www.sciencetranslationalmedicine.org/cgi/content/full/7/283/283ra52/DC1

Supplementary Materials for

STING agonist formulated cancer vaccines can cure established tumors resistant to PD-1 blockade

Juan Fu, David B. Kanne, Meredith Leong, Laura Hix Glickman, Sarah M. McWhirter, Edward Lemmens, Ken Mechette, Justin J. Leong, Peter Lauer, Weiqun Liu, Kelsey E. Sivick, Qi Zeng, Kevin C. Soares, Lei Zheng, Daniel A. Portnoy, Joshua J. Woodward, Drew M. Pardoll, Thomas W. Dubensky Jr.,* Young Kim*

*Corresponding author. E-mail: ykim76@jhmi.edu (Y.K.); tdubensky@adurobiotech.com (T.W.D.J.)

Published 15 April 2015, *Sci. Transl. Med.* **7**, 283ra52 (2015)
DOI: 10.1126/scitranslmed.aaa4306

The PDF file includes:

Supplementary Materials

Materials and Methods

Peptide vaccine: C57BL/6 mice or *goldenticket* mice were vaccinated subcutaneously with 75 µg CDA and 10 µg endotoxin-free ovalbumin protein formulated in 2% squalene-in-water emulsion (Addavax, Invivogen). Six days after the boost vaccination, spleen and blood were collected. ELISPOT assays were performed using $OVA_{257-264}$ and $OVA_{265-280}$ peptides loaded onto APC. OVA-specific IgG from the serum was measured by ELISA.

Cell toxicity assay: Tumor cells were incubated with CDN for 24 hours at 37^{o}C in a 5% CO_2 incubator. The dead cells were then stained with 7-AAD (eBioscience) for 5-15 minutes on ice before analysis on a flow cytometer. CDN-treated tumor cells were also incubated with annexin V-FITC (eBioscience) and propidium iodide (PharMingen) for 10 minutes. Apoptotic cells were defined as cells that were propidium iodide and annexin V positive.

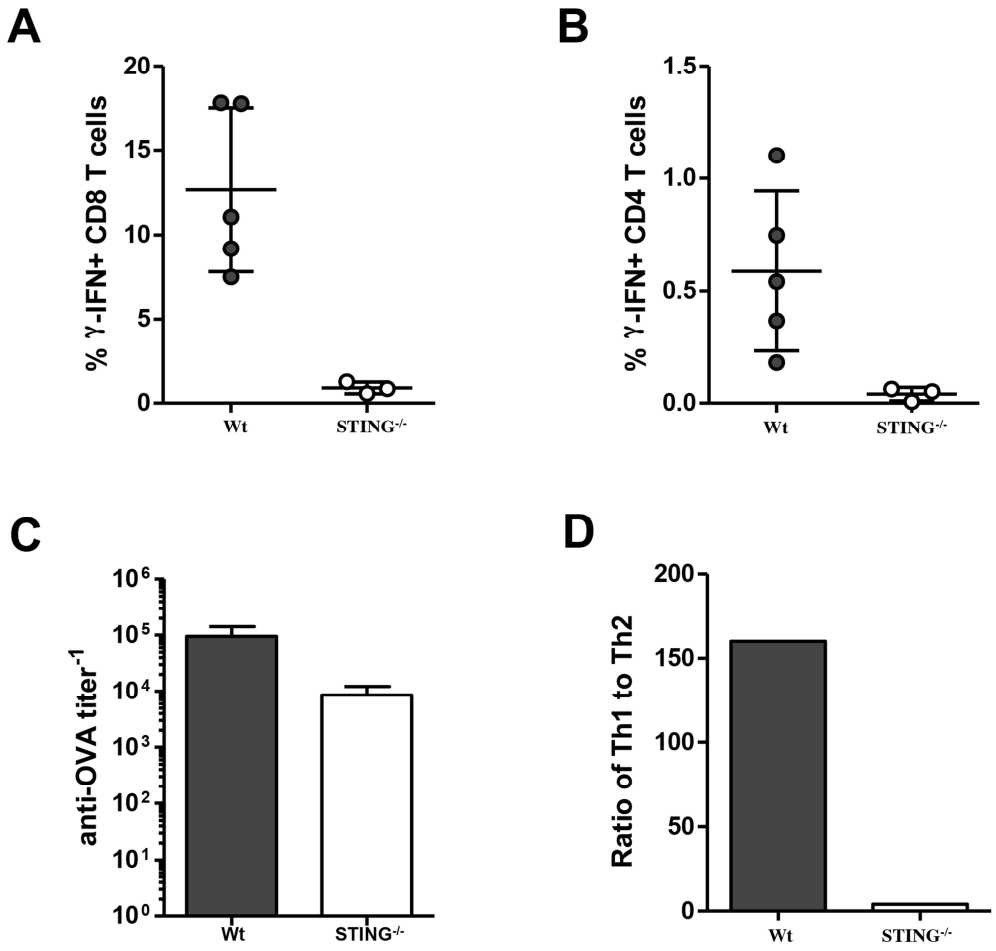
CDN preparation and synthesis: Synthesis of canonical CDA was initially accomplished by enzymatic production *in vitro* with recombinant His_6-tagged *B. subtilis* DisA protein. Briefly, a solution containing 1 mM ATP and 1 µM DisA in activity assay buffer (0.1 M NaCl, 40 mM Tris, pH 7.5, and 10 mM $MgCl_2$) was incubated for 24 h at 37 ^{o}C. At reaction completion (>95% of peak area was CDA as monitored by HPLC), 1 µL/10 mL reaction volume of calf intestinal phosphatase (New England Biolabs) was added to the reaction, which was then

incubated for 4 h at 37 ˚C. The reaction was inactivated in boiling water for 10 minutes. CDA was purified from the supernatant by anion exchange chromatography, eluted with 2 M ammonium acetate, and resuspended in PBS to 250 mM. LPS was removed by repeated Detoxi-Gel Endotoxin Removing Gel Column chromatography (Pierce) until the LPS content in the preparation was below 0.0125 EU/mL as determined by LAL assay (ToxinSensor, Genscript).

Modified CDN derivative molecules were synthesized by adaptation of the procedures of Gaffney et al. [44], using phosphoramidite linear coupling and H-phosphonate cyclization reactions (fig. S5A). Synthesis of dithio-CDN was accomplished by sulfurization reactions to replace the non-bridging oxygen atoms in the internucleotide phosphate bridge with sulfur atoms. For example, synthesis of dithio-[Rp,Rp]-c[A(2',5')pA(3',5')p] (ML RR-S2 CDA) (Fig. 3A) on a five millimole scale was achieved via suitably protected 2'-phoramidite **1** and 3'-H-phosphonate **2** nucleosides (fig. S5A). Sulfurization of phosphorus (III) intermediates generated during linear dimerization and cyclization gave **3** (X=S) and **4** (Y=S), respectively. After cyclization/sulfurization, the crude reaction mixture was purified by silica gel chromatography and subsequently deprotected to give bis-TBS-ML-S2-CDA as a mixture of RR- and RS-diastereomers. Separation of the diastereoisomers was performed by C-18 prep HPLC, and the isolated bis-TBS-ML-RR-S2-CDA was deprotected with TEA-3HF, neutralized with 1 M triethylammonium bicarbonate, and desalted on a C18 SepPak to give ML RR-S2 CDA as the bis-triethylammonium salt at >95% purity. Alternatively,

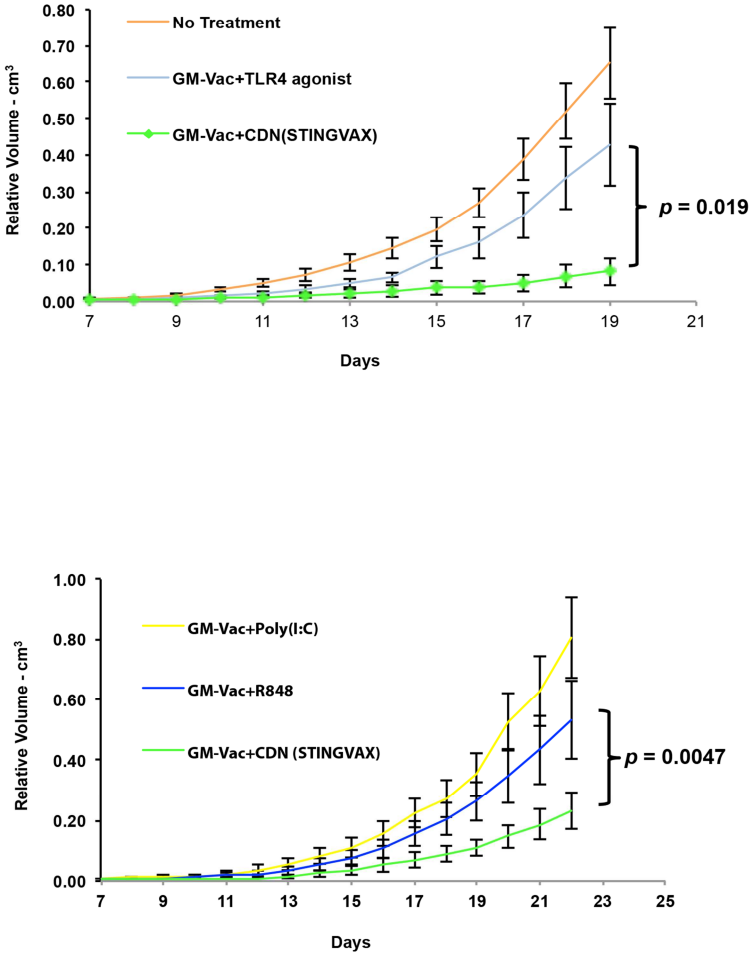
the TEA groups were exchanged with either sodium or ammonium counter ions by ion exchange, lyophilized, and resuspended in 10 mM Tris pH 7, 1 mM EDTA buffer to ~5 mg/mL, and filter-sterilized through a 0.2 micron filter, resulting in a final product that was $\geq$95% pure as determined by analytical HPLC (fig. S5B). High-resolution Fourier transform ion cyclotron resonance mass spectroscopy (FT-ICR) confirmed the expected elemental formula: $[M-H]^-$ calculated for $C_{20}H_{23}N_{10}O_{10}P_2S_2$ 689.0521; found 689.0514. The spectra for the ^{31}P NMR (y-axis of fig. S5C) were consistent with ML RR-S2 CDA. Direct evidence for the regiochemistry of the phosphodiester linkages was obtained by 1H-1H COSY (correlation NMR spectroscopy) for assignment of ribose protons (shown on x-axis of fig. S5C) in combination with a ^{1}H-^{31}P HMBC (heteronuclear multiple-bond correlation spectroscopy) experiment (fig. S5C). Before use in experiments, all synthetic CDN preparations were verified by LAL assay to be endotoxin-free (<1 EU/mg).

Supplementary Figure 1



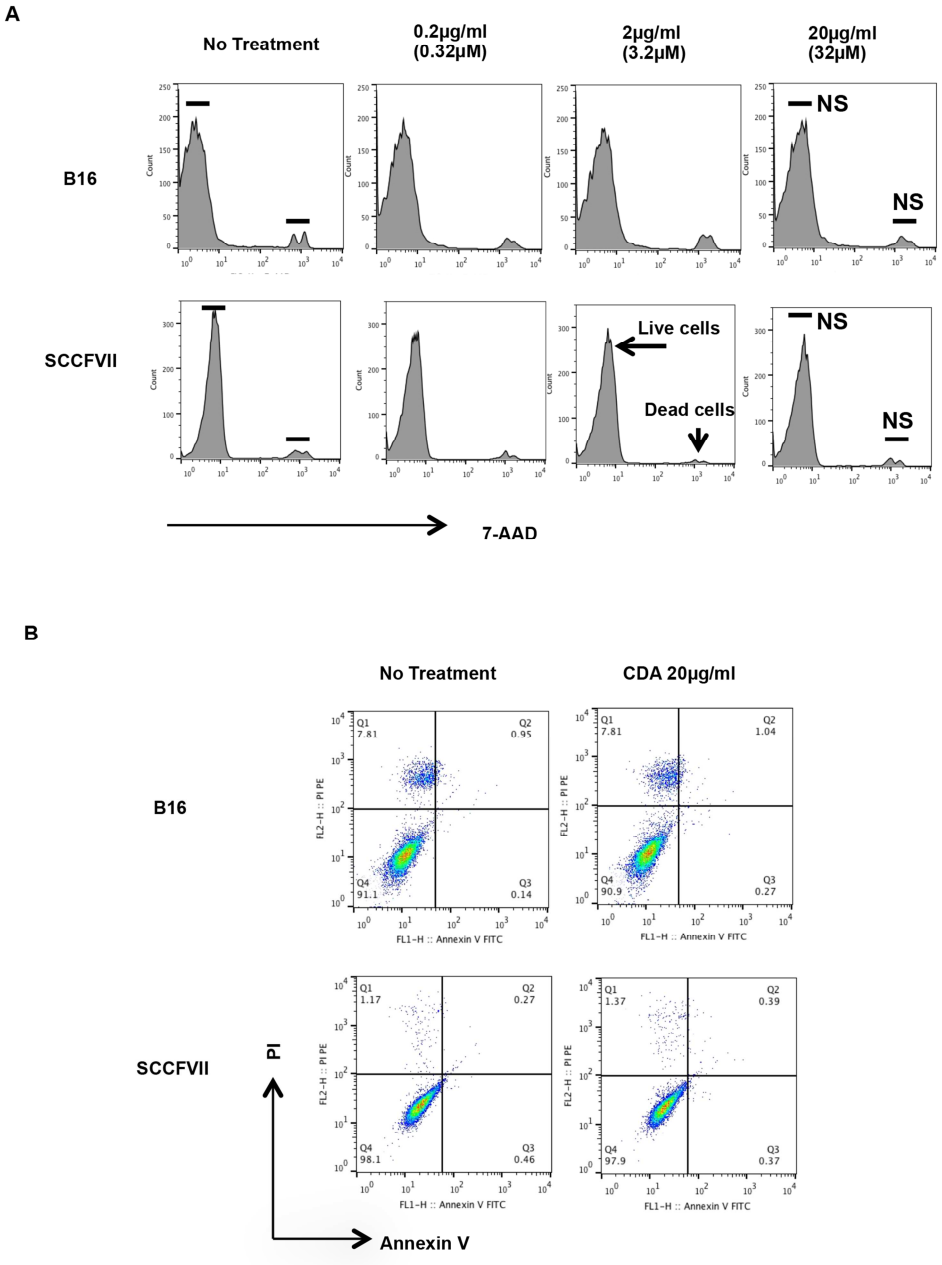
Supplementary Figure 1. **CDN can induce cellular and humoral immunity. (A,B)** On days 0 and 36, wt C57BL/6 mice (n=5, filled circles) and *goldenticket* (STING-/-) mice (n=3, open circles) were vaccinated, and OVA-specific $CD8^+$ (**A**, $OVA_{257-264}$) and $CD4^+$ (**B**, $OVA_{265-280}$) immune responses were measured in the spleens by intracellular cytokine staining. **(C,D)** Total OVA-specific IgG **(C)** or isotype-specific OVA-specific antibodies **(D)** were measured in serum by ELISA. Ratio of Th1 to Th2 was calculated by dividing the sum of OVA-specific IgG2a and IgG2b titers by the OVA-specific IgG1 titer.

Supplementary Figure 2



Supplementary Figure 2. CDNs are more potent adjuvants than TLR agonists. GM-vaccine cells were formulated with equimolar CDA or monophosphyl lipid A (MPL, a TLR4 agonist), R848 (TLR7/8 agonist), or poly (I:C) (TLR3 agonist) by simple incubation, then injected into mice. The anti-tumor effects of the adjuvants were compared in the B16 treatment model. STINGVAX (GM-vac with CDA) proved to have a more potent anti-tumor response *in vivo* when compared to GM-vac with MPL (top panel). STINGVAX also showed improved in vivo efficacy when compared to GM-vac formulated with poly(I:C) or R848 (bottom panel). Each group had 10 mice, and data represent mean +/- SEM. The graphs are representative of 3 experiments.

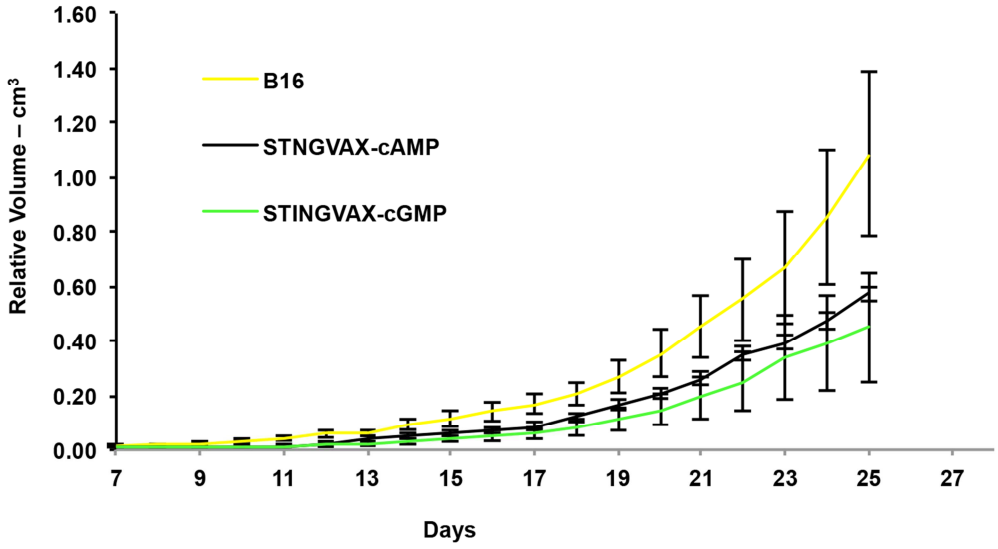
Supplementary Figure 3



Supplementary Figure 3. CDNs do not kill tumor cells directly.
A. B16 and SCCFVII cells were incubated with CDN (RR-S_2-CDA) at 0.2 µg/ml (0.32 µM), 2 µg/ml (3.2 µM), or 20 µg/ml (32 µM), and the number of dead and live cells was quantitated with 7-AAD. The FACS data show no significant differences in live and dead cells in all the groups. (NS=not statistically

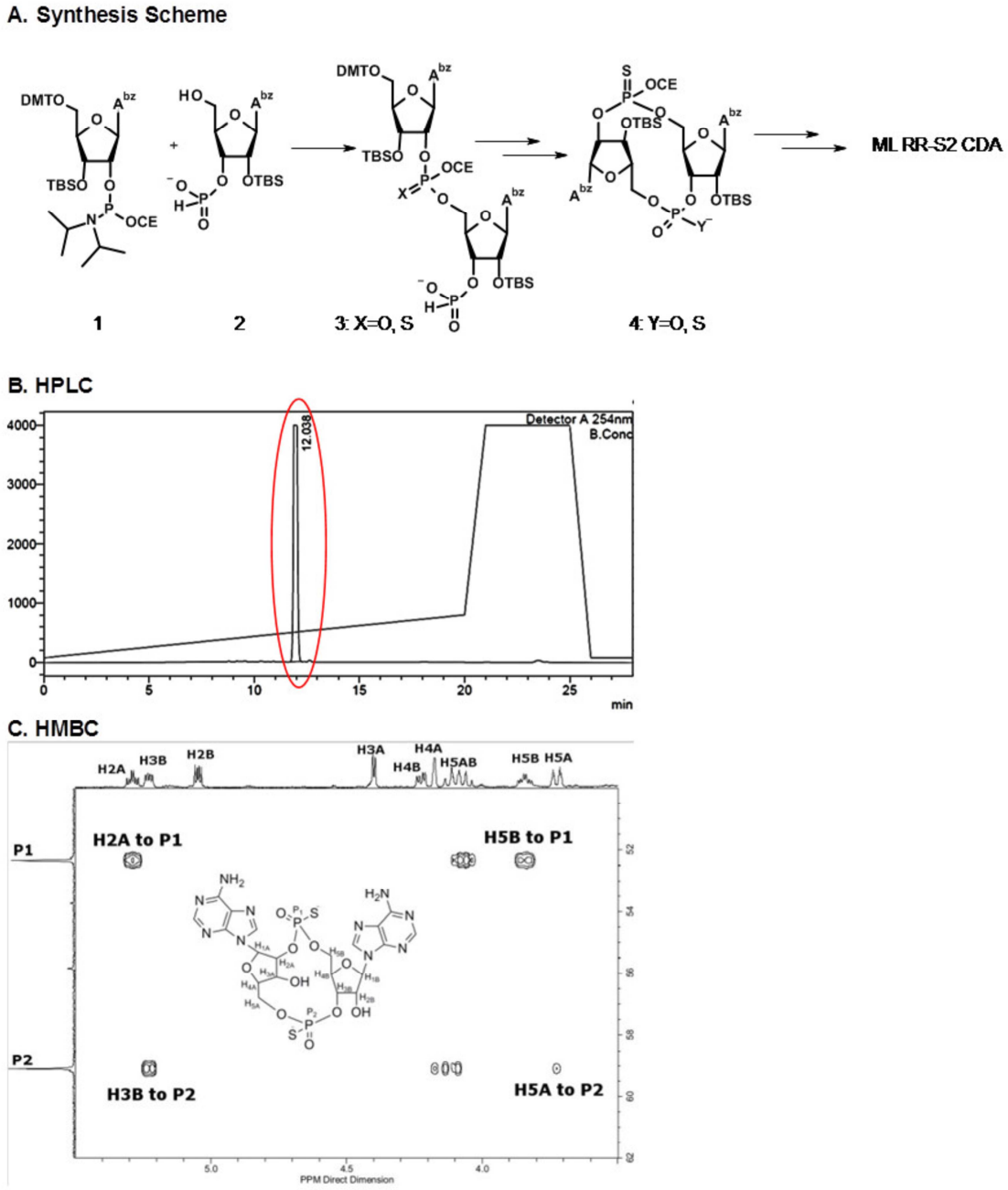
significant) **B.** Apoptosis assay with Annexin V staining showed no statistical differences in apoptosis between the treated cells and controls. Flow cytometry was used to detect apoptotic cells as defined by PI+ and annexin V+ cells.

Supplementary Figure 4



Supplementary Figure 4. CDA and CDG are equally effective when formulated into STINGVAX. STINGVAX was formulated with equimolar CDA and CDG and tested in the B16 treatment assay. Mice bearing palpable B16 tumors were treated with both formulations, and their tumor growth rate was measured. The growth curve rates were not statistically different between the two STINGVAX groups.

Supplementary Figure 5

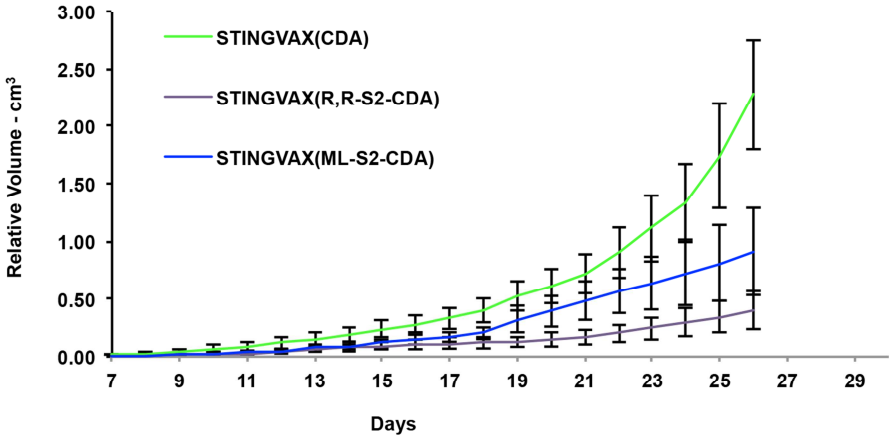


Supplementary Figure 5. CDNs with dithiophosphate backbone can be synthesized.

(A) Schematic of synthesis depicting the conversion of a non-canonical 2'-O-phosphoramidite (**1**) and 3'-H-phosphonate nucleosides (**2)** to a linear dimer (**3**) followed by ring closure via the H-phosphonate to give a cyclic dinucleotide (**4**).

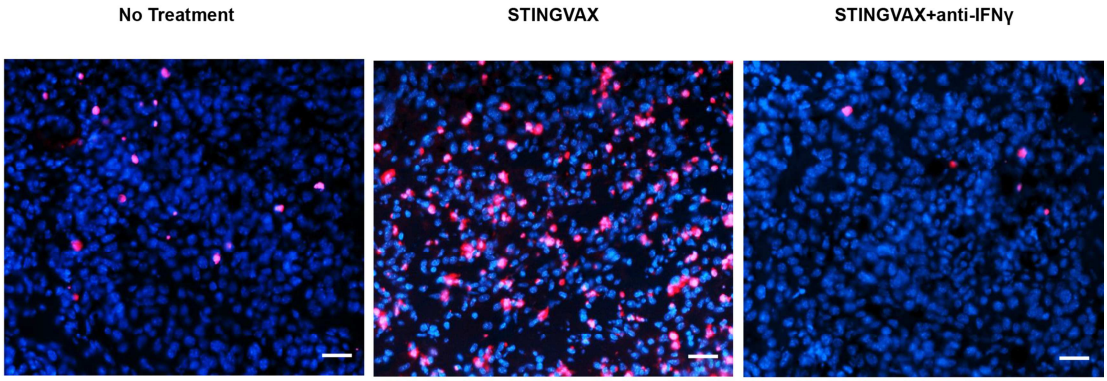
The phosphorus III intermediates generated at these two steps can either be oxidized or sulfurized. After deprotection and purification, the CDN product was characterized by one- and two-dimensional NMR spectroscopy in conjunction with LC/MS and high resolution mass spectroscopy. **(B)** HPLC chromatograph of ML RR-S2 CDA purification to $\geq$95%, using a 2% to 20% acetonitrile gradient in 10 mM triethylammonium acetate C-18 column, showing retention time of 12.04 min. **(C)** Two-dimensional ^{1}H-^{31}P Heteronuclear Multiple Bond Correlation (HMBC) of synthesized ML-RR-S2-CDA. The HMBC experiment revealed that the phosphorus nucleus, P-1, is correlated to the 2'-ribose proton (H-2A) as well as the 5' ribose methylene protons (H-5B). The other phosphorus nucleus, P-2, is correlated to the 3' ribose proton (H-3B) and to the 5' ribose methylene protons (H-5A) of the other adenosine. The combined 1H-1H COSY and 2D-HMBC results provide direct evidence that the regiochemistry of the phosphodiester linkages is 2',5'-3',5'.

Supplementary Figure 6



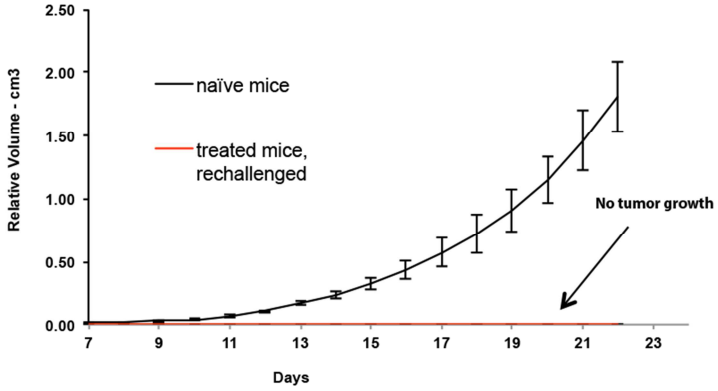
Supplementary Figure 6. 2′-3′ linkage did not have improved antitumor efficacy in the murine system. Mice bearing palpable B16 tumors were treated with STINGVAX formulated with CDA, RR-S2 CDA, or ML RR-S2 CDA. The mixed linked backbone derivative did not improve anti-tumor efficacy of CDN in this murine model. The difference between the two groups did not reach statistical significance by day 26. Each group had 10 mice, and data represent mean +/- SEM. The graph is representative of 5 experiments.

Supplementary Figure 7



Supplementary Figure 7. PD-L1 induction with STINGVAX treatment is IFNγ-dependent. B16 tumor-bearing mice were treated with STINGVAX (CDA) with or without neutralizing IFNγ antibody. The tumors were harvested, fixed, and stained first with rat anti-mouse PD-L1, then with anti-rat Cy3 conjugate and DAPI counterstain. Scale bars represent 100 μm.

Supplementary Figure 8



Supplementary Figure 8. STINGVAX-treated mice become immune to repeat tumor challenge. Naïve Balb/c mice and STINGVAX-treated Balb/c mice whose CT26 tumor had regressed were rechallenged with 10^5 tumor cells (10 mice/group). The tumor growth rates were followed daily. All the naïve mice showed tumor growth (black), but no tumors were seen among the previously treated mice (red).