Induction of necrotic cell death and activation of STING in the tumor microenvironment via cationic silica nanoparticles lead to enhanced antitumor immunity

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Electronic supplementary information (ESI)
EXPERIMENTAL SECTION

Materials and methods. All chemicals including SiNPs were purchased from Sigma-Aldrich unless noted otherwise. Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) and c-di-GMP control were purchased from InvivoGen (San Diego, CA) and dissolved in ddH2O. DY547-c-di-GMP was purchased from BIOLOG Life Science Institute. Murine MHC class I tetramers were obtained from MBL International Corporation (Woburn, MA). All other antibodies were purchased from eBioscience (San Diego, CA) or BD Bioscience (San Jose, CA).

Animals and cells. Animals were housed in the United States Department of Agriculture (USDA)-inspected Wayne State University animal facility under federal, state, local and NIH guidelines for animal care. Female C57BL/6 mice (6-8 weeks) were obtained from the Jackson Laboratory. B16-F10 cells were purchased from ATCC. Cells were cultured in complete medium (MEM, 10% fetal bovine serum (Greiner Bio-one), 100 U/mL penicillin G sodium and 100 μg/mL streptomycin (Pen/Strep).

Preparation of c-di-GMP loaded CSiNPs. Positively charged SiNPs were purchased from Sigma-Aldrich that were initially functionalized by triethoxysilylaminosilane. The size of purchased CSiNPs was about 30 nm and was confirmed by dynamic light scattering (DLS) measurements (Malvern Zetasizer). The CSiNPs solution was diluted 10 times using saline to decrease the nanoparticle density (0.116 g/mL) and mixed with the desired amount of c-di-GMP to prepare c-di-GMP-loaded CSiNPs. To facilitate the complexation process, probe-sonication was performed for 1 min with 2/2s on/off working cycle at a power output of 4 joules. c-di-GMP loaded CSiNPs were purified by repeated centrifugation and resuspension in PBS.
Size and zeta potential measurements. To measure the size and zeta potential of nanoparticles, dynamic light scattering (DLS, Zetasizer, Malvern) was used with He-Ne laser (633 nm) at 90° collecting optics at 25 °C.

Cell viability assay. Cellular viability was measured by the AlamarBlue assay (Thermo Fisher). Briefly, 5 x 10^4 B16-F10 cells were plated onto 96 multiwell plates (Costa, Corning, NY). After incubation with the indicated dose of nanoparticles for 24 h at 37 °C, cells were incubated with the AlarmaBlue agent for 4 h at 37 °C and the absorbance was measured at 570 nm, using 600 nm as a reference wavelength. The mean absorbance of non-exposed cells was the reference value for calculating 100 % cellular viability.

In vitro cellular uptake. To determine the intracellular uptake capacity of nanoparticles, the B16-F10 cells were seeded on glass coverslips in 6-well microscopy chamber at a density of 2 x 10^4 cells per well for 8 h at 37 °C. After 8 h, 2 µl of Mitochondria-Red Fluorescent Protein (RFP) (CellLight™ BacMam 2.0) per 10,000 cells was added into media to label mitochondria with RFP and cells were incubated for another 16 h at 37 °C. After total 24 h incubation, cells were treated with 10 µg/ml of fluorescence-labeled silica nanoparticles for 2 h or 24 h, and then washed with saline. For fixation, the glass that cells adhered to was immersed in 4 % paraformaldehyde in saline for 10 min at room temperature. Following fixation, the glass was washed with saline and mounted on a slide with nuclei staining by DAPI. Fluorescence images were obtained using a confocal microscope (Zeiss LSM-510) with a filter set of DAPI, FITC, and Mitochondria-RFP excitation/emission.
**Cellular staining with Fluorescent Probes.** The intracellular ROS production was measured by cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA, Thermo Fisher) staining following manufacturer’s instructions in B16-F10 after 2 h or 24 h exposure to nanoparticles at 37 °C.

**Sectioning and Immunohistochemistry.** Eight days after injection with 5 × 10$^5$ B16-F10 cells subcutaneously, mice were injected with formulations containing 5 μg of c-di-GMP in soluble or nanoparticle form directly into the tumor. Tumors were harvested 24 h later, placed in optimum cutting temperature (OCT) formulation (Sakura Finetek), and frozen in liquid nitrogen. Three-micrometer sections of the selected formalin-fixed, optimal cutting temperature (OCT)-embedded specimens were prepared and Gr-1 staining was performed using an anti-mouse Gr-1 Ab (BD Biosciences, 1:50). Stained histological sections were then imaged using an EVOS AMF4300 microscope.

**Sectioning and Immunofluorescence staining.** Eight days after injection with 5 × 10$^5$ B16-F10 cells subcutaneously into C57BL/6 mice, mice received a single intratumoral dose with formulations containing 5 μg c-di-GMP in soluble or nanoparticle form (5 μg c-di-GMP and 1.2 mg CSiNPs). Tumors were harvested 10 d later, placed in optimum cutting temperature (OCT) formulation (Sakura Finetek), and frozen in liquid nitrogen. For CD8$^+$ T cell infiltration assessment, samples were cryosectioned and stained with CD8-APC antibody (eBioscience). Slides were imaged using a Zeiss LSM 510 confocal microscope.

**Detection of necrosis in vivo by PI and Annexin V.** 5.0×10$^5$ B16F10 tumor cells were
inoculated in the flank of C57BL/6 mice (n=3/group) on day 0. When the size of tumor is 50 mm² (on day 8), the direct intratumoral injection of c-di-GMP (5 µg) or CSiNPs (1.2 mg) was given. After 24 h, mice were sacrificed and tumor tissues were isolated. Collected tumor tissues were then digested with 1.5 mL of freshly prepared enzyme mix composed of RPMI-1640 containing 0.8 mg/mL Collagenase/Dispase (Roche Diagnostics) and 0.1 mg/mL DNase (Roche Diagnostics) and cells were stained with PI and Annexin-V dye following manufacturer’s instructions. Percentages of PI and Annexin-V positive cells were determined by flow cytometry.

**Detection of necrosis by confocal microscopy.** B16-F10 cells were seeded on glass coverslips in 6-well microscopy chamber at a density of 2 x 10⁴ cells per well for 8 h at 37 °C. After 8 h, cells were treated with 10 µg/ml of fluorescence-labeled CSiNPs for 2 h or 24 h, and then washed with saline without a fixation step. The desired amount of PI dye dissolved in saline of 1 mL was added to each well for 10 min at 37 °C and then washed with saline. Following PI staining, the glass was washed with saline and mounted on a slide with nuclei staining by DAPI. Fluorescence images were obtained using a confocal microscope (Zeiss LSM-510) with a filter set of DAPI, FITC, and PI excitation/emission.

**Tumor model.** B16-F10 or B16.OVA cells (5.0 × 10⁵ cells) were subcutaneously inoculated into the right flank of 5-6-week-old C57BL/6 mice. When the tumor mass became palpable (7-8 mm, typically 8 days later), mice were divided into several treatment groups (n=8) and the tumor-bearing mice were intratumorally injected with 5 µg c-di-GMP in soluble or in nanoparticle form (5 µg c-di-GMP and 1.2 mg CSiNPs). For B16.OVA tumor study, c-di-GMP and OVA in soluble or in nanoparticle form (5 µg c-di-GMP, 5 µg OVA, and 1.2 mg CSiNPs)
were intratumorally injected into B16.OVA tumor-bearing C57BL/6 mice. Survival and tumor size were measured every day using a sliding caliper. Ten days after the intratumoral injection of vaccine formulations to B16.OVA bearing mice (day 18), mice were bled and peripheral blood mononuclear cells were evaluated by SIINFEKL/H-2Kb peptide-MHC tetramer staining and intracellular cytokines (IFN-gamma and TNF-alpha) staining.

**Statistical analysis.** Comparisons of mean values of two groups were performed using unpaired Student’s t-tests. To analyze the statistical difference between groups, a one-way analysis of variance (ANOVA) with Bonferroni post-test was used. All of the values were expressed as means ± standard deviations. GraphPad Prism software was used for all the statistical analyses. ***P<0.001, **P<0.01, *P<0.05. NS, not significant.

![Figure S1. Kinetics of CpG release from CS incubated in saline at 37 °C.](image)

Dye labeled c-di-GMP was complexed with CSiNPs. The nanoparticles were agitated in PBS in the dark at 37 °C. At varying time points, a small solution was sampled and centrifuged, the release of c-di-GMP was quantified by fluorescence measurement after centrifuge.
Figure S2. *In vitro* cell necrosis induced by cationic silica nanoparticles (CSiNPs). The detection of the necrotic cells was performed by flow cytometry with PI staining. Percentages of necrotic cells in PI-positive region are shown after 24 h exposure of B16-F10 cells with CSiNPs or negatively charged SiNPs (NSiNPs) at 37 °C.
Figure S3. *In vitro* preferential distribution of cationic silica nanoparticle (CSiNPs) to the mitochondria. B16F10 cells were incubated with CSiNPs at 37 °C for 2 h or 24 h. Cells were stained by nucleus staining with DAPI (4′,6-diamidino-2-phenylindole) followed by the mitochondria staining with a transfection agent of mitochondria-RFP at 37 °C for 16 h. The green color shows FITC-labelled CSiNPs. The red color indicates the mitochondria. The overlap between the fluorescence of mitochondria (red) and CSiNPs (green) appears as yellow and shows distribution of CSiNPs in the mitochondria of cells or CSiNPs selectively taken up
by the mitochondrial membrane of cells.

**Figure S4.** c-di-GMP/CSiNPs formulation increases tumor-infiltrating CD8+ T lymphocytes. a), B16-OVA tumors treated with either soluble c-di-GMP or c-di-GMP/CSiNPs were stained with aCD8-allophycocyanin (APC) and counterstained with DAPI (4’,6-diamidino-2-phenylindole). b), 3-D reconstruction of laser scanning confocal microscopy image of section sample from mice treated with c-di-GMP/CSiNPs. 10 confocal microscope images were achieved in the Z-stack mode by ZEISS LSM-510. Successive focal planes at the field depth of 24 µm were obtained along the z-axis at an interval of 2.67 µm depth.