Supporting Information

A Chromatin-Mimetic Nanomedicine for Therapeutic Tolerance Induction

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MATERIALS

All chemicals and proteins including keyhole limpet hemocyanin (KLH), ovalbumin(OVA), and uricase from Candida sp. were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted and were used as received. TLR9 antagonist, GpG (5'-T*G*A*C*T*G*T*G*A*A*G*G*T*T*A*G*A*G*A*T*G*A-3') with or without amino modification at 3'-end were synthesized by IDT with a phosphorothioate backbone. Methoxy polyethylene glycol succinimidyl carbonate, molecular weight (NHS-mPEG, 10 kDa MW) was purchased from Nanocs Inc. Carboxy betaine ester monomer terminated with tbutyl (tbCB) was synthesized following our previously published method[56]. LysoTracker Red DND-99 was purchased from Thermo Fisher Scientific. Amicon Ultra centrifugal filter was purchased from EMD Millipore (Billerica, MA). Amplex Red uric acid/uricase assay kit was purchased from Thermo Fisher Scientific (Waltham, MA). Anti-CD4-PE, anti-CD25-FITC and anti-Foxp3-Percep antibodies were purchased from Biolegend (San Diego, CA). Anti-mouse IgG secondary antibodies were purchased from Bethyl labs. Mouse cytokine (INF-γ, IL-5, IL-4, IL-13) quantikine ELISA kits were purchased from R&D systems. Mice monocytes RAW 264.7 and B cells LB 27.4 were purchased from ATCC.

METHODS

Cell uptake study. For the cell uptake study, GpG with amino-modification at 3' end was labeled with FITC. Briefly, the GpG dissolved in PBS solution (1mg/ml, pH 7.4) was reacted with FITC solution (50ul, 1mg/ml in DMSO) for 2h at room temperature. The FITC-labeled GpG was dialyzed using dialysis tubing (MWCO 1kDa) against PBS solution to remove free FITC dyes. Then DCN containing FITC-labeled GpG and Rhodamine-labeled KLH was prepared following the steps described above. RAW 264.7 and LB 27.4 were cultured in DMEM medium with 10% fetal bovine serum at 37 °C in humidified atmosphere containing 5% CO_2. The cell sub-culture was carried out once cells reached 70~80% confluence. RAW 264.7 (2×10^5 cells) and LB 27.4 (2×10^5 cells) were seeded into a 6-well plate containing coverslips in the wells and cultured for attachment, respectively. DCN or the mixture of free GpG and free KLH were added into wells at a final concentration of 50nM GpG/KLH for 2 h incubation at 37 °C. The cells were then washed for three time after removing the medium, and stained by Hoechst 33342 (Invitrogen) according to the manufacture's protocol. After 30min nuclear staining, cells were fixed with 4% formaldehyde in PBS for 20 min at room temperature, and then washed by PBS for three times. At last, the coverslips were covered onto glass slides with 20 μL aqueous mounting medium. Then the slides were observed using Confocal Laser Scanning Microscopes-FV1000 (CLSM, Olympus, Tokyo, Japan).

Lysotracker colocalization assay. 2 x10^5 HeLa cells were seeded in a 6 well plate and cultured for 24 h. DCN containing FITC-labeled GpG and unlabeled KLH was added into wells at a final concentration of 50nM GpG/KLH for incubation at 37 °C. After 4 h, the medium containing DCN was removed and washed three times with fresh PBS. LysoTracker Red DND-99 (Thermo Fisher Scientific) diluted in serum free medium at concentration of 500 nM was added to the cells, followed by incubation for 1 h at 37°C. Cells were washed three times after removing the medium and stained by Hoechst 33342 (Invitrogen) according to the

manufacture's protocol. After 30min nuclear staining, cells were fixed with 4% formaldehyde in PBS for 20 min at room temperature, and then washed by PBS for three times. At last, the coverslips were covered onto glass slides with 20 μL aqueous mounting medium. Then the slides were observed using Confocal Laser Scanning Microscopes-FV1000 (CLSM, Olympus, Tokyo, Japan).

Cell viability study. The cytotoxicity of DCN was evaluated by MTT assays in RAW264.7 and LB27.4 cells. Firstly, cells were seeded into a 96-well plate at a density of 1×10^4/well. Blanks were prepared by adding culture medium alone. Cells were cultured overnight for attachment. Subsequently, culture medium in each well was replaced with fresh medium containing DCN (GpG/KLH) at series of concentrations. Cells in wells without addition of DCN (GpG/KLH) were used as control group. Six replicates were included in each group. After another 48-h culture, 20 μL of sterile MTT solution (5 mg/mL) was added into each well to incubate at 37 °C for 4 h. After the removal of the unreacted MTT dye by aspiration, 150 μL of DMSO was added into each well to dissolve the produced formazan crystals, and the plate was gently shaken for 15 min. Finally, the optical density (OD) was measured at 420 nm using a plate reader (BioTek, USA).

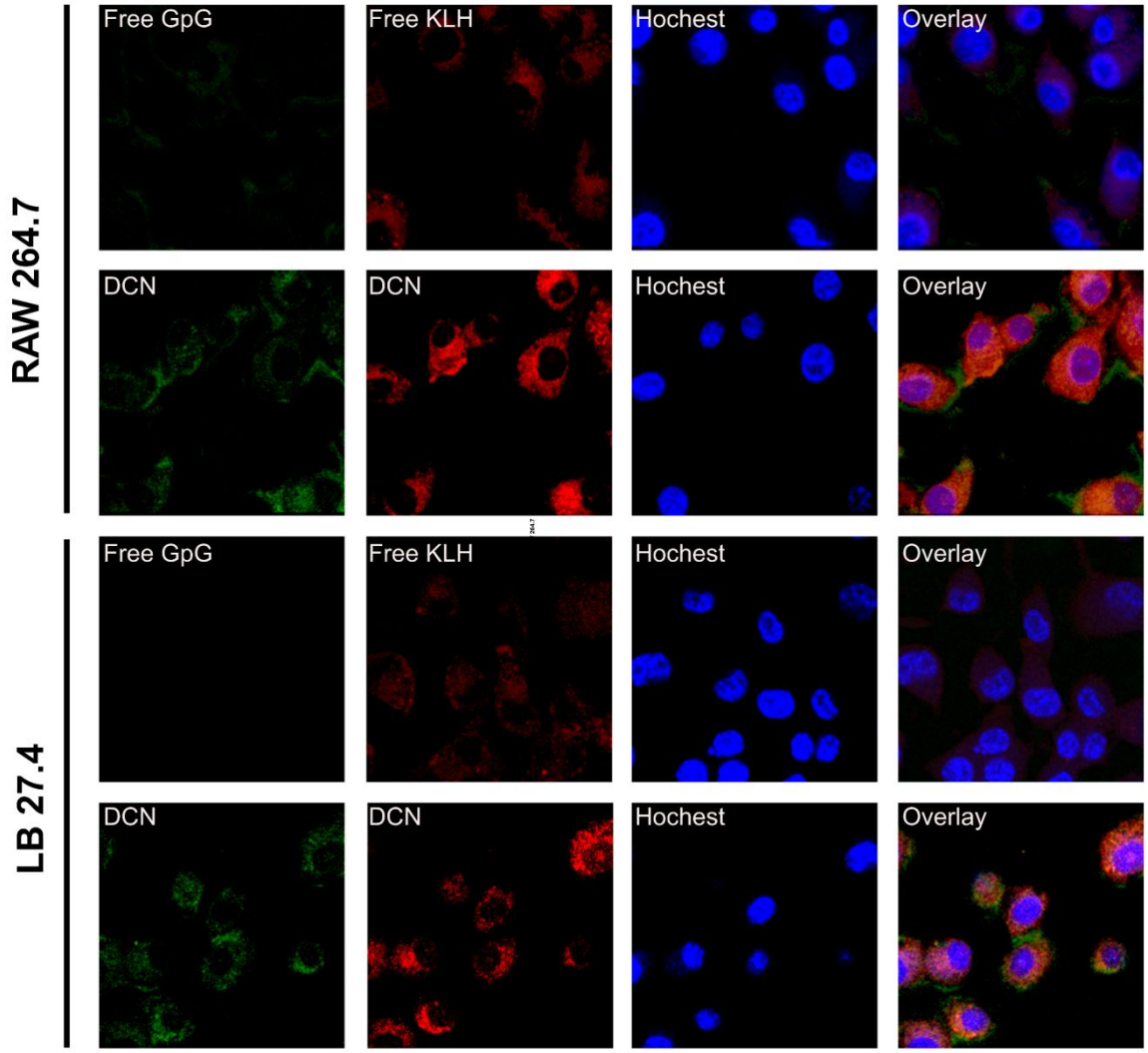

Figure S1. Cell uptake study. Confocal images of intracellular distribution of free GPG/free KLH and DCN(GpG/KLH) in RAW264.7 and LB27.4 cells after 2-h incubation at 37 °C. GpG was labeled with FITC and KLH was labeled with Rhodamine. Cell nuclei were stained with Hoechst 33342.

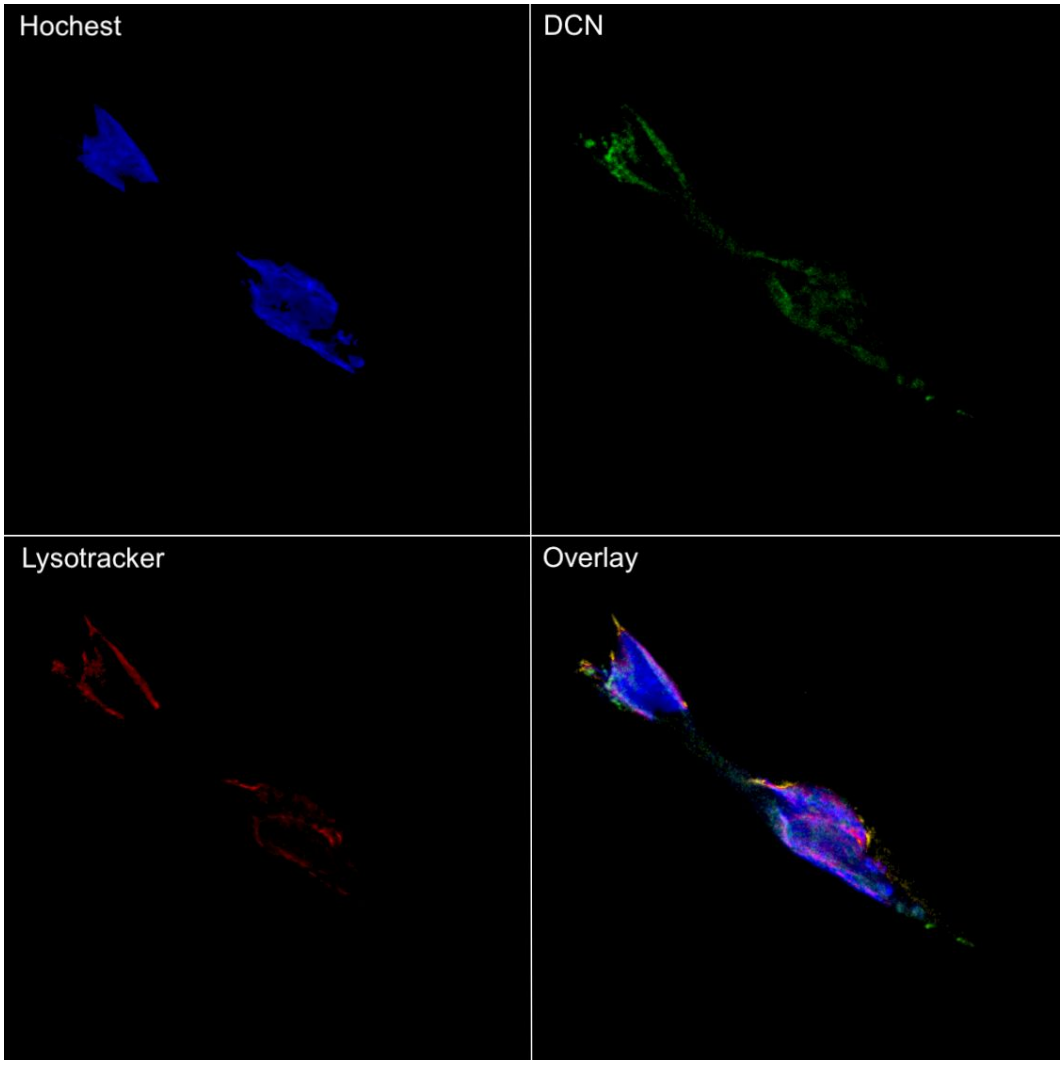

Figure S2. Co-localization of DCN and lysotracker in DC2.4 mouse dendritic cells. Confocal images of intracellular distribution of DCN(GpG/KLH) in DC2.4 mouse dendritic cells after 4-h incubation at 37 °C. DCN(GpG/KLH) was labeled with FITC and the location of endosomes was labeled with LysoTracker Red DND-99. Cell nuclei were stained with Hoechst 33342.

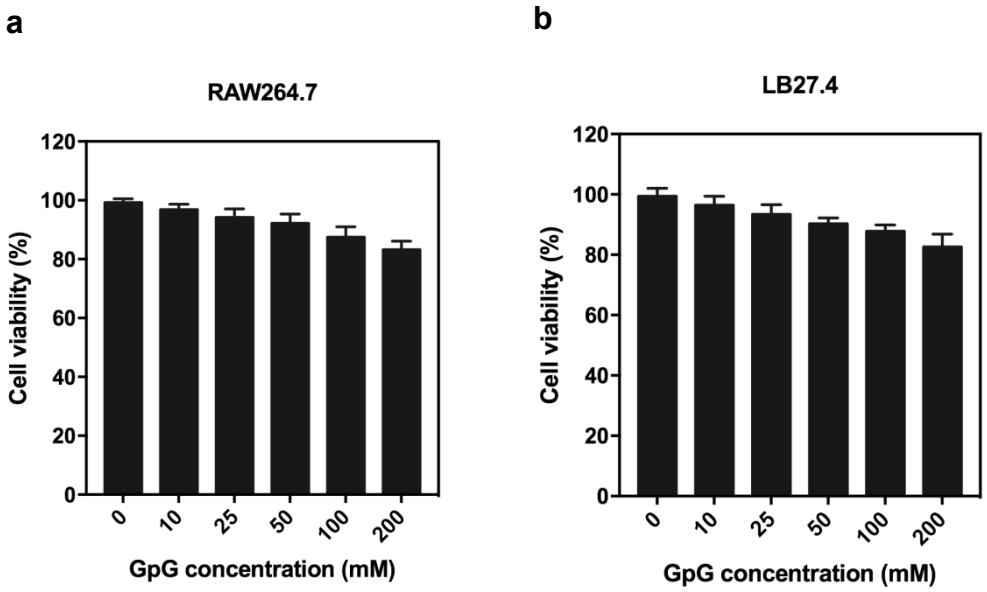

Figure S3. Cell viability study. MTT assay of DCN(GpG/KLH) synthesized at a M:G molar ratio of 3200:1 and a G:P weight ratio of 8:1 in RAW264.7 monocytes (a) and LB 27.4 B cells (b). Results are plotted as mean± s.d. (n=6).

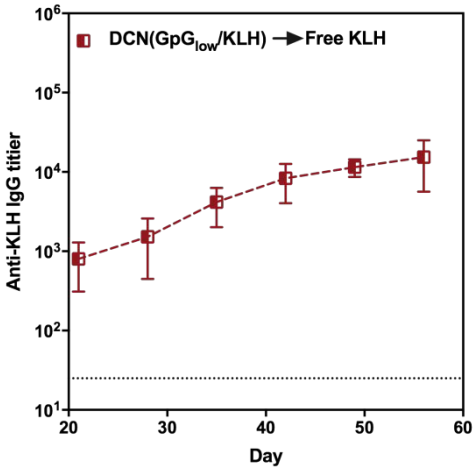

Figure S4. KLH tolerance study with DCN (GpG_{low}/KLH). Mice were IV. injected weekly with: DCN formulations containing a low dose of GpG (G:P weight ratio=0.5:1) for the first three weeks, followed by five weekly challenges of free KLH starting from day 21. The mice sera were collected on 21st, 28th, 35th, 42nd, 49th, and 56th days, and detected for IgG titer *via* ELISA test.

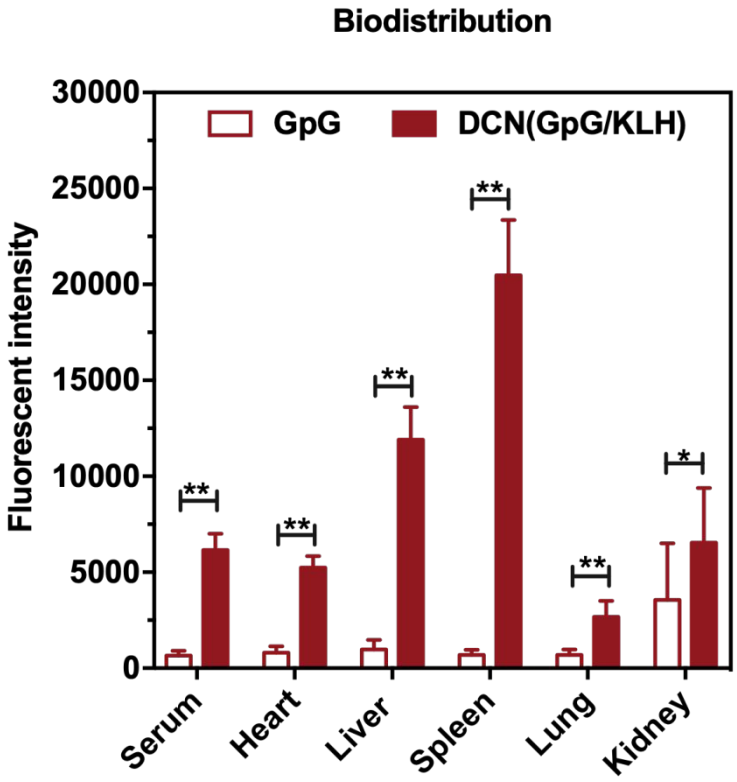

Figure S5. Biodistribution study. Biodistribution of free GpG and DCN(GpG/KLH) in main organs (serum, heart, liver, spleen, lung, and kidney) at 24h after the IV injection. Results are plotted as mean ± s.d. (n = 3). Statistical analysis was performed using student test. *P < 0.05; **P < 0.01.

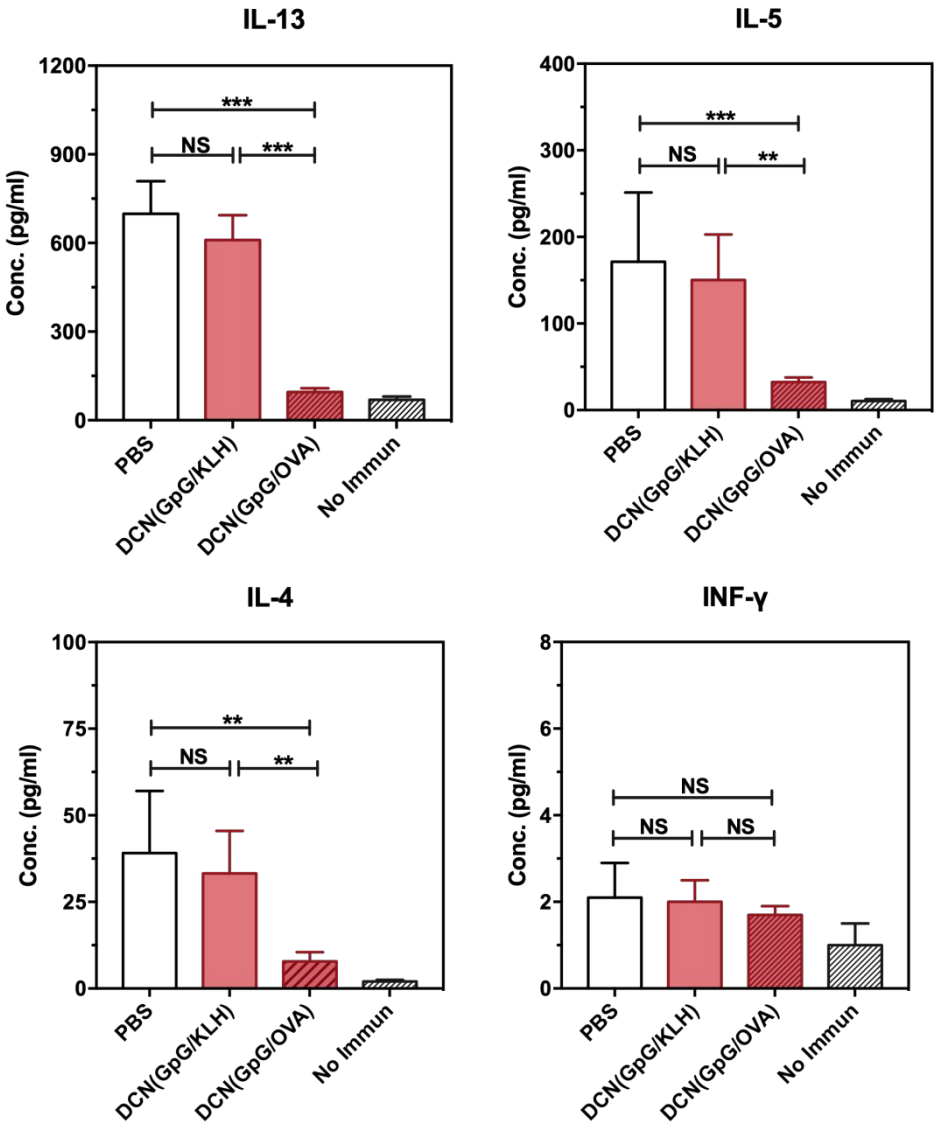

Figure S6. Cytokines secreted by splenocytes under OVA restimulation. Splenocytes were extracted from mice that were pretreated and then received immunizations. Levels of IL-13, IL-5, IL-4, and INF-γ were measured in supernatants of splenocytes restimulated ex vitro with OVA for 4 days. Results are plotted as mean ± s.d. (n = 5). All statistical analyses were performed using student test (*P < 0.05, **P<0.01, ***P<0.001).