Supporting Information

pH-responsive STING-activating DNA nanovaccines for cancer immunotherapy

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Experimental Section

Materials. poly(d,l-Lactide) with poly(ethylene glycol) with maleimide end (MAL-PEG$_{2000}$-*b*-PLA$_{3000}$) was purchased from JenKem Technology. Dithiothreitol (DTT) was purchased from Fisher Scientific. All oligonucleotides (see sequences in Table S1) were purchased from Integrated DNA Technologies. Sodium ascorbate was purchased from Sigma-Aldrich. c-di-GMP (CDG) was purchased from InvivoGen. Fluorescein-labeled CDG was purchased from BioLog Life Science Institute. All other chemicals and solvents were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification unless otherwise noted.

Instrumentation. UV-vis absorption spectra were recorded using the Thermo Scientific GENESYS™ 50 UV-Visible Light Spectrophotometer. Dynamic light scattering (DLS) measurements and the particle zeta potential values were determined using with a Zetasizer Nanoseries (Nano ZS90) instrument. Transmission Electron Microscopy (TEM) was conducted on the JEM-1400 Plus TEM. The samples in aqueous solutions (10 μL) were deposited onto carbon-coated copper grids, and were allowed to dry in air overnight. The production of proinflammatory factors IFN-α, lL-6 and IL-12 were determined using with a Synergy H1 Hybrid Multi-Mode Reader (BioTek). Fluorescence images of cancer cells were acquired by the laser scanning microscope Carl Zeiss LSM-710. Binding measurements were conducted using a NanoTemper Monolith NT.Automated Instrument (NanoTemper Technologies GmbH, Germany).

Synthesis of PEG-*b*-PLA NP. MAL-PEG$_{2000}$-*b*-PLA$_{3000}$ was dissolved in various organic solvents that are miscible with water. NPs were formed by adding the polymer solution dropwise to water. The resulting NP suspension was allowed to stir uncovered for 12 h at room temperature. NPs were purified by ultrafiltration (15 min, 3000 g, Amicon Ultra, Ultracel

membrane with 10,000 NMWL, Millipore, Billerica, MA, USA). The PEG-*b*-PLA NPs were resuspended, washed with water, and collected likewise.

Synthesis of i-motif-NP. Thiol modified i-motif (0.04 mM) was pretreated with DTT (0.1 M) in 1×PBS for 1 h at 37 °C to cleave the dithiol bond, and then NAP5 columns were used to purify the i-motif by removing the DTT and cleaved thiol-appending fragments in 1% sodium ascorbate buffer. The cleaved DNA products were mixed and reacted with MAL-PEG-*b*-PLA NPs dissolved in 1×PBS for 1 min at room temperature. i-motif-NPs were purified by ultrafiltration (15 min, 3000g, Amicon Ultra, Ultracel membrane with 10,000 NMWL, Millipore, Billerica, MA, USA). The i-motif-NPs were resuspended, washed with water, and collected likewise.

Preparation of CDG-loaded i-motif-NP. The i-motif-PEG-PLA NPs solution was mixed with the desired amount of CDG to prepare CDG-loaded NPs. The mixture was stirred at 37 °C for 12 h. CDG-loaded i-motif-NPs were purified by ultrafiltration (15 min, 3000g, Amicon Ultra, Ultracel membrane with 10,000 NMWL, Millipore, Billerica, MA, USA), and the filtrate was used to calculate drug loading capacity. The resulting products are denoted as STING-NVs. The CDG concentration in the filtrate was determined by a UV-Vis spectrophotometer at 254 nm to calculate the drug loading capacity. The drug loading capacity and loading efficiency were calculated by the following equations:

$$\text{Loading capacity} = (\text{CDG weight in NPs})/(\text{weight of NPs});$$

$$\text{Loading efficiency} = (\text{CDG weight in NPs})/(\text{total weight of CDG}).$$

CDG release from STING-NVs. First, STING-NVs were dispersed in 1 mL of 1×PBS at different pH and agitated at 200 rpm. Then the mixture was ultra-filtrated at each hour point. The filtrate was taken for CDG detection, and the same volume of fresh buffer was added back to the

residual mixture. The amount of released CDG in the filtrate was determined by measuring the absorption at 254 nm using a UV-Vis spectrometer.

Lipo-CDG Synthesis. DOTAP, cholesterol, and DSPE-PEG(2000) were purchased from Avanti Polar Lipids. Lipids were mixed at molar ratios of 1:1:0.2 DOTAP:cholesterol:DSPE-PEG(2000). Lipid mixtures were dried under a nitrogen stream in glass test tubes. The resulting lipid films were placed in a desiccation system overnight. Dry films were hydrated in a solution of 250 μg/mL CDG in DI water with six cycles of vortexing for 30 s every 5 min. Samples were then extruded using 21 passes in a mini-extruder (Avanti) through a 0.2 μm pore size polycarbonate filter (Whatman). Samples were placed in 10K MWCO dialysis cartridges (Thermo Scientific) and dialyzed against PBS for 6 h before use. The CDG content of the liposomes was determined by measuring the absorbance peak of the liposomes at 254 nm, corresponding to the maximum absorbance of CDG, subtracting the lipid contribution to absorbance at this wavelength.

MST and data analysis. MST was carried out in PBST (1× PBS, 0.05% Tween-20) at pH 7.4 or pH 5.0, using NT.Label-Free Standard treated capillaries. 5C-NPs or scramble DNA-NPs (0.5 μM DNA) and increasing CDG concentrations were mixed and incubated for 30 min at room temperature. Samples were then transferred into the NanoTemper Monolith NT.Automated Instrument. MST was carried out at high MST power for the measurements. The MST data of independent measurements were processed using MO.Affinity Analysis v2.3 software to calculate the dissociation constant (K_d), for which the fitting (e.g., Figure 2e) was processed using Sigmoidal (4PL, x is log(concentration)) in Prism GraphPad software.

Cell culture. THP-1 cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, 50 μM

2-mercaptoethanol, 1× non-essential amino acids and 10 mM HEPES. DC2.4 cells were cultured in RPMI medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/ml streptomycin. B16F10 and RAW264.7 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/ml streptomycin. All cells were cultured in 37 °C with 5% CO2.

Repolarization of M2 macrophages *in vitro*. $5\times10_5$/mL RAW264.7 cells were cultured in LPS (500 ng/ml) and IFN-γ (25 ng/ml) to induce an M1 phenotype (M1+) or IL-4 (25 ng/ml) to induce an M2 phenotype (M2+). On day 2, M2 cells were treated with indicated formulations for 24 h. On day 3, cells were harvested for gene expression analysis (M1/M2 biomarkers and costimulatory molecules) and flow cytometry; supernatants were collected for cytokine detection (ELISA/Luminex assay).

ELISA. DC2.4 cells were plated at densities of $5\times10_5$ cells/well in a 6-well plate. Cells were treated with the indicated formulations for 24 h. Secreted IFN-α was quantified with the LumiKine Xpress mIFN-α enzyme-linked immunosorbent assay kit (InvivoGen). Secreted IL-6 and IL-12 were quantified with the Mouse IL-6 Quantikine ELISA Kit (R&D Systems).

Flow Cytometry. In cell uptake experiments, cells were plated at $5\times10_5$ cells/mL per well of a 6-well plates and treated with various formulations of fluorescein-CDG at 0.5 μg/mL for the indicated time. Cells were analyzed for fluorescein signal (from fluorescein-modified CDG) on the CytoFLEX LX (Beckman Coulter) flow cytometer.

For immune activation studies, cells were stimulated for 24 h with 1.5 μg/mL equivalent CDG, collected and stained for viability and nucleated cells as above, and further stained with

Alexa647-conjugated anti-mouse CD80, PerCP-conjugated anti-mouse CD86, FITC-conjugated anti-mouse CD206, and PE-conjugated anti-mouse I-A/I-E before flow cytometry analysis.

Gene Expression Analysis. For *in vitro* gene expression studies, M1 or M2 Cells were lysed, and RNA was purified using a RNeasy Plus Mini Kit (Qiagen), quantified using a Nanodrop (Thermo Scientific), and transcribed into cDNA using the High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) for murine costimulatory molecules (Cxcl9 and Cxcl10), M1 markers (Tnf, IL6, IL12, Nos2, and Ifnb1), M2 markers (Ym1, Fizz1, Arg1, and Mrc1) were performed using Power SYBR® Green PCR Master Mix and Power SYBR® Green RT-PCR Reagents Kit (Applied Biosystems). Primer sequences are as follows:

mouse Ifnb1:
Forward Sequence, 5′-CGAGCAGAGATCTTCAGGAAC-3′
Reverse Sequence, 5′-TCACTACCAGTCCCAGAGTC-3′

mouse Gapdh:
Forward Sequence, 5′-CTTTGTCAAGCTCATTTCCTGG-3′
Reverse Sequence, 5′-TCTTGCTCAGTGTCCTTGC-3′

mouse Tnf:
Forward Sequence, 5'-GGTGCCTATGTCTCAGCCTCTT-3'
Reverse Sequence, 5'-GCCATAGAACTGATGAGAGGGAG-3'

mouse Cxcl10:
Forward Sequence, 5'-ATCATCCCTGCGAGCCTATCCT-3'
Reverse Sequence, 5'-GACCTTTTTTGGCTAAACGCTTTC-3'

mouse Cxcl9:
Forward Sequence, 5'-CCTAGTGATAAGGAATGCACGATG-3'
Reverse Sequence, 5'-CTAGGCAGGTTTGATCTCCGTTC-3'

mouse Fizz1:
Forward Sequence, 5'-TCCCAGTGAATACTGATGAGA-3'
Reverse Sequence, 5'-CCACTCTGGATCTCCCAAGA-3'

mouse Ym1:
Forward Sequence, 5'-GGGCATACCTTTATCCTGAG-3'
Reverse Sequence, 5'-CCACTGAAGTCATCCATGTC-3'

mouse ARG1:
Forward Sequence, 5'-CAGAAGAATGGAAGAGTCAG-3'
Reverse Sequence, 5'-CAGATATGCAGGGAGTCACC-3'

mouse NOS2:
Forward Sequence, 5'-TGCATGGACCAGTATAAGGCAAGC-3'
Reverse Sequence, 5'-GCTTCTGGTCGATGTCATGAGCAA-3'

mouse Mrc1:
Forward Sequence, 5'-GTTCACCTGGAGTGATGGTTCTC-3'
Reverse Sequence, 5'-AGGACATGCCAGGGTCACCTTT-3'

mouse IL-6:
Forward Sequence, 5'-GAGGATACCACTCCCAACAGACC -3'
Reverse Sequence, 5'-AAGTGCATCATCGTTGTTCATACA - 3'

mouse IL-12p40:
Forward Sequence, 5′-CAGAAGCTAACCATCTCCTGGTTTG-3′
Reverse Sequence, 5′-TCCGGAGTAATTTGGTGCTTCACAC-3′

For *in vivo* gene expression studies, at indicated time points after treatment, tumor tissue samples were collected and placed immediately into RNAlater solution (Thermo Scientific) and stored at 4 °C overnight. Tumor tissues were homogenized using 3.0 mm zirconium. Samples were centrifuged to remove debris and RNA was purified and analyzed as above by qPCR.

***In vitro* cellular uptake by confocal microscopy**. *In vitro* cell uptake of free CDG, Lipo-CDG, and STING-NVs were studied using confocal fluorescence microscopy. Cells were stained with LysoTracker Red DND-99 (Life Technologies) for 1.5 h, and were then stained with L Hoechst33342 (Life Technologies) for 0.5 h, and were finally treated with indicated formulation of fluorescein-CDG at 0.5 μg/mL for a specified time length. Cells were then washed with Dulbecco's PBS for three times prior to fluorescence imaging. Samples were imaged using an LSM 710 confocal microscope (Zeiss) at the VCU Microscopy Facility. Endosome escape was studied by quantifying the outside/inside ratio of FluoCDG fluorescence intensities in 10 representative cells using ZEISS Zen software.

Immune activation in THP-1 cells: THP-1 cells were seeded at densities of 1×10^4 cells/well in a 96-well plate. Cells were treated with CDG formulations (3 μg CDG equivalents) for 18 h. At the same time, HEK-Blue™ IFN-α/β cells (Invivogen) were seeded at densities of 1×10^4 cells/well in a 96-well plate. Then 100 μL THP-1 cell supernatant was added to HEK-Blue™ IFN-α/β cells for 24 h. 50 μL cell supernatant was collected from each sample and added to 150 μL of QUANTI-Blue SEAP detection medium (InvivoGen) and incubated for 2 h at 37 °C. SEAP activity was assessed by measuring the absorbance at 630 nm on a plate reader. The results were fit (Figure 4f) using log(agonist) *vs.* response (three parameters) in the Prism GraphPad software.

Animal studies. All work conducted on animals were cared for following NIH guidelines and in accordance with an approved protocol by the Virginia Commonwealth University Animal Care and Use Committee (IACUC). Female C57BL/6 mice (6-8 weeks) were purchased from the Charles Rivers.

B16F10 tumor-bearing mice (6-8 weeks old, female) were prepared by subcutaneously injection of 3×10^5 B16F10 cells in PBS (100 μL). When the tumor was established on day 7 (tumor volume ~60 mm^3) post tumor inoculation, mice started to be treated with specified regimens by intratumoral injection of CDG formulations (3 μg CDG equiv. in 30 μL PBS). Mice were treated for 5 times every 3 days. Tumor tissues were collected to analyze the immune milium in the tumor microenvironment. Tumor volume and mouse weight were monitored every 3 days. Mice were euthanized when any dimension of tumor was close to 2 cm or when mouse body weight was lost by over 20%. Tumor volume was calculated using the following formula:

$$\text{Volume} = (\text{length} * \text{width}^2) / 2$$

Statistical Analyses. Data were analyzed by nonparametric unpaired *t* tests (Mann-Whitney) or one-way ANOVA followed by Dunnett's multiple comparison tests using GraphPad Prism. $p < 0.05$ was considered statistical significance.

Supplementary Figures

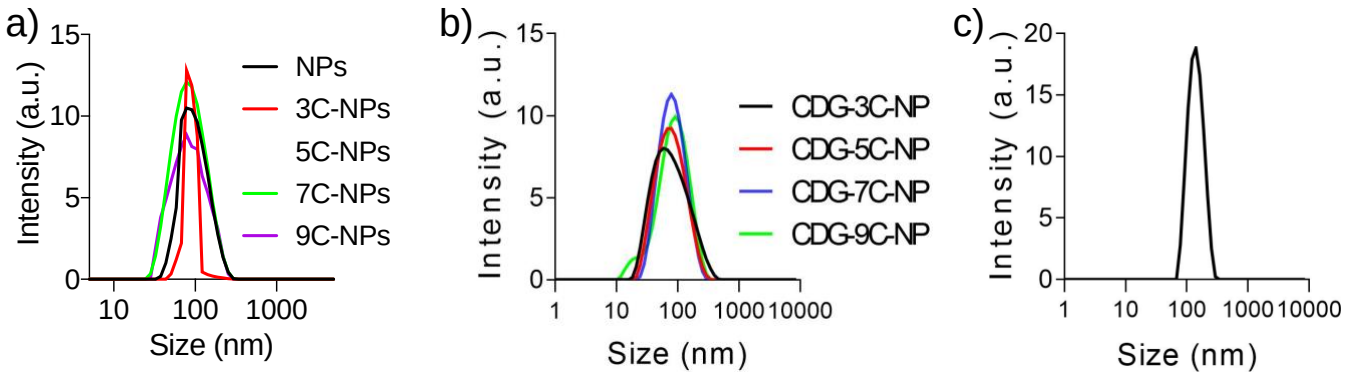

Figure S1. Size characterization of NPs. (**a**) DLS graphs showing the hydrodynamic diameters of PEG-*b*-PLA NPs (NPs, 78 ± 35 nm, PDI 0.195), and NPs modified with 3C-i-motif (3C-NPs, 82 ± 28 nm, PDI 0.125), 5C-i-motif (5C-NPs, 79 ± 34 nm, PDI 0.157), 7C-i-motif (7C-NPs, 83 ± 38 nm, PDI 0.201), and 9C-i-motif (9C-NPs, 84 ± 41 nm, PDI 0.212). (**b**) DLS graphs of CDG loaded 3C-NPs (CDG-3C-NPs), CDG loaded 5C-NPs (CDG-5C-NPs), CDG loaded 7C-NPs (CDG-7C-NPs), and CDG loaded 9C-NPs (CDG-9C-NPs). The hydrodynamic diameters of CDG-3C-NP, CDG-5C-NP, CDG-7C-NP, and CDG-9C-NP are 79 ± 40 nm, 81 ± 32 nm, 81 ± 36 nm, 82 ± 35 nm, and 88 ± 45 nm, respectively. The PDI of CDG-3C-NPs, CDG-5C-NPs, CDG-7C-NPs, and CDG-9C-NPs are 0.215, 0.131, 0.147, 0.225, and 0.231, respectively. (**c**) DLS of CDG-loaded liposome (Lipo-CDG). The hydrodynamic diameters of Lipo-CDG are 131 ± 18 nm. The PDI of Lipo-CDG is 0.057.

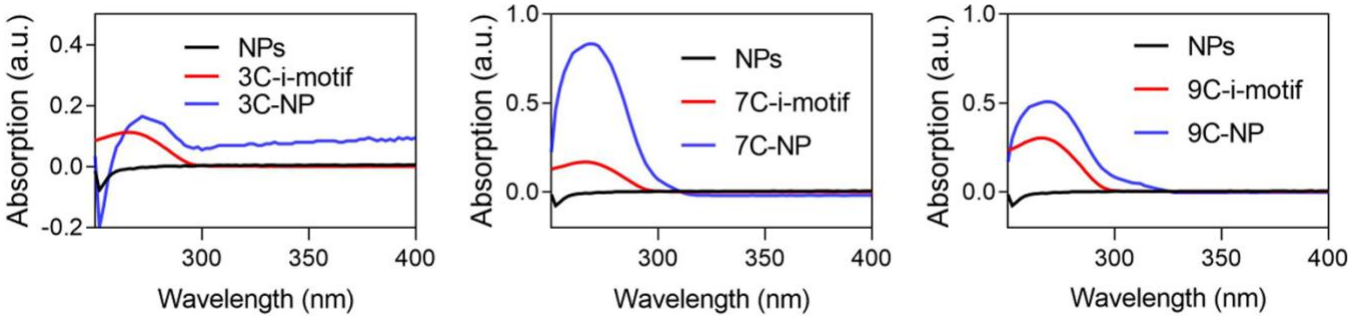

Figure S2. UV-vis absorption spectra of PEG-b-PLA NPs, DNA i-motif, and i-motif-NPs for 3C-i-motifs, 7C-i-motifs, and 9C-i-motifs, respectively.

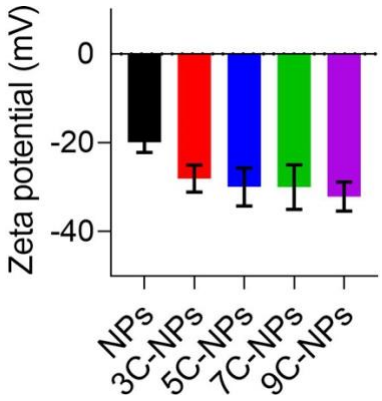

Figure S3. Zeta potential of NPs, 3C-NPs, 5C-NPs, 7C-NPs, and 9C-NPs. Data represent mean ± standard deviation (SD) (*n* = 3).

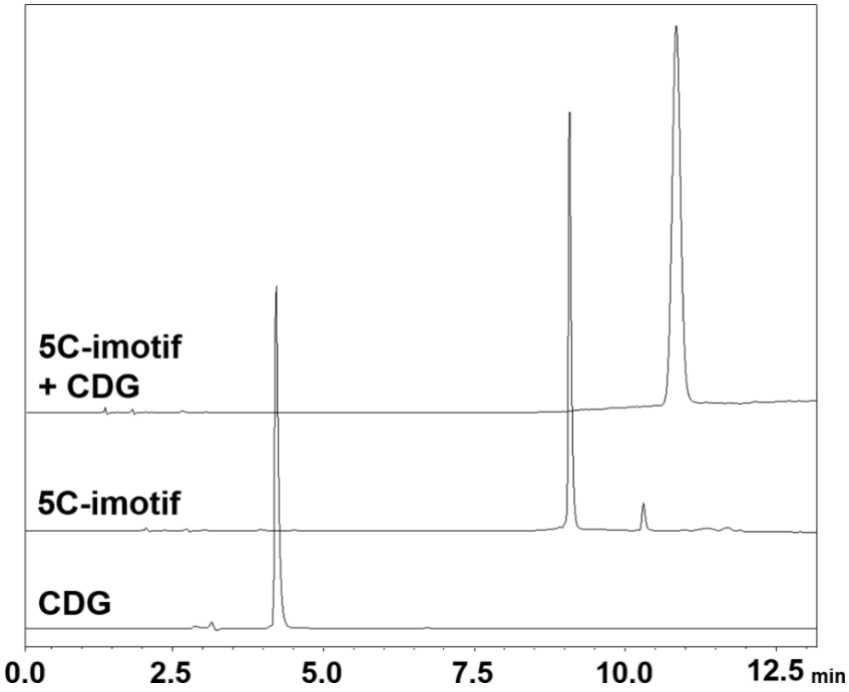

Figure S4. HPLC diagrams of free CDG, 5C-i-motif, and a physical mixture of CDG and 5C-i-motif (molar ratio: 2:1) at pH 7.4.

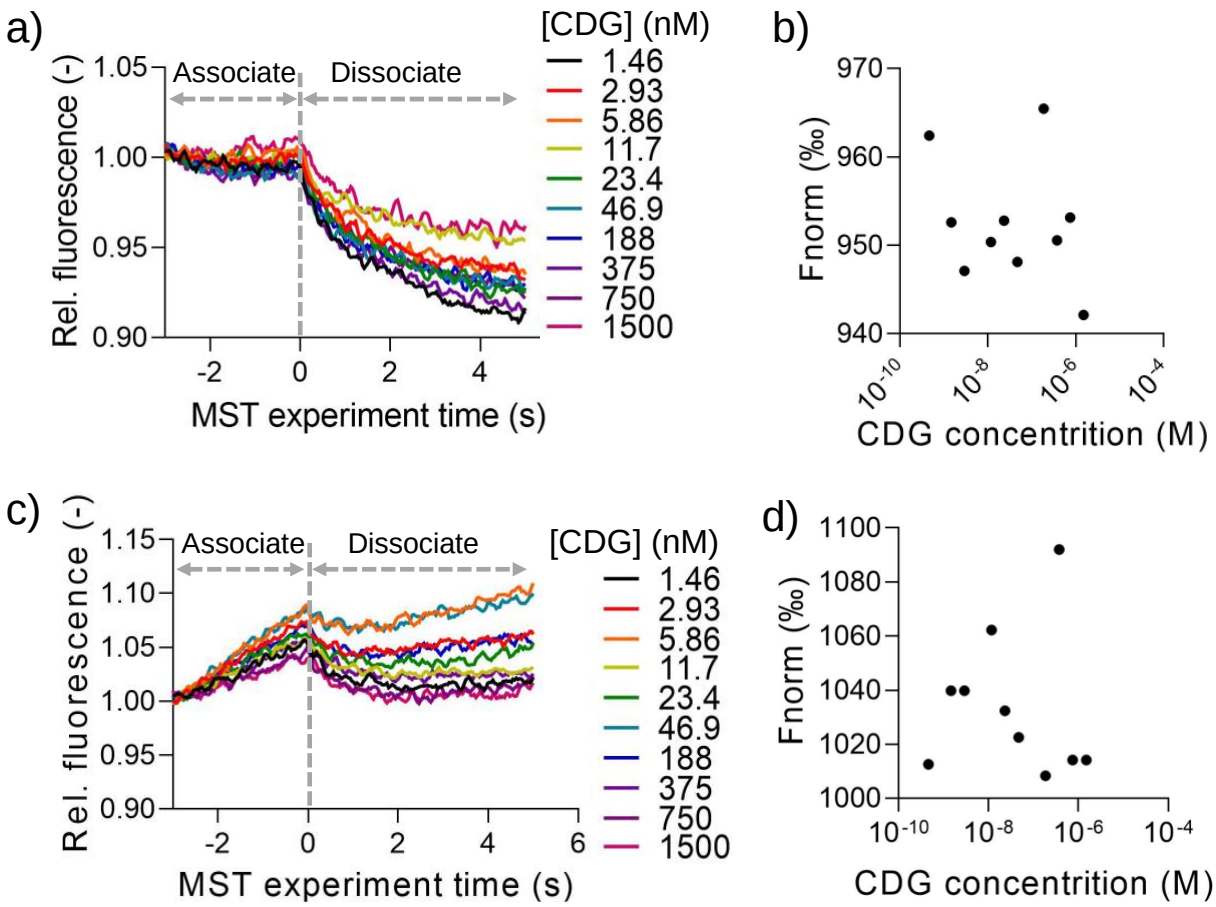

Figure S5. Microscale thermophoresis (MST) analysis of the kinetic association and dissociation between CDG and scramble DNA-NPs at pH 7.4 (**a**-**b**), and between CDG and 5C-NPs at pH 5.0 (**c**-**d**). (**a**, **c**) MST traces, (**b**, **d**) MTS quantification.

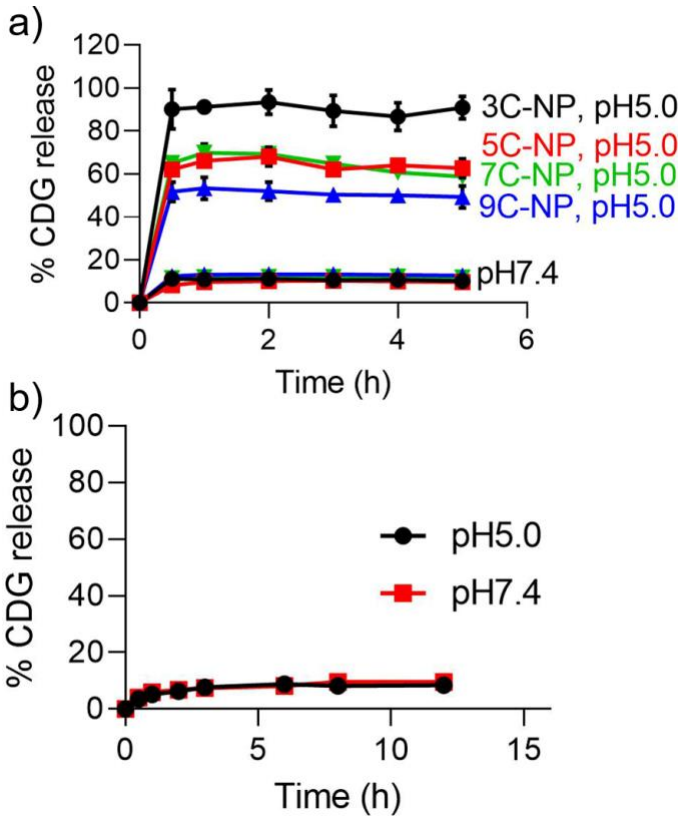

Figure S6. *In vitro* release profiles of CDG from (**a**) 3C-NPs, 5C-NPs, 7C-NPs, and 9C-NPs, as well as (**b**) Lipo-CDG at pH 5.0 and 7.4. Data represent mean ± SD (*n* = 3).

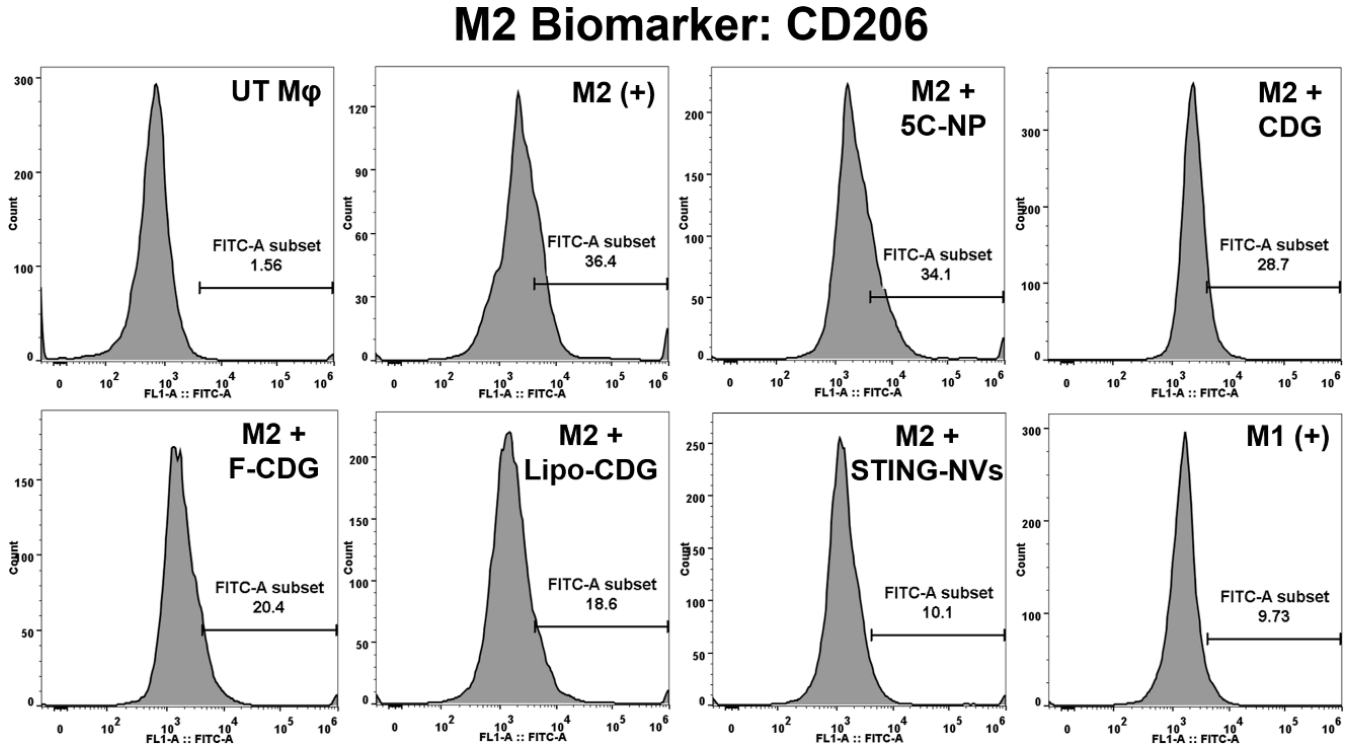

Figure S7. RAW264.7 macrophages were cultured in LPS plus IFN-γ for 24 hours to induce an M1-like phenotype and IL-4 to induce an M2-like phenotype. UT Mφ, untreated control. On day 2, M2 cells were treated with 1.5 μg/mL CDG delivered as free CDG, F-CDG, 5C-NPs, STING-NVs or Lipo-CDG. On day 3, the cells were treated with CD206 antibody and applied for flow cytometry. Flow cytometry results showing that M2 biomarker CD206 expression was downregulated in M2 phenotype cells treated with STING-NV for 24 h.

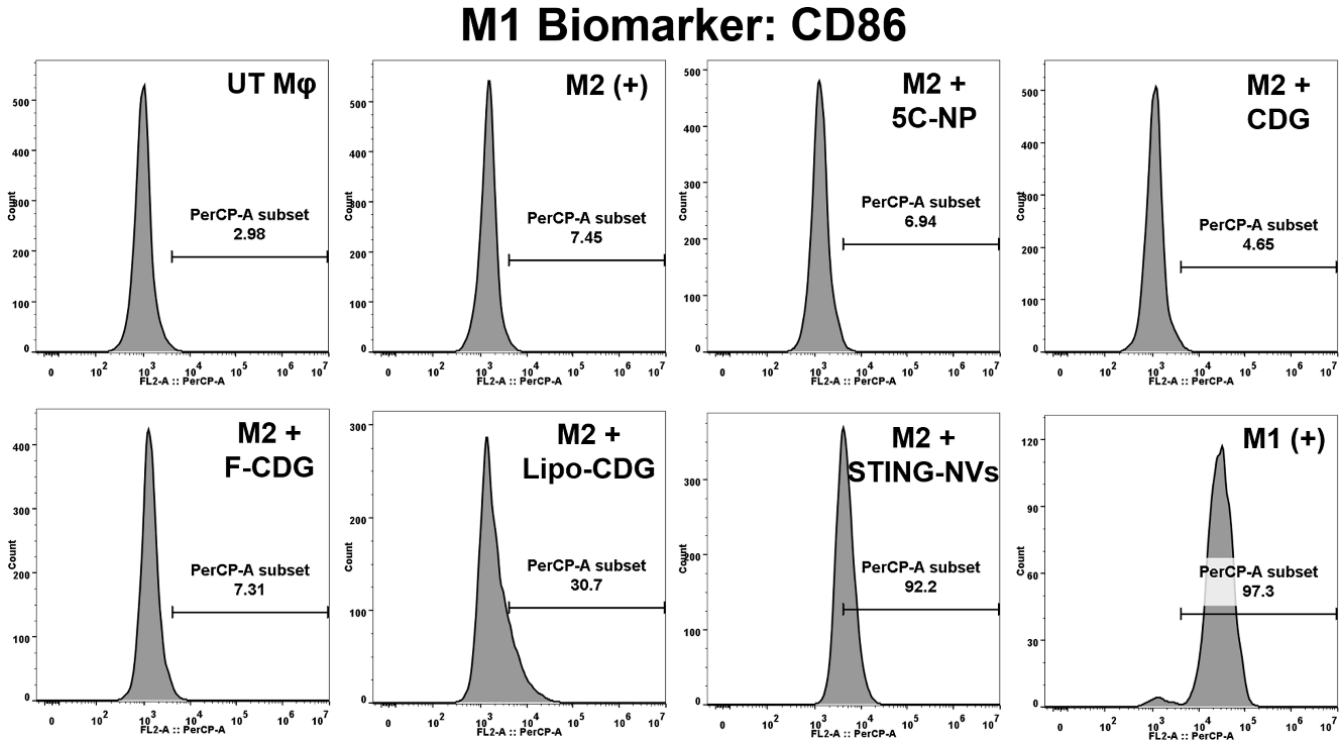

Figure S8. RAW264.7 macrophages were cultured in LPS plus IFN-γ for 24 hours to induce an M1-like phenotype and IL-4 to induce an M2-like phenotype. UT Mφ, untreated control. On day 2, M2 cells were treated with 1.5 μg/mL CDG delivered as free CDG, F-CDG, 5C-NPs, STING-NVs or Lipo-CDG. On day 3, the cells were treated with CD86 antibody and applied for flow cytometry. Flow cytometry results showing that STING-NV elevated the expression of M1 biomarker CD86 in M2 phenotype cells after 24 h.

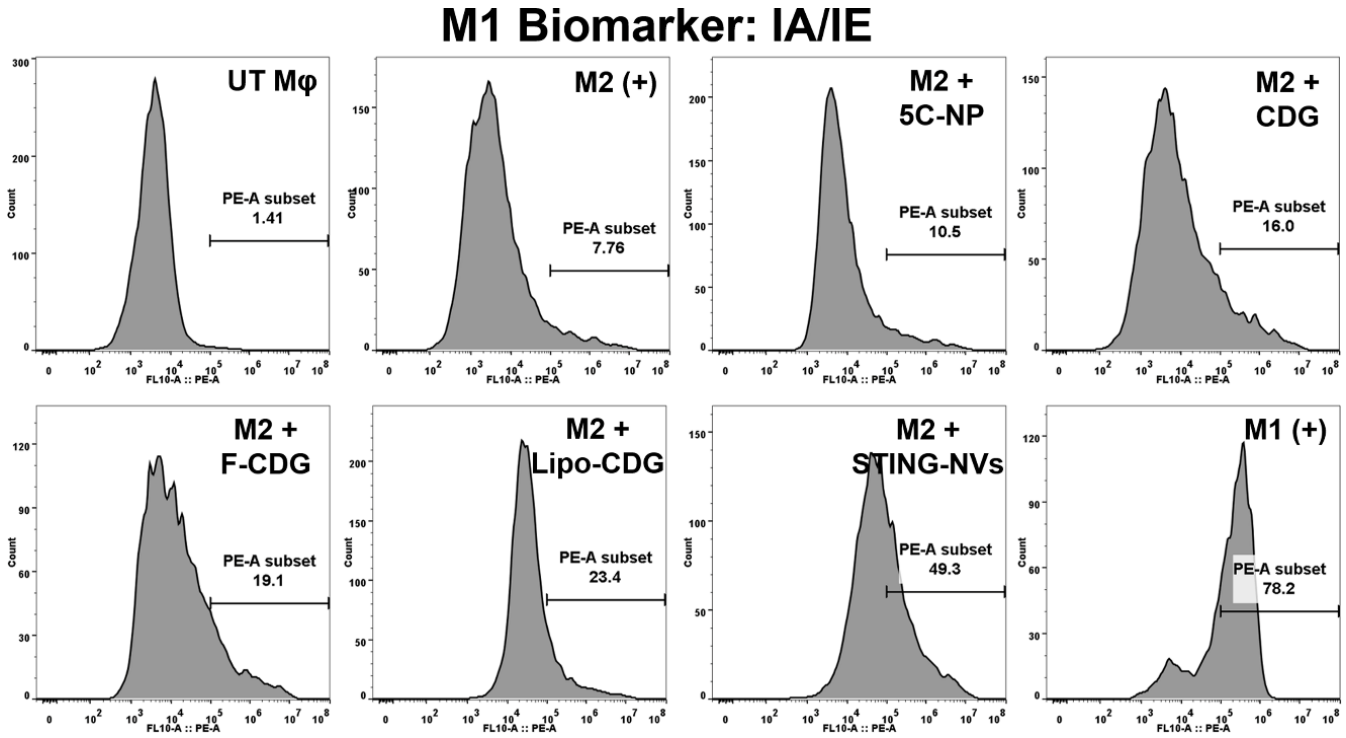

Figure S9. RAW264.7 macrophages were cultured in LPS plus IFN-γ for 24 hours to induce an M1-like phenotype and IL-4 to induce an M2-like phenotype. UT Mφ, untreated macrophage control. On day 2, M2 cells were treated with 1.5 μg/mL CDG delivered as free CDG, F-CDG, 5C-NPs, STING-NVs or Lipo-CDG. On day 3, the cells were treated with MHC class II (IA/IE) antibody and applied for flow cytometry. Flow cytometry results showing that STING-NVs elevated the expression of M1 biomarker MHC class II in M2 phenotype cells after 24 h.

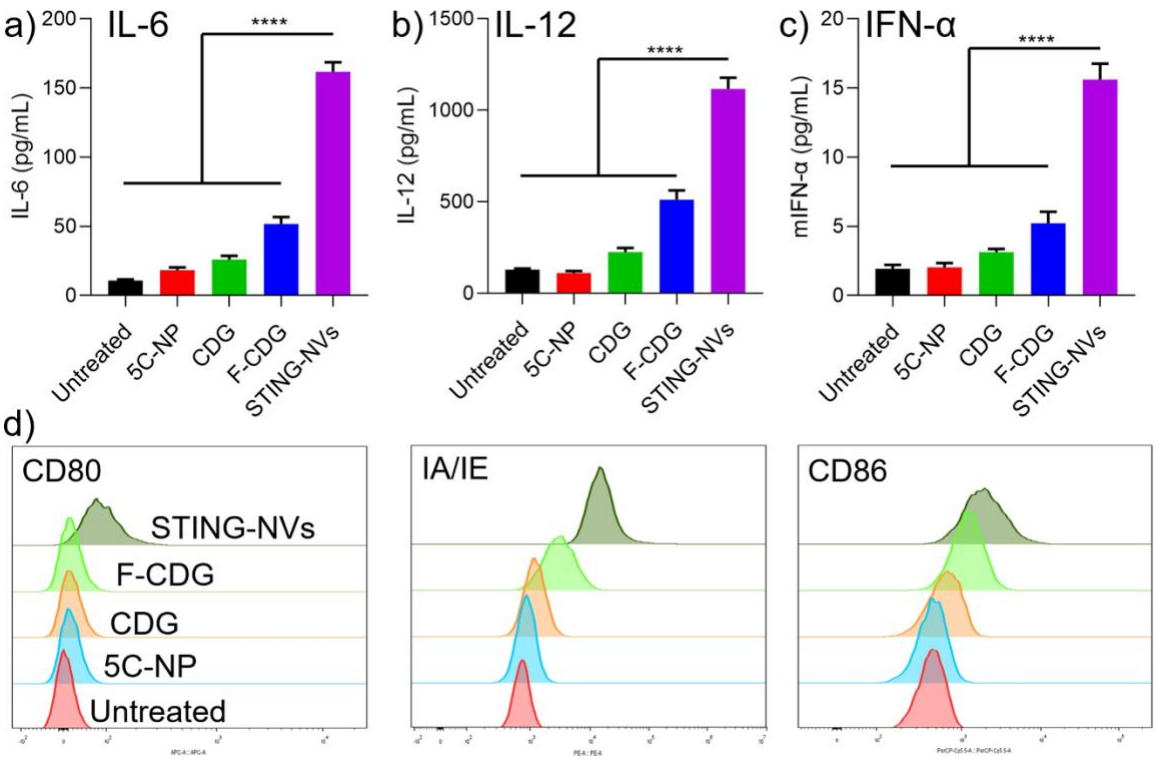

Figure S10. STING-NVs mediated potent immune stimulation in DCs. (**a-c**) STING-NVs elevated the production of proinflammatory factors IL-6 (**a**), IL-12 (**b**), and IFN-α (**c**) in DCs. DC2.4 cells were treated with 5C-NPs, CDG, F-CDG or STING-NVs (1 μg/mL CDG equivalents) for 24 h. ELISA results suggest STING-NVs induced DC2.4 cells to secrete significantly more IFN-α, IL-6, and IL-12p40 than CDG or even F-CDG, after 24 h. (**d**) STING-NVs elevated the expression of co-stimulatory factors and MHC molecules in DCs. DC2.4 cells were treated with 5C-NP, soluble CDG, F-CDG or STING-NVs (1 μg/mL CDG equivalents). After 24 h, the cells were treated with CD80, CD86, or MHC class II (IA/IE) antibodies and applied for flow cytometry. Flow cytometry results showing that STING-NVs elevated the expression of co-stimulatory and MHC molecules in DC2.4 cells after 24 h. Data represent mean ± SD. ****$p < 0.0001$, by one-way ANOVA with a Tukey's post hoc test.

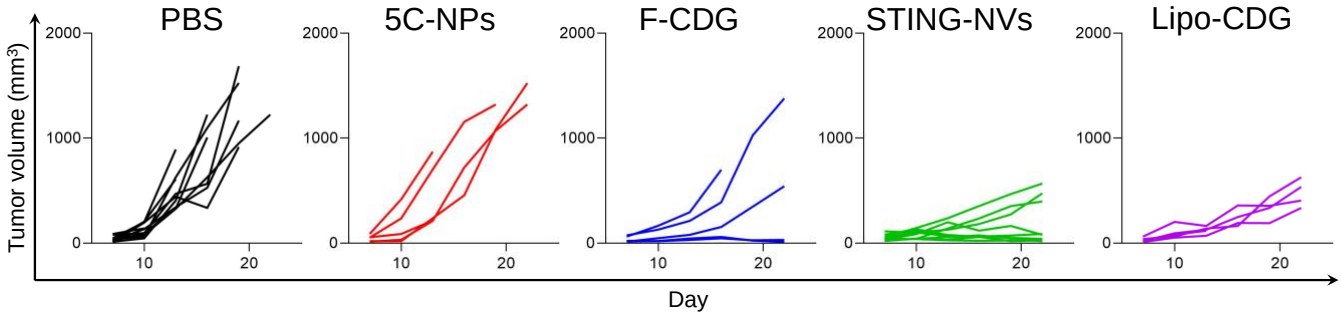

Figure S11. Individual tumor growth curves in the immunotherapy studies of STING-NVs in B16F10 melanoma.

Supplementary Tables

Table S1. The sequence of 3C-i-motif, 5C-i-motif, 7C-i-motif, 9C-i-motif, and scramble sequence, and the CDG loading efficiency and loading capacity of these primer modified NPs.

DNA	Sequences	Loading efficiency	Loading capacity
3C-i-motif	TTTTTTCCCTTACCCTTACCCTTACCC	14 %	0.8 %
5C-i-motif	**TTTTTTCCCCCTTACCCCCTTACCCCCTTACCCCC**	**53 %**	**2.9 %**
7C-i-motif	TTTTTTCCCCCCCCTTACCCCCCCCTTACCCCCCCCTTACCCCCCC	24 %	1.3 %
9C-i-motif	TTTTTTCCCCCCCCCCTTACCCCCCCCCCTTACCCCCCCCCCTTACCCCCCCCCC	25 %	1.4 %
Scramble	CACCACTCCTCACTCCTCTCCTCTCTCACACCTTC	2.3 %	0.1 %

Table S2. The i-motif conjugation efficiency, CDG loading efficiency and loading content of the different molar ratio of 5C-i-motif to PEG-*b*-PLA in the initial reaction mixture.

n(PEG-*b*-PLA): n(5C-i-motif)	Conjugation efficiency	Loading efficiency	Loading content	n(CDG): n(5C-i-motif)
2.5:1	81%	8%	3.2 %	0.63:1
5:1	92%	21%	4.2 %	1.3:1
10:1	85%	25 %	2.6 %	1.7:1
20:1	**93%**	**53 %**	**2.9 %**	**4.3:1**
50:1	87%	47 %	0.9 %	3.4:1