-suspended in pH 5.0 and pH 6.0 PBS and incubated at 37 ℃. The fluorescent scanning was performed at different time using a Shimadzu RF-6000 spectrofluorophotometer (Shimadzu, Japan). Free DOX at same concentration was set as positive control. The excitation wavelength is 465 nm.

Legumain-responsive size increase of D&H-A-A&C: 1 mL D&H-A-AK solution and 1 mL D&H-A-CABT solution was centrifuged at 12000 rpm/min for 10 min. Subsequently, the precipitate of D&H-A-AK and D&H-A-CABT were re-suspended

together in 1 mL of HEPES buffer (pH 5.0). The hydrodynamic size of D&H-A-A&C incubated with 2.5 μL legumain (1 mg/mL) at different time interval was monitored using DLS analysis. Similarly, the hydrodynamic diameter of D&H-A-P, H-A-A&C and D-A-A&C were determined under same condition.

Size characterization in HEPES containing FBS: 1 mL D&H-A-AK and 1 mL D&H-A-CABT were centrifuged at 12,000 rpm/min for 10 min. After aspirating the supernatant, the D&H-A-AK and D&H-A-CABT were re-suspended in HEPES (pH 5.0) containing 10% FBS. Similarly, D&H-A-P, H-A-A&C and D-A-A&C were prepared following the procedures described above. All samples were incubated at 37 ℃, 75 rpm/min, and their hydrodynamic sizes were determined at different time intervals.

Serum stability: 1 mL D&H-A-AK and 1 mL D&H-A-CABT were centrifuged at 12,000 rpm/min for 10 min. After aspirating the supernatant, the D&H-A-AK and D&H-A-CABT were re-suspended in HEPES (pH 5.0) containing 10%, 50%, and 90% FBS to obtain D&H-A-A&C. Similarly, D&H-A-P, H-A-A&C and D-A-A&C were prepared following the procedures described above. All samples were incubated at 37 ℃, 75 rpm/min, and their turbidities were determined at 560 nm at different time intervals. All nanoparticles re-suspended in FBS-free PBS were prepared as control.

Lysosomotropic property of D&H-A-A&C: C6 cells were seeded onto 6-well plate preplaced with 400 mm^2 coverslip at a density of 5×10^5 per well and allowed to grow until 60% confluency. After discarding culture medium, cells were washed with PBS twice and then incubated with D&H-A-A&C and control formulations for 24 h.

The administration dose of DOX and HCQ was at an equivalent concentration of 5 μg/mL and 30 μg/mL, respectively. For lysosomal labeling, Lysotracker Red (1 μM) was added into the culture medium at 30 min before incubation end. After incubation, the cells were washed with PBS twice and fixed with 4% paraformaldehyde for 20 min, and then counterstained with 0.5 μg/mL DAPI solution for 5 min. The coverslips were placed onto glass microscope slides with a drop of antifade mounting media (Beyotime Biotechnology, China). The fluorescence signal was observed using a confocal laser microscope (A1R+, Nikon, Japan).

***In vitro* cellular uptake:** C6 cells were seeded onto 6-well plate preplaced with a 400 mm^2 coverslip at a density of 5×10^5 per well and allowed to grow until 60% confluency. After discarding culture medium, cells were washed with PBS twice and then incubated with D&H-A-A&C, D&H-A-P and D-A-A&C 4 h and 24 h. The concentration of DOX was 5 μg/mL. After incubation, the cells were washed with PBS twice and fixed with 4% paraformaldehyde for 20 min, and then counterstained with 0.5 μg/mL DAPI solution for 5 min. The coverslips were placed onto glass microscope slides with a drop of antifade mounting media. The fluorescence signal was observed using a confocal laser microscope.

Cellular uptake after pre-incubation with protein corona: To prepare nanoparticles coated with protein corona in vitro, plasma was acquired by centrifugating the EDTA stabilized whole blood of male BALB/C mice at 2000 *g* for 20 min, then D&H-A-A&C, D&H-A-P and D-A-A&C were incubated with 20% and 50% plasma diluted in 20 mM HEPES buffer (pH 7.4) at 37 ℃ for 24 h respectively.

C6 cells were seeded onto 6-well plate preplaced with a 400 mm^2 coverslip at a density of 5×10^5 per well and allowed to grow until 60% confluency. After discarding culture medium, cells were washed with PBS twice and then incubated with nanoparticles-protein corona complexes for another 24 h. After incubation, the cells were washed with PBS twice and fixed with 4% paraformaldehyde for 20 min, and then counterstained with 0.5 μg/mL DAPI solution for 5 min. The coverslips were placed onto glass microscope slides with a drop of antifade mounting media. The fluorescence signal was observed using a confocal laser microscope.

eRFP-LC3-expressing cells: The eRFP-LC3-expressing C6 cells were constructed by eRFP-LC3 plasmid transfection. Briefly, C6 cells were seeded onto 6-well plate preplaced with 400 mm^2 coverslip at a density of 5×10^5 per well in DMEM culture medium and allowed to grow until 80% confluency. Then cells were transfected using lipofectamine 2000 refer to the manufacturer's instructions. For each well, 10 μg concentrated eRFP-LC3 plasmid were added into 1.5 mL OptiMEM medium without antibiotics and FBS, and then added with 10 μL lipofectamine 2000. The diluted plasmid and lipofectamine 2000 were pre-incubated for 10 min at room temperature to ensure a fully combination. Subsequently, the culture containing plasmid-cationic liposome complex was introduced into each well and allowed to incubate with C6 cells. The eRFP-LC3-expressing cells were obtained after 4 h incubation followed by the fresh FBS-free culture containing different formulations. After 24 h incubation, the cells were washed with PBS twice and fixed with 4% paraformaldehyde for 20 min, and then counterstained with 0.5 μg/mL DAPI solution for 5 min. The coverslips

were placed onto glass microscope slides with a drop of antifade mounting media. The fluorescence signal was observed using a confocal laser microscope.

Western blotting: C6 cells or GSC were seeded onto 6-well plate at a density of 5 × 10^5 per well and allowed to grow until 60% confluency. Then the medium was replaced with FBS-free DMEM culture medium containing D&H-A-A&C and control formulations. After 24 h incubation, cells were harvested and lysed in cell lysis buffer containing phenylmethanesulfonyl fluoride (PMSF, a protease inhibitor). The lysate was firstly underwent a quantitative detection by BCA protein assay kit (Thermo Scientific) to ensure an equivalent protein loading amount. 10 μL protein loading sample of each group was loaded on 12% SDS-PAGE gel for separation and then transferred onto a polyvinylidene difluoride (PVDF) membrane, which was pre-blocked in Tris-buffered saline (TBS) containing 5% non-fat milk and 0.5% Tween-20, followed by incubation with primary antibodies according to their recommended dilution at 4 ℃ overnight, then the membrane were incubated HRP-labeled secondary antibodies. The membranes were finally imaged by Immobilon Western HRP substrate (Millipore, USA) using a Bio-Rad Chemioc MP system (Bio-Rad Laboratories, USA).

***In vitro* TEM imaging of cells:** C6 cells were cultured in culture dish (Φ = 100 mm) until a density of 5 × 10^5 cells. Then the medium was replaced with FBS-free DMEM culture medium containing D&H-A-A&C and control formulation at an equivalent AuNPs concentration of approximate 100 μg/mL. After 24 h incubation, cells were washed twice with free culture meidum, and then were added with 1 mL

free culture medium. Cells were detached using cell scraper, harvested, and then added with 50 μL 2.5% glutaraldehyde solution. Afterwards, cells were centrifuged at 2500 rpm/min for 10 min. After removing the supernatant, 1 mL 2.5% glutaraldehyde was introduced slowly to infiltrate cells and stored at 4 ℃. The samples were polymerized at 60 ℃ for 24 h, cut into ultrathin sections using an ultramicrotome (Leica), and placed on 150-mesh copper grids. The ultrasection of cells was stained with uranyl acetate (2% in ethanol) for 10 min and then with lead citrate for 5 min. TEM images were captured using a Hitachi H-600 (Japan) at 200 kV.

ICP-OES analysis: The C6 cells were seeded onto 12-well plate at a density of 1×10^5 per well and were cultured until 80% confluency. Then the medium was replaced with fresh serum-free medium containing D&H-A-A&C, D&H-A-P, H-A-A&C and D-A-A&C at an equivalent AuNPs concentration approximate 60 μg/mL. After 24 h incubation, cells were harvested and digested overnight in 1 mL concentrated nitric acid containing 0.5 mL 30% hydrogen peroxide with a heating condition of 110 ℃ until a clear faint yellow solution is observed. All the digestion were solution was further diluted 20-fold using 2% nitric acid and the gold content was determined by 2100 DV ICP-OES (PE, USA).

MTT assay: C6 cells were seeded onto 96-well plate at a density of 2×10^3 per well and allow cells to grow to 50% confluency (5 wells enclosed in each concentration). D&H-A-A&C and control formulations in FBS-free culture medium with different dilutions were introduced to the well (100 μL) and incubated with cells for 24 h. After incubation, 10 μL MTT solution (5 mg/mL) was added to each well

and incubated for another 4 h. Then the solution was replaced with 150 μL DMSO and shock for 15 min under 37 ℃, 150 rpm/min. The absorbance at 570 nm was determined with a microplate reader (Thermo Scientific Varioskan Flash), cells without treatment serve as control.

Apoptosis assay: Apoptosis of C6 cells were conducted using the Annexin V-FITC/PI apoptosis kit (BD, USA). C6 cells were seeded onto 6-well plate at a density of 5×10^5 per well and allowed to grow until 60% confluency. After discarding culture medium, cells were washed with PBS twice and then incubated with D&H-A-A&C and control formulations. The administration dose of DOX and HCQ was at an equivalent concentration of 5 μg/mL and 30 μg/mL, respectively. After incubation for 24 h, cells treated without treatment were settled as control group. Subsequently, the analyzing procedures were conducted in accordance with the manufacturer's protocol. The Annexin V-FITC/PI double staining was measured by flow cytometry (FC500, Beckman Coulter, USA). The data were analyzed using Flowjo 7.6 software.

In vitro tube formation: 96-well plates were pre-coated with 50 μL Matrigel and incubated for 1 h at 37 ˚C. GSC cells (2×10^4 per well) were suspended with FBS-free DMEM medium containing D&H-A-A&C, D&H-A-P, H-A-A&C, D-A-A&C, free HCQ and free DOX respectively and seeded into wells (n = 3). GSC without treatment was set as control group. The administration dose of DOX and HCQ was at an equivalent concentration of 5 μg/mL and 30 μg/mL, respectively. After incubation for 12 h, each well was photographed by a microscopy at 10 ×

magnification. The percentage of tube formation was quantified by Image Pro Plus software 6.0.

CT imaging: C6 glioma-bearing mice were established following the procedures described in our previous literature.[2] In detail, the mice were firstly anesthetized by an intraperitoneal injection with 4% choral hydrate. C6 cells suspension (2.5×10^5 cells suspended in 5 μL PBS (pre-warmed to 37 ℃) were implanted into the right striatum (1.8 mm lateral, 0.6 mm longitudinal, 3 mm depth) of the mice using a brain stereotactic fixation device with a mouse adapter. 14 days after implantation, the intracranial C6 glioma-bearing mice were randomly divided into two groups (three mice per group): D&H-A-A&C and D&H-A-P. Each mouse was intravenously injected with different nanoparticles at an equivalent gold dose of 30 mg/kg through the tail vein. *In vivo* cross-sectional scanning of mice were performed with nominal resolution (pixel size) of 36 μm, employing an aluminum filter 0.2 mm thick and an applied x-ray tube voltage of 45 kV. Surface-rendered 3D models were constructed for 3D imaging of the analyzed mice. Volume rendered 3D imaging were generated using an RGBA transfer function in SkyScan CT-Volume (CTVol) software. (Skyscan 1176, Bruker micro-CT, Kontich, Belgium), (NRecon v.1.6.9, Bruker micro-CT).

In vivo anti-glioma effect: C6 glioma-bearing mice were established as described above. Ten days after implantation, glioma-bearing mice were randomly divided into seven groups (13 mice per group). On 10th, 12nd, 14th, 16th and 18th day, mice of each group were intravenously injected with D&H-A-A&C, D&H-A-P, H-A-A&C, D-A-A&C, Free HCQ, Free DOX and normal saline, the administration dose of DOX

and HCQ were 2.5 mg/kg and 15 mg/kg, respectively. For all groups, the overall survival was monitored. Moreover, three mice were sacrificed on the day after the last administration, brains and major organs were sampled for H&E staining, TUNEL staining and CD34-PAS double-staining.

Combination therapy of D&H-A-A&C and Anti-PD-L1 antibody: C6-glioma bearing mice were established as described above. Mice were randomly divided into four groups: D&H-A-A&C plus anti-PD-L1 antibody (n = 24), anti-PD-L1 antibody (n = 14), D&H-A-A&C (n = 14) and normal saline (n = 14). On 10th, 12nd, 14th, 16th and 18th day, mice received anti-PD-L1 antibody at a dose of 100 μg *via* intraperitoneal injection and D&H-A-A&C *via* intravenous injection at a dose of 2.5 mg/kg DOX and 15 mg/kg HCQ. The overall survival was monitored. After the last administration on day 20, four mice every group were selected randomly to collect spleens for flow cytometry analysis of $CD83^+CD11c^+$ and $CD86^+CD11c^+$ DCs. Brains were collected for H&E staining and IHC staining with anti-CD4 antibody, anti-CD8 antibody, and anti-Foxp3 antibody, respectively.

Tumor rechallenge: To evaluate the memory immune response induced by combination therapy, mice survived over 65 days were rechallenged with C6 glioma cells at the contralateral hemisphere using the same method as described above. Each groups has 6 mice, and on day 11 after inoculation, three mice were sacrificed for collecting brains, spleens, peripheral blood and draining cervical lymph nodes. Brains were prepared for H&E staining and IHC staining assays ($CD4^+$, $CD8^+$, $Foxp3^+$). Spleens and draining cervical lymph nodes were prepared for immune cells

characterization using flow cytometry analysis. Peripheral blood was prepared for detecting released cytokines using ELISA.

Flow cytometry analysis of immune cells: To acquire single-cell suspensions, spleens and lymph nodes were grinded and mechanically homogenized with collagen IV (175 U/mL) in fresh RPMI medium, then filtered with 70 μm filter. The resulting cells were washed with PBS and lyzed by red blood cell lysis buffer. To prepare peripheral blood mononuclear cells (PBMCs), peripheral blood was collected in heparin anticoagulant tubes and diluted with PBS in a 1:1 ratio, then gently added onto the top of 3-fold volume Ficoll-paque PREMIUM sterile solution. PBMCs were separated from the milky layer after centrifugation. The above single-cell suspensions were counted and resuspended in PBS containing 0.5% FBS at a concentration of 1×10^7 cells/mL. Cells were incubated with antibodies in dark at 4 ℃ for 30 min. After washing twice with PBS containing 0.5% FBS, the samples were analyzed by flow cytometry (FC500, Beckman Coulter, USA). All data were analyzed using Flowjo 7.6 software.

Statistical analysis: A comparison between experiment group and control group was analyzed by the non-paired Student's t-test. The statistical analysis for survival was analyzed using Kaplan-Meier survival plot (SPSS 16.0), and the overall survival was compared using the log-rank (Mantel-Cox) test. $p < 0.05$, 0.01 and 0.001 were considered a statistically significant difference and remarked with *, ** and ***, respectively.

SUPPLEMENTARY FIGURES

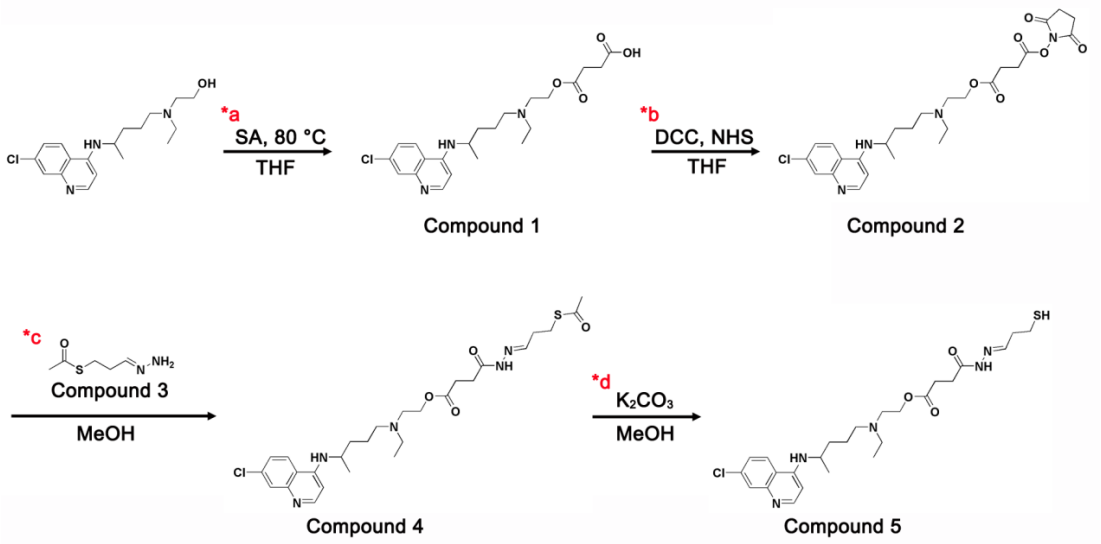

Figure S1. Synthetic route of pH-sensitive HCQ. *a: succinic anhydride (SA) and tetrahydrofuran (THF). *b: Dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS). *c: Compound 3 was pre-synthesized following the procedures described in our previous study.[2] *d: potassium carbonate was pre-dissolved in anhydrous methanol under water bath ultrasonic.

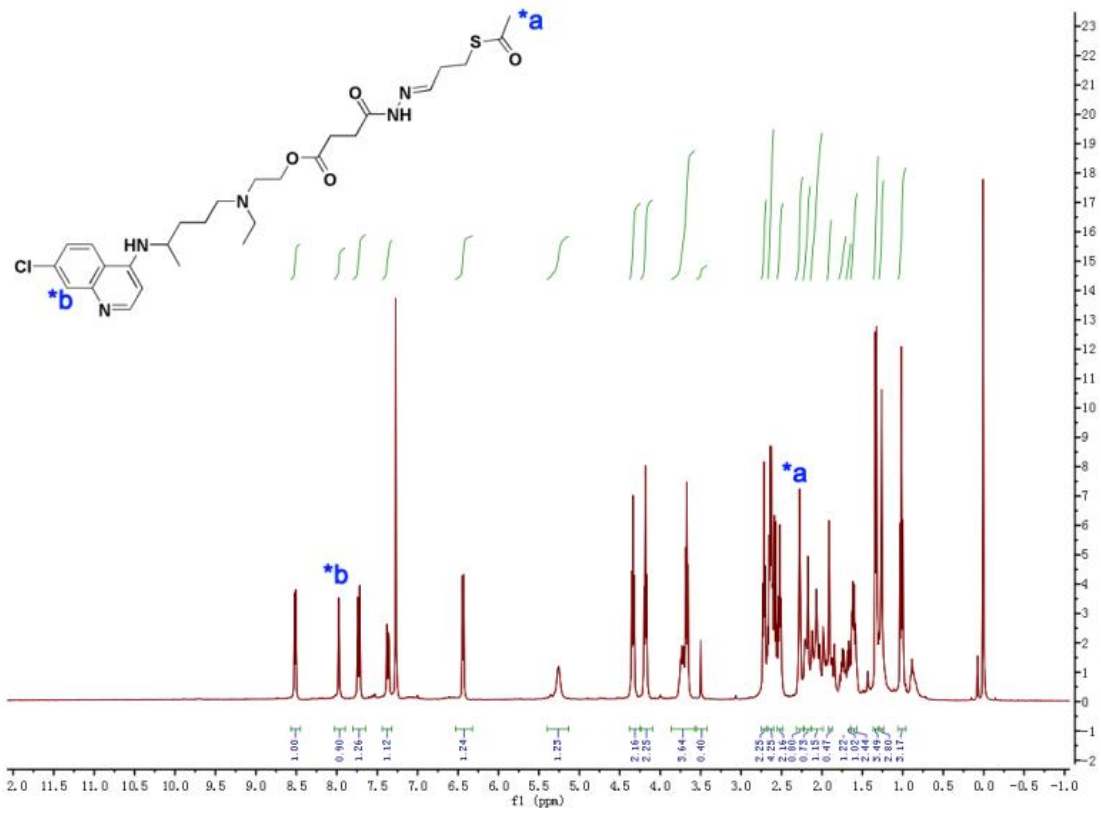

Figure S2. ^{1}H NMR spectrum of pH-sensitive HCQ (compound 4) in $CDCl_3$, *a and *b indicate the corresponding representative group magnetic displacement.

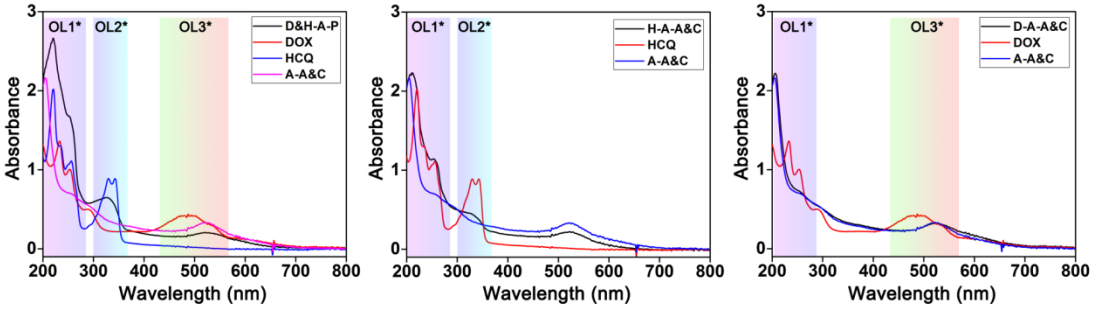

Figure S3. UV-Vis spectral of D&H-A-P, H-A-A&C, D-A-A&C, A-A&C, free DOX and free HCQ. OL1*, OL2* and OL3* indicate the characteristic absorption peaks of D&H-A-P, H-A-A&C, D-A-A&C overlaid with A-A&C, free DOX and free HCQ, respectively.

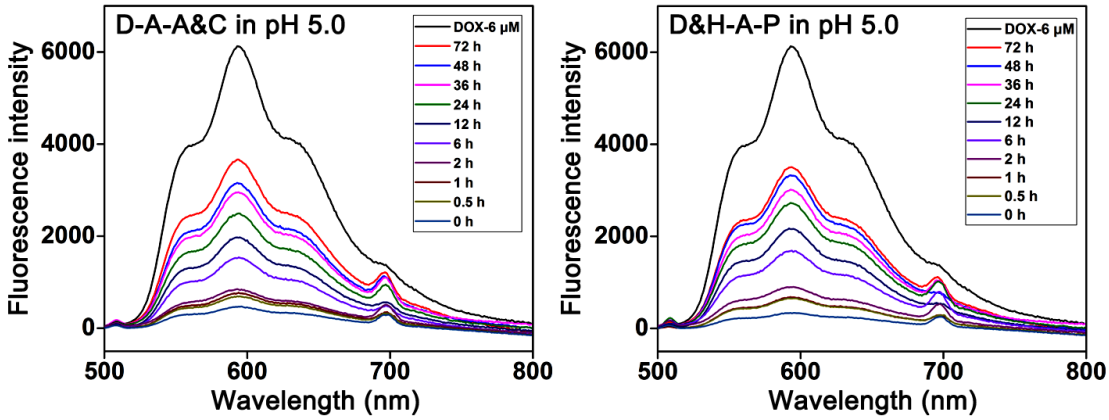

Figure S4. Fluorescent recovery of D-A-A&C and D&H-A-P in PBS (pH 5.0) at different time intervals, respectively, 6 μM free DOX was settled as positive control.

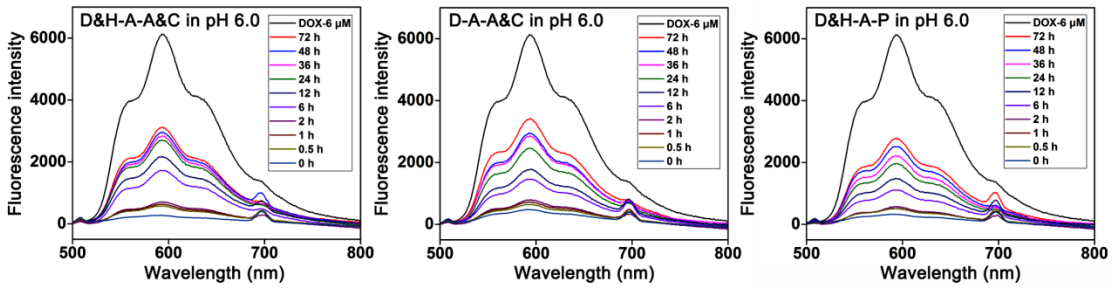

Figure S5. Fluorescent recovery of D&H-A-A&C, D-A-A&C and D&H-A-P in PBS (pH 6.0) at different time intervals, respectively, 6 μM free DOX was settled as positive control.

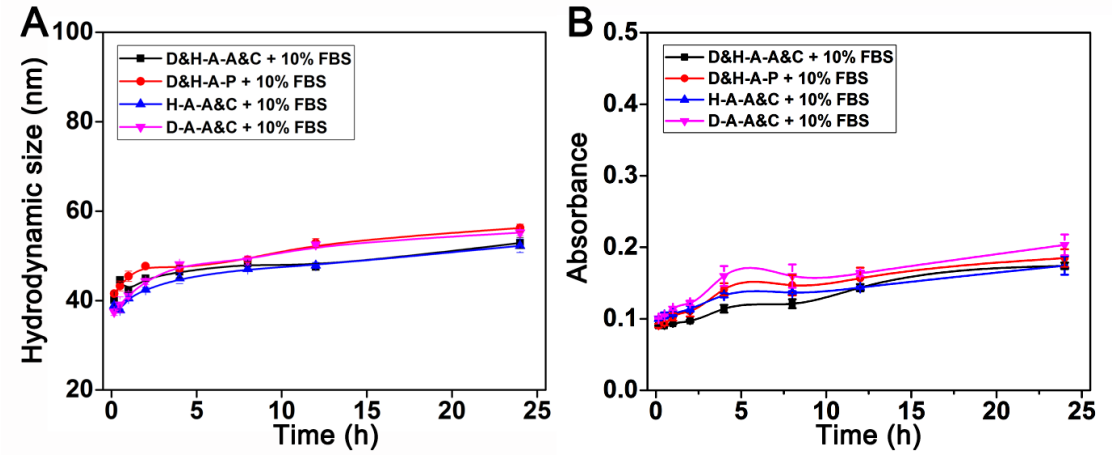

Figure S6. (A) The hydrodynamic size of D&H-A-A&C and control nanoparticles

after incubation with 10% FBS at different time intervals. (B) The turbidity change of D&H-A-A&C and control nanoparticles after incubation with 10% FBS at different time intervals.

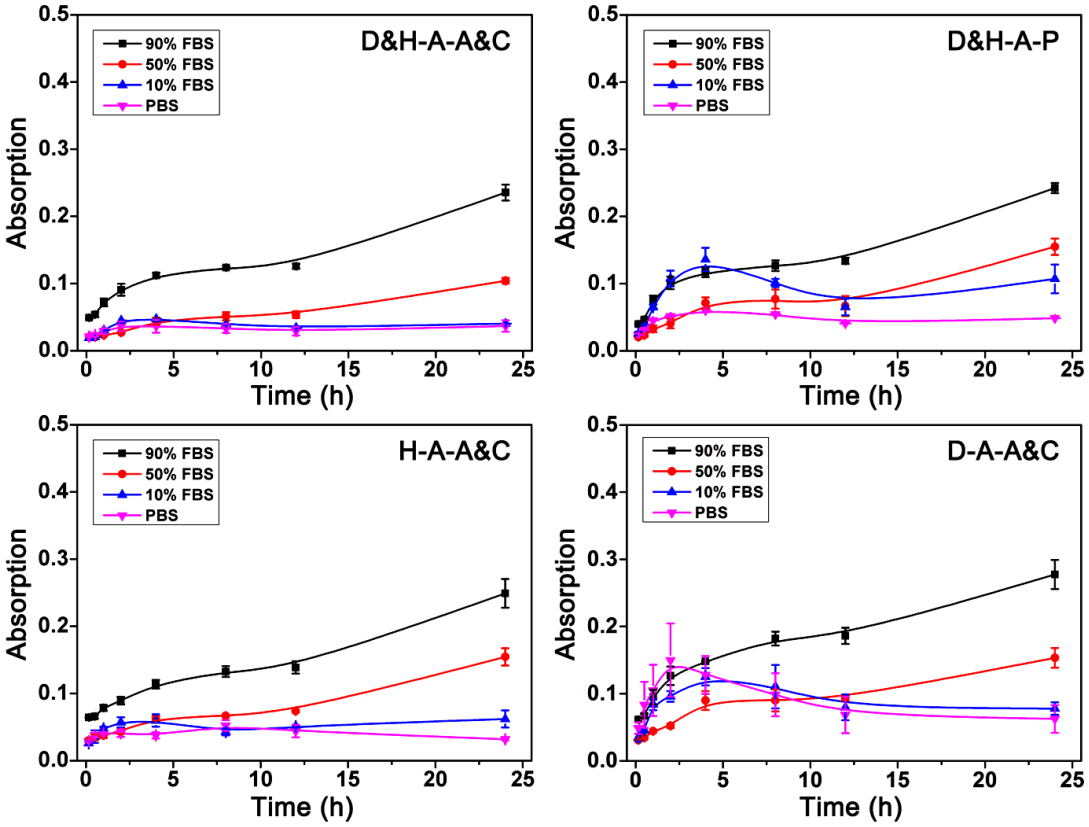

Figure S7. The turbidity change of D&H-A-A&C and control nanoparticles incubated with 10%, 50% and 90% FBS at different time intervals.

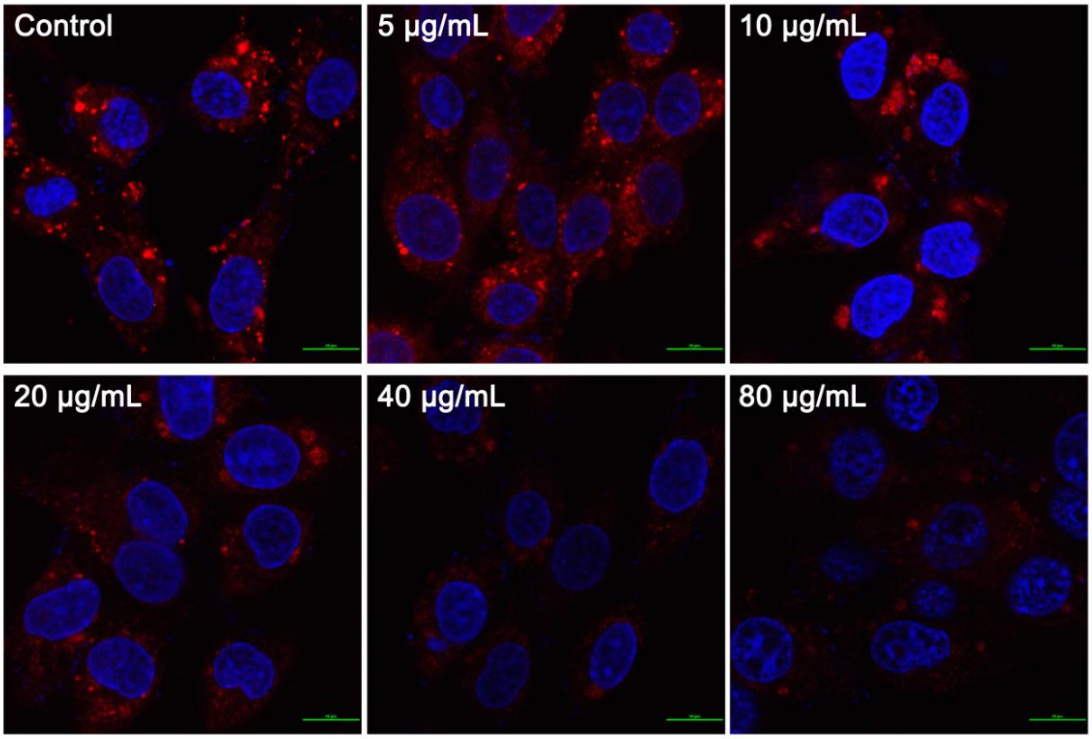

Figure S8. Confocal images of C6 cells incubated with free HCQ at different concentrations for 4 h and labeled with Lysotracker Red, bar represents 10 μm.

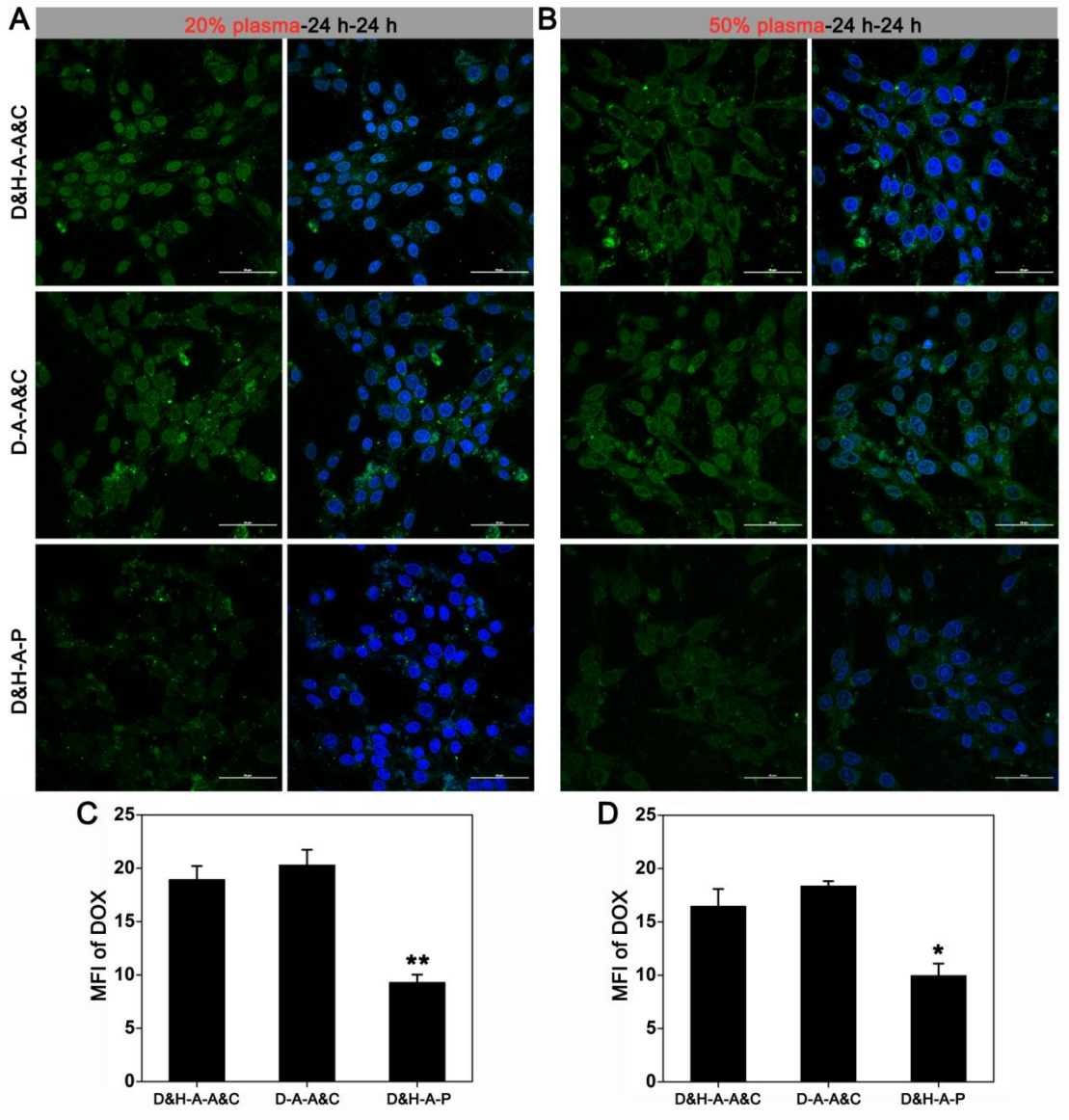

Figure S9. In vitro evaluation of cellular uptake after coating with protein corona. (A) Confocal images of C6 cells treated with D&H-A-A&C, D-A-A&C and D&H-A-P for 24 h, these nanoparticles were preincubated with 20% mouse plasma for 24 h, bar represents 50 μm. (B) Confocal images of C6 cells treated with D&H-A-A&C, D-A-A&C and D&H-A-P for 24 h, these nanoparticles were preincubated with 50% mouse plasma for 24 h, bar represents 50 μm. (C) Semiquantitative mean fluorescent intensity (MFI) of DOX calculated from A, $^{**}p < 0.01$ indicate the statistic difference versus D&H-A-A&C (n = 3). (D) Semiquantitative mean fluorescent intensity (MFI)

of DOX calculated from A, *p < 0.05 indicate the statistic difference versus D&H-A-A&C (n = 3).

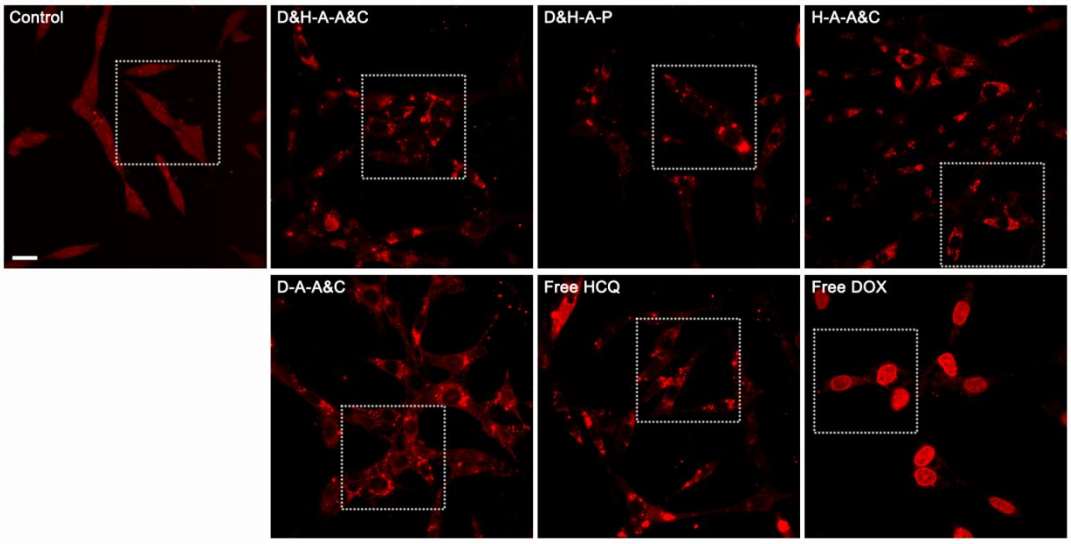

Figure S10. Confocal images of eRFP-LC3-expressing C6 cells after incubation with different formulations for 24 h, dotted boxes indicate the selected area demonstrated in Figure 2A, red puncta represent autophagosomes, bar represents 20 μm.

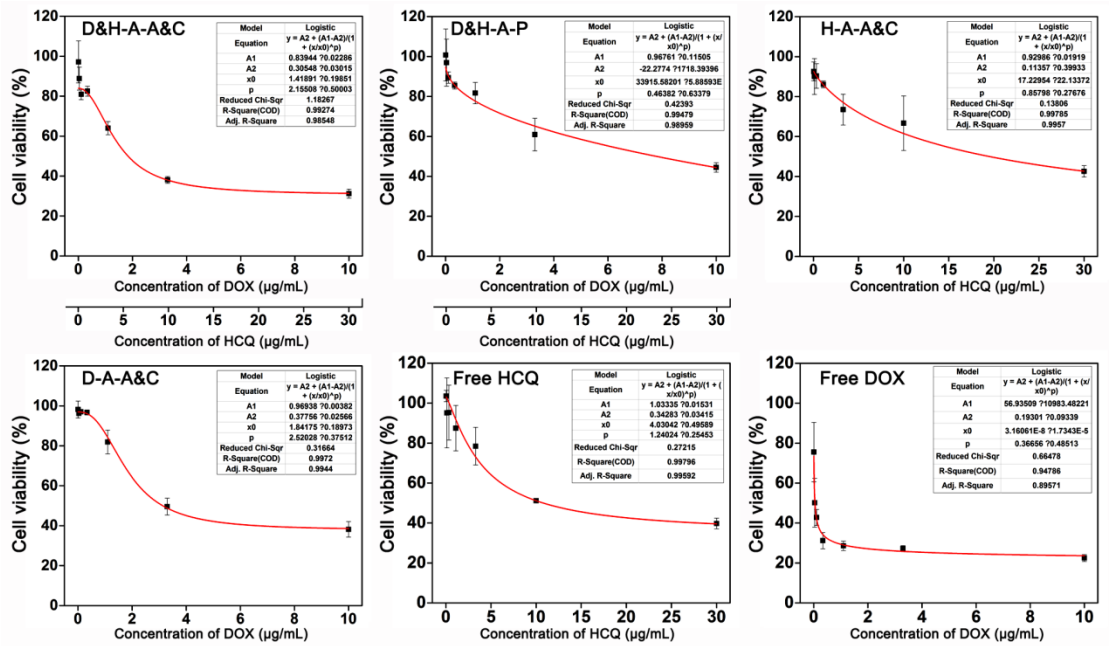

Figure S11. MTT assay of C6 cells treated with different formulation at different concentrations for 24 h.

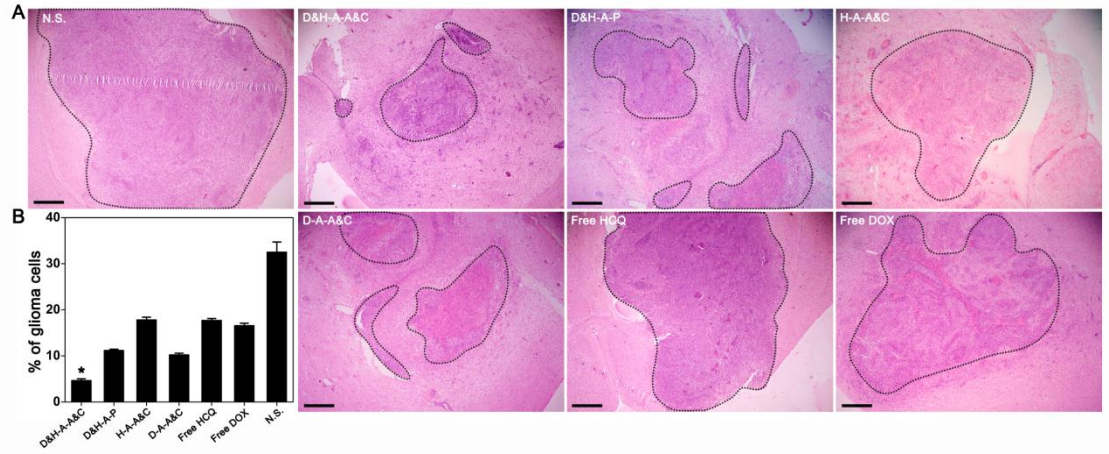

Figure S12. (A) H&E staining of mice brains collected on the day after last administration with different formulations, dash lines represent the glioma cells nuclei and bar represents 200 μm. (B) Percentage of healthy brain tissue occupied by glioma cells after different treatment calculated from A using photoshop, $*p < 0.05$.

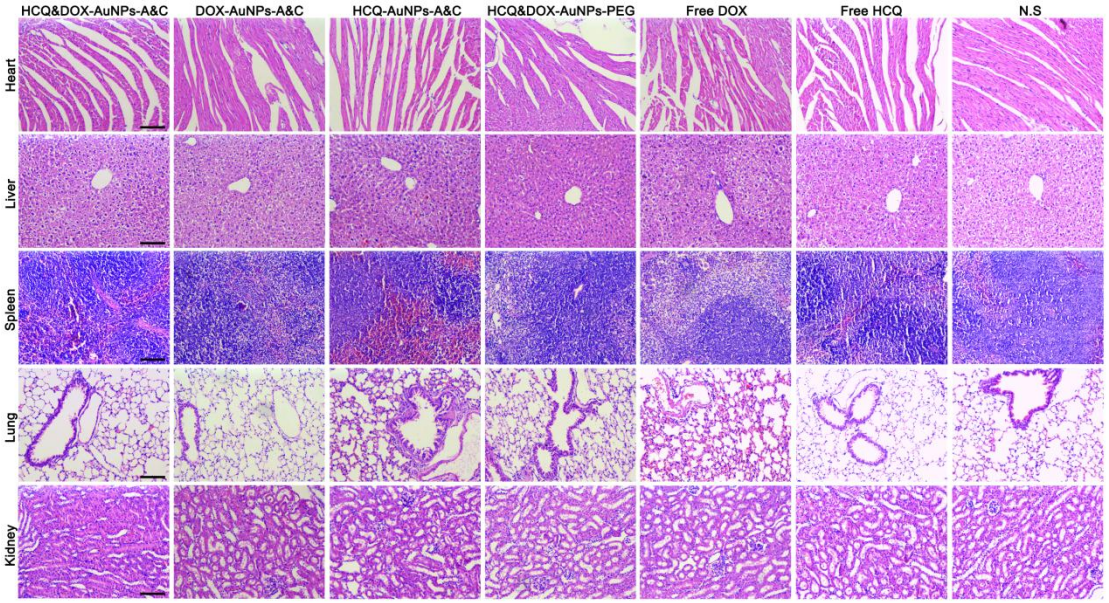

Figure S13. H&E staining of major organs from C6 glioma-bearing mice collected on

the day after last administration with different formulations, bars represent 100 μm.

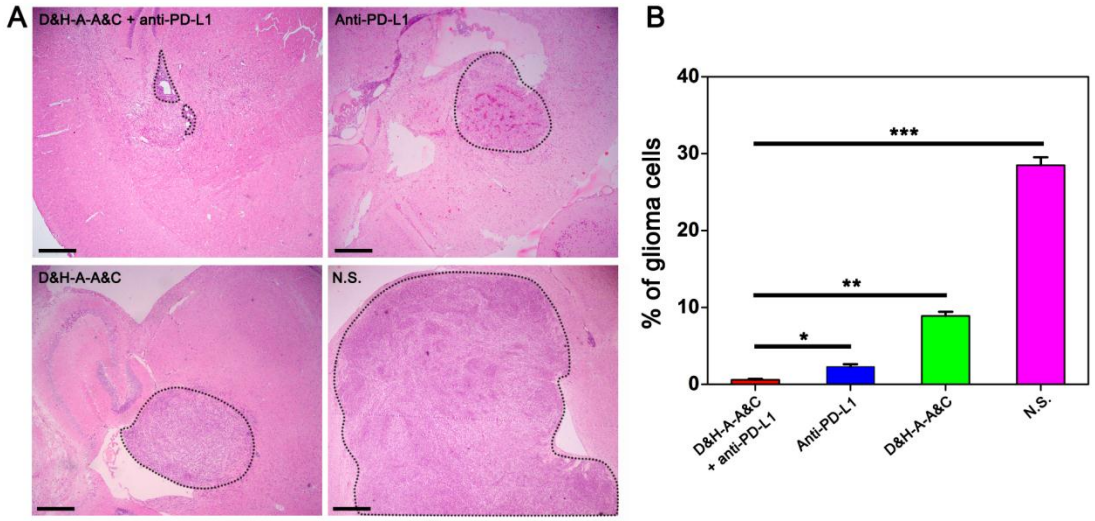

Figure S14. (A) H&E staining of mice brains collected on the day after last administration, dash line indicates the glioma foci, bar represents 200 μm. (B) Percentage of healthy brain tissue occupied by glioma cells after different treatment calculated from A using photoshop, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

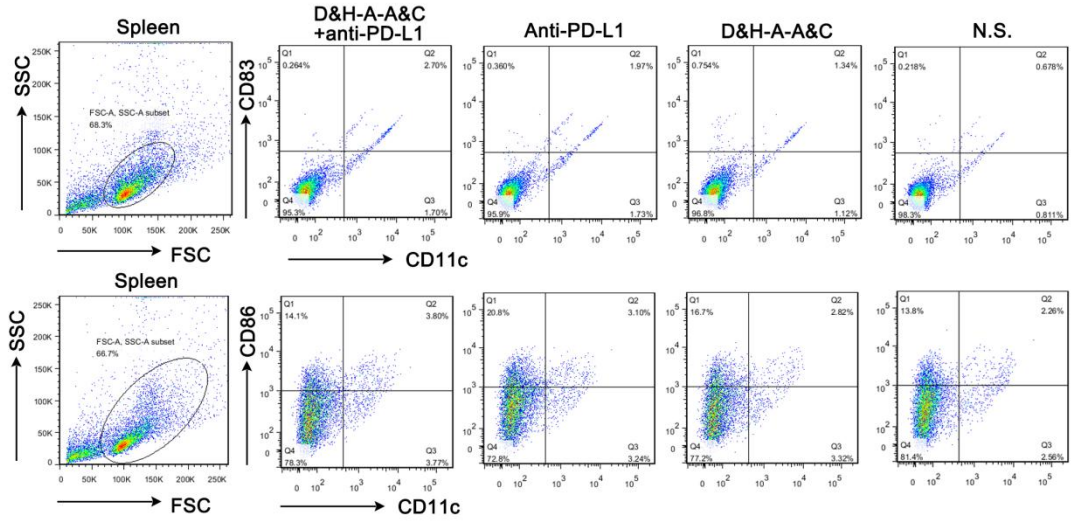

Figure S15. Gating strategy used for flow cytometry analysis of mature DCs ($CD83^+CD11c^+$ and $CD86^+CD11c^+$) population in spleen after treatment.

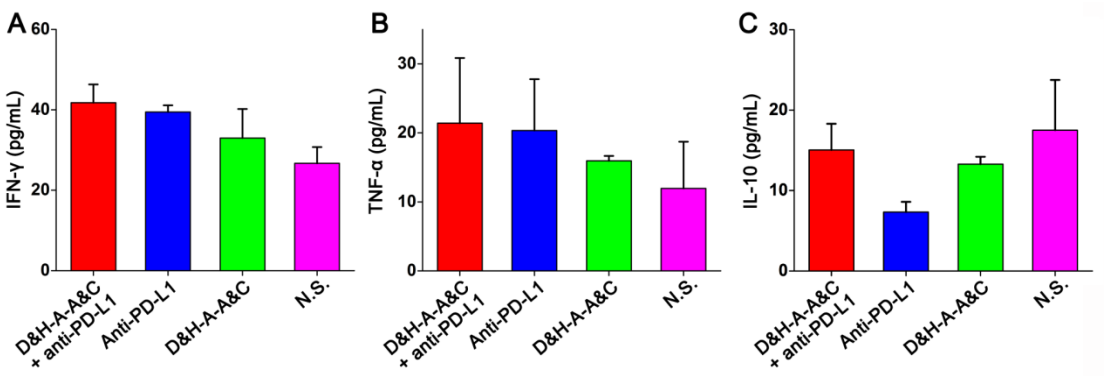

Figure S16. ELISA assay of cytokines release in serum on the second day after administration with D&H-A-A&C plus anti-PD-L1 antibody, anti-PD-L1 antibody, D&H-A-A&C. (A) IFN-γ; (B) TNF-α; (C) IL-10.

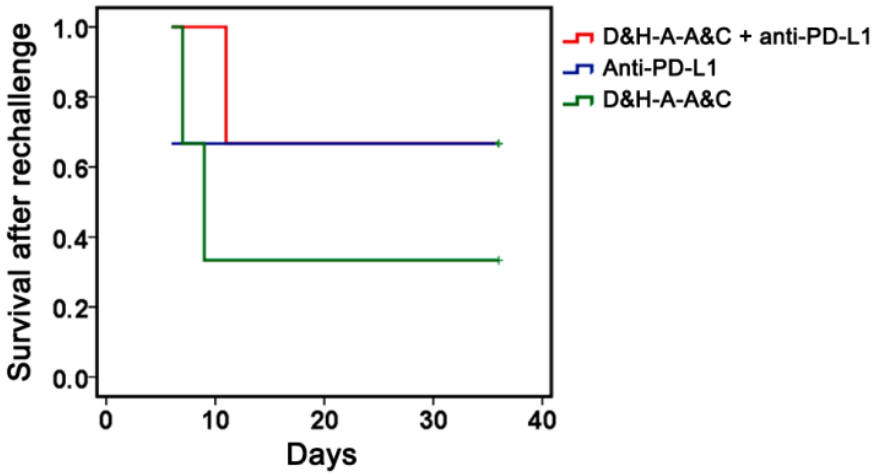

Figure S17. Kaplan-Meier survival analysis of mice surviving over 65 days were rechallenged with C6 glioma cells (n = 3).

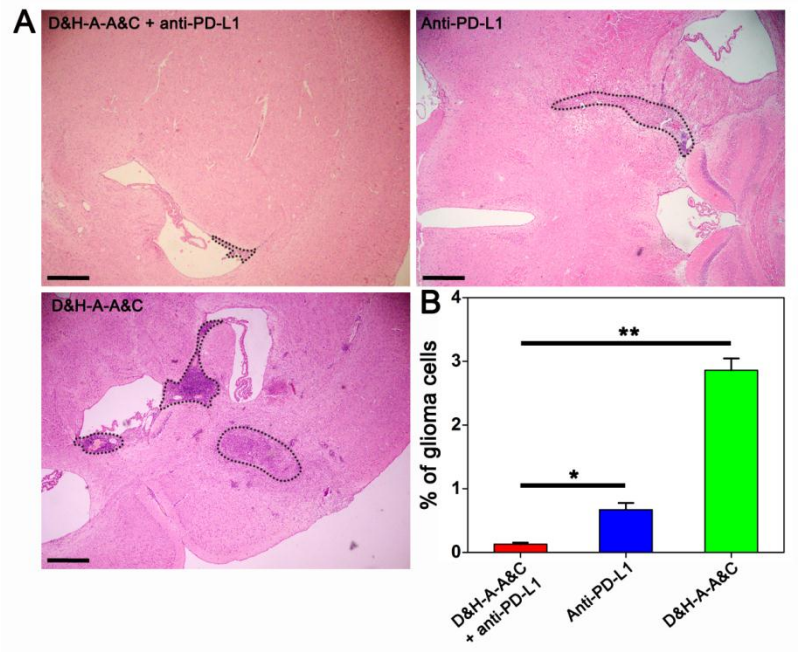

Figure S18. (A) H&E staining of mice brains collected at day 20 after the last administration, dash lines represent glioma cells area, bar represents 200 μm. (B) Percentage of healthy brain tissue occupied by glioma cells after glioma rechallenge calculated from A using photoshop, *$p < 0.05$, **$p < 0.01$.

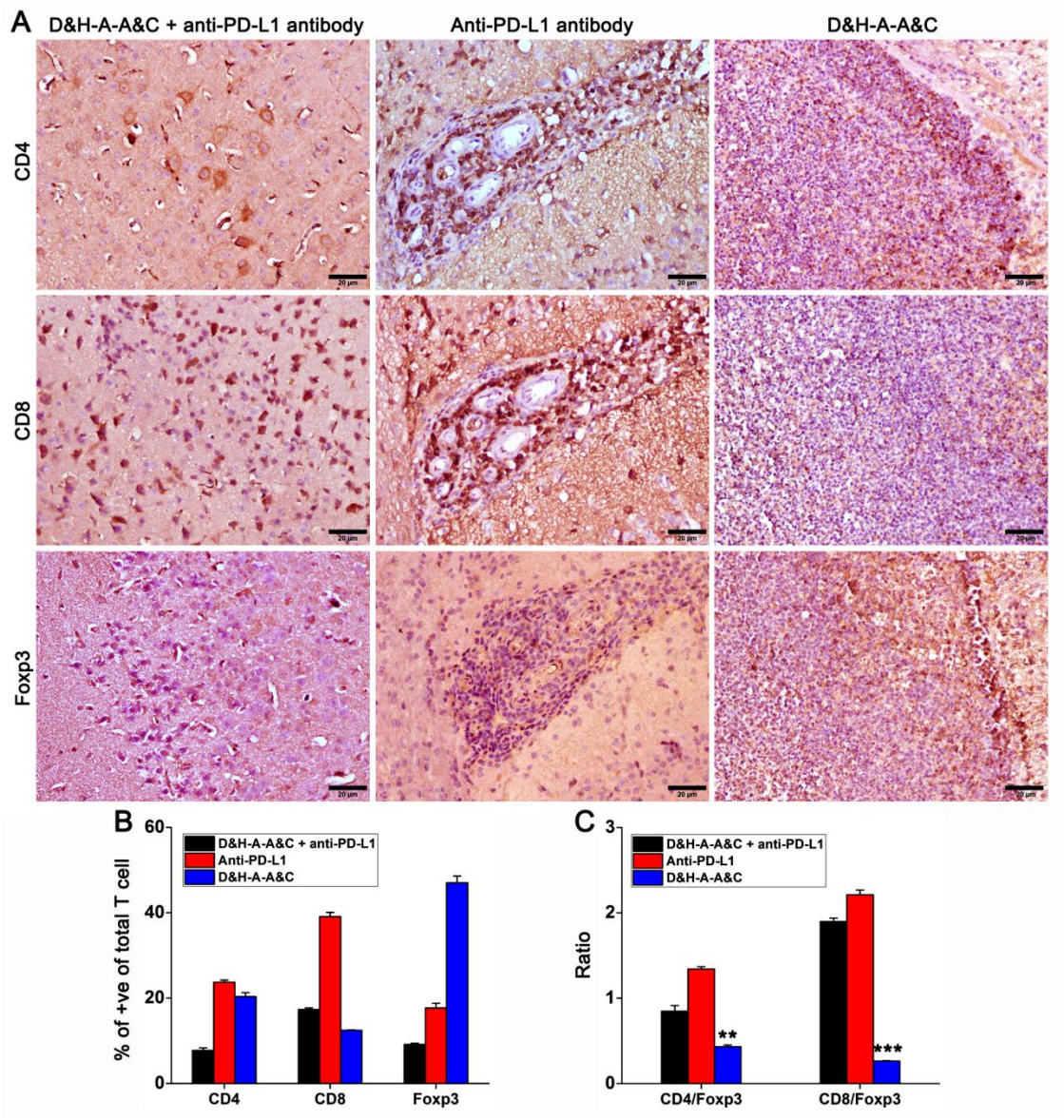

Figure S19. Evaluation of T cells population after glioma rechallenge. (A) IHC staining of mice brains collected the day after last administration, CD4, CD8 and Foxp3 were staining brown, scale bars represent 20 μm. (B) Semi-quantitative analysis of percentage of $CD4^+$ T cells, $CD8^+$ T cells and $Foxp3^+$ Treg of total T cells using ImageJ IHC Profiler analysis (n = 3). (C) Ratio of $CD4^+$ T cells to $Foxp3^+$ Treg and $CD8^+$ T cells to $Foxp3^+$ Treg, $^{**}p < 0.01$, $^{***}p < 0.001$ represent statistical significance versus D&H-A-A&C + anti-PD-L1 antibody group (n = 3).

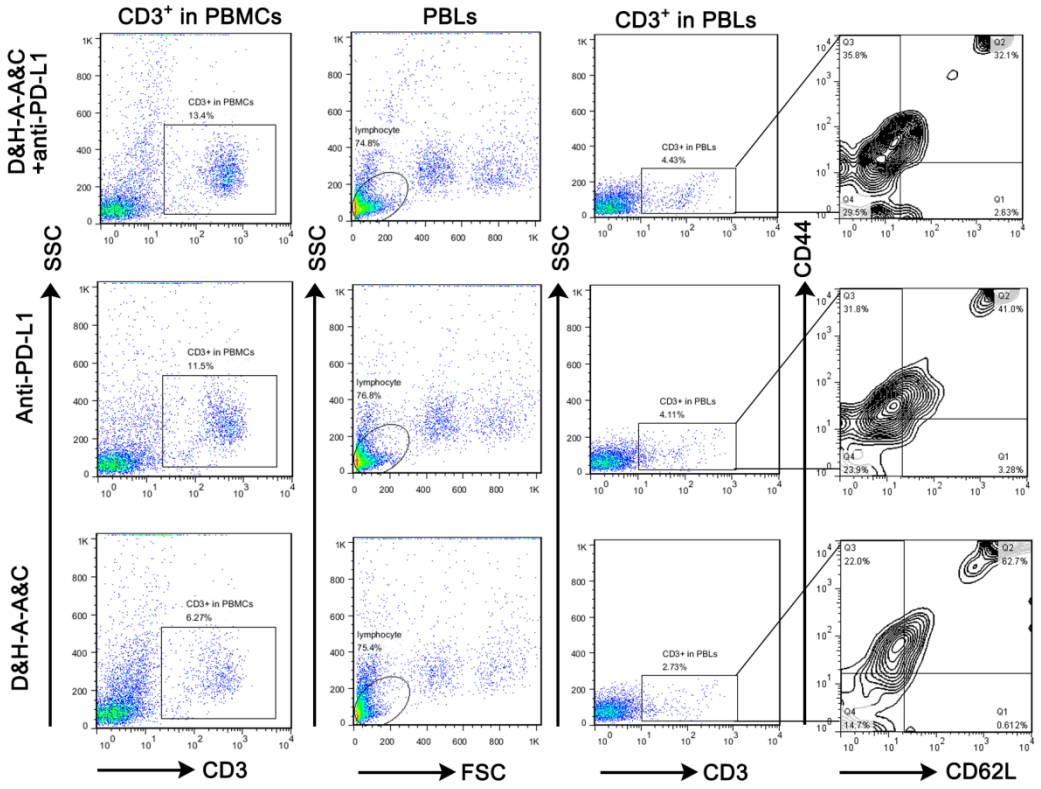

Figure S20. Gating strategy used for flow cytometry analysis of $CD3^+$ lymphocytes and T memory lymphocytes ($CD3^+CD44^{[high]}$ $CD62L^{[low]}$) in peripheral blood after treatment.

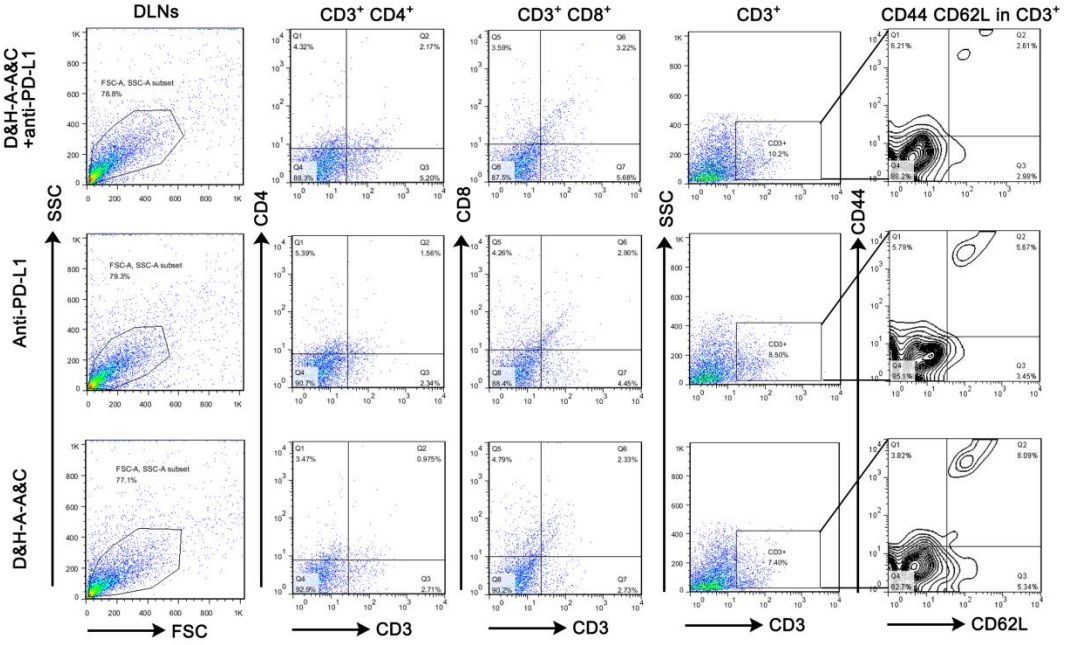

Figure S21. Gating strategy used for flow cytometry analysis of $CD3^+$ lymphocytes, T memory lymphocytes ($CD3^+CD44^{[high]}$ $CD62L^{[low]}$), T helper cells ($CD3^+CD4^+$) and cytotoxic T cells ($CD3^+CD8^+$) in DLNs after treatment.

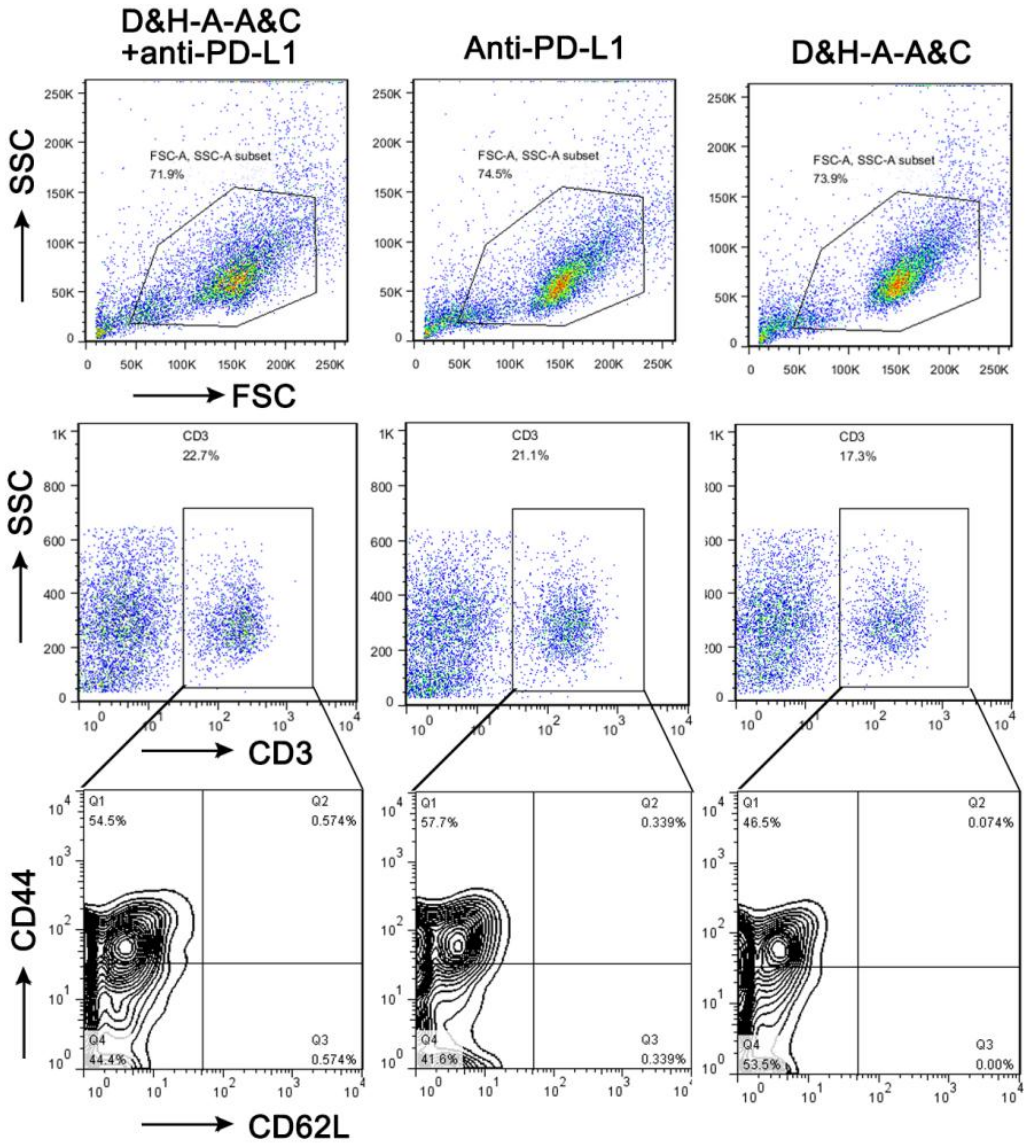

Figure S22. Gating strategy used for flow cytometry analysis of $CD3^+$ lymphocytes and T memory lymphocytes ($CD3^+CD44^{[high]}$ $CD62L^{[low]}$) in spleen after treatment.

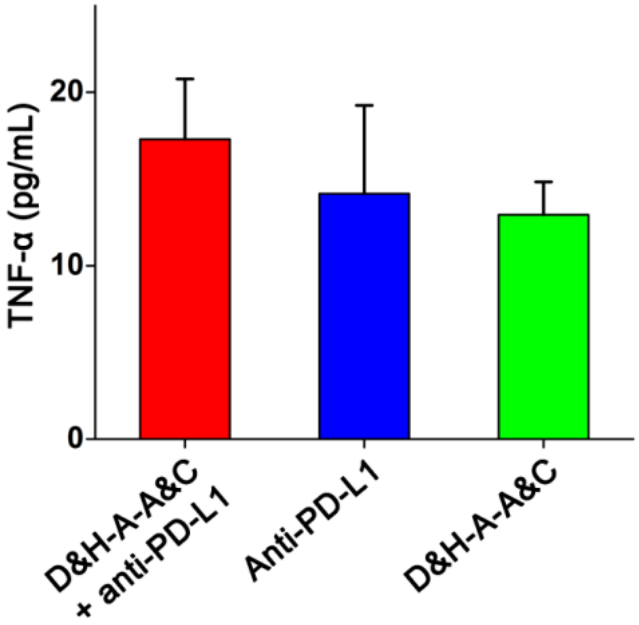

Figure S23. TNF-α secretion in serum determined by ELISA assay at day 11 after C6 glioma rechallenge (n = 3).

SUPPLEMENTARY TABLES

Table S1. Hydrodynamic diameter, PDI, ζ-potential and drug loading capacity of different nanoparticles (n = 3)

Formulations	Number mean (nm)	PDI	ζ-potential (mV)	DLC of DOX (%)	DLC of HCQ (%)
D&H-A-AK	32.47 ±1.3	0.362 ±0.045	-6.35 ±1.12	9.36 ±0.35	37.38 ±0.46
D&H-A-CABT	32.12 ±0.9	0.353 ±0.029	-6.16 ±0.89	9.43 ±0.29	38.45 ±0.61
D&H-A-P	35.93 ±1.3	0.328 ±0.041	-8.21 ±0.63	9.22 ±0.21	39.72 ±0.65
D-A-AK	34.49 ±0.6	0.328 ±0.034	-10.73 ±0.85	9.67 ±0.35	-
D-A-CABT	33.72 ±0.9	0.341 ±0.022	-11.19 ±1.26	9.59 ±0.37	-
H-A-AK	34.65 ±1.1	0.339 ±0.015	-8.45 ±1.37	-	38.90 ±0.52
H-A-CABT	33.83 ±0.8	0.314 ±0.048	-7.66 ±0.54	-	37.66 ±0.43

*Note: PDI indicates polydispersity index, DLC indicates drug loading capacity.

Table S2. Hydrodynamic size, PDI and ζ-potential of D&H-A-A&C and control nanoparticles (n = 3)

Formulations	Number mean (d. nm)	PDI	ζ-potential (mV)
D&H-A-A&C	38.10 ±1.6	0.463 ±0.062	-5.23 ±1.56
D&H-A-PEG	37.91 ±2.4	0.511 ±0.058	-5.97 ±1.39
D-A-AK	35.39 ±2.1	0.489 ±0.048	-8.62 ±1.46
H-A-AK	36.52 ±1.8	0.428 ±0.053	-6.14 ±0.98

Table S3. Median survival of C6 glioma-bearing mice treated with D&H-A-A&C, D&H-A-P, H-A-A&C, D-A-A&C, free HCQ, free DOX and N.S. (n = 10).

Group	Median (day)	Standard error (day)	Significance	Increased survival time
D&H-A-A&C	56.0	12.6	-	115%
D&H-A-P	42.0	11.6	0.272	62%
H-A-A&C	38.0	11.0	0.073	46%
D-A-A&C	44.0	14.2	0.545	69%
Free HCQ	30.0	7.9	0.010	15%
Free DOX	36.0	5.2	0.054	38%
N.S	26.0	4.9	0.003	-

Table S4. Pairwise comparison for survival using log-rank (Mantel-Cox) test (n = 10).

	1		2		3		4		5		6		7	
	a	b	a	b	a	b	a	b	a	b	a	b	a	b
1			0.367	0.545	1.209	0.272	3.209	0.073	6.669	0.010	3.704	0.054	8.895	0.003
2	0.367	0.545			0.208	0.649	1.624	0.203	4.063	0.044	2.107	0.147	6.134	0.013
3	3.209	0.073	1.624	0.203			1.171	0.279	2.243	0.134	0.245	0.620	4.364	0.037
4	1.209	0.272	0.208	0.649	1.171	0.279			3.508	0.061	1.271	0.260	6.094	0.014
5	3.704	0.054	2.107	0.147	1.271	0.260	0.245	0.620			2.579	0.108	4.255	0.039
6	6.669	0.010	4.063	0.044	3.508	0.061	2.243	0.134	2.579	0.108			1.459	0.227
7	8.985	0.003	6.134	0.013	6.049	0.014	4.364	0.037	1.459	0.227	4.255	0.039		

1 represents D&H-A-A&C; 2 represents D&H-A-P; 3 represents H-A-A&C; 4 represents D-A-A&C; 5 represents free HCQ; 6 represents free DOX; 7 represents N.S.; a represents Chi-square; b represents significance.

Table S5. Mean survival of C6 glioma-bearing mice treated with D&H-A-A&C + anti-PD-L1, anti-PD-L1, D&H-A-A&C and N.S. (n =10).

Group	Mean (day)	Standard error (day)	Significance	Increased survival time
D&H-A-A&C + anti-PD-L1	60.9	2.8	-	166%
Anti-PD-L1	51.3	6.7	0.146	124%
D&H-A-A&C	47.0	7.0	0.047	105%
N.S.	22.9	4.6	0.000	

Table S6. Pairwise comparison for survival using log-rank (Mantel-Cox) test (n = 10).

	1		2		3		4	
	a	b	a	b	a	b	a	b
1			2.115	0.146	3.942	0.047	25.791	0.000
2	2.115	0.146			0.158	0.691	7.888	0.005
3	3.942	0.047	0.158	0.691			5.822	0.016
4	25.791	0.000	7.888	0.005	5.882	0.16		

Note: 1 represent D&H-A-A&C + anti-PD-L1; 2 represent anti-PD-L1; 3 represent D&H-A-A&C; 4 represent N.S.; a represents Chi-square; b represents significance.

Table S7. Mean survival of mice surviving over 65 days rechallenged with C6 glioma cells (n =10).

Group	Mean (day)	Standard error (day)	Significance
D&H-A-A&C + anti-PD-L1	27.7	6.8	-
Anti-PD-L1	26.0	8.2	0.886
D&H-A-A&C	17.3	7.6	0.302
N.S.	-	-	-

Table S8. Pairwise comparison for survival using log-rank (Mantel-Cox) test (n = 10).

	1		2		3		4	
	a	b	a	b	a	b	a	b
1			0.020	0.886	1.066	0.302	-	-
2	0.020	0.886			0.216	0.642	-	-
3	1.066	0.302	0.216	0.642			-	-
4	-	-	-	-	-	-	-	-

Note: 1 represent D&H-A-A&C + anti-PD-L1; 2 represent anti-PD-L1; 3 represent D&H-A-A&C; 4 represent N.S.; a represents Chi-square; b represents significance.

SUPPLEMENTARY REFERENCES

(1) Ruan, S.; Yuan, M.; Zhang, L.; Hu, G.; Chen, J.; Cun, X.; Zhang, Q.; Yang, Y.; He, Q.; Gao, H. Tumor Microenvironment Sensitive Doxorubicin Delivery and Release to Glioma Using Angiopep-2 Decorated Gold Nanoparticles. *Biomaterials* **2015**, 37, 425-435.

(2) Ruan, S.; He, Q.; Gao, H. Matrix Metalloproteinase Triggered Size-Shrinkable Gelatin-Gold Fabricated Nanoparticles for Tumor Microenvironment Sensitive Penetration and Diagnosis of Glioma. *Nanoscale* **2015**, 7, 9487-9496.

(3) Ruan, S.; Hu, C.; Tang, X.; Cun, X.; Xiao, W.; Shi, K.; He, Q.; Gao, H. Increased Gold Nanoparticle Retention in Brain Tumors by in Situ Enzyme-Induced Aggregation. *ACS Nano* **2016**, 10, 10086-98.