Electronic Supplementary Information

A dual-responsive doxorubicin-indoximod conjugate for programmed chemoimmunotherapy

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Materials

Doxorubicin (DOX, 98%) was purchased from Beijing Huafeng (China); 4-dimethylaminopyridine (DMAP, 99%), 9-fluorenylmethyl chloroformate (Fmoc-Cl, 98%) were purchased from Alfa (UK); *N*,*N*-diisopropylethylamine (DIPEA), hydroxybenzotriazole hydrate (HOBt), Fmoc-Asp-OH, Fmoc-Val-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Asp(OtBu)-OH, Boc-2-(methylamino)ethylcarbamate (95%), 4-nitrobenzyl chloroformate, dimethyl sulfoxide-d6 (DMSO-d_6) were purchased from Inno-Chem (China); 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl), benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP, 97%), *tert*-butyl carbazate (95%), 9-fluorenylmethyl carbazate (95%), triisopropylsilane were purchased from Aladdin (China); succinic anhydride (99%), triethylamine (TEA, 99%), trifluoroacetic acid (TFA, 99%) were purchased from J&K Scientific (China). All chemicals were used as received.

D-1-Methyltryptophan (4a, IND). To a 500 mL three-necked flask containing D-tryptophan (3.00 g, 13.8 mmol) in dimethyl sulfoxide (DMSO, 250 mL) at 0 °C under N_2, sodium hydride (497 mg, 20.7 mmol) was added. The resulting mixture was stirred at room temperature for 1 h. Methyl iodide (2.20 g, 15.2 mmol) dissolved in 20 mL DMSO was then added dropwise and the resulting mixture was stirred at room temperature for 24 h and concentrated *in vacuo*. The residue was dissolved in water and the pH was adjusted to 7 to yield a brownish yellow precipitate. The crude product was collected, washed with acetone and water, dried *in vacuo* to give **6b** (2.5 g, 78% yield) as a white solid: ^{1}H NMR (400 MHz, D_2O) δ 7.74 (dt, *J* = 8.0, 1.0 Hz, 1H), 7.52 (dt, *J* = 8.4, 1.0 Hz, 1H), 7.35 (ddd, *J* = 8.3, 7.0, 1.2 Hz, 1H), 7.28– 7.18 (m, 2H), 4.05 (dd, *J* = 7.9, 4.9 Hz, 1H), 3.81 (s, 3H), 3.48 (ddd, *J* = 15.3, 4.9, 0.8 Hz, 1H), 3.31 (dd, *J* = 15.3, 7.9 Hz, 1H).

Fmoc-IND-OH (3a). To a 100 mL round bottom flask containing **4a** (1.00 g, 4.58 mmol) in 10% Na_2CO_3 aqueous solution, Fmoc-Cl (2.40 g, 9.16 mmol) dissolved in acetone (10 mL) was added dropwise and the resulting solution was stirred at room temperature overnight and concentrated *in vacuo*. The residue was with diluted with water and dichloromethane (DCM). The organic layers were combined, dried with anhydrous Na_2SO_4, and concentrated *in vacuo*. The crude product was purified with flash chromatography (DCM/MeOH = 10:1 v/v) to give **3a** (1.9 g, 90% yield) as a brown solid: ^{1}H NMR (400 MHz, DMSO-d6) δ 12.63 (s, 1H), 7.88 (d, *J* = 7.6 Hz, 2H), 7.66 (dd, *J* = 8.5, 6.5 Hz,

3H), 7.58 (dt, *J* = 7.9, 1.0 Hz, 1H), 7.46–7.34 (m, 3H), 7.29 (dtd, *J* = 16.2, 7.4, 1.1 Hz, 2H), 7.19–7.09 (m, 2H), 7.08–6.97 (m, 1H), 4.34–4.09 (m, 4H), 3.18 (dd, *J* = 14.6, 4.7 Hz, 1H), 3.02 (dd, *J* = 14.6, 9.5 Hz, 1H).

DEVD-IND (2a). Fmoc-IND (1.00 g, 2.28 mmol) and DIPEA (522 μL, 3.00 mmol) were dissolved in DCM (30 mL). The mixture was poured into a peptide reaction vessel containing pretreated 2-chlorotrityl chloride resins (2 g). The reaction mixture was shaken overnight (the vessel was left on a shaker) and the resins were washed with DCM (30 mL) twice. 8 mL MeOH and 1 mL DIPEA (1.0 mL) in DCM (30 mL) was added and the reaction mixture was shaken for 1 h. The resins was washed with *N*,*N*-dimethylformamide (DMF 30 mL) for 3 times. Fmoc-Asp-OH (3.2 g, 9.0 mmol), PyBOP (4.7g, 9.0 mmol) and HOBt (1.1g, 9.0 mmol) were dissolved in a solution of collidine in DMF (20% v/v, 15 mL), and the resulting solution was added to the reaction vessel. The reaction mixture was bubbled with N_2 for 1 h. For monitoring the reaction, a small amount of the resins was sampled for a ninhydrin test. Fmoc-Val-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Asp(OtBu)-OH, and Fmoc-NHNHCOCH$_2$CH$_2$-COOH were successively conjugated *via* Fmoc solid synthesis. After conjugation, the resins were washed with DCM (30 mL) and the product was cleaved from the resins with a solution of hexafluoro-2-propanol (HFIP) in DCM (20% v/v, 30 mL). After the solvent was removed, the residue was stirred in a solution (TFA: H_2O: triisopropylsilane = 95:2.5:2.5 v/v/v) for 4 h. After deprotection, the reaction mixture was concentrated *in vacuo*. Purification with reversed-phase high performance liquid chromatography (RP-HPLC) and lyophilization gave **2a** (210mg, 12% yield) as a white solid: ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.63 (s, 4H), 8.35 (d, *J* = 10.6 Hz, 2H), 8.02 (d, *J* = 7.5 Hz, 2H), 7.90 (d, *J* = 7.8 Hz, 1H), 7.76 (d, *J* = 7.7 Hz, 1H), 7.52 (d, *J* = 7.8 Hz, 1H), 7.34 (d, *J* = 8.1 Hz, 1H), 7.15 – 7.07 (m, 2H), 6.99 (t, *J* = 7.4 Hz, 1H), 4.56 (dtd, *J* = 18.4, 8.1, 5.2 Hz, 2H), 4.30 (d, *J* = 12.1 Hz, 1H), 4.18 – 4.01 (m, 3H), 3.71 (d, *J* = 4.9 Hz, 4H), 3.56 (s, 4H), 2.47 – 2.19 (m, 6H), 1.95 (dq, *J* = 19.2, 6.9 Hz, 3H), 0.82 (t, *J* = 7.0 Hz, 6H), two of the peaks (for 2 H) is obscured by DMSO and water peaks. ESI-MS (m/z) calcd. for $C_{34}H_{47}N_8O_{14}$ $[M+H]^+$ 791.3, found 791.4 .

DOX-DEVD-IND (DOXIND, 1a). To a 10 mL flask, **2a** (20 mg, 0.025 mmol) and DOX (23.3 mg, 0.040 mmol) dissolved in 3 mL DMSO was added and the resulting solution was stirred in dark at room temperature for 3 d. Purification with RP-HPLC (water containing 50 mM NH_4OAc (pH

7.4)/acetonitrile) and lyophilization gave **1a** as red solid (12mg, with residual NH_4OAc, ~32% yield) as a red solid: ESI-MS (m/z) calcd. for $C_{61}H_{74}N_9O_{24}$ $[M+H]^+$ 1316.4841 $[M+H]^+$, found 1316.4844. The purity of the final product was confirmed by analytical HPLC with multiple detectors (Figure S1). The concentration of **DOXIND** for use was determined by the UV absorption of DOX moiety at 480 nm.

HPLC analysis of DOXIND

HPLC analysis was performed on a DIONEX ultimate 3000 HPLC instrument with a UV detector, a fluorescence detector and an Agilent RP-C18 column (250 mm × 4 mm ID, 5 μm). Deionized water containing 20 mM ammonium acetate (pH 7.4) and acetonitrile were used as eluents for analysis. UV absorption at 254 nm was used for the detection of DOX and **DOXIND**. Fluorescence emission at 280 nm (excitation: 230 nm) was used for the detection of IND and **2a**.

Cell culturing

4T1 murine breast cancer cells and HeLa human cervical cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Human peripheral blood mononuclear cells (PBMCs) were obtained from Cell Applications, Inc. and cultured according to recommended protocols.

Cytotoxicity assessment

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were used to evaluated cell viabilities. HeLa and 4T1 cells were seeded in 96-well plates at a density of 6.5×10^3 cells/well and incubated for 24 h. Fresh media containing different formulas were added to reach various final concentrations as indicated. 48 h later, the media in each well was replaced by 100 μL of serum-free media containing 1 mg/mL MTT. After 4 h incubation, the media were replaced by 100 μL DMSO to dissolve the formazan in each well. The absorbances at 490 nm and 569 nm of each well was measured on a MultiSkan FC microplate reader immediately and used for assessing cell viabilities.

Evaluation of caspase-3 activity in HeLa cells

Caspase-3 activity in HeLa cells was evaluated with a commercial Caspase 3 Activity Assay Kit (C1115, Beyotime). Briefly, HeLa cells were seeded in a 6-well plate at a density of 1×10^5 HeLa cells per well and allowed to grow to a final density of 1.5×10^6 cells per well. Then HeLa cells were treated with 2 μM DOX. 6 h later, the cells were collected and lysed with the reagents from Caspase 3 Activity Assay Kit. The proteins were extracted and incubated with Ac-DEVD-pNA at 37°C. After 3 h incubation, the absorbance at 405 nm of each sample was measured and used for assessing caspase-3 activity in HeLa cells.

Confocal laser scanning microscopy (CLSM)

HeLa cells were seeded in 27 mm glass bottom dishes (Thermo Scientific™ Nunc™) and cultured overnight. Then the cells were incubated with DOX or **DOXIND** (2.5 μM) for 4 h. Then stained by Hoechst 33342 and Lyso Tracker Green for another 20 min, washed with PBS for three times. Images were acquired on a confocal laser scanning microscope (Leica, TCS SP5).

CRT surface expression[1]

HeLa cells were seeded in a 6-well plate at a density of 1×10^5 HeLa cells per well and allowed to grow until a final density of 1.5×10^6 cells per well was reached. Then HeLa cells were treated with 2 μM DOX, 2 μM **DOXIND**, and PBS. 6 h later, the cells were trypsinized and washed with PBS, followed by stained with a primary anti-CRT antibody (CST) at 37 ℃ for 1.5 h. After washed in cold PBS three times, the cells were incubated with an Alexa Fluor 680-conjugated secondary antibody for 1 h. The cells were washed with cold PBS for three times and subjected to flow cytometry.

Mixed leukocyte reaction assays[2]

HeLa cells were seeded in 96-well plates at a density of 1×10^3 cells per well and incubated for 12 h before hIFN-γ was added to reach a final concentration of 100 ng/mL. After 12 h incubation, the cells were treated with different formulas as indicated for 24 h. Peripheral blood mononuclear cells (PBMCs) pretreated with 10 μg/mL phytohemagglutinin-M (PHA-M) for 12 h and stained with CellTrace™ Blue Cell Proliferation Kit (CFSE) were co-cultured (2×10^5 cells/well) with the HeLa cells for another 3 d. Then the PBMCs in each well were collected by centrifugation, resuspended in

PBS, labelled with PE-Cy7-A conjugated anti-CD4 antibody or APC-A conjugated anti-CD8 antibody, and analyzed by flow cytometry. Signals from 10,000 events were collected for each sample. The data were analyzed with the FlowJo software.

Animal ethics

All animal procedures were in accordance with the National Institute of Health Guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of Xiamen University.

Assessments of *in vivo* therapeutic efficacy

BALB/c mice (6 weeks old, 18-22g) were purchased from the Laboratory Animal Center of Xiamen University. Tumors were inoculated by injecting 4T1 cells (1×10^7) into subcutaneous tissues. The mice were randomly separated to four groups (three for each group) when the tumor sizes approached ~100-150 mm^3. The mice of each group were treated with PBS, DOX, **DOXIND**, or DOX+IND (IND: DOX = 1:1, molar ratio) via intravenous injection every two days for 4 times at the beginning of the treatment. The dosage for all drugs is 3 mg/kg DOX per body weight. Tumor sizes were carefully monitored by a digital caliper.

Immunohistochemistry (IHC) analysis[3]

BALB/c mice bearing 4T1 tumors (~100-150 mm^3) were treated with PBS, DOX, **DOXIND**, or DOX+IND (IND : DOX = 1:1, molar ratio) via intravenous injection every two days for four times. The dosage for all drugs is 3 mg/kg DOX per body weight. 24 h after the last injection, the tumor tissues were collected, stored in 10% formalin fixing solution (Sangon Biotech), and sent for subsequent immunohistochemical analysis (Univ-bio Co.).

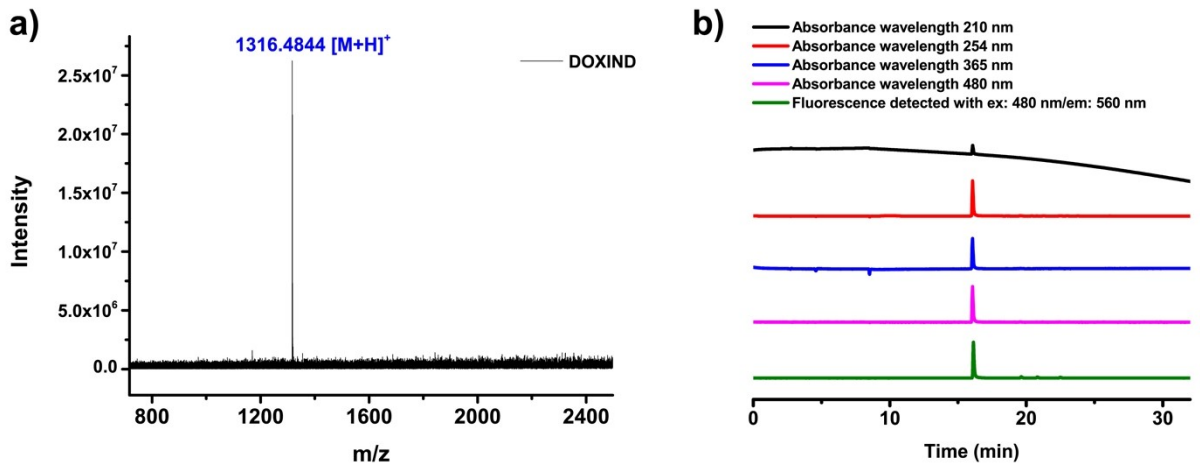

Figure S1. (a) A high resolution mass spectrum (HR-MS) of **DOXIND** ($[M+H]^+$, $C_{61}H_{74}N_9O_{24}$). (b) HPLC chromatograms of **DOXIND** (10 μM) with multiple detectors as indicated.

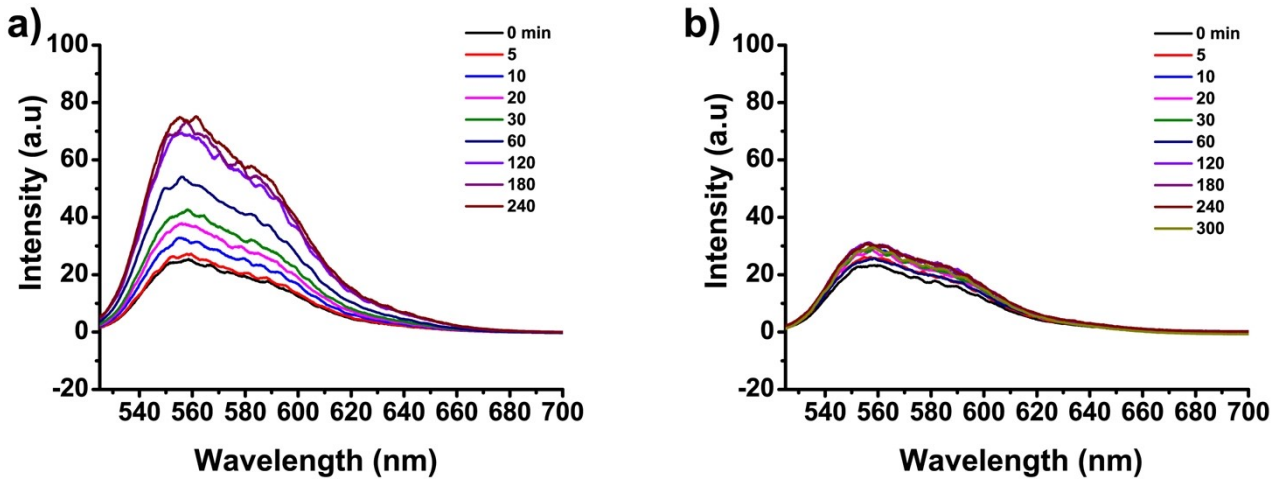

Figure S2. Fluorescence intensity changes of **DOXIND** (5 μM) incubated in (a) pH 5.4 or (b) pH 7.4 PBS buffers over time. Excitation: 480 nm.

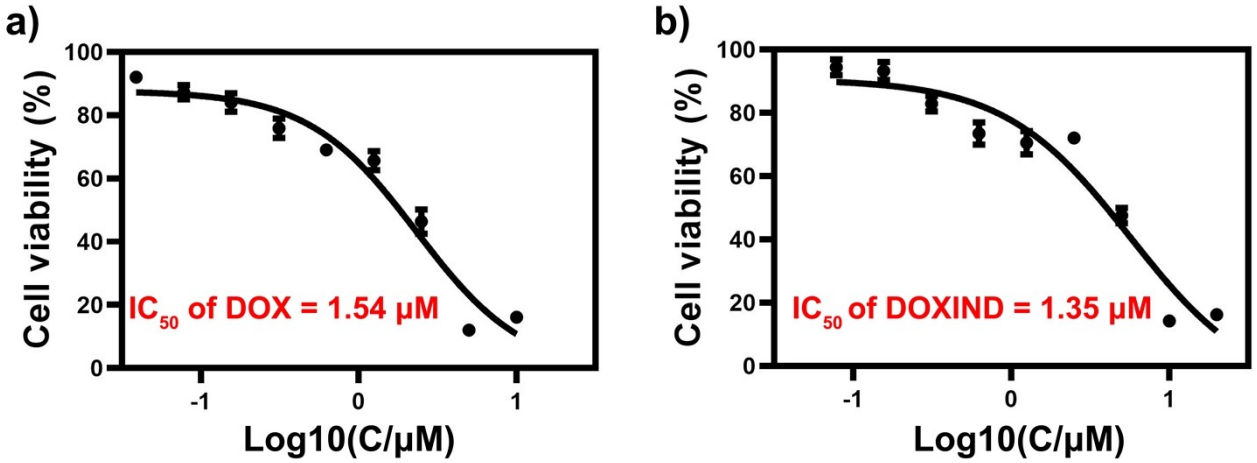

Figure S3. Cytotoxic assessment of DOX (a) and **DOXIND** (b) against 4T1 cells, evaluated with MTT assays.

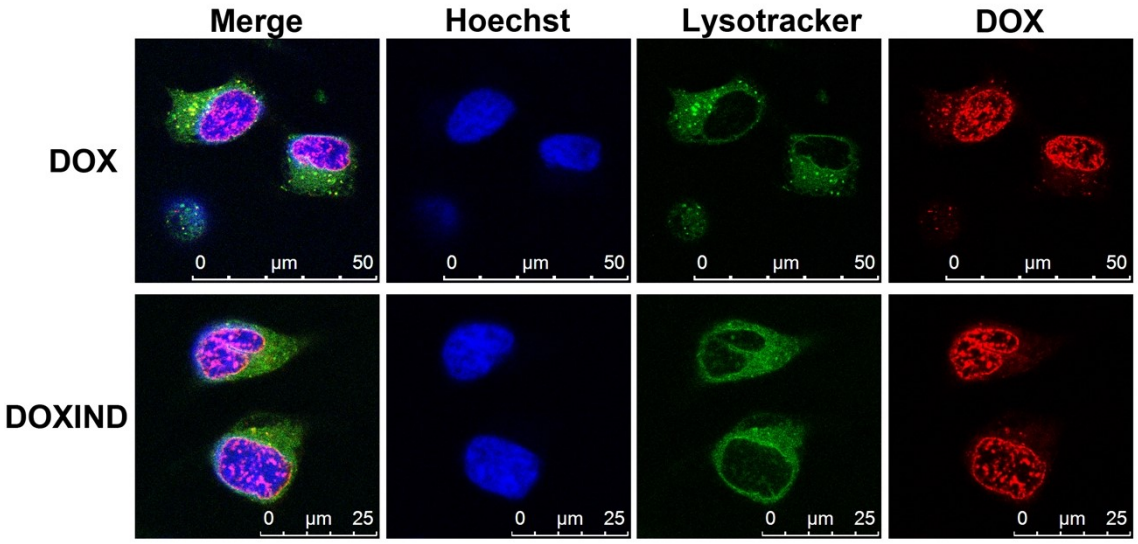

Figure S4. Confocal laser scanning microscopy (CLSM) images of HeLa cells incubated with **DOXIND** (2.5 µM) and DOX (2.5 µM) for 4 h.

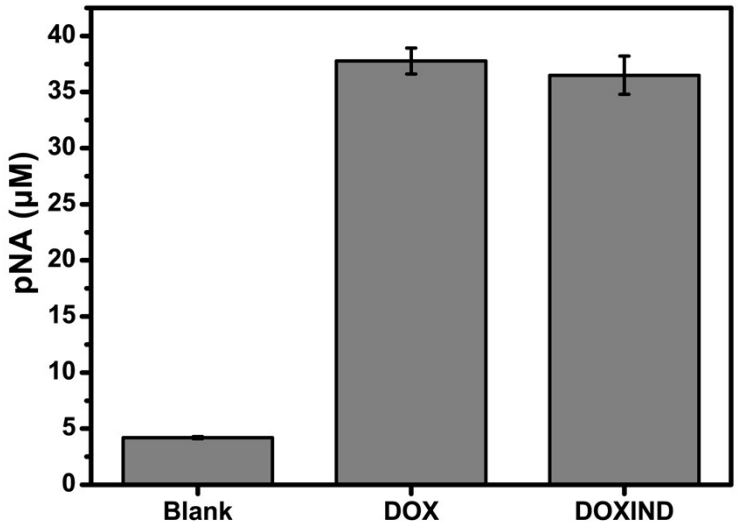

Figure S5. Evaluation of caspase-3 activity in HeLa cells treated with PBS, DOX (2 μM), and **DOXIND** (2 μM).

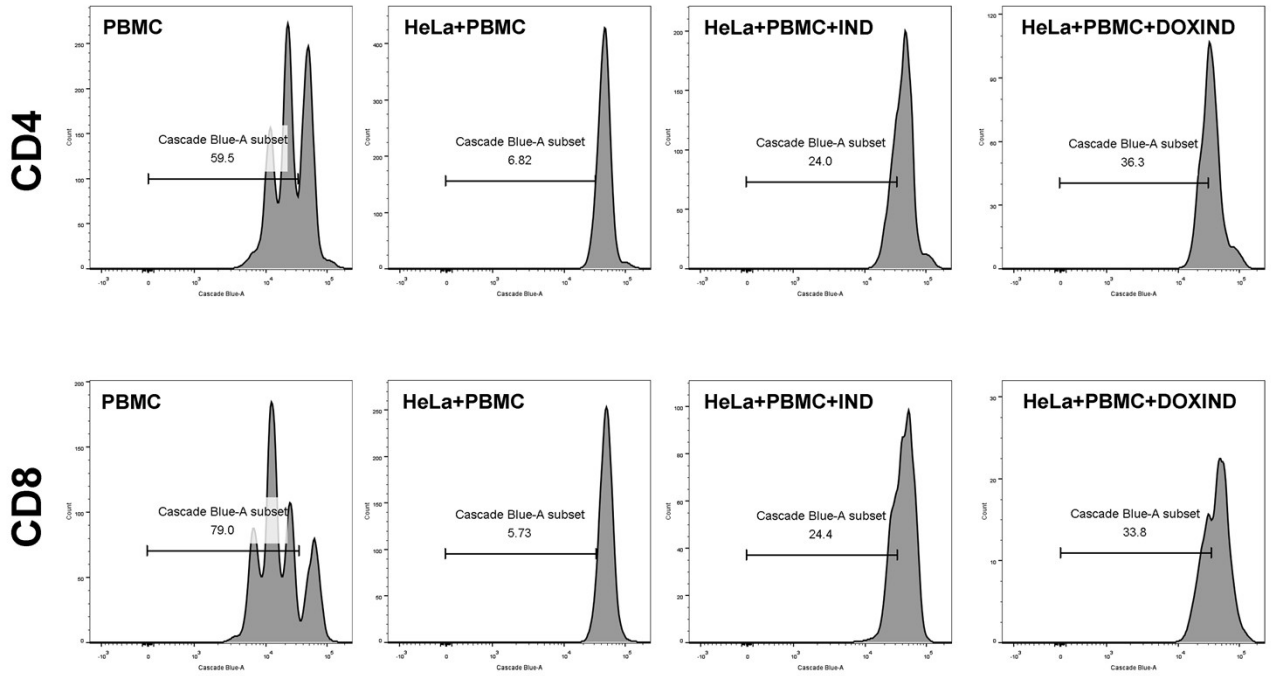

Figure S6. Flow cytometry profiles of CFSE-labeled CD4+ and CD8+ T cells before and after indicated treatments.

References:

1. J. Lu, X. Liu, Y. P. Liao, F. Salazar, B. Sun, W. Jiang, C. H. Chang, J. Jiang, X. Wang, A. M. Wu, H. Meng and A. E. Nel, *Nat. Commun.*, 2017, **8**, 1811.

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