SUPPLEMENTARY INFORMATION

for

Endosomolytic Polymersomes Increase the Activity of Cyclic Dinucleotide STING Agonists to Enhance Cancer Immunotherapy

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Supporting Data:

Table S1 | Characterization of diblock copolymer properties, self-assembly behavior, and cGAMP encapsulation efficiency.

| Polymer            | DEAEMA (%)<sup>a</sup> | BMA (%)<sup>a</sup> | PDSMA (%)<sup>a</sup> | PDI<sup>b</sup> | EE<sup>c</sup> | W<sub>PEG</sub> (%) | Morphology<sup>d</sup> |
|--------------------|--------------------------|---------------------|-----------------------|-----------------|---------------|---------------------|------------------------|
| PEG<sub>2kDa</sub>-b-DB<sub>5kDa</sub> | 36.3                     | 36.3                | 0                     | 1.23            | 44.8 ± 8      | 28.6                | V                      |
| PEG<sub>2kDa</sub>-b-DB<sub>7.8kDa</sub> | 61.7                     | 38.3                | 0                     | 1.09            | 0             | 20.4                | P                      |
| PEG<sub>2kDa</sub>-b-DBP<sub>4.5kDa</sub> | 57                       | 35.2                | 7.8                   | 1.01            | 38 ± 3        | 30.7                | V                      |
| PEG<sub>2kDa</sub>-b-DB<sub>10.8kDa</sub> | 67.4                     | 32.6                | 0                     | 1.08            | N/A           | 15.6                | P                      |
| PEG<sub>2kDa</sub>-b-DB<sub>20kDa</sub> | 62.1                     | 37.9                | 0                     | 1.06            | N/A           | 9.1                 | P                      |
| PEG<sub>2kDa</sub>-b-DB<sub>36kDa</sub> | 61.6                     | 38.4                | 0                     | NS              | N/A           | 5.3                 | P                      |
| PEG<sub>5kDa</sub>-b-DB<sub>9.2kDa</sub> | 65                       | 35                  | 0                     | 1.14            | 16 ± 11       | 35.2                | F                      |
| PEG<sub>5kDa</sub>-b-DB<sub>14.3kDa</sub> | 59.5                     | 40.5                | 0                     | 1.23            | 18 ± 12       | 25.9                | F+S                    |
| PEG<sub>10kDa</sub>-b-DB<sub>21.2kDa</sub> | 58.9                     | 41.1                | 0                     | 1.14            | 16 ± 11       | 32.1                | S                      |
| PEG<sub>10kDa</sub>-b-DB<sub>30.2kDa</sub> | 57.3                     | 42.7                | 0                     | 1.23            | 12 ± 5        | 24.8                | F+S                    |

<sup>a</sup> Molar percent determined NMR
<sup>b</sup> Determined by GPC, NS: Not soluble in DMF mobile phase
<sup>c</sup> Encapsulation efficiency and morphology from direct hydration formulation. Determined via HPLC. Data are presented as mean ± SD. n=3 independent samples.
<sup>d</sup> Morphology determined by TEM. V = Vesicles, F = Fibrillar micelles, S = Spherical micelles, P = Macroscopic Precipitation
Figure S1 | Characterization of *in situ* polymersome crosslinking. a) UV-vis spectroscopic detection of the release of 2-pyridinethione (343 nm) following addition of different equivalents of DTT to PEG-b-DBP polymersomes. b-d) Dynamic light scattering histograms of crosslinked (CL) and non-crosslinked (NC) polymersomes. All experiments were repeated once with similar results.
Figure S2 | Morphological characterization of self-assembled colloids from PEG-b-DB diblock copolymers by transmission electron microscopy. Representative TEM images of colloidal suspensions assembled via modified direction hydration of PEG-b-DB copolymers of indicated block molecular weight and weight fraction of PEG (w_{PEG}). All experiments were repeated once with similar results.
Figure S3 | STING-NPs enhance cGAMP delivery to dendritic cells. a) ELISA quantification of IFN-α secretion by DC2.4 cells after 24h treatment with indicated formulation (n=4 biologically independent samples). PEG-DB: cGAMP delivered with polymersomes assembled using non-crosslinkable PEG2kDa-DB5kDa chains; Mix: physical mixture of empty crosslinked PEG2kDa-b-DBP4.5kDa polymersomes and free cGAMP (n=4 biologically independent samples). b-f) Flow cytometric quantification of DC maturation surface marker expression in bone marrow derived dendritic cells 24h following treatment with STING-NP, free cGAMP, or PBS. n=3 biologically independent samples. One-way ANOVA with Tukey test. All data are presented as mean ± SD.

Figure S4 | Comparison of activity between STING-NP and non-vesicular PEG-b-DB/cGAMP formulations in THP-1 ISG human reporter monocytes. a) Formulations of higher molecular weight PEG-b-DB polymers without vesicular encapsulation of 2’3’-cGAMP do not result in significant activation of IRF signaling in THP-1 ISG reporter cells. n=4 biologically independent samples. b) A negligible IFN-I response above baseline was detected in THP-1 ISG reporter cell lines 24h following treatment with empty polymersomes. A 150 nM dose of cGAMP with STING-NPs corresponds to 6.6 µg/mL of polymer. n=4 biologically independent samples. All data are presented as mean ± SD.
Figure S5 | Ifnb1 and Cxcl9/Cxcl10 expression are correlated in STING-NP treated tumors. Normalized Cxcl9 or Cxcl10 transcript levels plotted against normalized Ifnb1 transcript levels in each tumor and fitted with a linear regression calculated by the method of least squares. p-value describes if the slope of a fitted line is significantly different from zero (two-tailed F test). For STING-NP and cGAMP, n= 11, 10 biologically independent samples.
Figure S6 | Evaluation of IT administered STING-NP and cGAMP toxicity. 
a) Longitudinal measurement of mouse body weight following IT administration of three doses of indicated formulation including 10 µg cGAMP per dose. Arrows denote treatment dates. For PBS, NP, cGAMP, Mix, and STING-NP, n=6, 6, 6, 5, and 5 biologically independent samples. Data are presented as mean ± SEM. b-c) Blood levels of alanine aminotransferase and total bilirubin. Blood was harvested when mice reached tumor size endpoints. Creatinine levels of all tested mice were below 0.5 mg/dL. For PBS, cGAMP and STING-NP, n=5, 6, and 6 biologically independent samples. d) Representative images H&E stained liver sections harvested at sacrificial endpoints. The most commonly observed pathology was vacuolar degeneration, a sign of reversible cell injury. The severity of vacuolation was scored from 0-3 in a masked fashion by a board-certified veterinary pathologist, guided by INHAND criteria for evaluation of the mouse hepatobiliary system and Haschek and Rousseaux’s Handbook of Toxicologic
Pathology, 3rd ed. This experiment was performed once and is representative of 3 biologically independent samples. e) Representative images demonstrating the 0-3 scaling system used for evaluation of vacuolation. f) Scoring of vacuolation severity for mice treated with STING-NP, cGAMP, or PBS (n=3 biologically independent samples). All data, unless otherwise stated, are presented as mean ± SD.

Figure S7 | Nanostring analysis of upregulated genes following IT STING-NP or cGAMP treatment. cGAMP treatment triggers upregulation of several of the same genes as does STING-NP treatment, but mostly with a lower magnitude fold change and higher p value. Fold change is calculated with respect to PBS treated control tumors. Genes with a greater than 10 fold change are denoted. n=4 biologically independent samples, one-way ANOVA with Tukey test.
Figure S8 | STING-NP treatment does not effect infiltration of IL-4 and IL-10 secreting T cells. Subcutaneous B16.F10 tumors were injected intratumorally STING-NPs or cGAMP at doses equivalent to 10 µg of cGAMP or PBS. Two days following treatment, tumors were harvested and prepared into single cell suspensions. Samples were stimulated with PMA/ionomycin and brefeldin A for 4h followed by ICCS for IL-10 and IL-4 and analysis by flow cytometry (For STING-NP, cGAMP, and PBS, n= 10, 10, and 9). Data are presented as mean ± SD.
Figure S9 | Evaluation of IV administered STING-NP and cGAMP toxicity. a) Longitudinal measurement of mouse body weight following IV administration of three doses of indicated formulation including 20 µg cGAMP per dose (n=10 biologically independent samples). Arrows denote treatment times. Data are presented as mean ± SEM. b-c) Blood levels of alanine aminotransferase and total bilirubin of mice. Blood was harvested when mice reached tumor size endpoints. Creatinine levels of all tested mice were below 0.5 mg/dL. For STING-NP + ICB, STING-NP, mix+ICB, cGAMP+ICB, cGAMP, ICB, and PBS, n=5, 8, 8, 8, 8, 8, and 8, respectively. d) The severity of liver vacuolation was scored from 0-3 in a masked fashion by a
board-certified veterinary pathologist as described in Figure S6. For STING-NP + ICB, STING-NP, mix+ICB, cGAMP+ICB, cGAMP, ICB, and PBS, n=5, 8, 8, 8, 8, and 8, respectively. e) Randomly selected H&E staining of liver sections harvested from mice at sacrificial endpoints. This experiment was performed once. All data are presented as mean ± SD unless otherwise noted.
Figure S10 | Characterization of pH-responsive diblock polymers. a) Representative $^1$H NMR spectrum of poly[(ethylene glycol)-b-((2-(diethylamino)ethyl methacrylate)-c-(butyl methacrylate))] polymer (PEG-b-DB). b) Representative spectrum of poly[(ethylene glycol)-b-((2-(diethylamino)ethyl methacrylate)-c-(butyl methacrylate)-c-(pyridyl disulfide ethyl methacrylate))] polymer. c) Stacked plot of PEG$_{2k}$-b-DB$_{x}$ $^1$H NMR spectra. d) GPC chromatograms of PEG$_{2k}$-DB$_{x}$ polymers and PEG$_{2k}$-DBP$_{4.5k}$. These experiments were performed once.
Scheme S1: Synthesis of 2’3’-cGAMP

1. DMOCP/Pyr
2. I₂, H₂O

1. Pyr.TFA/ACN,H₂O
2. t-BuNH₂
3. DCA/H₂O

1. ACN
2. I-BuOOH
3. DCA/H₂O

1. CH₃NH₂
2. TEAxFHF

13
Figure S11 | $^1$H-NMR spectrum of synthesized 2’3-cGAMP. $^1$H-NMR (DMSO-d$_6$) δ 10.64 (broad s, H-8), 8.45 (s, 1H, H-9), 8.16 (s, 1H, H-10), 7.93 (s, 1H, H-6), 7.47 (broad s, 2H, H-11), 6.59 (broad s, 1H, H-7), 5.92 (d, 1H, $J$ = 8 Hz, H-1’), 5.90 (d, 1H, $J$ = 8 Hz, H-1’’), 5.17 (m, 1H, H-2’), 5.01 (m, 1H, H-2’’), 4.92 (m, 1H, 2’’-OH), 4.32 (m, 1H, H3’), 4.24 (m, 1H, H-3’’), 4.16 (m, 1H, H-3’-OH), 4.14 (m, 2H, H4’ and H4), 4.05 (m, 1H, 3’-OH), 3.86 (m, 2H, H5’ and H5’’).
Figure S12 | LCMS characterization of 2’3’-cGAMP. Mass calculated for C$_{20}$H$_{24}$N$_{10}$O$_{13}$P$_2$, 674.4; m/z found, 675.0 [M + H]$^+$, Rf = 0.08.
Figure S13 | Comparison of biological activity of synthesized and commercially available 2’3’-cGAMP. A) Dose response curve of STING-NPs formulated with 2’3-cGAMP produced in house or purchased from Invivogen in THP-1 ISG reporter cell lines. n=4 biologically independent samples. b) Activity dose curve of free 2’3’-cGAMP in THP-1 ISG reporter cell lines. n=4 biologically independent samples. All data are presented as mean ± SD.
Figure S14 | Gating scheme for flow cytometric analysis of immune cell populations in the TME. Cells were stained separately with distinct panels of antibody conjugated fluorophores for analysis.