Cell Reports
Supplemental Information

Direct activation of STING in the tumor microenvironment leads to potent and systemic tumor regression and immunity

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Figure S1, related to Figure 1.

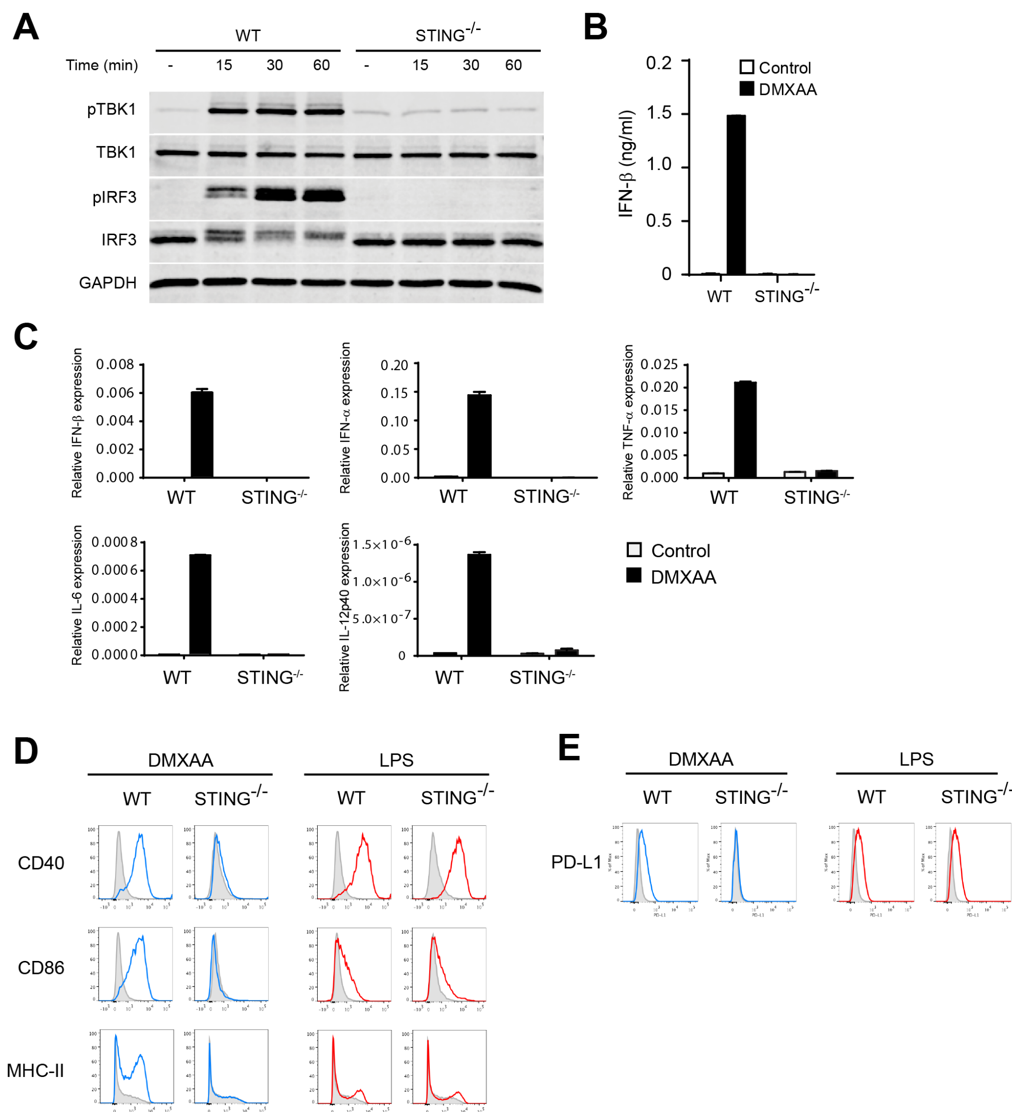


Figure S1. DMXAA activates the STING pathway and promotes the activation of BM-DCs. (A) Bone marrow-derived DCs (BM-DC) from WT or STING$^{-/-}$ mice were stimulated with 25 μg/ml of DMXAA for the indicated time points. The amount of pTBK1, total TBK1, pIRF3, total IRF3 and GAPDH was measured by Western blot. (B-D) WT or STING$^{-/-}$ BM-DCs were stimulated with 25 μg/ml of DMXAA. Amount of secreted IFN-β was measured by ELISA after 12 hours (B); expression of innate cytokines was measured by q-RT-PCR after 4 hours (C); and expression of co-stimulatory molecules on the cell membrane was measured by staining with specific antibodies against CD11c, CD40, CD86, MHC class II (D); and PD-L1 (E). Cells were acquired in the LSRII-Blue Cytometer and analyzed with the FlowJo software (Treestar).

Figure S2, related to Figure 2.

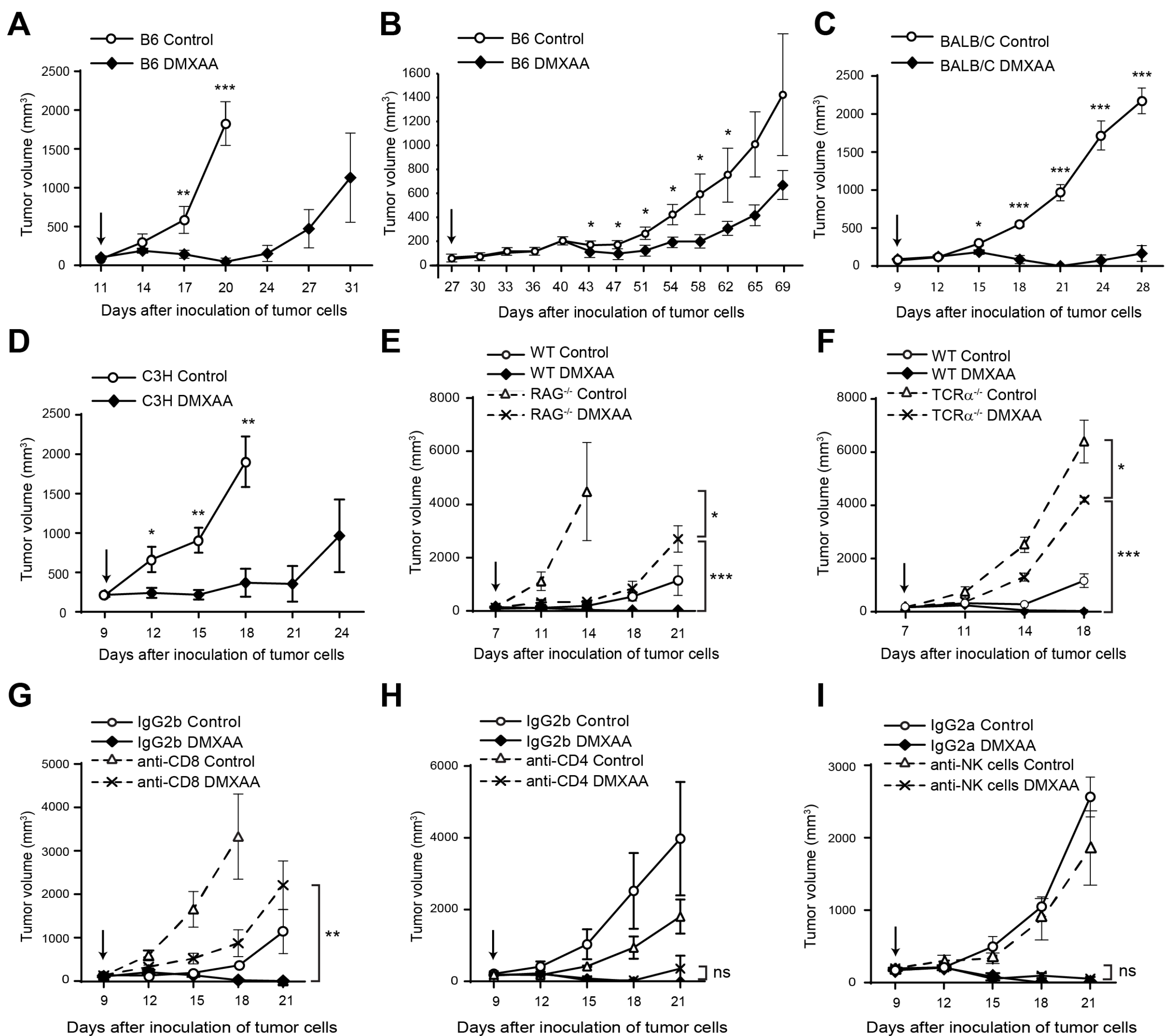
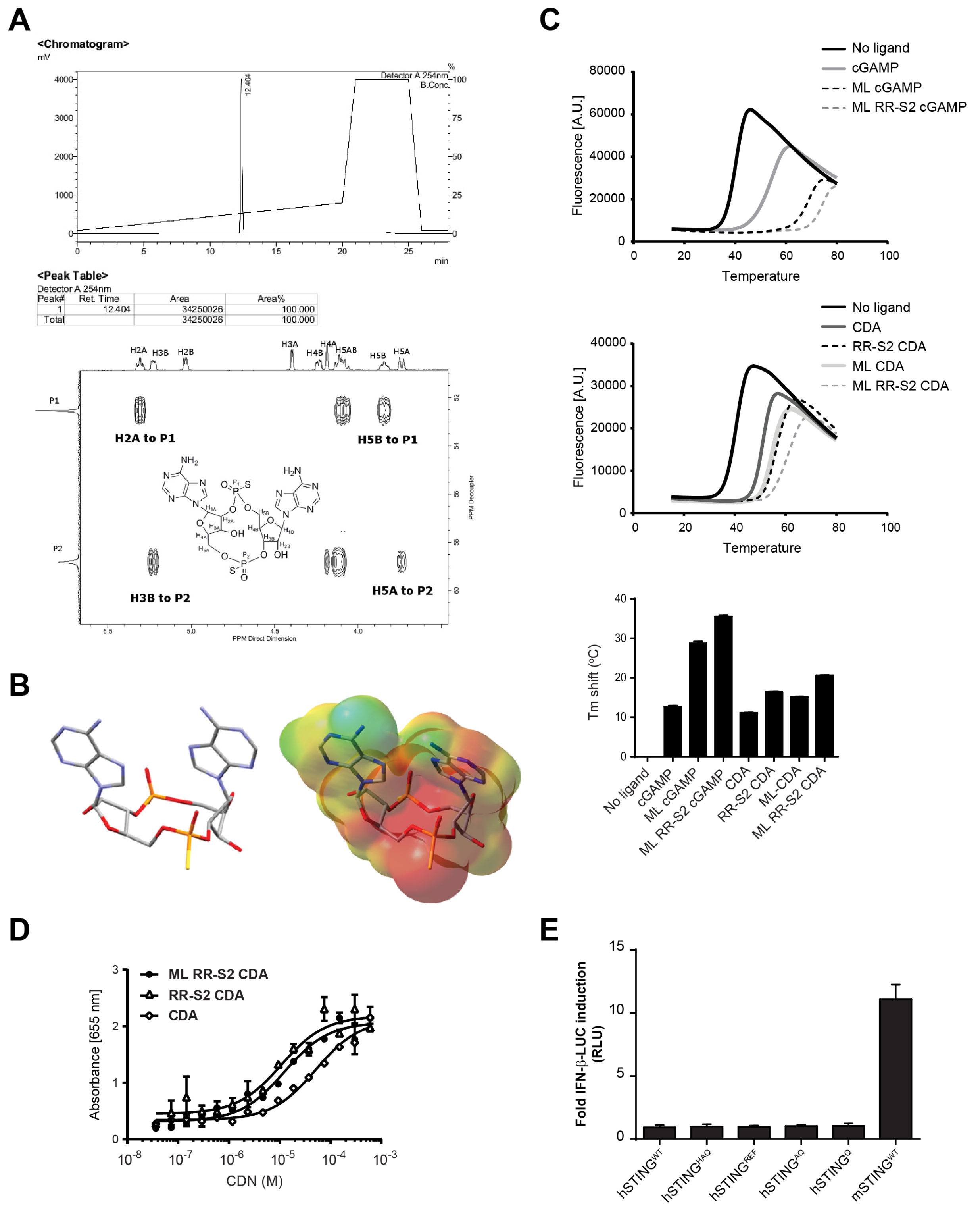


Figure S2. DMXAA has therapeutic effect in different mouse tumor models and requires the adaptive immune response. (A-D) WT mice were inoculated with 10^6 B16.F10 (A) or TRAMP-C2 (B) into C57BL/6 mice, 4T-1 into BALB/C mice (C), or Ag104L into C3H mice (D). (E-F) WT and $RAG^{-/-}$ C57BL/6 mice (E) or WT and $TCR\alpha^{-/-}$ mice (F) were inoculated with 10^6 B16.SIY cells in the left flank. (G-I) WT C57BL/6 mice were depleted of $CD8^+$ T cells by a weekly injection of 250 μg of anti-CD8 antibody (clone 2.43), or isotype IgG2b as control (G); 250 μg of anti-CD4 antibody (clone GK1.1), or isotype IgG2b as control (H); or anti-NK1.1 antibody (clone PK136), or isotype IgG2a as control (I). Two days after the first injection of antibodies mice were challenged with 10^6 B16.SIY cells in the left flank. When tumor volumes were 100-200 mm^3 they received a single IT dose of 500 μg of DMXAA or

saline (as arrows indicate). Tumor volume was measured at different time points. Results are shown as mean tumor volume ± s.e.m. (n = 5 animals per group). Data represent at least two independent experiments. *$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure S3, related to Figure 3.



Peak#	Ret. Time	Area	Area%
1	12.404	34250026	100.000
Total		34250026	100.000

Figure S3. Structure of cyclic dinucleotides and binding and activation of human STING. (A) (Upper panel) HPLC chromatograph of ML-RR-CDA purification to ≥95%, using a 2% to 20% acetonitrile gradient in 10 mM triethylammonium acetate C-18 column, showing retention time of 12.40 min. (Lower panel) Two- dimensional ^{1}H-^{31}P Heteronuclear Multiple Bond Correlation (HMBC) of synthesized ML RR-S2 CDA. Two dimensional ^{1}H- ^{31}P HMBC revealed that the phosphorus nucleus, P-1 is correlated to the 2'-ribose proton (H-2A) as well as the 5' ribose methyelene protons (H-5B). The other phosphorous nucleus, P-2, is correlated to the 3' ribose proton (H-3B) and to the 5' ribose methyelene 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' according to the structure shown. (B) (Left panel) X-ray crystallographic structure (stick model) of ML RR-S2 CDA, confirming the R,R diastereomer configuration and regiochemistry of the 2'-5'-3'-5' phosphodiester linkages. Color scheme: carbon (white); nitrogen (blue); oxygen (red); sulfur (yellow). (Right panel) Electrostatic surface potential of ML RR-S2 CDA displayed with green (positive), yellow (neutral), and red (negative). (C) Purified murine STING binding to CDNs was analyzed by thermal shift assay. Temperature curves are the average from a representative experiment of three independent experiments performed in duplicate. Tm shift values are mean values + s.e.m. (Lower panel). (D) THP-1-Blue™ ISG cells were incubated with the indicated concentrations of CDNs for 30 minutes, washed and incubated overnight. Supernatants were assayed for secreted embryonic alkaline phosphatase (SEAP) activity as a readout for type I interferon production. EC_{50} values were calculated to be 12.3 μM for ML RR-S2 CDA, 10.7 μM for RR-S2 CDA and 51.3 μM for CDA. Data are representative of two independent experiments. Data were analyzed in triplicate and plotted as the mean +/- s.e.m. (E) HEK293T cells stably expressing the indicated STING alleles were transfected with an IFN-β-luciferase reporter construct. After 24 hours, cells were stimulated for 6 hours with 100 μg/ml DMXAA before measuring luciferase gene reporter activity. Data are representative of two independent experiments. Data were analyzed in triplicate and plotted as the mean +/- SD.

Figure S4, related to Figure 3.

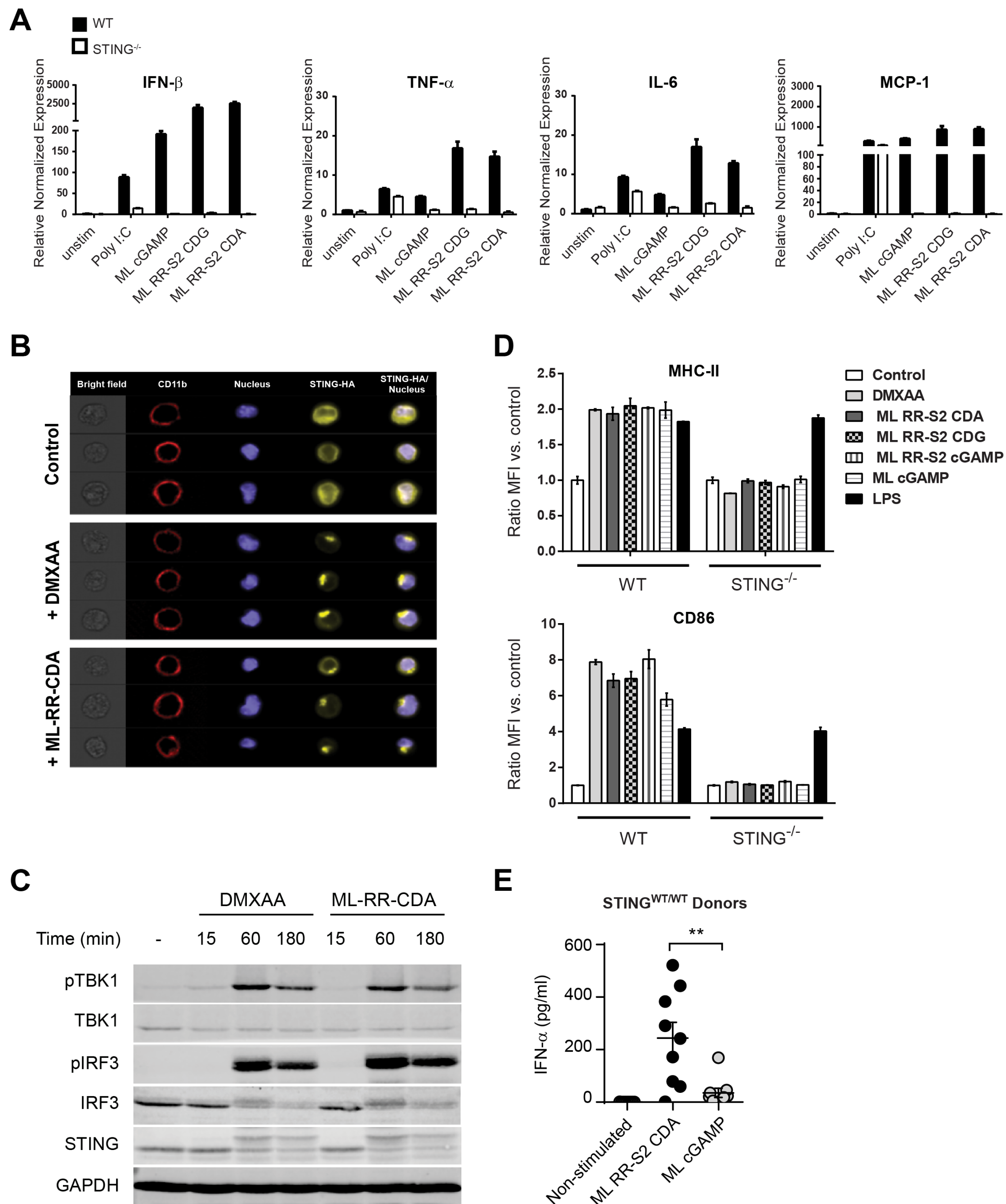


Figure S4. Induction of pro-inflammatory cytokines and activation of the STING pathway by CDNs. (A) CDNs were added to BMMs isolated from C57BL/6 or from STING$^{-/-}$ mice at 5 μM. After 6 hour incubation, induced expression of IFN-β, TNF-α, IL-6 and CCL2/MCP-1 was assessed by q-RT-PCR, and relative normalized expression was determined by comparison with unstimulated C57BL/6 BMMs, and Gapdh and Ywhaz reference genes. Data are representative of two

independent experiments. Data were analyzed in triplicate and plotted as the mean +/- SD. (B) $STING^{-/-}$ macrophages expressing STING-HA were stimulated for 1 hour with 50 mg/ml DMXAA or 50 μM ML RR-S2 CDA then stained with specific antibodies against HA tag and CD11b along with DAPI. Single cell images were acquired in the ImageStream and data were analyzed with the IDEAS software (Amnis, Millipore). (C) WT macrophages were stimulated with 50 μg/ml of DMXAA or 50 μM ML RR-S2 CDA for the indicated time points. The amount of pTBK1, total TBK1, pIRF3, total IRF3, STING and GAPDH was measured by Western blot. (D) BM-DCs derived from WT or $STING^{-/-}$ mice were stimulated in media with 10 μM of the indicated CDNs, 1 μg/ml LPS, or 100 μg/ml DMXAA. After 24 hours, expression of MHC class II or CD86 was measured by FACS gated on $CD11c^{+}$ DCs. (E) Human PBMCs from donors with $STING^{WT/WT}$ alleles (n = 9) were stimulated with 10 μM of ML RR-S2 CDA or ML cGAMP. After 6 hours stimulation, IFN-α was measured from cell supernatants by cytometric bead array. Data were plotted as the mean +/- SD. ** $P < 0.01$.

Figure S5, related to Figure 4 and to Figure 5.

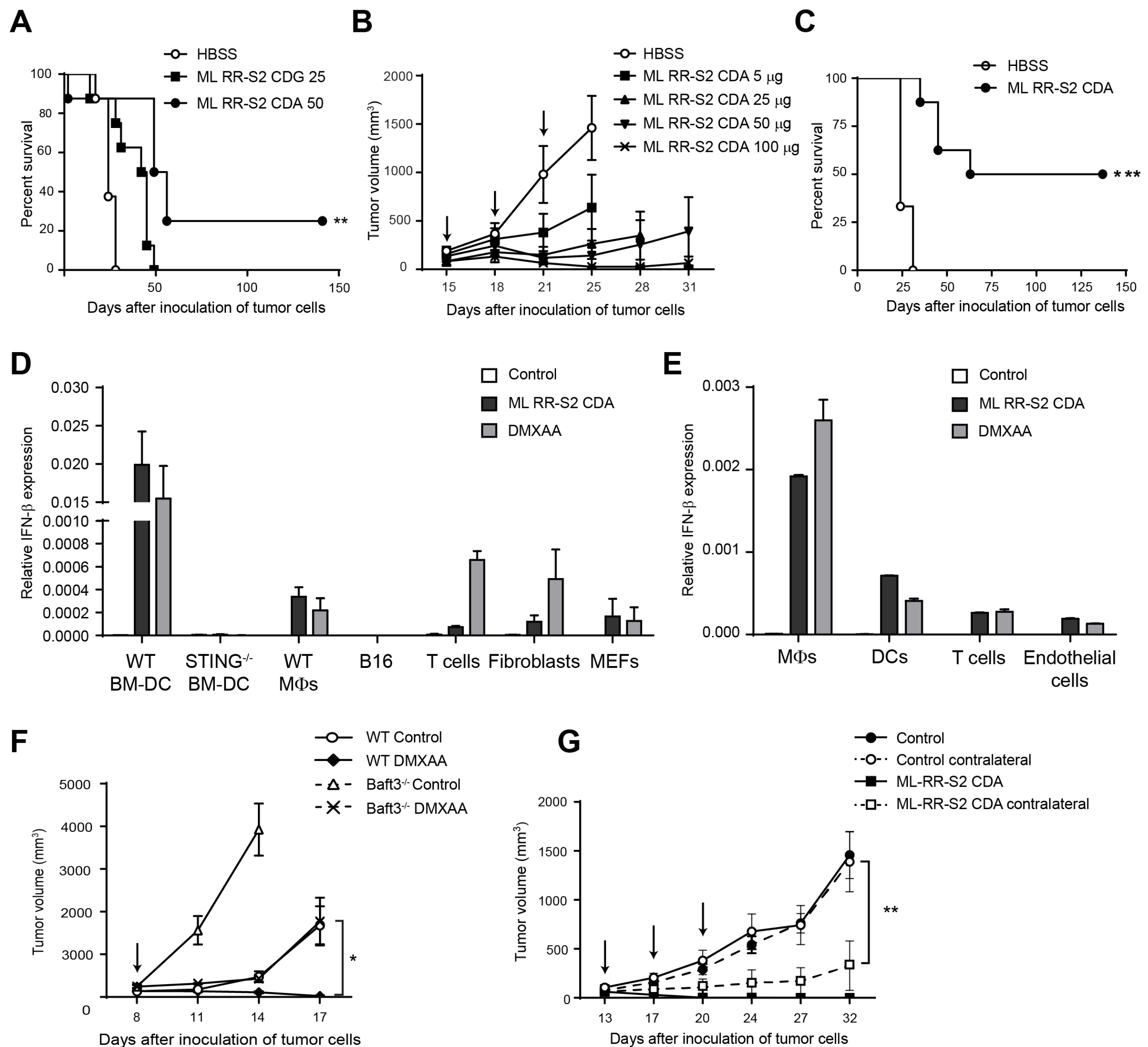


Figure S5. Therapeutic effect of ML RR-S2 CDA in tumor-bearing mice and characterization of the cell subsets that produce IFN-β in response to STING agonists. (A) WT C57BL/6 mice were inoculated with 5 x 10^4 B16.F10 cells in the left flank (n=8). When tumor volumes were 100 mm^3 mice received three IT doses of either ML RR-S2 CDG (25 μg), ML RR-S2 CDA (50 μg), or HBSS as control. (B) WT C57BL/6 mice were inoculated with 5 x 10^4 B16.F10 cells in the left flank (n=5). When tumor volumes were 100 mm^3 they received three IT doses of ML RR-S2 CDA at 5, 25, 50 or 100 μg or HBSS as control. (C) WT C57BL/6 mice were

inoculated with 5 x 10^4 B16.F10 cells in the left flank (n=8). When tumor volumes were 100 mm^3 they received three IT doses of 100 μg ML RR-S2 CDA or HBSS as control. Treatments were administered on days 13, 17 and 20 and tumor measurements were taken twice weekly. Results are shown as percent survival by Log-rank (Mantel-Cox) test (A and C). ** $P < 0.01$; *** $P < 0.001$ Data are representative of at least two independent experiments. (D) BM-DCs, macrophages, B16 tumor cells, T cells, and primary adult skin or embryonic fibroblasts were stimulated for 4 hours with 25 μg/ml DMXAA or 10 μM ML RR-S2 CDA. Expression of IFN-β was measured by q-RT-PCR. (E) Macrophages ($CD45^+$ MHC-II^+ $CD11b^+$ $F4/80^+$), DCs ($CD45^+$ $MHCII^+$ $CD11c^+$), T cells ($CD45^+$ $CD3^+$) and endothelial cells ($CD45^-$ $CD31^+$) were sorted from 7 days pre-established B16 tumors. These cells were stimulated for 4 hours with 25 μg/ml DMXAA or 10 μM ML RR-S2 CDA, and expression of IFN-β was measured by q-RT-PCR. (F) WT and $Batf3^{-/-}$ mice were inoculated with 10^6 B16.SIY cells in the left flank (n = 5). When tumor volumes were 100-200 mm^3 animal received a single IT dose of 500 μg of DMXAA or saline. Tumor volume was measured at the indicated time points. Data represent mean ± s.e.m. of three independent experiments. * $P < 0.05$. (G) WT BALB/c mice were implanted with $1x10^5$ of 4T-1 tumor cells on both flanks. On the days indicated, mice were treated in one flank only with ML RR-S2 CDA (50 μg), or HBSS vehicle control (n = 8). Data are representative of at least two independent experiments. Results are shown as mean ± s.e.m. ** $P < 0.01$.

Supplemental Methods

Cells

The prostate cancer TRAMP-C2 cells (from ATCC) and the fibrosarcoma Ag104L cell line (gifted by Dr. Hans Schreiber, University of Chicago)) were maintained at 37°C with 7.5% CO_2 in DMEM with 10% heat-inactivated FCS, penicillin, streptomycin, L-arginine, L-glutamine, folic acid, and L-asparagine.

Bone marrow-derived dendritic cells (BMDCs) from WT and STING$^{-/-}$ mice were generated by culturing cells from the tibiae and femurs in the presence of rmGM-CSF (20 ng/ml; BioLegend) for 9 days. After the incubation, the phenotype of cells with specific antibodies confirmed that >90% of the cells were CD11c^{+}, CD11b^{+} or CD11b^{-}, and CD8^{-},Cd4^{-} and CD19^{-}.

C57Bl/6 primary mouse embryonic fibroblasts (MEFs) were obtained from 13.5 d post coitus embryos and cultured in DMEM supplemented with 10% FBS, 1% non-essential amino acids, and 1% penicillin/streptomycin for no more than five passages. Primary fibroblasts were obtained from (Cell Biologics) and maintained in basal medium supplemented with 10% FBS, hydrocortisone, L-Glutamine, FGF, and antibiotic-antimycotic Solution (Cell Biologics).

Expression of activation markers by flow cytometry. BM-DCs from WT or STING$^{-/-}$ mice were stimulated with 25 μg/ml DMXAA or 100 ng/ml LPS for 12 hours, or with 50 μM of each CDN for 24 hours. After stimulation, cells were pre-incubated for 15 min with anti-CD16/32 monoclonal antibody (93) to block potential nonspecific binding and then with specific antibodies: anti-CD11c-Pe-Cy7 or APC (N418), anti-CD11b-PerCP-Cy5.5 (M1/70), anti-CD40-PE (3/23), anti-CD80-APC (16-10A1), anti-CD86-FITC or PE (GL1), anti-IA/IE-PB or FITC (M5/114.15.2) and anti-PD-L1-APC (10F.9G2). Stained cells were analyzed using LSR II cytometer

with FACSDiva software (BD) or FACSVerse with FACSuite software. Data analysis was conducted with FlowJo software (Tree Star).

Type I IFN bioassay. THP1-Blue™ ISG cells, which express an interferon regulatory factor (IRF)-inducible secreted embryonic alkaline phosphatase (SEAP) reporter construct (Invivogen) were treated with CDNs in 50 mM HEPES (pH 7.0), 100 mM KCL, 3 mM $MgCl_2$, 0.1 mM DTT, 85 mM sucrose,0.2% BSA, 1 mM ATP, 0.1 mM GTP. After 30 minutes, cells were washed and plated in a 96-well dish in RPMI media containing 10% FBS, and incubated at 37°C with 5% CO_2. Cell culture supernatants from each sample were collected after 16 hour incubation, and measured for SEAP activity using QUANTI-Blue reagent (Invivogen) to evaluate type I interferon protein levels. Absorbance readings at 655 nm were measured with a SpectraMax M2 plate reader (Molecular Devices). Data was fit to a three parameter dose response curve to determine the EC_{50} using Graph Pad Prism (Graph Pad Software Inc., San Diego).

Measurement of IFN-α protein. Cryopreserved hPBMCs were thawed and $1x10^6$ cells per well were plated in a 96 well plate. Cells were stimulated with 10 μM ML RR-CDA or ML cGAMP for 6 hours and supernatants were harvested. Supernatants were diluted 1:2 and assayed for IFN-α protein using Cytometric Bead Array (CBA) Human Flex Set according to the manufacturer's instructions (BD). Data was collected using a FACSVerse cytometer and analyzed by FCAP Array Software (BD).

Depletion of $CD8^+$ or $CD4^+$ T cells or NK cells. For depletion of $CD8^+$, $CD4^+$ T cells or NK cells, mice were injected IP weekly with rat mAb to mouse CD8

(43.2), rat mAb to mouse CD4 (GK1.5), rat anti-mouse NK1.1 (PK136) or isotype controls IgG2b or IgG2a (BioXcell) at a dose of 250 μg per mouse. These regimens resulted in > 99% depletion of $CD8\alpha^+$, $CD4^+$ T cells or NK cells from the peripheral blood, as evaluated by flow cytometry.

Isolation of different cell populations from tumors. $2x10^6$ B16-SIY tumor cells were inoculated in both flanks of WT C57BL6 mice. After one week, tumors were harvested and incubated with collagenase (50 unit/ml; Worthington Biochemical Corporation) for 30 minutes at 37° C. Single suspensions of tumor-derived cells were prepared by homogenization using cell strainer and then Ficoll-Hypaque centrifugation. Cells were incubated with fluorocrome-conjugated antibodies against mouse CD45-Alexa Fluor 488 (30-F11), MHC-II-PE-Cy7 (M5/114.15.2), CD11b-PerCP-Cy5.5 (M1/70), F4/80-Pacific Blue (Bm8), CD11c-PE (N418), CD3-APC eFluor 780 (17A2), CD31-APC (390) (all antibodies from BioLegend), and Fixable Viability Dye eFluor 450 (eBioscience). Cells were sorted using the FACs AriaII sorter devise (BD).

Preparation of natural cyclic dinucleotide STING ligands and synthetic derivative molecules. Modified CDN derivative molecules were synthesized according to modifications of the "one-pot" Gaffney procedure, described previously. Synthesis of CDN molecules utilized phosphoramidite linear coupling and H-phosphonate cyclization reactions. Synthesis of dithio CDNs 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)-[cyclic[A(2',5')pA(3',5')p]], shown as ML RR-S2 CDA in **Supplementary**

Fig. 4B, on a five millimole scale was achieved with 5'-O-DMTr-3'-O-TBDMS-Adenosine (N-Bz)-2'-CEP and the H-phosphonate derived from 5'-O-DMTr-2'-O-TBDMS-Adenosine (N-Bz)-3'-CEP. The phosphorus III intermediates generated upon formation of the linear dimer (phosphite triester stage) and cyclic dincucleotide (H-phosphonate diester stage) were sulfurized by treatment with 3-((N,N-dimethylaminomethylidene)amino)-3H-1,2,4-dithiazole-5-thione (DDTT) and 3-H-1,2-benzodithiol-3-one, respectively. The crude reaction mixture obtained after the second sulfurization was chromatographed on silica gel to generate a mixture of the RR- and RS-diastereomers of fully protected ML S2 CDA. Benzoyl and cyanoethyl deprotection using methanol and concentrated aqueous ammonia generated bis-TBS-ML-S2 CDA as a mixture of RR- and RS-diastereomers which were separated by C-18 prep HPLC. The purified 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 in >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 pH7/1 mM EDTA buffer to ~5 mg/mL, and filter sterilized through a 0.2 micron filter, resulting in a final product that was ≥95% purity as determined by analytical HPLC (**Figure S4A**). 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 both ^1H NMR (data not shown) and the ^{31}P NMR (y-axis of **Figure A4A**) 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 **Figure S4**) in combination with a ^1H-^{31}P HMBC

(heteronuclear multiple-bond correlation spectroscopy) experiment. Prior to use in experiments, all synthetic CDN preparations were verified by LAL assay to be endotoxin free (<1 EU/mg).

ML RR-S2 CDA Crystal Structure and Electrostatic Potential Surface. The X ray structure was determined at UC Berkeley College of Chemistry X-ray Crystallography Facility (Antonio DiPasquale, PhD). X-ray quality crystals were grown from a saturated wet ethanol solution followed by the slow vapor diffusion of acetone, which was then followed by the slow vapor diffusion of hexane to deposit the crystalline material. A colorless plate 0.050 x 0.040 x 0.010 mm in size was mounted on a Cryoloop with Paratone oil. Data were collected in a nitrogen gas stream at 100(2) K using phi and omega scans. Crystal-to-detector distance was 60 mm and exposure time was 10 seconds per frame using a scan width of 1.0°. Data collection was 100.0% complete to 67.000° in θ. A total of 113285 reflections were collected covering the indices, $-19<=h<=19$, $-24<=k<=24$, $-26<=l<=29$. 14929 reflections were found to be symmetry independent, with an R_{int} of 0.0445. Indexing and unit cell refinement indicated a primitive, orthorhombic lattice. The space group was found to be P 21 21 21 (No. 19). The data were integrated using the Bruker SAINT software program and scaled using the SADABS software program. Solution by iterative methods (SHELXT) produced a complete heavy-atom phasing model consistent with the proposed structure. All non-hydrogen atoms were refined anisotropically by full-matrix least-squares (SHELXL-2014). All hydrogen atoms were placed using a riding model. Their positions were constrained relative to their parent atom using the appropriate HFIX command in SHELXL-2014. Absolute stereochemistry was unambiguously determined to be *R* at all chiral centers.

Gaussian 09 (Revision A.02) was used to optimize the structure of the dianion monomer using the B3LYP/6-31G(d) level of theory starting from the coordinates determined from the X-ray diffraction experiment. Once a stationary point in the optimization was found, an electrostatic potential surface was calculated for the optimized structure.

Table 1

Gene	Forward	Reverse	Probe
Cytokines			
IFN-β	GGAAAGATTGACGTGGGAGA	CCTTTGCACCCTCCAGTAAT	CTGCTCTC
TNF-α	CTGTAGCCCACGTCGTAGC	GGTTGTCTTTGAGATCCATGC	CCAGGAGG
IL-6	GCTACCAAACTGGATATAATCAGGA	CCAGGTAGCTATGGTACTCCAGAA	TTCCTCTG
IL-12p40	CCTGCATCTAGAGGCTGTCC	CAAACCAGGAGATGGTTAGCTT	GACTCCAG
Pan IFN-α	CCTGAGAGAGAAGAAACACAGCC	TCTGCTCTGACCACCTCCCAG	
STING alleles			
hSTING exon 3	GCTGAGACAGGAGCTTTGG	AGCCAGAGAGGTTCAAGGA	
hSTING exon 6	GGCCAATGACCTGGGTCTCA	CACCCAGAATAGCATCCAGC	
hSTING exon 7	TCAGAGTTGGGTATCAGAGGC	ATCTGGTGTGCTGGGAAGAGG	

Table 1. List of primers used in real time PCR and for sequencing STING alleles.