Supporting Information

The Content of CpG-DNA in Antigen-CpG Conjugate Vaccines Determines Their Cross-Presentation Activity

Ryan M. Clauson,† Brendan Berg‡ and Beata Chertok*,†‡

†Department of Pharmaceutical Sciences, College of Pharmacy, University of Michigan, Ann Arbor, Michigan 48109, United States

‡Department of Biomedical Engineering, College of Engineering, University of Michigan, Ann Arbor, Michigan 48109, United States

* Corresponding Author: Dr. Beata Chertok; Address: College of Pharmacy, 428 Church St, Ann Arbor MI 48109; Phone: 734-763-2656; Email: beata@umich.edu
S1. Experimental Section

Materials. All reagents were used as obtained from commercial sources without further purification. Ovalbumin (OVA) was purchased from MP Biomedical. Succinimidyl 3-(2-pyridylthio) propionate) (SPDP) was obtained from CovaChem. CpG oligonucleotides with a 3' terminal disulfide bridge modification (3' Thiol Modifier C3 S-S; 1-propanol-disulfide, 1'-succinyl-lcaa-CPG) were purchased from IDT DNA Technologies (CpG 1826 (5') TCCATGACGTTCCTGACGTT-(CH2)3-S-S-CH3 (3')). Chlorophenol Red-β-D-galactopyranoside (CPRG), tris(2-carboxyethyl) phosphine hydrochloride (TCEP-HCl), ammonium persulfate and N,N,N',N'-tetramethylethylenediamine (TEMED) were acquired from Sigma-Aldrich. SYBR Gold Nucleic Acid Gel Stain (10,000X) and the microBCA protein assay kit were obtained from Thermo Fisher. DC2.4 mouse dendritic cell line was purchased from Millipore-Sigma. B3Z T-cell hybridoma cells were generously donated by Dr. John-Demian Sauer (University of Wisconsin-Madison).

Synthesis and purification of Ovalbumin-CpG conjugate (OVA-CpG). We first assessed the purity of OVA using mass spectrometry (MALDI-TOF, Bruker Autoflex Speed) and Dynamic Light Scattering (DLS, Malvern Zetasizer Nano-ZS). Mass spectrogram displayed a dominant peak at m/z of ~44.5 kDa, corresponding to monomeric OVA 1, 2 (Figure S1, A). Peak area analysis revealed that monomeric OVA was ~95 % pure from multimeric aggregates. Volume-weighted size distribution by DLS displayed a single peak at ~6 nm hydrodynamic diameter (Figure S1, B) corresponding to monomeric OVA (Stokes radius of ~3 nm 3), and a ~99.9% purity from aggregates within the 10 nm – 1 µm diameter range. Based on these results, we proceeded to use the commercially obtained OVA without further purification.

OVA-CpG covalent conjugates were synthesized using a three-step disulfide coupling chemistry. In the first step, OVA was reacted with SPDP to produce functionalized OVA-PDP. The reaction was carried out as previously described 4 with minor modifications. Briefly, OVA (1 mg/mL in 100 mM PBS buffer, pH 7.4) was reacted with a molar excess of SPDP (20 mM SPDP, dimethylsulfoxide DMSO) for 12 hours at room temperature. The molar ratios of SPDP to OVA were varied to produce conjugates with different degrees of functionalization (5-fold to 15-fold molar excess). OVA-PDP was purified sequentially by anion exchange chromatography (HiTrap Q FF, 5 mL) and ultrafiltration (10,000 MWCO) to remove unreacted species. Purified OVA-PDP was analyzed by microBCA and pyridine-2-thione spectrophotometric assays 5 to determine the PDP content of the functionalized protein. In the second step, CpG oligonucleotides modified with a terminal protected disulfide were reacted with a reducing agent (TCEP-HCL, 100 mM, overnight) to produce oligonucleotides with a free terminal sulfhydryl group (CpG-SH). CpG-SH was purified by anion exchange chromatography (HiTrap Q FF, 1
mL), followed by desalting with ultrafiltration (1,000 MWCO). The purified oligonucleotides were quantified by spectrophotometric analysis (NanoQuant Plate, Tecan M1000 PRO). 6 In the third step, OVA-CpG covalent conjugates were produced by reacting OVA-PDP (1 mg/mL in 100 mM PBS, pH 7.4) with CpG-SH (at 1:1 molar ratio of PDP:CpG) at room temperature for 48 hours. To isolate OVA-CpG conjugates with defined CpG content, the reaction mixture was fractionated using anion exchange chromatography (HiTrap Q FF, 5 mL) with a stair-step sodium chloride (NaCl) elution gradient (0-2 M, NaCl). Fractions were desalted and purified of unreacted oligonucleotides via centrifugal filtration (30,000 MWCO, 15 mL) using PBS buffer (100 mM, pH 7.4) as a solvent.

**Characterization of Ova-CpG conjugates.** To confirm isolation of major conjugate species from heterogeneous product mixtures, purified species were characterized by anion exchange chromatography. Anion exchange chromatography was carried out using quaternary ammonium solid phase (HiTrap Q FF, 5 mL) with a stair-step sodium chloride (NaCl) elution gradient (0-2 M, NaCl). To construct elution profiles, the eluted fractions (1 mL) were collected and analyzed for protein concentration using microBCA assay (Thermo Scientific). Molecular weights were estimated by SDS polyacrylamide gel electrophoresis (SDS-PAGE, 10% crosslinking, non-reducing conditions, 75 V, 1.5 - 2 hours) and visualized with silver staining. 7 Migration distances on SDS-PAGE electrophoregrams were quantified by Image J and used as a relative measure of the molecular weight. Calibration was performed using protein standards of known molecular weights (Bio-Rad). The calibration curve was linear (R² = 0.98) in the range of 28-78 kDa. The propensity of proteins to aggregation was determined by native PAGE electrophoresis (N-PAGE, 10% crosslinking, 75 V, 2 hours) followed by gel densitometry image analysis. 8 Image analysis was carried out using ImageJ and Matlab R2016b software. For qualitative analysis, pixel densities of individual lanes were averaged axially and the means plotted as a function of relative migration distance. For quantitative analysis, the density peaks below the defined migration threshold for aggregation intermediates (<0.6) were integrated and normalized by total peak density. 8 To determine the oligonucleotide content per unit protein in conjugate species, conjugates were analyzed using SYBR Gold 9, 10 and microBCA assays (Thermo Scientific) to quantify single-stranded DNA and protein, respectively.

**In vitro cross-presentation assay using B3Z CD8 T cell hybridoma.** The ability of OVA-CpG conjugates to facilitate antigen cross-presentation was evaluated by an in vitro cross-presentation assay using the CD8⁺ T-cell hybridoma B3Z cell line and the immortalized murine dendritic cells, DC2.4. The B3Z T-cell hybridoma is a genetically modified T-cell line that can be triggered to produce β-Galactosidase (β-Gal) via activation by antigen-presenting cells that display the OVA epitope SIINFEKL in the context of a murine K⁰ MHC Class I molecule. 11 In
this way, the B3Z T-cell hybridoma assay provides a measure of the extent of antigen cross-presentation and T-cell activation. DC2.4 cell line recapitulates most characteristic features of dendritic cells (DC) including the mechanisms of cross-presentation, while allowing facile expansion of homogeneous cell populations in culture. Therefore, DC2.4 provides a suitable DC-like antigen-presenting cell model for evaluating cross-presentation.

Prior to beginning experiments, DC2.4 and B3Z T-cell hybridoma cells were cultured under previously described conditions. Briefly, DC2.4 cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 10 mM HEPES, 100 µM non-essential amino acids and 50 µM β-mercaptoethanol. B3Z cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mM sodium pyruvate, 2 mM glutamine and 50 µM β-mercaptoethanol. All cells were maintained at 37°C, 5% CO2/ air atmosphere. The cross-presentation assay was conducted essentially according to the protocol of Bouwer et al. with minor modifications. Briefly, DC2.4 cells (1 x 10^5 cells / well) were plated in 96-well U-bottom plates in complete RPMI medium supplemented with 10% (v/v) FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. Cell suspensions were spiked with OVA-CpG conjugates or controls at a final concentration of 500 µg/mL (OVA-equivalent) and incubated for 5 hours (37°C, 5% CO2). Subsequently, the media was replaced to remove the conjugates, and the DC2.4 cells were co-cultured with B3Z T-cell hybridoma cells (1 x 10^5 cells /well) for 18 hr (37°C, 5% CO2). The activity of β-Gal was quantified by the CPRG spectrophotometric assay (at 570 nm) using purified β-Gal enzyme with known enzymatic activity for calibration.

To rule out chemical degradation of di-sulfide linked conjugates during the assay we evaluated chemical stability of the conjugates under the assay conditions. To assess chemical stability, we compared the CpG content of the OVA-CpG conjugates before (in 100 mM PBS, pH 7.4) and after incubation of the conjugates in assay media (RPMI, 10% FBS, 1% Pen/Strep) for 5 hr at 37C. Samples were analyzed by agarose electrophoresis (3%, 75 V, 1 hr) and CpG was visualized using SYBR Gold. CpG content in bands corresponding to OVA-CpG conjugates was quantified by densitometry. All three OVA-CpG conjugates retained most of their original CpG content (OVA-1-CpG: 93 ± 3 %, OVA-2-CpG: 91 ± 9 %, OVA-3-CpG: 87 ± 8 %) and did not differ significantly in percent retained CpG (p = 0.67, Figure S2). These results revealed that the conjugates preserved chemical stability in the assay media and confirmed conjugate compatibility with the assay conditions.

**Statistical analysis.** Data are expressed as mean ± S.D. Means of multiple groups were compared with the one-way ANOVA, followed by post hoc Tukey’s pairwise comparisons. All
probability values are two-sided, and values of $p < 0.05$ were considered statistically significant. Statistical analyses were carried out using the GraphPad Prism 7 software package.

S2. Supplementary Figures

Figure S1. Characterization of commercially obtained OVA. A. Representative mass spectrum of OVA measured by MALDI-TOF using sinapinic acid matrix. B. Representative volume-weighted size distribution of OVA (100 mM PBS, pH 7.4) measured by Dynamic Light Scattering.
Figure S2. Chemical stability of OVA-CpG conjugates under the cross-presentation assay conditions. Conjugates (500 µg/mL) were freshly prepared in PBS (100 mM, pH 7.4) or incubated in assay media (RPMI, 10% FBS, 1% Pen/Strep) for 5 hr at 37°C. Conjugates were analyzed by agarose gel electrophoresis and their CpG content visualized with SYBR GOLD and quantified by densitometry. Retained CpG content (%) was calculated as the CpG content in samples subjected to the assay conditions normalized by the CpG content in freshly prepared samples. Data represent mean ± S.D., n = 3
Figure S3. Generation of OVA-PDP species with controlled extent of PDP functionalization. The content of PDP functional groups per unit OVA was quantified using the BCA and the pyridine-2-thione spectrophotometric assays to determine the contents of protein and the PDP functional group, respectively. Data represent mean ± S.D., n = 3.

Figure S4. Polyacrylamide gel electrophoresis (SDS-PAGE) of crude OVA-CpG conjugates. Representative image of SDS-PAGE analysis (10%, 75V, 90 minutes) for OVA (lane 1), crude OVA-1-CpG (lane 2) and crude OVA-2-CpG (lane 3) conjugates prior to purification by anion exchange chromatography. Molecular weights were analyzed using the ladder of protein standards.
Figure S5. Representative anion exchange chromatogram for crude OVA-3-CpG species. Anion exchange chromatography was carried out using quaternary ammonium solid phase with a stair-step sodium chloride (NaCl) elution gradient (dashed line).

REFERENCES:

1. Wang, Y.; He, C. H.; Zheng, H.; Zhang, H. B. (2012) Characterization and Comparison of Fumonisin B-1-Protein Conjugates by Six Methods. *Int J Mol Sci* 13, 84-96.
2. Sugimoto, Y.; Sanuki, S.; Ohsako, S.; Higashimoto, Y.; Kondo, M.; Kurawaki, J.; Ibrahim, H. R.; Aoki, T.; Kusakabe, T.; Koga, K. (1999) Ovalbumin in Developing Chicken Eggs Migrates from Egg White to Embryonic Organs While Changing Its Conformation and Thermal Stability. *J Biol Chem* 274, 11030-11037.
3. Erickson, H. P. (2009) Size and Shape of Protein Molecules at the Nanometer Level Determined by Sedimentation, Gel Filtration, and Electron Microscopy. *Biol Proced Online* 11, 32-51.
4. Carlsson, J.; Drevin, H.; Axen, R. (1978) Protein Thiolation and Reversible Protein-Protein Conjugation. N-Succinimidyl 3-(2-Pyridyldithio)Propionate, a New Heterobifunctional Reagent. *The Biochemical journal* 173, 723-37.
5. Slutter, B.; Soema, P. C.; Ding, Z.; Verheul, R.; Hennink, W.; Jiskoot, W. (2010) Conjugation of Ovalbumin to Trimethyl Chitosan Improves Immunogenicity of the Antigen. *J Control Release* 143, 207-14.
6. Lucena-Aguilar, G.; Sanchez-Lopez, A. M.; Barberan-Aceituno, C.; Carrillo-Avila, J. A.; Lopez-Guerrero, J. A.; Aguilar-Quesada, R. (2016) DNA Source Selection for Downstream Applications Based on DNA Quality Indicators Analysis. *Biopreservation and Biobanking* 14, 264-270.
7. Wray, W.; Boulikas, T.; Wray, V. P.; Hancock, R. (1981) Silver Staining of Proteins in Polyacrylamide Gels. *Anal Biochem* 118, 197-203.
8. Pathak, M.; Dutta, D.; Rathore, A. (2014) Analytical Qbd: Development of a Native Gel Electrophoresis Method for Measurement of Monoclonal Antibody Aggregates. *Electrophoresis* 35, 2163-2171.
9. Tuma, R. S.; Beaudet, M. P.; Jin, X.; Jones, L. J.; Cheung, C. Y.; Yue, S.; Singer, V. L. (1999) Characterization of Sybr Gold Nucleic Acid Gel Stain: A Dye Optimized for Use with 300-Nm Ultraviolet Transilluminators. *Anal Biochem* 268, 278-88.
(10) Goldshtein, H.; Hausmann, M. J.; Douvdevani, A. (2009) A Rapid Direct Fluorescent Assay for Cell-Free DNA Quantification in Biological Fluids. *Ann Clin Biochem* 46, 488-94.

(11) Karttunen, J.; Sanderson, S.; Shastri, N. (1992) Detection of Rare Antigen-Presenting Cells by the LacZ T-Cell Activation Assay Suggests an Expression Cloning Strategy for T-Cell Antigens. *Proc Natl Acad Sci U S A* 89, 6020-4.

(12) Mant, A.; Chinnery, F.; Elliott, T.; Williams, A. P. (2012) The Pathway of Cross-Presentation Is Influenced by the Particle Size of Phagocytosed Antigen. *Immunology* 136, 163-175.

(13) Shen, Z. H.; Reznikoff, G.; Dranoff, G.; Rock, K. L. (1997) Cloned Dendritic Cells Can Present Exogenous Antigens on Both Mhc Class I and Class Ii Molecules. *J Immunol* 158, 2723-2730.

(14) Hari, A.; Ganguly, A.; Mu, L. B.; Davis, S. P.; Stenner, M. D.; Lam, R.; Munro, F.; Namet, I.; Alghamdi, E.; Furstenhaupt, T., et al. (2015) Redirecting Soluble Antigen for Mhc Class I Cross-Presentation During Phagocytosis. *Eur J Immunol* 45, 383-395.

(15) Hargadon, K. M.; Forrest, O. A.; Reddy, P. R. (2012) Suppression of the Maturation and Activation of the Dendritic Cell Line Dc2.4 by Melanoma-Derived Factors. *Cell Immunol* 272, 275-282.

(16) Fu, R. H.; Hran, H. J.; Chu, C. L.; Huang, C. M.; Liu, S. P.; Wang, Y. C.; Lin, Y. H.; Shyu, W. C.; Lin, S. Z. (2011) Lipopolysaccharide-Stimulated Activation of Murine Dc2.4 Cells Is Attenuated by N-Butylidenephthalide through Suppression of the Nf-Kappa B Pathway. *Biotechnol Lett* 33, 903-910.

(17) Higgins, D. E.; Shastri, N.; Portnoy, D. A. (1999) Delivery of Protein to the Cytosol of Macrophages Using Escherichia Coli K-12. *Mol Microbiol* 31, 1631-1641.

(18) Bouwer, H. G. A.; Alberti-Segul, C.; Montfort, M. J.; Berkowitz, N. D.; Higgins, D. E. (2006) Directed Antigen Delivery as a Vaccine Strategy for an Intracellular Bacterial Pathogen. *P Natl Acad Sci USA* 103, 5102-5107.

(19) Sanderson, S.; Campbell, D. J.; Shastri, N. (1995) Identification of a Cd4+ T-Cell-Stimulating Antigen of Pathogenic Bacteria by Expression Cloning. *J Exp Med* 182, 1751-1757.