Supporting Information

Programming immunogenic cell death in breast tumors with designer DNA frameworks

Tian Tian[1]‡, Bei Zhao[1]‡, Huizhen Wei[1], Mengru Sun[1], Zhaoshuai Gao[2], Min Lv[4], Haozhi Lei[3]*, Zhilei Ge[2]*, Guangbo Ge[1]*

1. Shanghai Frontiers Science Center of TCM Chemical Biology, Institute of Interdisciplinary Integrative Medicine Research, Shanghai University of Traditional Chinese Medicine, Shanghai, 201203, China

2. School of Chemistry and Chemical Engineering, Frontiers Science Center for Transformative Molecules and National Center for Translational Medicine, Shanghai Jiao Tong University, Shanghai 200240, China

3. Institute of Molecular Medicine, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200127, China

4. College of Chemistry and Materials Science, Shanghai Normal University, Shanghai 200234, China

‡ These authors contributed equally

* E-mail: geguangbo@shutcm.edu.cn (Guangbo Ge), gezhilei@sjtu.edu.cn (Zhilei Ge), kflhz@mail.ustc.edu.cn (Haozhi Lei)

Supplementary Methods

Reagents: DNA oligonucleotides were synthesized and purified by Sangon Biotech Co., Ltd. (Shanghai, China). Cyclo(RGDyK) modified with alkyne group was obtained from ChinaPeptides Co., Ltd. (Shanghai, China). Doxorubicin (DOX) was purchased from Sigma-Aldrich. GelRed DNA gel stain solution was purchased from Biotium. LysoTracker Green and Hoechst 33258 were purchased from Invitrogen. Murine IL-4 and murine GM-CSF were purchased from PeproTech. Antibodies used in this study include the following: PE-conjugated anti-CD11c (Biolegend), PE/Cyanine7-conjugated anti-CD86 (Biolegend), PerCP/Cyanine5.5-conjugated anti-CD80 (Biolegend), anti-CD16/32 (Biolegend), APC-conjugated anti-CD3 (Biolegend), Alexa Fluor 488-conjugated anti-CD8a (Biolegend), PE/Cyanine7-conjugated anti-granzyme

B (Biolegend), PE-conjugated anti-perforin (Biolegend), PerCP/Cyanine5.5-conjugated anti-CD4 (Biolegend), PE-conjugated anti-CD25 (Biolegend), Alexa Fluor 488-conjugated anti-calreticulin (Abcam) and α-PD-1 (RMP1-14, InVivoMAb anti-mouse PD-1, BioXCell). Other chemical reagents were purchased from Signpharm Chemical Reagent Co., Ltd. (Shanghai, China).

Cell lines and animals: 4T1 cell line was purchased from ATCC and were cultured in RPMI 1640 Medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco), penicillin (100 U/mL, Invitrogen), and streptomycin (100 μg/mL, Invitrogen) at 37˚C under a humidified atmosphere containing 5% CO_2. Bone marrow derived dendritic cells (BMDCs) were obtained from female BALB/c mice (6-8 weeks old) by isolating bone marrow cells and differentiating bone marrow cells into BMDCs with culturing for 7 days in the presence of IL-4 (10 ng/mL) and GM-CSF (20 ng/mL).

Female BALB/c mice (6–8 weeks old, weighing 20–22 g) were obtained from the Laboratory Animal Center of Shanghai University of Traditional Chinese Medicine (Shanghai, China) and maintained under specific-pathogen-free conditions with free access to food and water. All animal experiments were approved by the Animal Ethics Committee of Shanghai University of Traditional Chinese Medicine and animals were

treated in accordance with the Guidelines for the Care and Use of Laboratory Animals.

Cytotoxicity assay: The cytotoxicity of DOX, CpG-TDOX and RGD-CpG-TDOX were evaluated by CCK-8 assay. 4T1 cells were seeded in 96-well plates and cultured for 24 h. Different concentrations of DOX (0, 0.5, 1, 2, 4 μM) were incubated with cells for 48 h. Meanwhile, CpG-TDOX and RGD-CpG-TDOX with the same concentrations of DOX were separately incubated with cells for 48 h. After incubation, cell viability was detected with CCK-8 kit according to the instruction.

ICD *in vitro*: CRT exposure induced by DNA nanostructures was observed using an immunofluorescence assay. 4T1 cells were seeded in confocal culture plates and cultured for 24 h at 37˚C. Afterwards, PBS, RGD-CpG-T, DOX and RGD-CpG-TDOX were separately incubated with 4T1 cells for 24 h. After incubation, cells were washed three times with PBS and incubated with Alexa Fluor 488-conugated anti-calreticulin antibody for 30 min at 4 ˚C. Subsequently, cells were washed three times with PBS, fixed with 4% paraformaldehyde, and stained with Hoechst 33258. Then cells were observed by confocal microscope setup (Leica TCS SP8, Germany).

To determine the ATP secretion and HMGB-1 release, 4T1 cells were seeded into 24-well culture plates and cultured for 24 h at 37˚C. Afterwards, PBS, RGD-CpG-T,

DOX and RGD-CpG-TDOX were separately incubated with 4T1 cells for 24 h, and then the supernatants were taken for quantitative analysis by ATP assay system (Promega) and HMGB-1 ELISA Kit (Novus) according to the instructions.

Immunostimulatory effect *in vitro*: 4T1 cells were seeded in 12-well culture plates and cultured for 24 h at 37˚C. PBS, RGD-CpG-T, DOX and RGD-CpG-TDOX were separately incubated with 4T1 cells for 6 h, then the culture media was removed and replaced with new media without preparations and continued to incubate for 18 h. Subsequently, BMDCs were added and co-incubated for 24 h. Finally, cells were harvested and suspended in FCM staining buffer, then stained with PE-conjugated anti-CD11c, PE/Cyanine7-conjugated anti-CD86 and PerCP/Cyanine5.5-conjugated anti-CD80 antibody for 20 min for DCs analysis by FCM (CytoFLEX LX, Beckman coulter).

Abscopal effect: To investigate the effects of combination of DNA nanostructures and ICB therapy on tumor treatment and anti-tumor recurrence, a bilateral mouse tumor model of 4T1 cells was established. Firstly, the primary tumors were subcutaneously implanted in the left flank regions of female BALB/c mice. After primary tumors reached 50 mm^3, different preparations (saline, RGD-CpG-T, DOX, α-PD-1 and RGD-CpG-TDOX & α-PD-1) were intravenously administrated every 2 days for a total of

five injections. Besides the injection of RGD-CpG-TDOX, mice in the combined treatment group (RGD-CpG-TDOX & α-PD-1) received injection of α-PD-1 antibody every 2 days for a total of four injections. Subsequently, the secondary tumors were subcutaneously implanted in the right flank regions of BALB/c mice, and were monitored as distant tumors for two weeks. Tumor sizes were measured by digital vernier caliper, and the volume was calculated by the formula of length × width^2 /2. Tumor tissues were harvested and weighted. Distant tumor tissues were cut into small pieces and incubated with digestion solution for 1 h to make single cell suspensions. Subsequently, tumor cells were blocked with anti-CD16/32 antibody, and then stained with APC-conjugated anti-CD3, Alexa Fluor 488-conjugated anti-CD8a for analyzing $CD8^+$ T cells by FCM.

Statistical Analysis: All statistical data were presented as mean ± S.D., statistical analysis was analyzed using an unpaired Student's *t* test. *P* values < 0.05 were considered statistically significant.

Supplementary figures

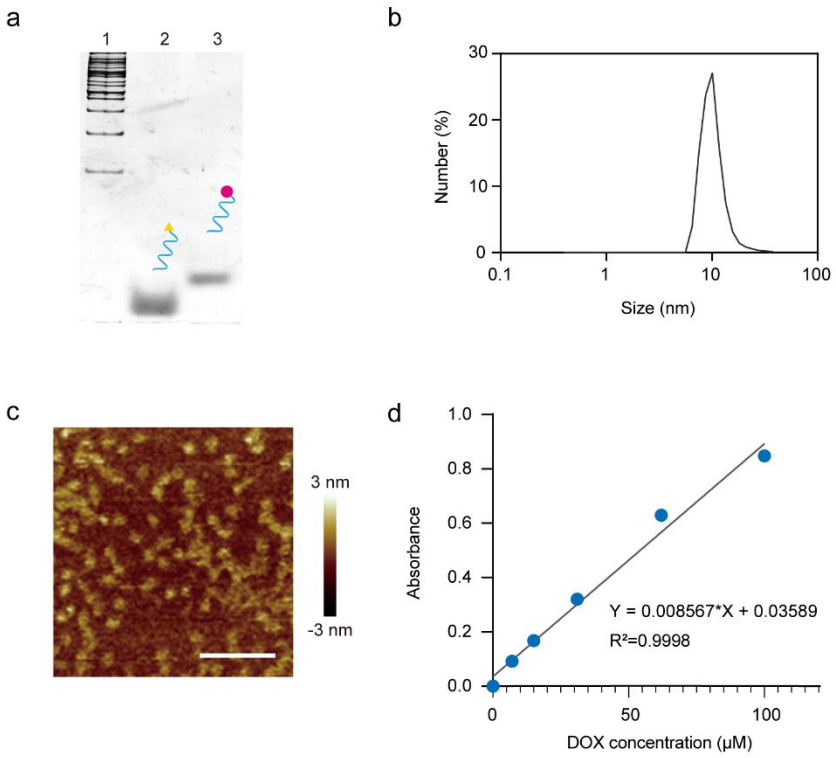

Figure S1. (a) PAGE result of identification for RGD-ssDNA. Line 1: DNA marker, line 2: ssDNA, line 3: RGD-ssDNA. (b) DLS result of TDF. (c) AFM imaging of CpG-TDOX. Scale bar: 100 nm. (d) The concentration-absorption standard curve of DOX.

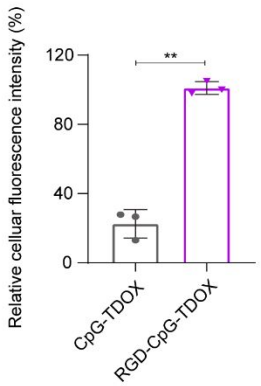

Figure S2. Statistical analysis for fluorescence intensity of cellular uptake of nanostructures in 4T1 cells.

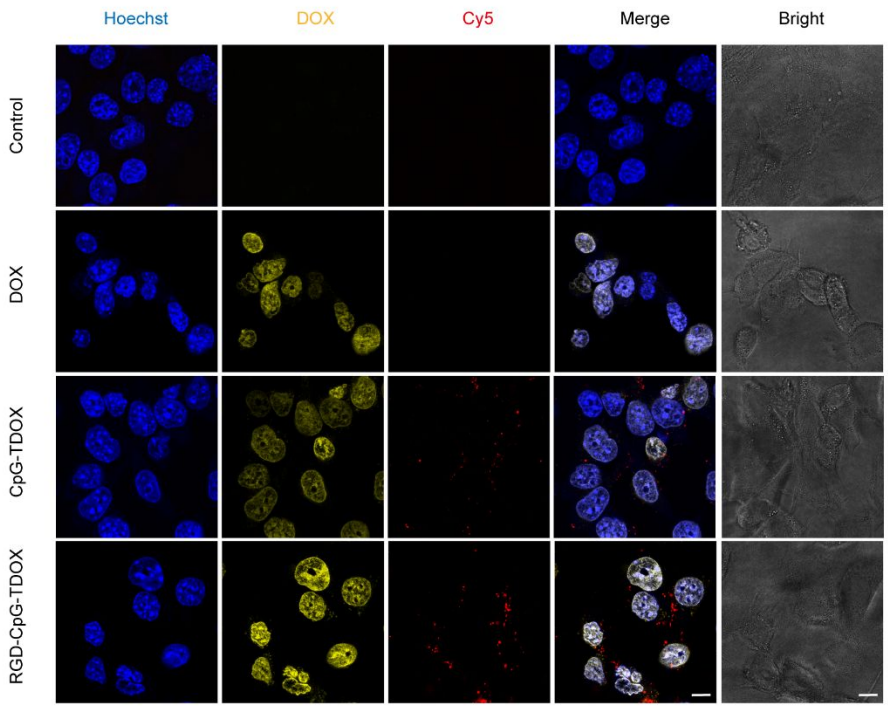

Figure S3. Intercellular internalization of DOX, CpG-TDOX and RGD-CpG-TDOX in 4T1 cells. Blue: nucleus, yellow: DOX, red: Cy5 labeled nanostructures. Scale bar: 10 μm.

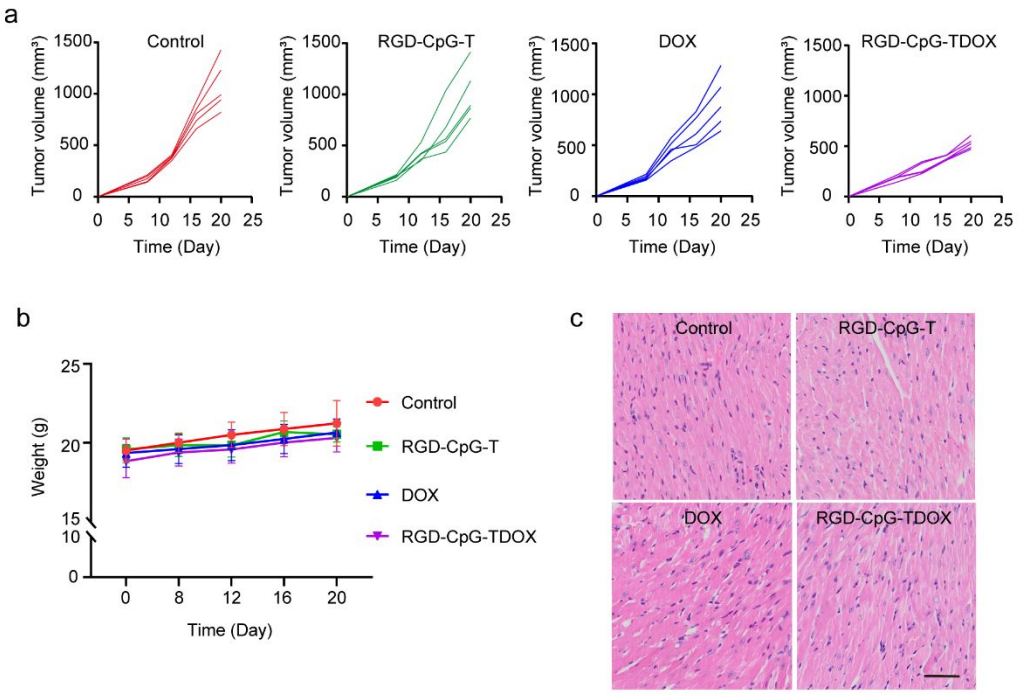

Figure S4. (a) Tumor growth curves of individual mouse in different treatment groups. (b) Body weight curves of mice in different treatment groups (n = 5). (c) H&E staining of mice heart tissues in different treatment groups. Scale bar: 50 μm.

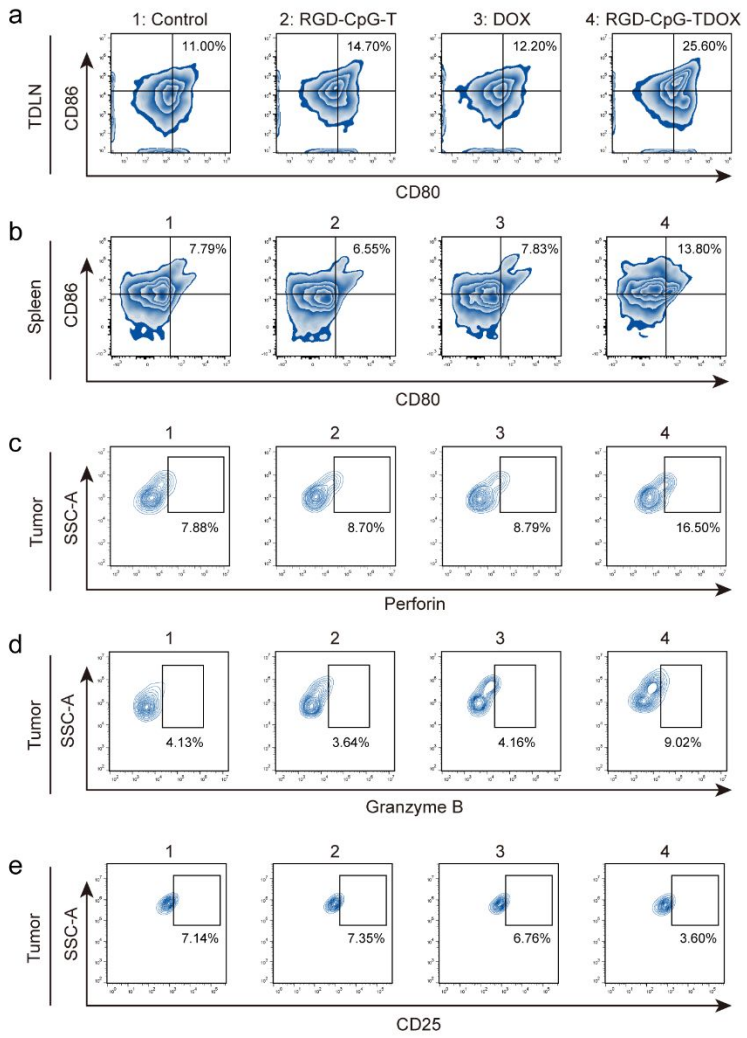

Figure S5. (a) Representative FCM images of mature DCs ($CD80^+$ $CD86^+$) in different groups of TDLN (gated on $CD11c^+$). (b) Representative FCM images of mature DCs ($CD80^+$ $CD86^+$) in different groups of spleen (gated on $CD11c^+$). (c, d) Representative FCM images of perforin (c) and granzyme B (d) in different groups (gated on $CD3^+$ $CD8^+$). (e) Representative FCM images of Tregs ($CD25^+$) in different groups (gated on $CD3^+$ $CD4^+$).

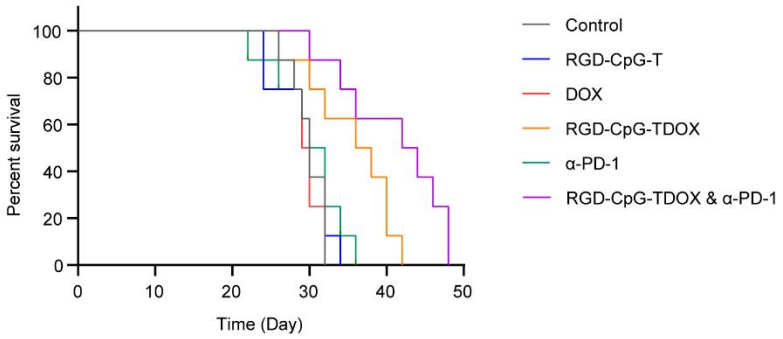

Figure S6. Survival rates of bilateral 4T1-tumor-bearing mice with different treatment (n=8).

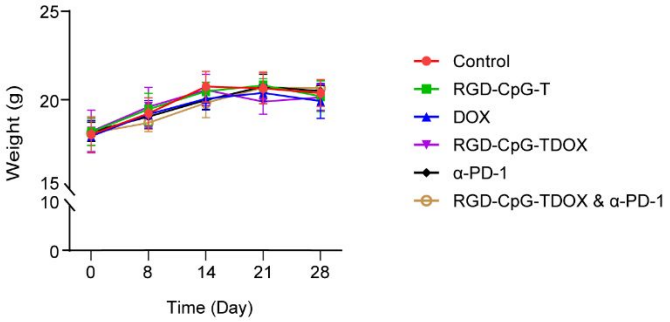

Figure S7. Body weight curves of mice in different treatment groups (n = 5).

Supplementary tables

Table S1. Oligonucleotide sequences used in this study.

Oligo names	Sequence (5' to 3')
S1	TTTTTTTTTTTTTTTTTTTTACATTCCTAAGTCTG AAACATTACAGCTTGCTACACGAGAAGAGCCG CCATAGTA

S2	TCCATGACGTTCCTGACGTTTTTTTTATCACCAG GCAGTTGACAGTGTAGCAAGCTGTAATAGATGC GAGGGTCCAATAC
S3	TCCATGACGTTCCTGACGTTTTTTTTCAACTGCC TGGTGATAAAACGACACTACGTGGGAATCTACT ATGGCGGCTCTTC
S4	TTCAGACTTAGGAATGTGCTTCCCACGTAGTGT CGTTTGTATTGGACCCTCGCAT
680-S4	S4 labeled with Alexa Fluor 680 at the 3’ end
Cy5-S4	S4 labeled with Cy5 at the 3’ end
A20	AAAAAAAAAAAAAAAAAAAA
A20-N_3	A20 modified with azide group at the 3’ end