Supporting Information for the manuscript:

Conjugation Chemistry-Dependent T-Cell Activation with Spherical Nucleic Acids

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2-(2-Pyridyldithio)ethanol. 2-mercaptoethanol (2.24 g, 28.7 mmol) was added to a solution of 2,2'-dipyridyldisulfide (9.49 g, 43.1 mmol) in methanol (30 mL). The mixture was stirred at room temperature for 12 hours, then the reaction solvent was evaporated under reduced pressure and reconstituted in dichloromethane. The solution was washed with 10% sodium hydroxide in water and saturated sodium chloride solution. The product was purified on a silica gel column with diethyl ether/hexanes solvent system and isolated as a yellow oil (4.13 g, 77%). ^{1}H NMR (400 MHz, $CDCl_3$, δ): 8.51 (ddd, *J* = 5.0, 1.9, 1.0 Hz, 1H), 7.58 (ddd, *J* = 8.1, 7.4, 1.8 Hz, 1H), 7.40 (m, 1H), 7.15 (ddd, *J* = 7.4, 4.9, 1.0 Hz, 1H), 3.80 (t, 2H, *J* = 5.1 Hz), 2.95 (t, 2H, *J* = 5.1).

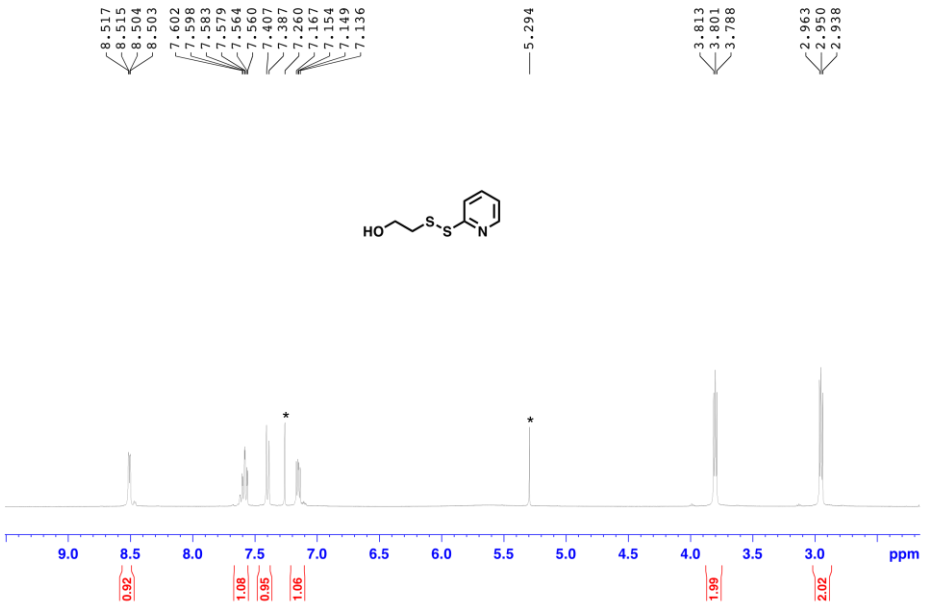

Figure S1. ^{1}H NMR of 2-(2-pyridyldithio)ethanol. Solvent peaks indicated by asterisk: $CHCl_3$, CH_2Cl_2.

4-Nitrophenyl 2-(2-pyridyldithio)ethyl carbonate (NDEC). 2-(2-Pyridyldithio)ethanol (2.72 g, 14.5 mmol) was combined with triethylamine (2.23 mL, 16.0 mmol) in anhydrous dichloromethane under a nitrogen atmosphere. 4-Nitrophenyl chloroformate (3.5 g, 17.4 mmol) was added to the solution and left to stir at room temperature overnight. Solvent was removed under reduced pressure and the crude mixture was purified using silica chromatography with ethyl acetate/dichloromethane solvent system. The product was isolated as a yellow oil (1.75 g, 25%). ^{1}H NMR (400 MHz, $CDCl_3$, δ): 8.50 (m, 1H), 8.28 (m, 2H), 7.65 (m, 2H), 7.38 (m, 2H), 7.12 (ddd, *J* = 6.4, 4.8, 2.1 Hz, 1H), 4.57 (t, 2H, *J* = 6.4 Hz), 3.16 (t, 2H, *J* = 6.4 Hz).

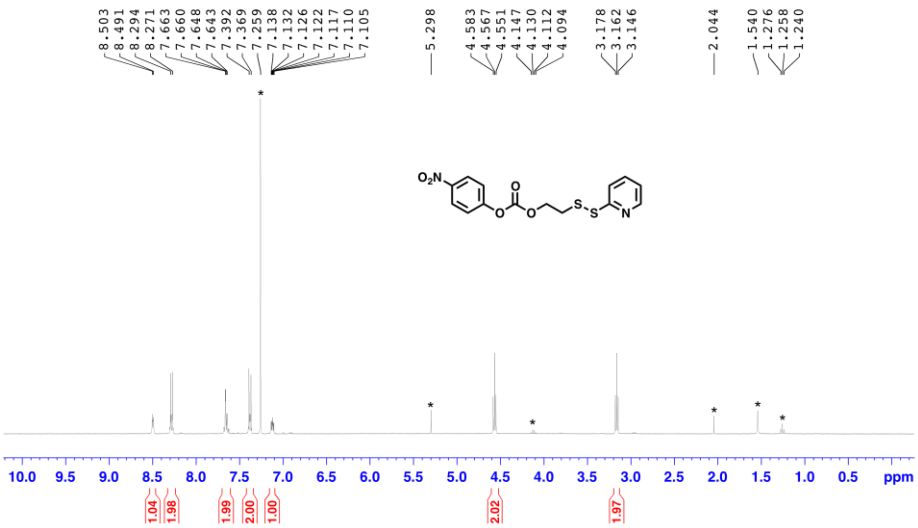

Figure S2. ^{1}H NMR of NDEC linker. Solvent peaks indicated by asterisk: $CHCl_3$, CH_2Cl_2, ethyl acetate, and water.

General procedure for synthesis of gp100–DNA conjugates. To prepare gp100-DNA conjugates, linkers were attached to the gp100 peptide first, followed by attachment of thiol-modified DNA. The gp100 peptide (5.8 mg, 5 μmol) was dissolved in anhydrous dimethyl formamide (200 μL) to which was added the linker (1 μmol) and diisopropylethylamine (5 μmol). The reaction was shaken at room temperature overnight. Afterwards, the peptide product was precipitated and washed thrice with 2 mL of 5% acetic acid in diethyl ether. The remaining acetic acid and diethyl ether were evaporated under reduced pressure.

In order to add the DNA, the crude gp100-linker conjugate was combined with thiol modified DNA (1 equivalent) in 400 μL of 1:1 solution of water:dimethylformamide and 0.1 M EPPS buffer at pH 8.0. The reaction was shaken overnight at room temperature. Following this, the reaction was diluted with water and washed five times with water using an Amicon 3 kDa-0.5 mL molecular weight cut-off filter. This product was purified using denaturing polyacrylamide gel electrophoresis, and further washed eight times with water using an Amicon 3 kDa-15mL filter (32% yield over two steps).

AlexaFluor 488-modified conjugates were synthesized as described above, then incubated with NHS-ester activated AlexaFluor dye (10 equivalents) for 12 hours and purified by washing ten times with water in a molecular weight cut-off filter (3 kDa-0.5mL, Amicon).

General procedure for SNA synthesis. SNA synthesis was carried out in three independent steps: duplex formation, liposome synthesis, and SNA assembly. To form duplex strands, the gp100-DNA conjugate was mixed with an equimolar amount of complementary strand labeled with Cy5 and bearing a 3´-cholesterol group. The solution was lyophilized and reconstituted in buffer (1x duplex buffer, IDT) to a concentration of 200 μM by duplex. This solution was heated to 70°C for 10 minutes, allowed to cool to room temperature and incubated at 4°C overnight.

Liposomes were synthesized by drying a film of 50 mg of DOPC in chloroform (Avanti Polar Lipid 850375C) in a glass vial using dry nitrogen gas followed by overnight lyophilization. The phospholipids were hydrated with 5 mL of PBS followed by vortexing and five freeze/thaw cycles, followed by extrusion through 200 nm, 100 nm, 80 nm and 50 nm polycarbonate filters, consecutively (Sterlitech). After concentration, diameters of liposomes were measured by DLS using a Zetasizer Nano ZS.

SNAs were assembled by mixing the duplex with liposomes in a 75:1 ratio and diluting with 1x PBS to a concentration of 100 μM by duplex (or 1.33 μM by SNA). The solution was shaken at 33 °C overnight and then used without further purification. We assume that linker identity does not impact cholesterol anchor intercalation into the liposome and thus the SNA loading.

Table S1. Oligonucleotide sequences.

CpG Anchor (PS)	5´ - TCC ATG ACG TTC CTG ACG TT (Cy5) $(Sp18)_2$ Cholesterol - 3´
Conjugate (CpG complement, PO)	5´ - AAC GTC AGG AAC GTC ATG GA (Sp18) C3Thiol - 3´
FRET conjugate (PO)	5´ - AAC GTC AGG AAC GTC ATG GA (Sp18) (Eclipse Quencher) C3Thiol - 3´

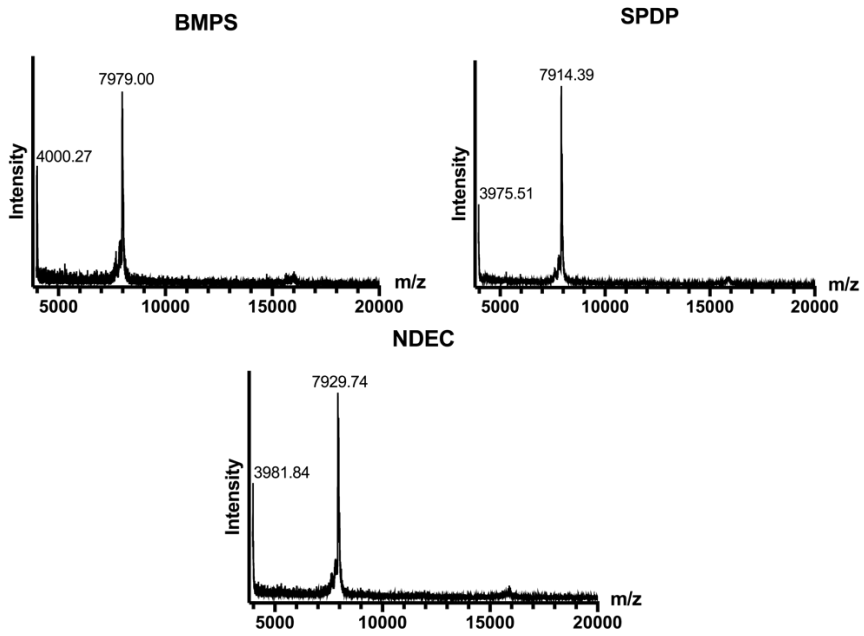

Figure S3. MALDI-TOF spectrum of peptide-DNA conjugates, collected with 2',6'-dihydroxyacetophenone (DHAP) matrix in negative linear mode. Expected masses of conjugates are 7980 Da (BMPS conjugate), 7915 Da (SPDP conjugate), and 7931 (NDEC conjugate).

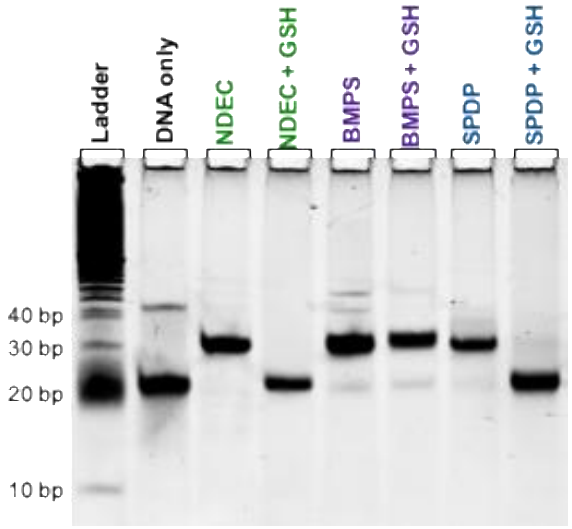

Figure S4. The three gp100–DNA conjugates were treated with 10 mM glutathione (GSH) in 1x PBS (pH 7.4) for 2 hours at room temperature. Cleavable conjugates NDEC and SPDP show a shift in electrophoretic mobility indicative of disulfide cleavage, while non-cleavable BMPS shows no change. Gel visualized with Sybr Gold DNA stain.

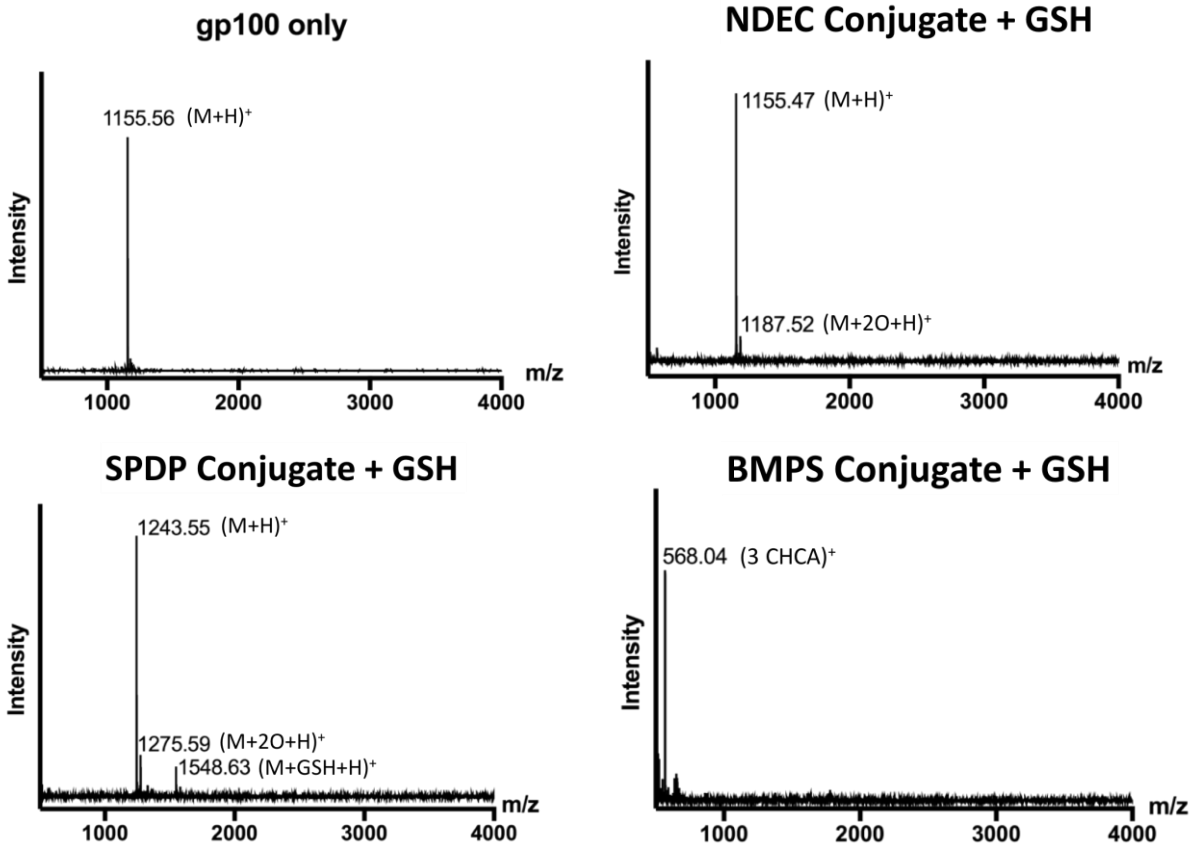

Figure S5. MALDI-TOF spectra of conjugates before and after treatment with 10 mM glutathione in 2x PBS buffer (pH 7.4) for 24 hours at room temperature. Reactions were purified with C18 ZipTips before spotting on plate with α-cyano-4-hydroxycinnamic acid (CHCA) matrix, samples were collected in positive reflectron mode.

Quantification of cleavage kinetics. Cleavage kinetics of the conjugate were quantified by incubating conjugates **1**–**3** at 200 nM concentration in 1x PBS with 20 mM GSH at room temperature, and monitoring the emission at 520 nm while exciting at 485 nm. No increase in fluorescence was observed for the samples incubated in PBS only, while samples **1** and **2** showed increase in fluorescence in the presence of GSH. Following 90 minutes of incubation, TCEP was added to the reactions, to a 9 mM concentration, to reduce any remaining disulfides and establish a fully-cleaved maximum fluorescence. The PBS only samples served to correct for background fluorescence. A non-linear exponential decay, with the plateau constrained to zero, was used to fit the data and extract the half-lives of 31 (30–32) and 54 (53–56) minutes (95% CI) for **1** and **2**, respectively. We expect the small difference in rates of cleavage to be biologically insignificant and not account for the observed differences in T-cell proliferation.

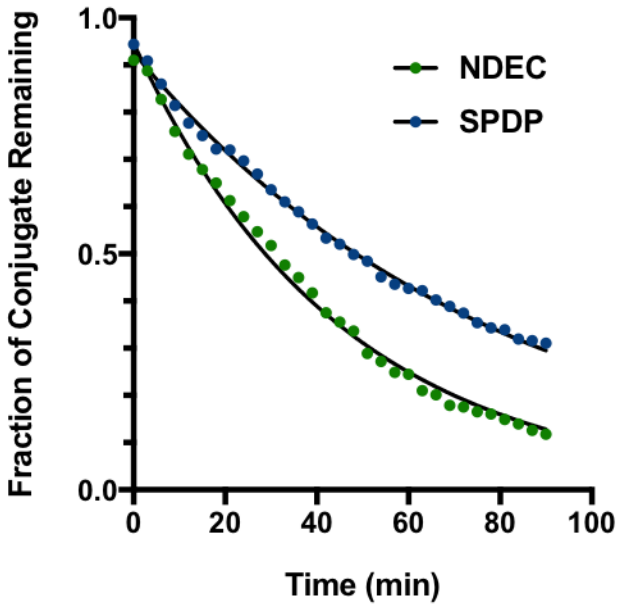

Figure S6. Cleavage kinetics of the conjugates characterized using a fluorophore-quencher system.

Toxicity Assay. Cytotoxicity of the NDEC-conjugate was assayed using an MTT cell proliferation kit (Roche, Cat. No 11465007001) to ensure that the released linker degradation products were not cytotoxic. Dendritic cells isolated from mice bone marrow were selected by Biotin positive selection kit (Stem Cell Catalog # 18556) and plated in a 96 well-plate with $1x10^4$ cell confluency. Then cells were incubated with **1**-SNA at different concentrations for 24 hours at 37 °C and 5% CO_2. Measurements were carried out according to the manufacturer's instructions. No significant toxicity was observed at the tested conditions. Results are shown below, error bars show standard deviations of three replicates.

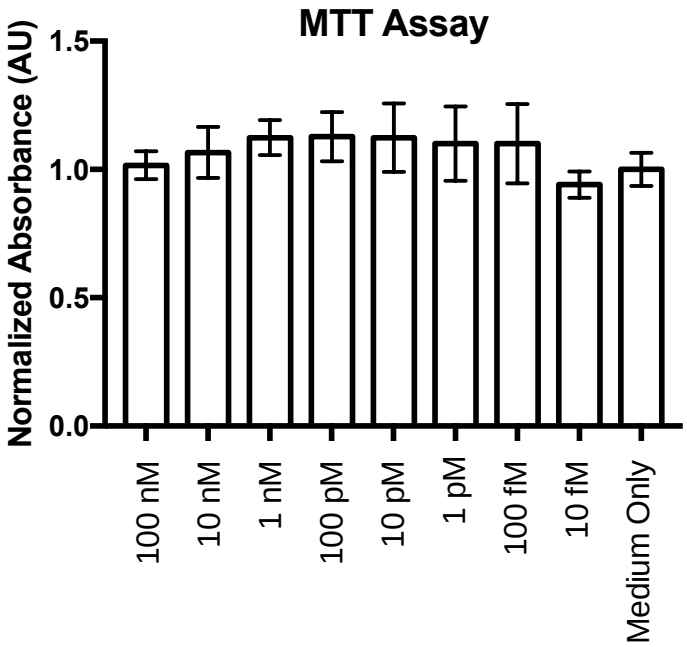

Figure S7. MTT assay results for treatment with **1**-SNA.

Co-delivery. To measure the uptake of SNAs compared with linear counterpart, we used bone marrow-derived dendritic cells (DCs) that were cultured and stimulated by GM-CSF for 6 days. After that, we used biotin-positive selection kit (Stem Cell Catalog # 18556) to select DCs with the CD11c marker. Then 5E5 cells were treated with **1**-SNA or a linear mixture in an incubator (37 °C, 5% CO_2) for 15 minutes before measuring gp100 and CpG uptake. Flow cytometry data shows that SNAs achieve higher uptake measurements compared with linear mixtures. All experiments were preformed in triplicate.

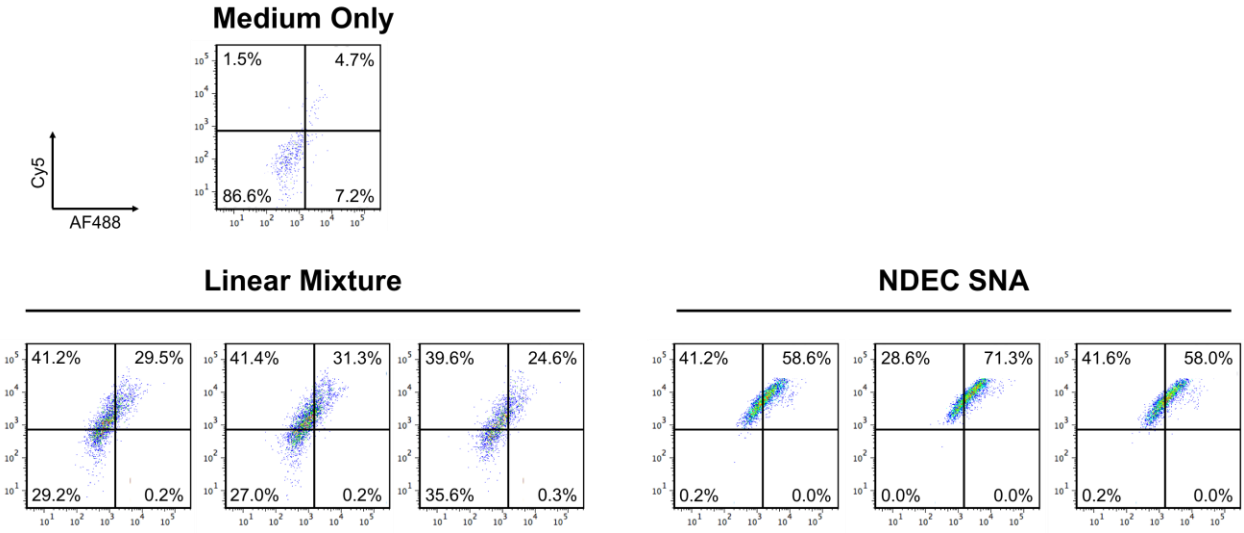

Figure S8. Raw flow cytometry dot plots of adjuvant and antigen co-delivery in mouse DCs, Q2 signifies cells showing co-delivery of both entities.

T-Cell Proliferation. Antigen specific T-cell proliferation was measured using genotyped pmel mice. Whole splenocytes were harvested from the mice, stained with eFluor 450 dye, and cultured under the different treatment conditions for 72 hours. Following treatment, the CD8 marker was stained and flow cytometry was run to measure the proliferation ratio of CD8+ T-cells. Gating stagnate was based on read-out from a medium only treatment group. All experiments were carried out in triplicate.

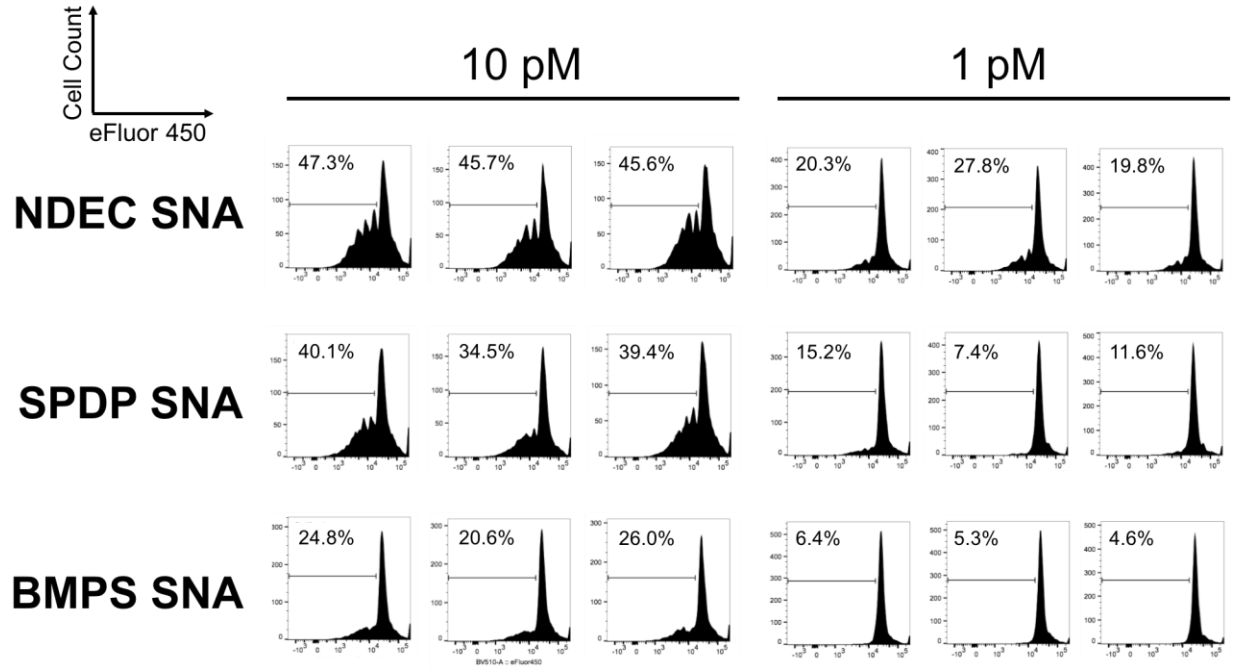

Figure S9. Raw flow cytometry data of T-cell proliferation using the eFluor 450 assay showing triplicate measurements for the three different SNA types at 10 pM and 1 pM concentrations by gp100 peptide.

APC activation and SNA uptake. APC activation after treatment with SNAs was measured using bone marrow-derived DCs that were cultured and stimulated by GM-CSF for 6 days prior to treatment. Biotin-positive selection kit (Stem Cell Catalog # 18556) was used to select DCs with the CD11c marker. Then, 3E5 cells were treated with three different SNAs at a 100 nM concentration in an incubator (37°C, 5% CO_2) for 24 hours before measuring the activation markers. Flow cytometry data shows that all SNAs achieved indistinguishable APC activation via CD40 and CD80 expression. Uptake was measured by comparing the amount of Cy5 fluorescence in DCs using flow cytometry. Cells treated with the three SNAs show indistinguishable levels of Cy5 median fluorescence.

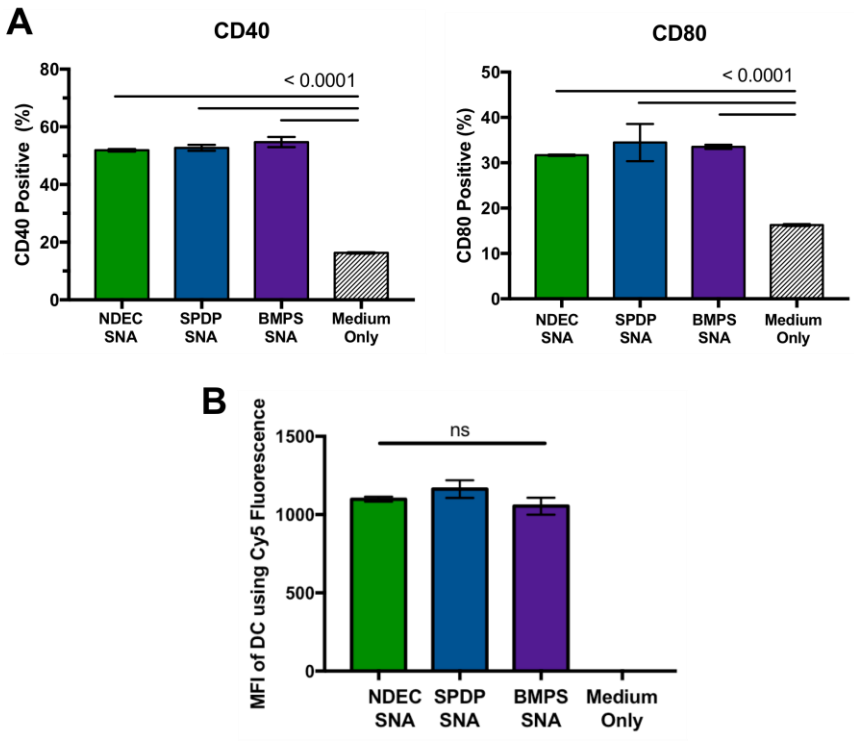

Figure S10. (A) Activation of mouse bone marrow derived DCs, using CD40 and CD80 markers, after treatment with different SNA structures at a 100 nM concentration or a medium only control. (B) Uptake of SNAs into mouse bone marrow derived dendritic cells measured by MFI of Cy5-conjugated CpG under the same treatment conditions.