Supporting Information

Sustained delivery of carfilzomib by tannic acid-based nanocapsules helps develop antitumor immunity

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Materials and Methods

Materials

Carfilzomib (CFZ) was purchased from Shenzhen Chemical Co. LTD. (Shanghai, China). Tannic acid, iron chloride, 2-hydroxypropyl-β-cyclodextrin, human serum albumin (≥96% agarose gel electrophoresis) and Irgacure 2959 were purchased from Sigma–Aldrich (St. Louis, MO, USA). Polyethyleneglycol dimethylacrylate (PEGDA, MW: 3400 Da) was purchased from Alfa Aesar (Haverhill, MA, USA). Mouse interferon-gamma (IFN-γ) ELISA kit was purchased from Invitrogen (Eugene, OR, USA). GM-CSF was purchased from PeproTech (Rock Hill, NJ, USA). Purified rat anti-mouse CD16/CD32 (Fc block), APC anti-mouse CD11c, FITC anti-mouse CD40, PE anti-mouse CD86, FITC anti-mouse CD4, APC anti-mouse CD8a, and Zombie Violet were purchased from BioLegend (San Diego, CA, USA). AH1 peptide was purchased from Anaspec (Fremont, CA, USA). All other materials were of analytical grade and purchased from Thermo Fisher Scientific (Waltham, MA, USA).

TA/Fe^{3+}-stabilized CFZ nanocore preparation and albumin coating

CFZ was mixed with TA in 30 μL ethanol at a CFZ to TA molar ratio of 2.4. To this mixture, 10 mL water containing 100 μg $FeCl_3$ (molar ratio of TA to Fe^{3+}: 1.1) was added and mixed briefly with simple agitation to form nanocapsules. The particles were centrifugated at 43,400 rcf for 20 min at 4 °C to remove excess TA and $FeCl_3$. The formed nanocapsules were called CFZ-pTA, where pTA stands for polymerized tannic acid crosslinked via Fe^{3+}. The CFZ-pTA nanocapsules were further incubated in albumin solution (2 mg/mL) in water at CFZ-pTA to albumin weight ratio of 1:2, with mild rotation at room temperature (RT) for 4 h. The particles were centrifuged at 43,400 rcf for 20 min at 4°C to remove unadsorbed albumin and washed twice by repeated centrifugation. The albumin-coated CFZ-pTA nanocapsules were called CFZ-pTA-alb. As controls, pTA (TA/Fe^{3+} nanoassemblies) and pTA-alb (albumin-coated pTA) were prepared in the same method as above omitting CFZ. Bare CFZ particles (with no pTA) were prepared by adding water to ethanolic solution of CFZ. The particles were suspended in water and stored at 4 °C.

Nanocapsule characterization

Particle size and surface charge

The hydrodynamic diameter (z-ave), polydispersity index (PDI) and zeta potential of particles were measured by dynamic light scattering (DLS) in sodium phosphate buffer (10 mM, pH 7.4) using a Zetasizer Nano-ZS90 (Malvern Instruments, Worcestershire, UK).

Morphology

Particle morphology was examined by transmission electron microscopy (TEM). An aqueous suspension of freshly prepared particles was placed on a carbon coated copper grid (400 mesh), negatively stained with 1% uranyl acetate and allowed to dry in air. The dried grid was examined under an FEI Tecnai T20 transmission electron microscope (Hillsboro, OR, USA). To visualize the capsule structure of CFZ-pTA, the core part of the particles was dissolved in PBS containing 0.2% Tween 80 for 6 h. The particles were collected by centrifugation, resuspended in water, put on the grid and processed as above.

CFZ and TA contents in CFZ-pTA

The CFZ content in CFZ-pTA was determined by C18 reverse phase HPLC (25 cm × 4.6 mm, particle size: 5 μm). CFZ-pTA with a premeasured mass was added to an aqueous solution (pH 7.4) containing ethylenediaminetetraacetic acid (EDTA) (100 mM, to remove Fe^{3+}) and urea solution (5M, to disrupt hydrogen bonding between TA and CFZ), mixed briefly by vortexing, and mixed with additional acetonitrile (to dissolve CFZ), making the final particle concentration ~50 μg/mL. The resulting solution was filtered on 0.45 μm syringe filter prior to analysis. CFZ was analyzed according to the previously reported method[1] with slight modifications: the mobile phase was composed of water and acetonitrile containing 0.05% trifluoroacetic acid and run in an acetonitrile gradient of 40-80% over 22 min at 0.7 mL/min. CFZ was detected with a UV detector at a wavelength of 210 nm.

The TA content in CFZ-pTA was estimated by bicinchoninic acid (BCA) assay (Pierce® BCA assay kit). CFZ-pTA 0.1 mg was suspended in 200 μL of 0.05 N HCl to dissolve TA. The BCA reagent was mixed with the sample in 8:1 v/v ratio at 37 °C for 30 min. The absorbance of the solution was read at 570 nm by a SpectraMax M3 microplate reader (Molecular Device, Sunnyvale, CA, USA). CFZ solution at an equivalent concentration was treated same way, and the absorbance was subtracted from the reading of CFZ-pTA. The difference was compared to a calibration curve drawn with TA to determine the TA concentration in the sample.

Albumin content in CFZ-pTA-alb

The albumin content in CFZ-pTA-alb was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). CFZ-pTA-alb with a premeasured mass or standard albumin solutions were prepared in Laemmli buffer containing β-mercaptoethanol and heated at 95 °C for 5 min. The samples were resolved on a 12% SDS-PAGE run at 120 V for 30 min. After staining with QC Colloidal Coomassie Stain and imaged with Azure c300 (Dublin, CA, USA), the band intensity was analyzed by densitometry (AzureSpot Analysis Software). The albumin content was determined by comparing the band intensities of CFZ-pTA-alb samples and standard albumin solutions.

Size stability

CFZ-pTA or CFZ-pTA-alb (at a concentration equivalent to CFZ 60 μg/mL, n=3 per group) were incubated in 50% fetal bovine serum (FBS) at 37 °C for 24 h. The suspensions were sampled periodically to measure their particle size by the Zetasizer.

In vitro CFZ release kinetics

CFZ-pTA or CFZ-pTA equivalent to 10 μg of CFZ were suspended in 0.25 mL of 10% PEG dimethylacrylate (PEGDA, 3400 Da) solution in PBS. The suspensions were illuminated under UV (365 nm) for 10 min in the presence of 25 μL of irgacure solution (20% w/v in methanol) to crosslink PEGDA. The crosslinked PEGDA matrix was briefly rinsed with water to remove free particles and immersed in 1 mL of RPMI-1640 medium supplemented with 10% FBS as a release medium (n=3 per group). The matrix was then incubated at 37 °C on an orbital shaker, and the release medium was completely removed for HPLC analysis and replaced with 1 mL of fresh medium at predetermined time points.

Cytotoxicity of CFZ

B16F10 (derived from murine melanoma; ATCC, Manassas, VA, USA) or HCC-1937 (derived from human triple negative breast cancer; ATCC, Manassas, VA, USA) were grown in RPMI 1640 or DMEM medium, respectively, supplemented with 10% FBS and penicillin (100 IU/mL) and streptomycin (100 μg/mL). Cells were seeded in a 96 well plate at a density of 4,000 cells per well. After 24 h incubation, the culture medium was replaced with fresh medium containing CFZ (as a stock solution in DMSO), CFZ-pTA or CFZ-pTA-alb. The cells were subject to either continuous treatment (in drug-containing media at concentrations equivalent to CFZ 10 - 400 nM for 72 h) or pulse treatment (in drug-containing media at concentrations equivalent to CFZ 50 - 800 nM for 2 - 24 h, followed by washout and additional incubation in drug-free medium up to 72 h). Cell viability was estimated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Culture medium was replaced with 100 μL of fresh medium and 15 μL of MTT solution (5 mg/mL), and cells were incubated for 4 h. The stop/solubilizing solution was added to dissolve formazan crystals, and the absorbance was read at the wavelength of 562 nm by the SpectraMax M3 microplate reader. The cell viability was calculated by normalizing the measured absorbance to that of control cells that did not receive treatments.

The cytotoxicity of CFZ was also measured on splenocytes. The spleen was freshly harvested from a male C57BL/6 mouse, cut into pieces, and filtered through 70 μm and 40 μm cell strainers sequentially to obtain a single cell suspension. The cell suspension was incubated with 1 mL ammonium-chloride-potassium (ACK) lysis buffer for 1 min to remove red blood cells. The splenocytes, suspended in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 IU/mL) and streptomycin (100 μg/mL), were seeded at 200,000 cells per well in a 96 well plate and treated with CFZ solutions for 24 h or 2.4 h with additional 21.6 h incubation in drug-free medium. Cell viability was measured as described above.

***In vitro* metabolic stability of CFZ**

Metabolic stability of CFZ in different formulations (CFZ-CD, CFZ dissolved in 10 mM citrate buffer (pH 3.1) with 20% (w/v) 2-hydroxypropyl-β-cyclodextrin),[2] CFZ-pTA and CFZ-pTA-alb) was measured in whole blood. The blood was collected from C57BL/6 male mice and stored in a heparinized tube on ice. CFZ formulations were added to the cold blood at a final concentration of 67 μM (n=3 per group) and incubated at 37 °C for 30 min. The blood sample was mixed with 200 μL of 5% Triton-X 100 and vortexed for 10 min. To this mixture, 600 μL of acetonitrile was added, bath-sonicated for 10 min, and centrifuged at 4500 rcf for 30 min to remove precipitated proteins. The supernatant was analyzed by HPLC.[1] A calibration standard curve was prepared with CFZ doped in blood at final concentrations of 4 - 278 μM and treated in the same way as the samples immediately.

Cellular uptake of CFZ-pTA or CFZ-pTA-alb

Fluorescently-labeled CFZ-pTA (CFZ*-pTA) or CFZ-pTA-alb (CFZ*-pTA-alb) were prepared by adding DiI in the CFZ/TA mixture at a concentration of 0.5 wt%. The amount of incorporated DiI was determined by measuring the fluorescence intensity ($\lambda_{ex}/\lambda_{em}$: 549 nm/565 nm) of DiI after dissolving nanocapsules in acetonitrile. B16F10 or HCC 1937 cells were seeded in a 35 mm glass bottom dish with a 14 mm micro-well, at a density of 300,000 cells per dish. After 24 h, the medium was replaced with 1 mL of fresh serum-supplemented medium containing CFZ*-pTA, CFZ*-pTA-alb (particle concentration: 100 μg/mL), or free DiI, at equivalent fluorescence intensity. After 2 or 4

h incubation with the treatments, cells were rinsed with PBS twice followed by fixation with 4% paraformaldehyde for 10 min. Cells were incubated with Hoechst 33342 (5 μg/mL) for 5 min for nuclei staining. After rinsing, cells were imaged with a Nikon-A1R confocal microscope (Nikon America Inc., NY, USA). Cell nuclei were detected with $\lambda_{ex}/\lambda_{em}$ of 407 nm/450 nm, and fluorescent nanocapsules with $\lambda_{ex}/\lambda_{em}$ of 561 nm/595 nm.

Protein-capturing ability of CFZ-pTA-alb

B16F10 cells were treated with 10 μM CFZ solution in serum-free RPMI 1640 medium (supplemented with penicillin 100 IU/mL and streptomycin 100 μg/mL) for 48 h. The cells were centrifuged at 300 rcf for 5 min to separate soluble protein antigens and DAMPs released from dying cells. CFZ-pTA-alb was incubated in the collected supernatant at a NP concentration of 1 mg/mL for 2 h at 37 °C with rotation. The particles were centrifuged at 16,100 rcf for 20 min at 4 °C, washed with water twice, resuspended in water at a concentration of 4 mg/mL and analyzed by SDS-PAGE. Protein bands were excised and analyzed by LC-MS/MS according to the method described previously.[3]

CFZ-pTA-alb - dendritic cell interaction

Bone marrow cells were collected from healthy male C57BL/6 as reported previously.[4] Mice at the age of 6-8 weeks were sacrificed using CO_2 asphyxiation. Using a syringe, the cavities of femur bones were flushed with RPMI 1640 medium containing penicillin/streptomycin. The collected bone marrow was pipetted several times and passed through a 40 μm cell strainer to obtain single cell suspension. The cells were collected by centrifugation at 500 rcf for 8 min, treated with ACK buffer, rinsed, and suspended in IMDM medium supplemented with 10% FBS, penicillin/streptomycin, 20 ng/mL GM-CSF, and 10 mM β-mercaptoethanol at a density of 2×10^6 cells per 10 mL to differentiate into dendritic cells (DCs). Additional medium was supplemented three days later, and the floating and loosely adherent cells were collected by centrifugation on day 6. The DC phenotype was confirmed by CD11c staining. In a 6-well plate, 10^5 DCs were cocultured for 4 or 24 h with 4×10^5 DiI-labeled B16F10 cells (denoted as *B16F10 cells; DiI stains the whole cell[6]), which had been left untreated or treated with blank pTA-alb, CFZ solution or CFZ-pTA-alb (at a concentration equivalent to 10 μM CFZ) for 24 h and rinsed once. The co-cultured cells were collected, resuspended in staining buffer, incubated with Fc block for 5 min, and stained with APC-labeled anti-mouse CD11c antibody to determine the extent of phagocytic uptake of *B16F10 cells by dendritic cells. Separately, the co-cultured cells (with unlabeled B16F10 cells) were stained with APC-labeled anti-mouse CD11c, FITC-labeled anti-mouse CD40 and PE-labeled anti-mouse CD86 antibodies to determine the activation status of DCs. The stained cells were analyzed by flow cytometry (Accuri C6, BD Biosciences, San Jose, CA, USA). Fluorescence-minus-one (FMO) controls were used for compensation and gating. The extent of phagocytosis was expressed as the percentage of DiI^+CD11c^+ cells per total $CD11c^+$ cells. The DC activation was assessed by $CD11c^+CD40^+$ or $CD11c^+CD86^+$ per total $CD11c^+$ cells.

Local administration of CFZ-pTA-alb

All animal procedures were approved by Purdue Animal Care and Use Committee, in conformity with the NIH guidelines for the care and use of laboratory animals.

Antitumor effect in B16F10 tumors in C57BL/6 mice

Male C57BL/6 mice (5-6 week old, ~20 g) were purchased from Envigo (Indianapolis, IN, USA) and acclimatized for 1 week prior to tumor inoculation. One million B16F10 melanoma cells were inoculated subcutaneously to each C57BL/6 mouse in the upper flank of right hind limb. When tumors reached 50-100 mm^3 on the average, mice were randomly assigned to 3 groups and treated with an intratumoral injection of 50 μL of PBS, CFZ-CD or CFZ-pTA-alb (at a dose equivalent to 1.2 μg CFZ). The tumor volume and body weight were measured every day. Animals showing >20% body weight loss or with tumors greater than 10% of the body weight were humanely sacrificed prior to the end of the study. The tumor length (L) and width (W) were measured using a digital caliper and the volume (V) was calculated as: $V= (L \times W^2)/2)$. The specific growth rate of the tumor was calculated as $\Delta \log V/\Delta t$ (t: time in days) [7]. The mice were sacrificed at 7 days after the treatment.

B16F10 tumors were collected, cut into small pieces, mechanically disrupted, and filtered through 70 μm and 40 μm cell strainers sequentially to obtain a single cell suspension. The cell suspension was incubated with 3 mL of ACK lysis buffer for 1 min to remove red blood cells. The cells were rinsed with PBS, resuspended in cell staining buffer at a density of 10^6 cells per 100 μL, incubated with Fc block for 5 min, and stained with PE-labeled anti-mouse CD8a antibody for 1 h at 4 °C. The stained cells were analyzed by flow cytometry (Accuri C6, BD Biosciences, San Jose, CA).

CFZ retention in B16F10 tumor

Fifty microliters of CFZ-CD or CFZ-pTA-alb (at a dose equivalent to 1.2 μg CFZ) were injected intratumorally into 100 mm^3 B16F10 tumors inoculated in C57BL/6 mice. Two hours later the mice were sacrificed, blood was collected via cardiac puncture and put in a heparinized tube. Tumors were harvested, washed with PBS and homogenized in cold PBS (pH 7.4) at 100 mg tissue per 400 μL with an Omni Tissue Master 125 homogenizer. A 100 μL of the tumor homogenate was vortex-mixed with 200 μL of 5% Triton-X 100 for 2 min and then mixed with tert-butyl methyl ether (TBME) (1800 μL) containing carfilzomib-d8 (Cayman Chemical, Ann Arbor, MI, USA) as an internal standard (250 ng/mL) by rotation for 40 min to extract CFZ. The mixture was centrifuged at 4,500 rcf for 15 min, and the organic layer was separated, transferred to a glass vial, and dried in vacuum. The dried sample was dissolved in 100 μL of DMSO, filtered through 0.45 μm syringe filter and analyzed by LC-MS/MS (Agilent triple quadrupole mass spectrometer coupled with the Agilent 1200 Rapid Resolution HPLC, operated in a positive ion mode).[8] A calibration standard curve was prepared with tumor homogenates doped with CFZ at a concentration of 0.99 – 500 ng/mL and processed in the same way as the samples. For quantification of CFZ absorbed to the system, 5 μL of plasma was mixed with 40 μL of acetonitrile, containing 250 ng/mL carfilzomib-d8 as an internal standard, and made up to 20 μL with water. The mixture was vortexed for 5 min then centrifuged at 13,000 g for 10 min. The supernatant was analyzed by LC-MS/MS.[8] A calibration standard curve was prepared with plasma doped with CFZ at a concentration of 3.26 to 416.7 ng/mL and processed in the same way as the samples.

Effects on remote B16F10 tumors in C57BL/6 mice or athymic nude mice

C57BL/6 mice were inoculated with 10^6 B16F10 cells subcutaneously in the upper flank of right hind limb. Seven days later, the mice were inoculated with 3×10^5 B16F10 cells on the contralateral side. When the first tumor on the right side reached 50 mm^3 on the average, mice were randomly assigned to 3 groups. Each mouse received an intratumoral injection of 20 μL of PBS, CFZ-CD or CFZ-pTA-alb (at a dose equivalent to 60 μg CFZ) in the first tumor. The treated tumor and the second inoculation site on the left were monitored every other day. The same procedure was repeated with athymic nude ($Foxn1^{nu}$) mice.

Anti-tumor effect and tumor immunophenotyping in CT26 tumors in Balb/c mice

10^5 CT26 mouse colon carcinoma cells (ATCC, Manassas, VA, USA) were inoculated subcutaneously in the mammary fat pad of each female Balb/c mouse. When tumors reached 50-100 mm^3 on the average, mice were randomly assigned to 3 groups and received an intratumoral injection of 50 μL of PBS, CFZ-CD or CFZ-pTA-alb (at a dose equivalent to 1.2 μg CFZ). The tumor volume and body weight were measured every day for 7 days. A separate group of CT26 tumor-bearing Balb/c mice (3×10^5 CT26 cells inoculated subcutaneously to the upper flank of the right hind limb) were treated with PBS, CFZ-CD or CFZ-pTA-alb (at a dose equivalent to 60 μg CFZ) and sacrificed at 6 days post treatment. Tumors were digested in 10 mL of complete RPMI 1640 containing 1 mg/mL collagenase I for 1-2 h, passed through a 70 μm cell strainer to remove large debris, and then pelleted. The samples were treated with 3 mL ACK buffer for 2 min at room temperature, rinsed with 10 volumes of PBS, passed through a 70 μm cell strainer and incubated with Zombie Violet and Fc block at room temperature in dark for 10 min. BV605-labeled anti-mouse CD45 and APC-labeled anti-mouse CD8a antibodies were added to the samples and incubated for 30 min at 4 °C. The stained cells were rinsed with PBS and resuspended in 1 mL of 10% neutral buffered formalin for flow cytometry analysis (BD LSRFortessa, San Jose, CA, USA).

Tumor-specific immunity in CT26 tumors in Balb/c mice

Female Balb/c mice were inoculated with 3×10^5 CT26 cells subcutaneously in the upper flank of right hind limb, followed by 10^5 CT26 cells on the left hind limb 7 days later. When tumors on the right limb grew to ~50 mm^3, the mice were randomly assigned to 3 groups and treated with an intratumoral injection of 20 μL of PBS, CFZ-CD or CFZ-pTA-alb (at a dose equivalent to 60 μg CFZ). The tumor size was monitored for 22 days. At sacrifice, the spleens were collected to evaluate the response to AH1 peptide, a CT26-related peptide antigen. Splenocytes were prepared as described previously, plated at a density of 3×10^5 cells per well in a 96-well plate, and stimulated with 10 μg/mL of AH1 peptide in the presence of 20 ng/mL of GM-CSF. After 72 h incubation, the cells were centrifuged at 300 rcf for 5 min to separate a supernatant. The concentration of interferon-γ (IFN-γ) in each supernatant was measured by ELISA (Invitrogen, Carlsbad, CA, USA). The IFN-γ production from the AH1 peptide-challenged splenocytes was compared with that of the non-challenged cells collected from the same mouse.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 7 (La Jolla, CA, USA). All *in vitro* data were analyzed by unpaired two-way t-test, one-way or two-way ANOVA test to determine the difference of means among groups, followed by the recommended multiple

comparisons tests. *In vivo* data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test unless specified otherwise. The comparison of survival curves was conducted with the Log-rank (Mantel-Cox) test. The p-value was indicated for each comparison. A value of $p < 0.005$ was considered statistically significant, and p-values between 0.05 and 0.005 were referred as suggestive, according to the recommendation of the American Statistical Association.[9]

Table S1. Particle size and zeta potential of NPs (at least n = 4 independent samples; mean ± s.d.)

NPs	Suspending medium	Z-average (nm)	PDI	Zeta potential (mV)
pTA	Phosphate buffer	174 ± 32	0.11 ± 0.01	-26 ± 4
CFZ	Water	161 ± 1	0.17 ± 0.03	17 ± 8
	Phosphate buffer	3473 ± 1265	0.57 ± 0.16	-3 ± 2
CFZ-pTA	Phosphate buffer	164 ± 1	0.16 ± 0.02	-27 ± 4
CFZ-pTA-alb	Phosphate buffer	179 ± 6	0.09 ± 0.01	-21 ± 3

Phosphate buffer: 10 mM, pH 7.4

Table S2. NP compositions (n = 3 independent samples; mean ± s.d.)

	CFZ (%)	TA (%)	Fe (%)	HSA (%)
CFZ-pTA	51 ± 1	49 ± 7	4.6 ± 0.02	-
CFZ-pTA-alb	41 ± 2	not measured	3.0 ± 0.0	15 ± 1

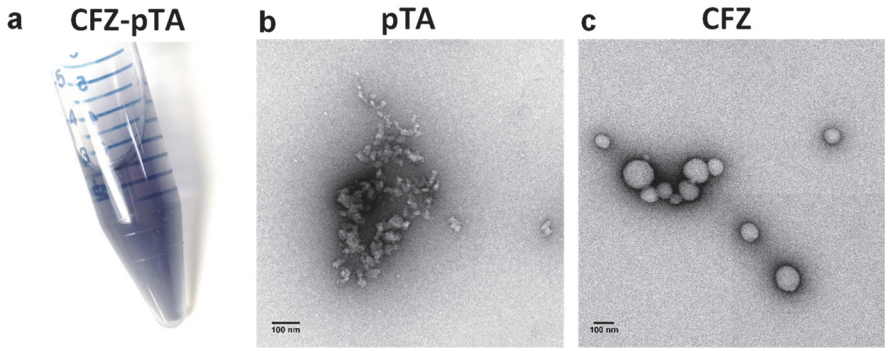


Figure S1. (a) CFZ-pTA suspension with a unique blue color. TEM images of (b) pTA assemblies (without CFZ) and (c) CFZ nanodroplets (without TA and $FeCl_3$).

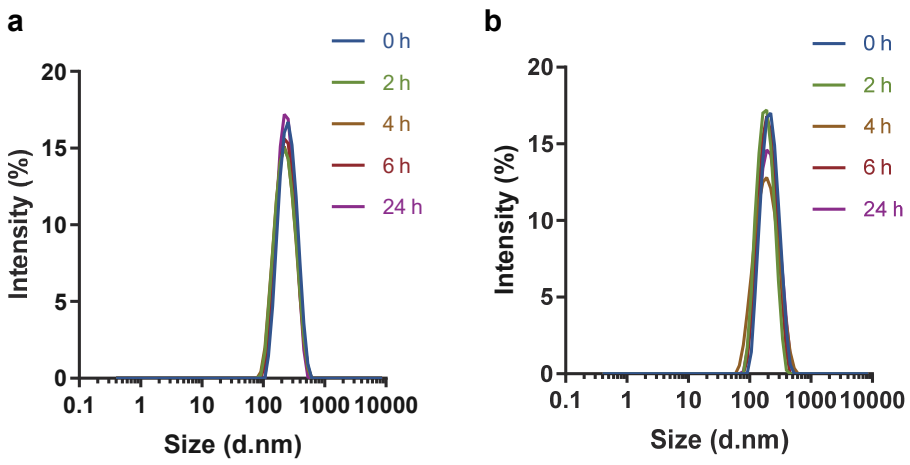


Figure S2. Size distribution of (a) CFZ-pTA and (b) CFZ-pTA-alb, incubated in 50% FBS at 37 °C for different time periods. Measured by dynamic light scattering. Representative of multiple batches.

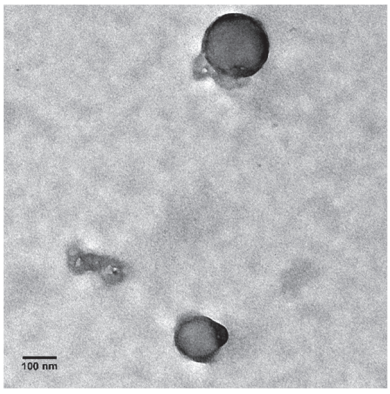


Figure S3. TEM image of CFZ-pTA incubated in 100% FBS for 24 h.

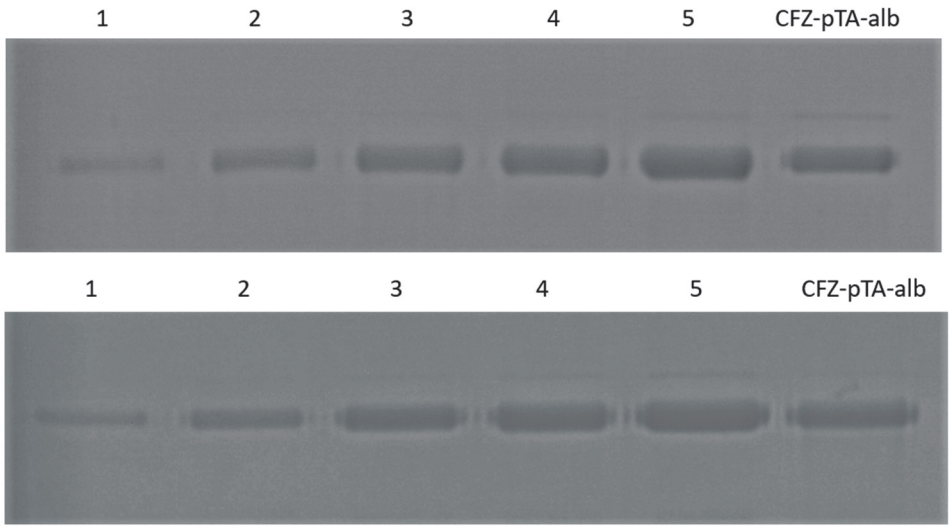


Figure S4. Two representative SDS-PAGE gels used for determination of albumin content in CFZ-pTA-alb. Lanes 1, 2, 3, 4 and 5: 10, 25, 50, 75 and 100 µg/mL albumin.

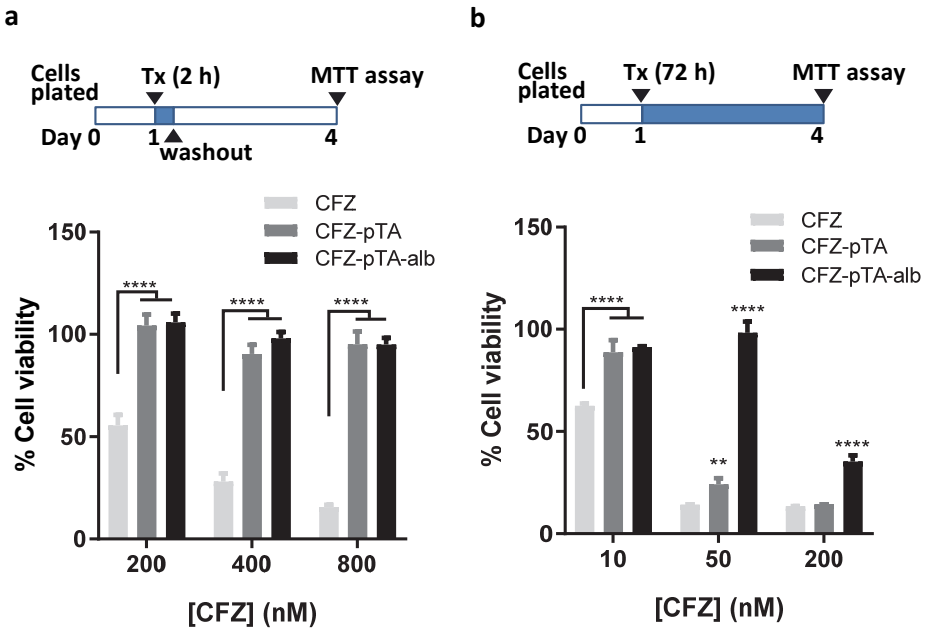


Figure S5. Viability of HCC-1937 cells (a) exposed to CFZ, CFZ-pTA or CFZ-pTA-alb at equivalent CFZ concentrations (200, 400 or 800 nM) for 2 h, followed by incubation in drug-free medium with the total incubation time of 72 h, or (b) exposed to CFZ, CFZ-pTA or CFZ-pTA-alb at equivalent CFZ concentrations (10, 50 or 200 nM) for 72 h. **: $p < 0.01$ and ****: $p < 0.0001$, two-way ANOVA with Dunnett's multiple comparisons test versus CFZ.

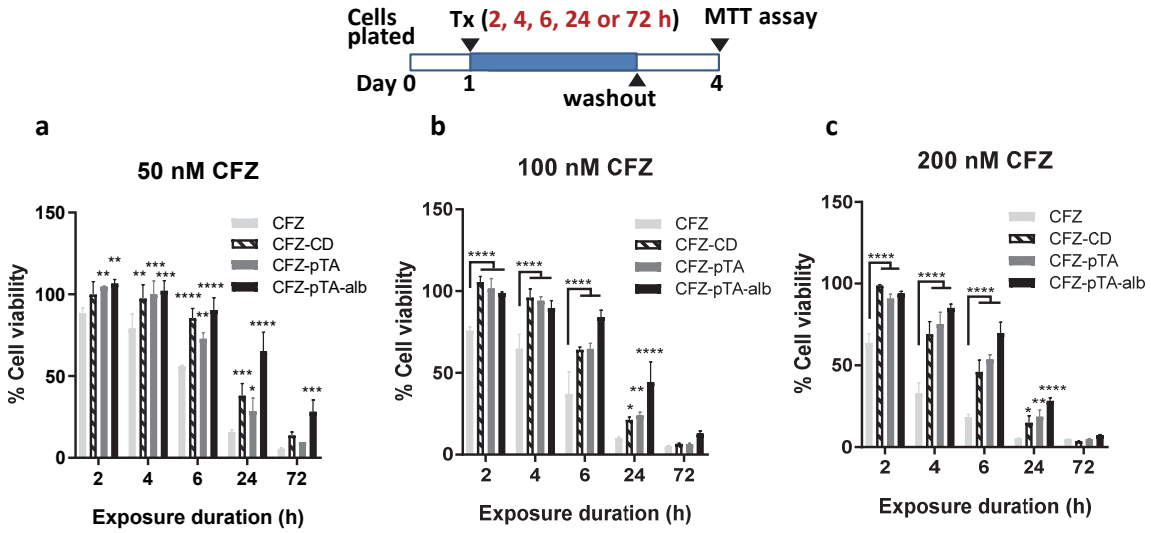
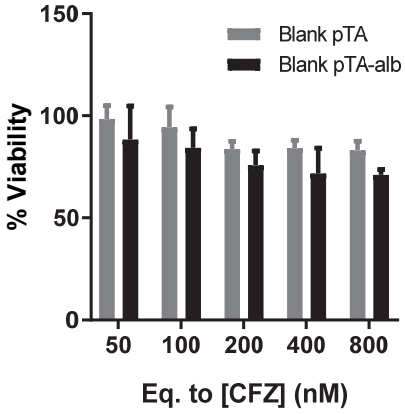


Figure S6. Viability of B16F10 cells exposed to CFZ, CFZ-CD, CFZ-pTA or CFZ-pTA-alb at concentrations equivalent to (a) 50 nM, (b) 100 nM, or (c) 200 nM of CFZ for 2, 4, 6, 24 or 72 h, followed by incubation in drug-free medium with the total incubation time of 72 h.

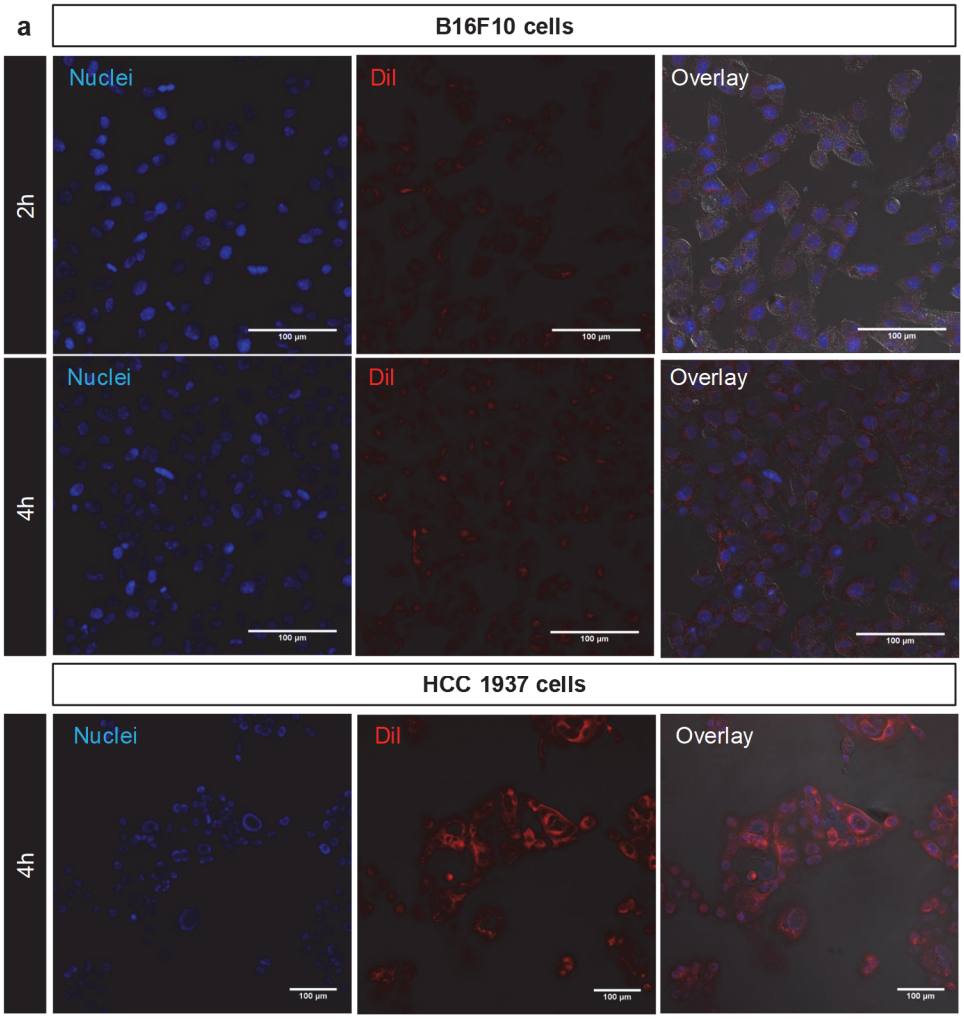
a



b

Equivalent to CFZ (nM)	Blank pTA (µg/mL)	Blank pTA-alb (µg/mL)
50	0.036	0.054
100	0.072	0.108
200	0.144	0.216
400	0.288	0.432
800	0.576	0.864

Figure S7. (a) Viability of B16F10 cells exposed to blank pTA or blank pTA-alb at concentrations equivalent to their respective contents in corresponding CFZ nanocapsules for 72 h. (b) Concentrations of blank pTA and blank pTA-alb used in cytotoxicity study equivalent to their respective contents in corresponding CFZ nanocapsules.


a
B16F10 cells
2h
Nuclei
DiI
Overlay
4h
Nuclei
DiI
Overlay
HCC 1937 cells
4h
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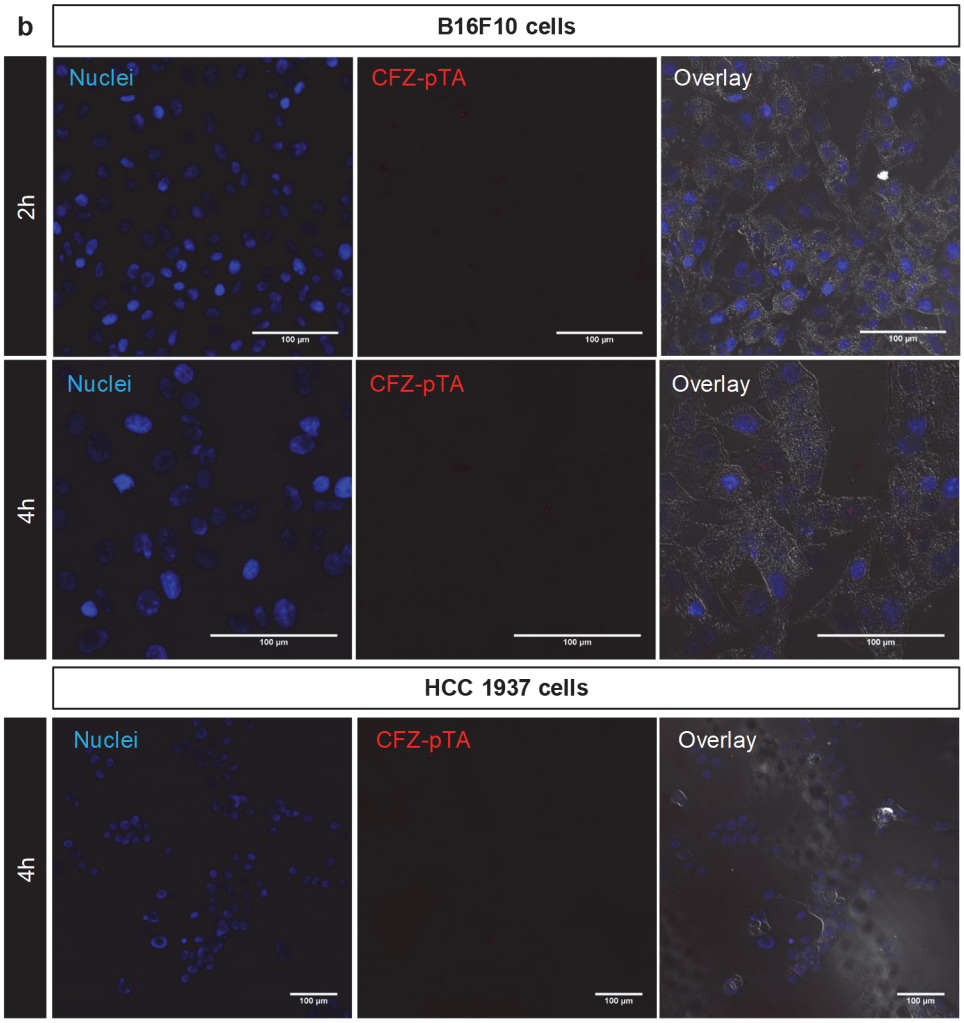
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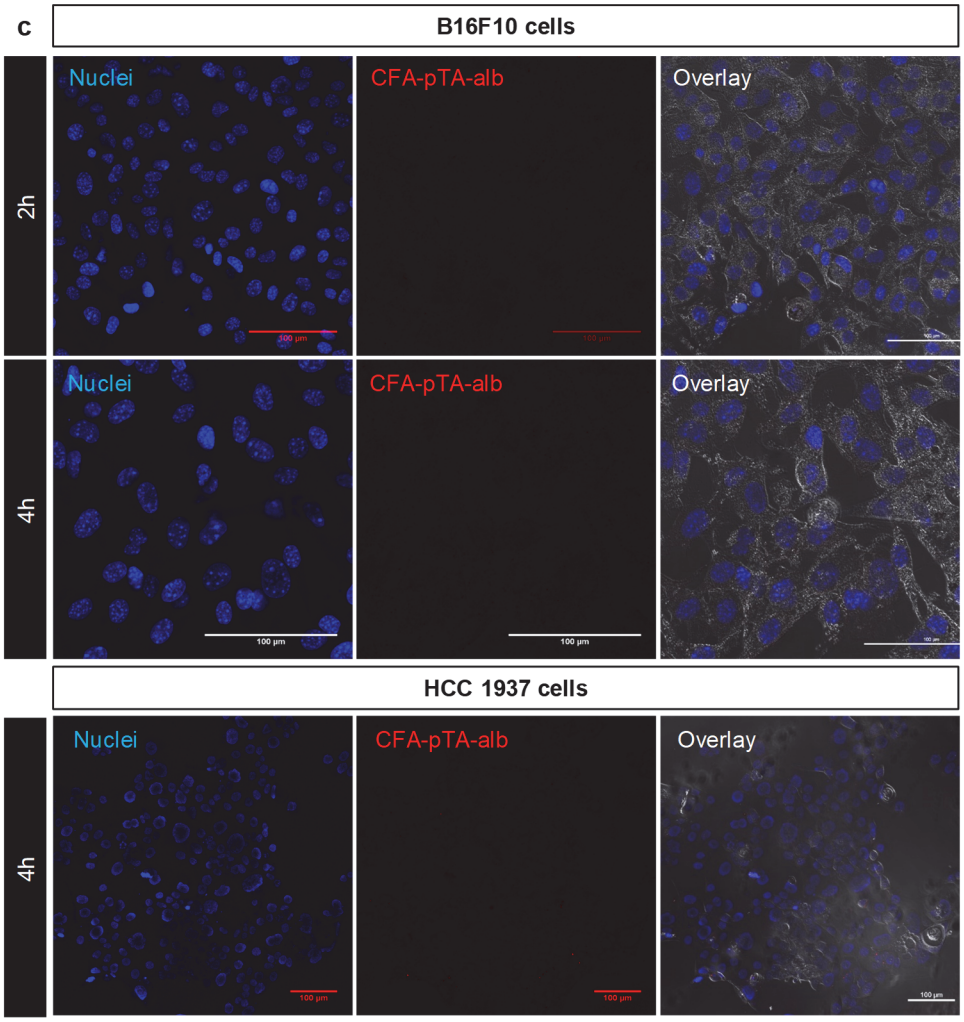

Figure S8. Confocal images of B16F10 and HCC 1937 cells after 2 or 4 h incubation with (a) DiI dye, (b) DiI-labeled CFZ-pTA, or (c) DiI-labeled CFZ-pTA-alb (at equivalent fluorescence intensity). Nanocapsules were given at 0.1 mg/mL. Red: DiI or DiI-labeled CFZ-pTA-alb; blue: nuclei stained with Hoechst 33342. Scale bars: 100 μm.

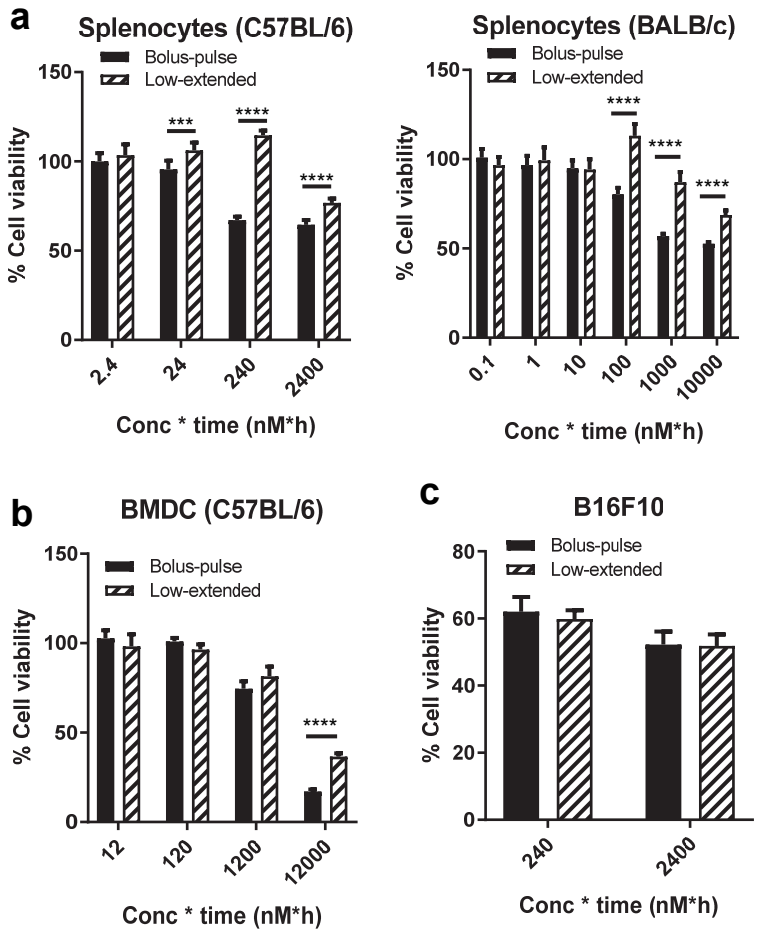

Figure S9. Viability of (a) spleen cells from Balb/c or C57BL/6 mice, (b) dendritic cells derived from C57BL/6 mouse bone marrow, and (c) B16F10 cells, after bolus-pulse or low-extended treatments. Bolus-pulse: Cells were incubated with CFZ at different CFZ concentrations for 2.4 h then washed and incubated in drug-free medium for additional 21.6 h making the total incubation time of 24 h. Low-extended: Cells were incubated with at $1/10^{th}$ CFZ concentrations for 24 h. Cell viability was measured by the MTT assay at the end of 24 h. % cell viability was calculated by normalizing to the viability of control cells treated with the equivalent amount of vehicle for the same period of time. X-axis = CFZ concentration × exposure time. **: $p < 0.01$, ***: $p < 0.001$ and ****: $p < 0.0001$, two-way ANOVA with Sidak's multiple comparisons test.

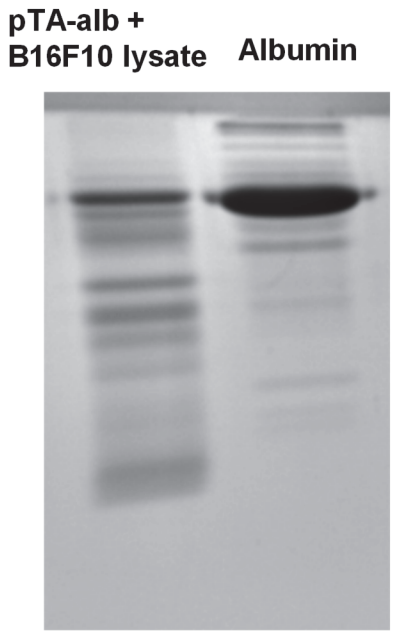

Figure S10. SDS-PAGE gel of pTA-alb incubated in B16F10 cell lysate (B16F10 cells treated with 10 μM CFZ in 10% FBS containing medium for 24 h and lysed by 3 cycles of freezing and thawing. Lysate includes both cell debris and medium.) for 2 h at 37 ºC then rinsed twice with water; Albumin control to locate albumins from pTA-alb particles and FBS.

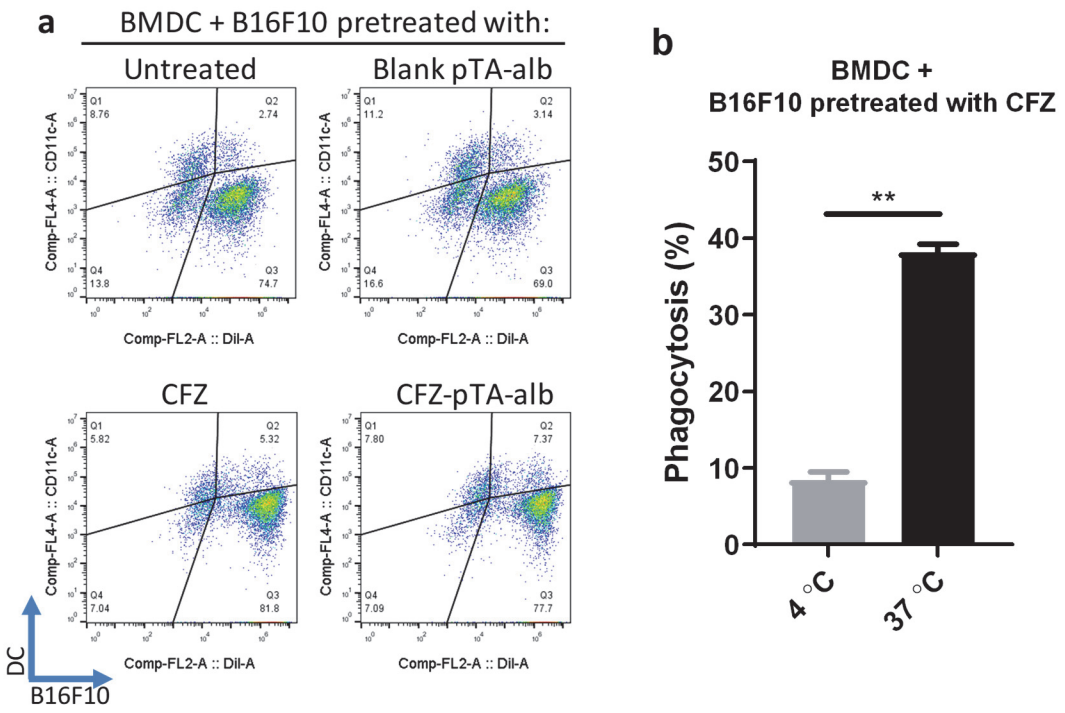

Figure S11. Phagocytosis of B16F10 cells by BMDCs: (a) DiI-stained B16F10 cells were pretreated with blank pTA-alb, CFZ or CFZ-pTA-alb (at a concentration equivalent to 10 μM CFZ) for 24 h, rinsed once and cocultured with BMDCs for 4 h at 37 ºC. BMDCs were identified with APC-anti mouse CD11c antibody and analyzed by flow cytometry. Representative flow cytometry plots are shown. Upper right quadrant (Q2, DiI^+ $CD11c^+$ cells) indicates BMDCs engulfing B16F10 cells (or cell fragments) receiving different treatments. (b) Phagocytosis (%): fraction of BMDCs engulfing B16F10 cells (pretreated with 10 μM CFZ) after 4 h coculture at 4 ºC or 37 ºC. **: $p < 0.01$, unpaired two-tailed *t*-test.

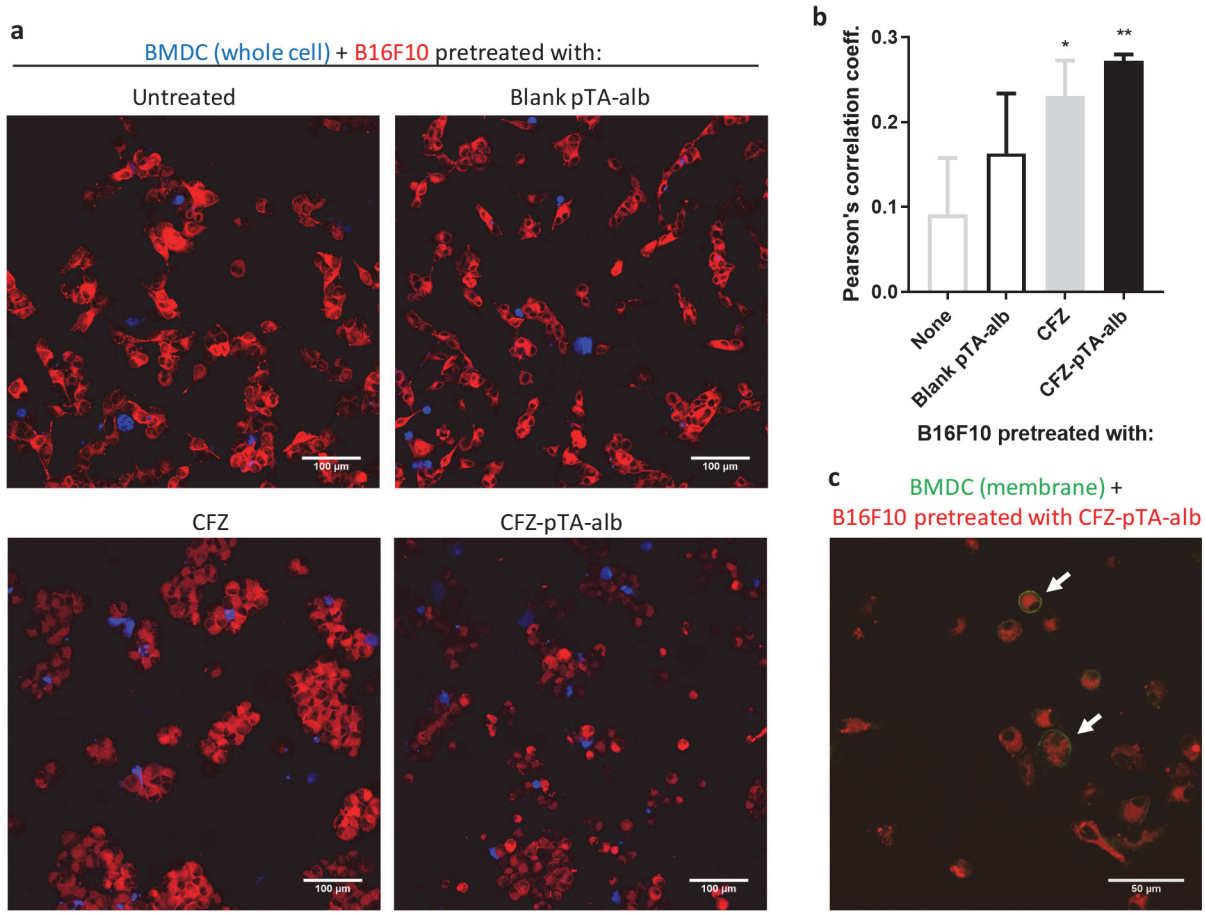

Figure S12. Confocal microscope images of BMDCs engulfing B16F10 cells after 4 h coculture: (a) B16F10 cells and BMDCs were prelabeled with DiI (red) and CellTracker™ Blue CMAC dye (blue), respectively. Scale bars: 100 µm. (b) Pearson's correlation coefficients representing colocalization of red and blue signals (i.e., B16F10 cells taken up by BMDCs) in (a), calculated by NIS Elements viewer software. Averages ± standard deviations of 3-4 images per sample. (c) B16F10 cells were prelabeled with DiI (red), and the surface of BMDCs was stained with FITC-anti-mouse CD11c antibody (green). Scale bar: 50 µm.

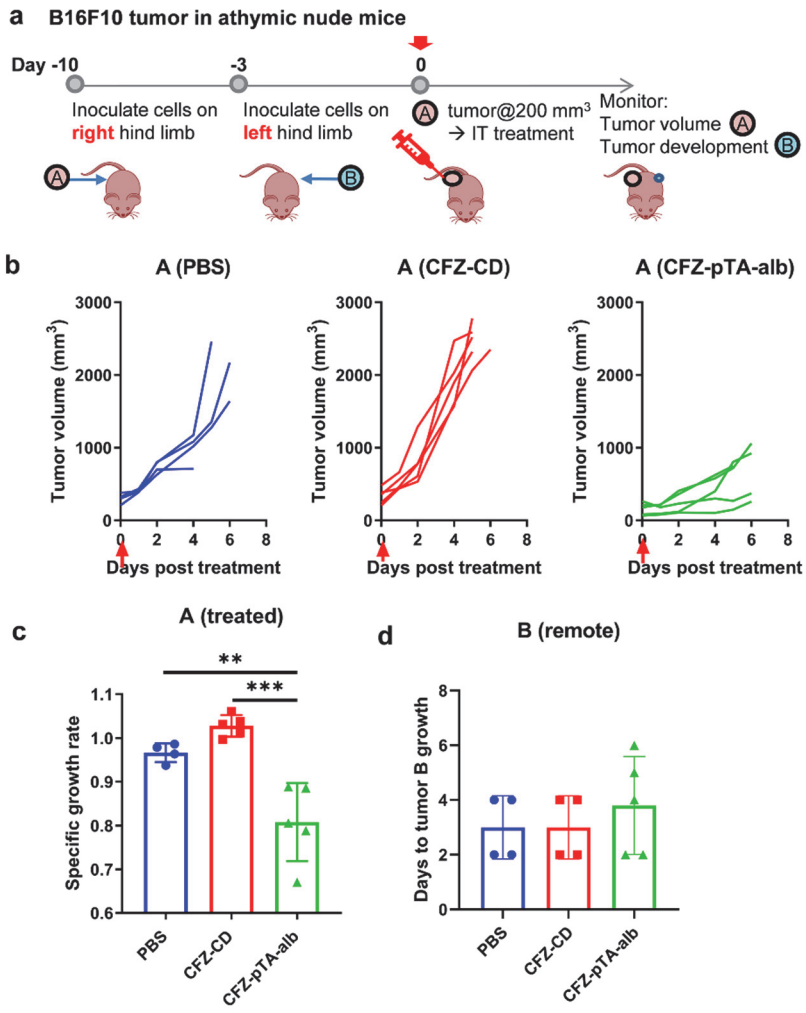

Figure S13. The effect of CFZ-pTA-alb vs. CFZ-CD, administered as a single IT injection at a dose equivalent to 60 µg CFZ, on the treated (A) and remote (B) tumors in an immune compromised B16F10@athymic nude (Foxn1^{nu}) mouse model. (a) Schedule of B16F10 tumor inoculation in athymic nude (Foxn1^{nu}) mice with deficient T cell function and treatment injection. (b and c) Antitumor activity in the treated tumor A: (b) Individual growth curves of tumors treated with PBS (n=4), CFZ-CD (n=4) or CFZ-pTA-alb (n=5). Arrow indicates the treatment time. (c) Specific growth rate of treated tumor (ΔlogV/Δt). (d) Days to appearance of tumor B (remote, untreated). **: p<0.01 and *** p<0.001, one-way ANOVA with Tukey's multiple comparisons test.

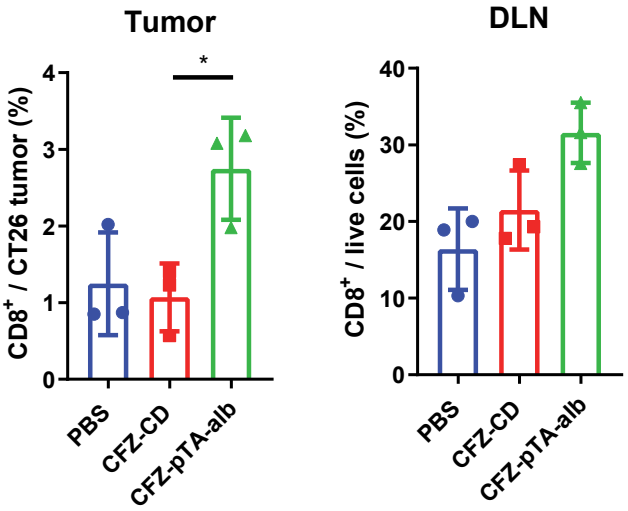

Figure S14. % $CD8^+$ T cells of total live cells in CT26 tumors or draining lymph nodes of mice receiving a single IT injection of CFZ-CD or CFZ-pTA-alb (n=3 per group) at a dose equivalent to 60 µg CFZ, 6 days post treatment. *: $p<0.05$, one-way ANOVA with Tukey's multiple comparisons test.

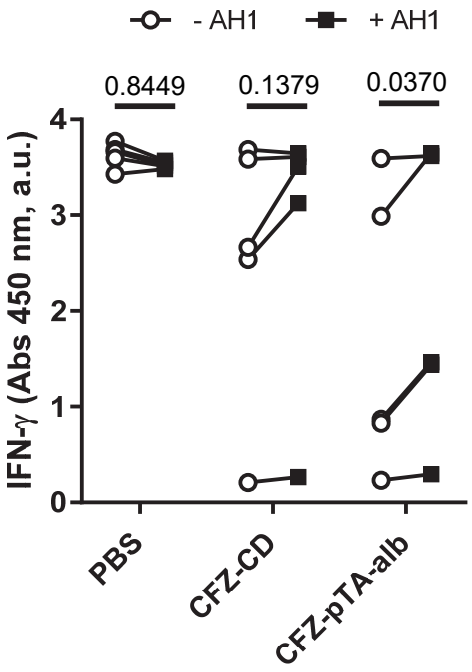

Figure S15. IFN-γ secretion from splenocytes of Balb/c mice bearing CT26 tumors with treatments, in response to AH1 peptide. Balb/c mice were inoculated with 300,000 CT26 cells on the right hind limb, followed by 100,000 CT26 cells on the left hind limb 7 days later. Right tumors (tumor A) reaching 50 mm^3 were treated by a single IT injection of CFZ-CD, CFZ-pTA-alb (a dose equivalent to 60 μg CFZ) or PBS (n=5 per group). Splenocytes were harvested on day 22 post-treatment and challenged with AH1 peptide to measure IFN-γ secretion. Statistical analysis by repeated measures two-way ANOVA with Sidak's multiple comparisons test.

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