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Electronic supplementary information for

Exploration of graphene oxide as intelligent platform for cancer vaccine

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Supporting experimental section

Materials

Bicinchoninic acid (BCA) Kit was from Pierce. Alum adjuvant (aluminium hydroxide gel) was kindly provided by Hualan Biological Engineering Incorporation. SIINFEKL peptide was synthesized by GL Biochem. Penicillin, streptomycin, mitomycin, Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640, and fetal bovine serum (FBS) wre ordered from Gibco. Granulocyte macrophage colony-stimulating factor (GM-CSF) and intereukin-4 (IL-4) were from Peprotech. G-418 (Geneticin), 4, 6-diamidino-2-phenylindole (DAPI), Lyso Tracker Red DND-99, Alexa Fluor 488-labeled goat anti-rat, carboxyfluorescein diacetate succinimidyl ester (CFSE), and IgG Dynal CD8 negative isolation kit were all purchased from Invitrogen. Fluorescein isothiocyanate (FITC)-CD80, Phycoerythrin (PE)-CD86, Allophycocyanin (APC)-Cy7-CD11c, PE-Cy7-CD8α, and PerCP-Cy5.5-CD3 anti-mouse mAbs were bought from Biolegend. Allophycocyanin (APC) anti-mouse OVA257-264 (SIINFEKL)-H-2kb (OVA specific MHC I Ab), eFluor 450 anti-mouse MHC Class II, anti-mouse IFN-γ Abs, and intracellular Fixation/Permeabilization buffer set were from eBioscience. Cytometric Bead Array (CBA) Mouse Inflammation Kit was obtained from BD Biosciences. Rabbit anti-mouse Beclin 1 antibody was purchased from Abcam. Dyhigh 549 goat anti-rabbit IgG was from KPL. Recombinant IL-2, rabbit anti-mouse LC3A/B Ab, Horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG, and 3,3'-N-Diaminobenzidine Tertrahydrochloride (DAB)- HRP Color Development Kit were ordered from Cell Signalling Technology. Bicinchoninic acid (BCA) Protein Assay Kit was purchased from Pierce. Lactate

dehydrogenase (LDH) Cytotoxicity Assay Kit was from TAKARA. All the other reagents were of analytic grade.

Cells and animals

BMDC were harvested from the C57BL/6 mice. In brief, BMDC were generated by flushing tibia and femurs of mice, and cultured in complete RPMI 1640 medium containing 20 ng/mL GM-CSF, 10 ng/mL IL-4, and 10% (v/v) FBS for 6-8 days. The purity of BMDC was analyzed by using the ADP 9 color flow cytometry (FACS, Beckman) after staining with APC-Cy7-CD11c. Mouse lymphoma EL-4 cell line and E.G7 cells (OVA-expressing derivative of EL4) were supplied from ATCC (American Type Culture Collection). EL-4 was cultured in RPMI 1640 supplemented with 10% FBS, while E.G7 were maintained in RPMI 1640 medium containing 10% FBS, 0.05 mM 2-mercaptoethanol, and 0.4 mg/ml G418. All cells were grown in a humidified incubator at 37 °C and 5% CO_2.

The C57BL/6 male mice (6-8 weeks) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products. The OT-1 (TCR recognizes OVA 257-264) mice were ordered from The Jackson Laboratory. All animal experiments were in compliance with the Institutional Ethical Committee for animal care guidelines.

Evaluation of antigen internalization and intracellular trafficking

BMDC were challenged with OVA or GO-OVA (with an equivalent dose of OVA loaded on GO) for 12 h, and then post-cultured for 48 h after antigen removal. Relative internalization of OVA (dyed with FITC) in BMDC was assayed on FACS. Data were normalized to 24 h for OVA control. For lysosome staining, BMDC were directly stained

with LysoTracker Red for 30 min at 37 ^{o}C, and imaged by SP5 laser scanning confocal microscope (CLSM, Leica).

Westernblot analysis and TEM* (*transmission electron microscopy) imaging of autophagy

For western blot assay, cells were seeded in 24-wells plate and incubated for 24 h. The expressions of Beclin 1 and the conversion of LC3-I (cytosolic form) to LC3-II (membrane-bound lipidated form) were detected by the corresponding antibodies and immunoblotting after 36 h. β-actin was used as a loading control, and the blotted proteins were stained using the HRP-DAB detection method.

To clearly observe the interaction between the GO and cells, BMDC were allowed to adhere in 6-well plates. Following 12 h exposure, cells were rinsed, detached, and then fixed in 2.5% glutaraldehyde (pH 7.4) for 1 h at room temperature. Afterward, samples were post-fixed, serially dehydrated with ethanol, and embedded in Epon. Serial sections were cut on a Reichert Ultracut microtome (Leica), and the samples were imaged by using a JEM-1400 (JEOL) transmission electron microscopy.

Detection of specific anti-OVA IgG levels

Blood samples were collected on days 14 and 21, and anti-OVA IgG levels were determined by enzyme-linked immunosorbent assay (ELISA) analysis. A vaccine formulated with 500 μg alum adjuvant plus 20 μg soluble OVA was used as a positive control.

Safety evaluations of GO based vaccine

Blood samples were collected 24 days after the first immunization for normal mice. The lactate dehydrogenase LDH, blood urea nitrogen BUN, alanine aminotransferase ALT, aspartate aminotransferase AST, and alkaline phosphatase ALP levels were determined

spectrophotometrically using an automated analyzer (Hitachi-917). For histological analysis, the heart, liver, spleen, lung, and kidney were collected and fixed in 10% (v/v) formaldehyde. These tissues were processed by hematoxylin and eosin (H&E) staining at Health Science Center of the Peking University. The optical images of the tissue slices were taken by the Vectra platform (Caliper Life Sciences, Hopkinton).

Statistical analysis

Statistical evaluations of data were performed by Student's t-test for multiple groups. All results were expressed as mean±standard (s.d.) error. Asterisks denote significant differences (*$p < 0.05$; **$p < 0.01$).

Supplementary Data

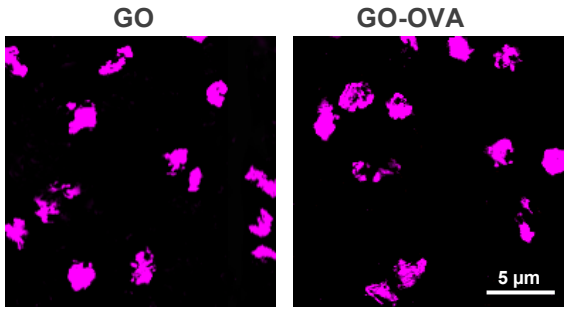

Fig. S1. The CLSM images of the well-distributed GO before and after OVA adsorption.

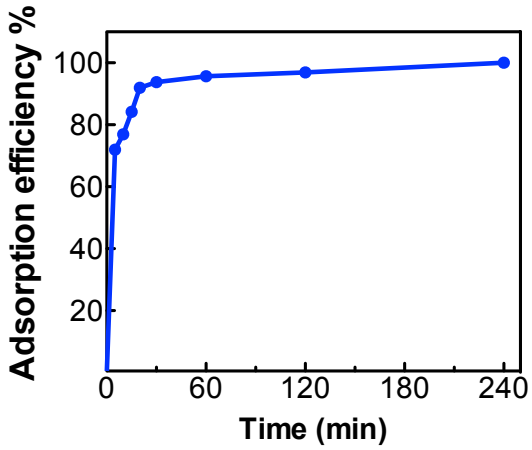

Fig. S2. Adsorption efficiency of ovalbumin on the 2 μm GO sheets.

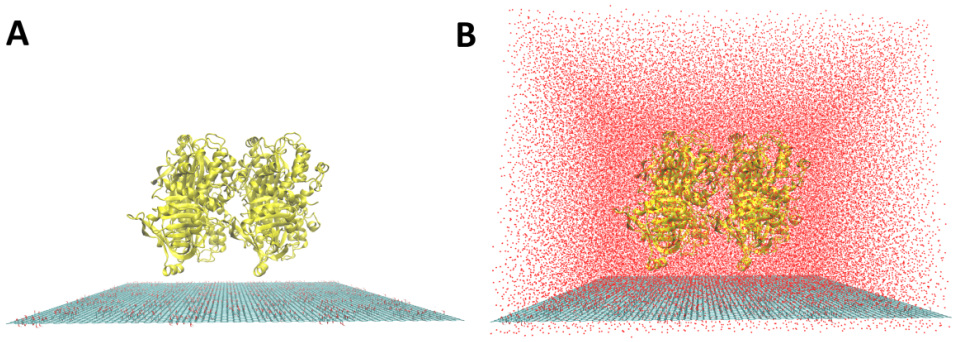

Fig. S3. Initial configuration for molecular dynamics simulations, with the system shown without solvating water (A), and with water (red dots) (B). The initial distance between OVA and GO was set to 1.0 nm and the simulation was carried out with multiple trajectories, each lasting for 100 ns.

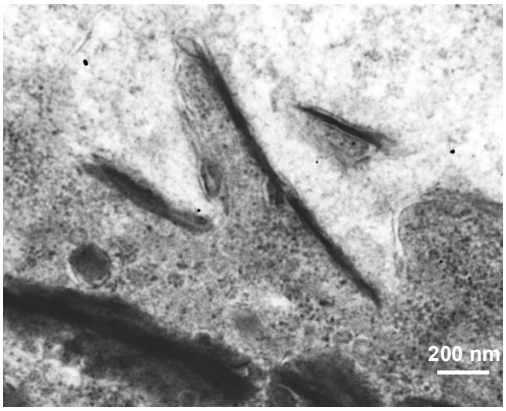

Fig. S4. TEM images showing that GO straightly penetrated into the cell membrane without being sequestered in endosomes.

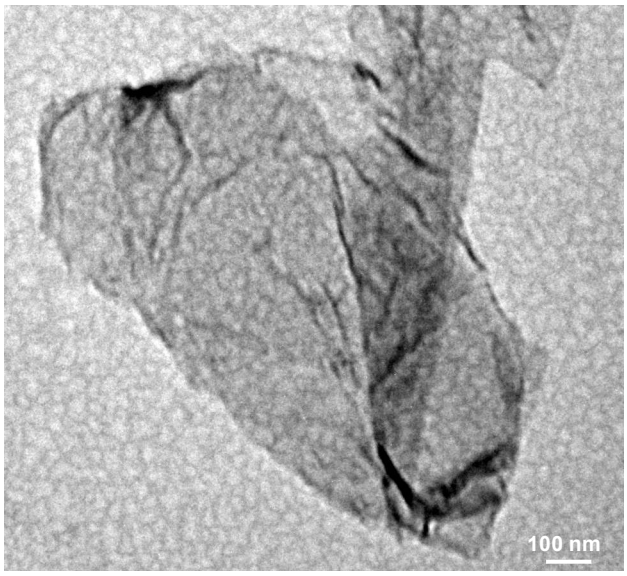

Fig. S5. The TEM image showing that the original GO was in a planar morphology.

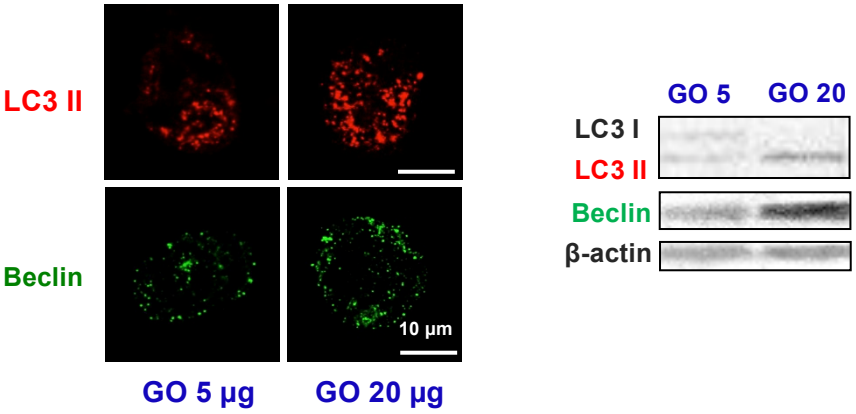

Fig. S6. Dose effect on GO induced autophagy phenomenon in BMDC. CLSM and Western blot results showing the autophagy phenomenon of DCs after treatment with 5 μg/ml GO and 20 μg/ml GO for 12 h.

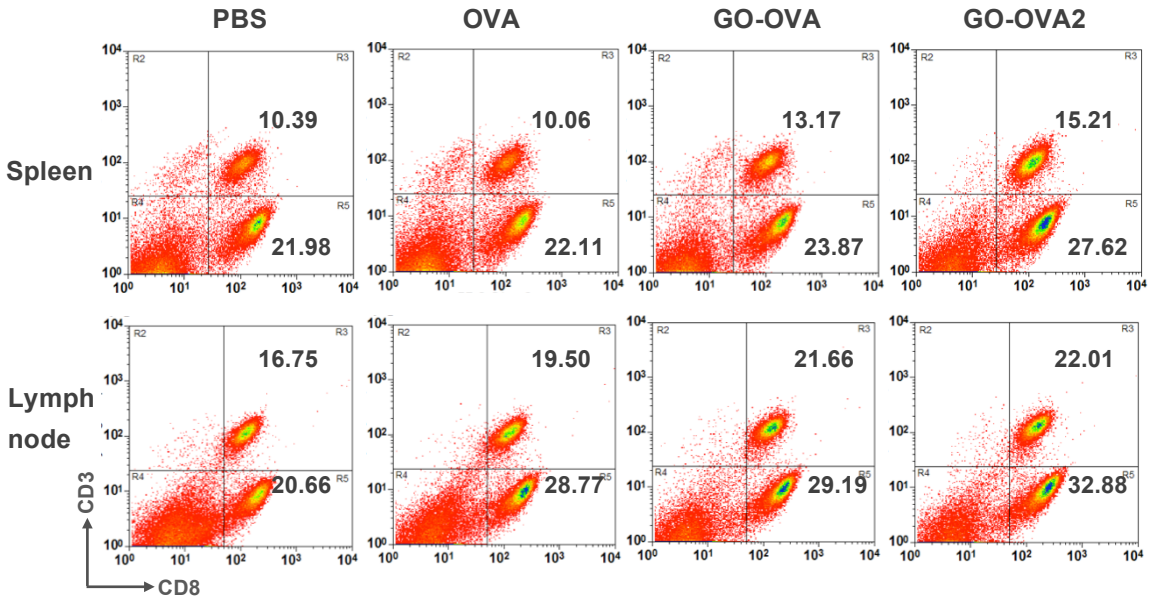

Fig. S7. Effect of the formulation on the T-cell subsets in spleen and lymph node. The percentages of $CD3^+CD8^+$ (CD8 T) and $CD3^+$ T were analyzed by FACS on day 14. Compared with PBS or OVA immunization, the GO-OVA immunization resulted in efficient upregulation of CD8 T and $CD3^+$ T cells in both spleen and lymph node. These results suggest that GO-OVA vaccination may help construct effective immune defense system and lead to potent tumor-suppression.

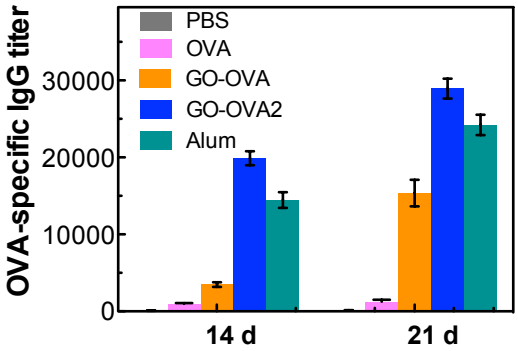

Fig. S8. Efficient humoral response after GO-OVA vaccination. The OVA specific IgG antibody titer of mice blood serum.

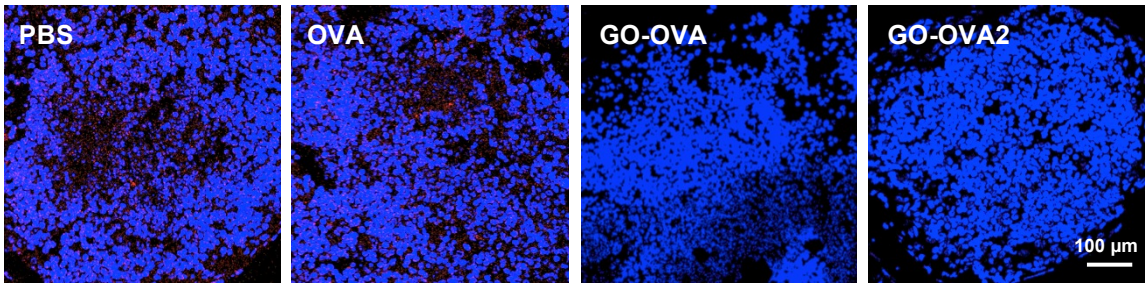

Fig. S9. Efficient inhibition of tumor metastasis after GO-OVA vaccination. Immunofluorescent images showing the OVA expressing cells in lymph node 24 days after tumor bearing in different group. OVA expressing cells (E.G7 cells, indicated by red signals) dispersed into the lymph node at 24 day in PBS or OVA group, showing signs of metastasis. On the contrary, the GO-OVA vaccinations result in no penetration of tumor cells.

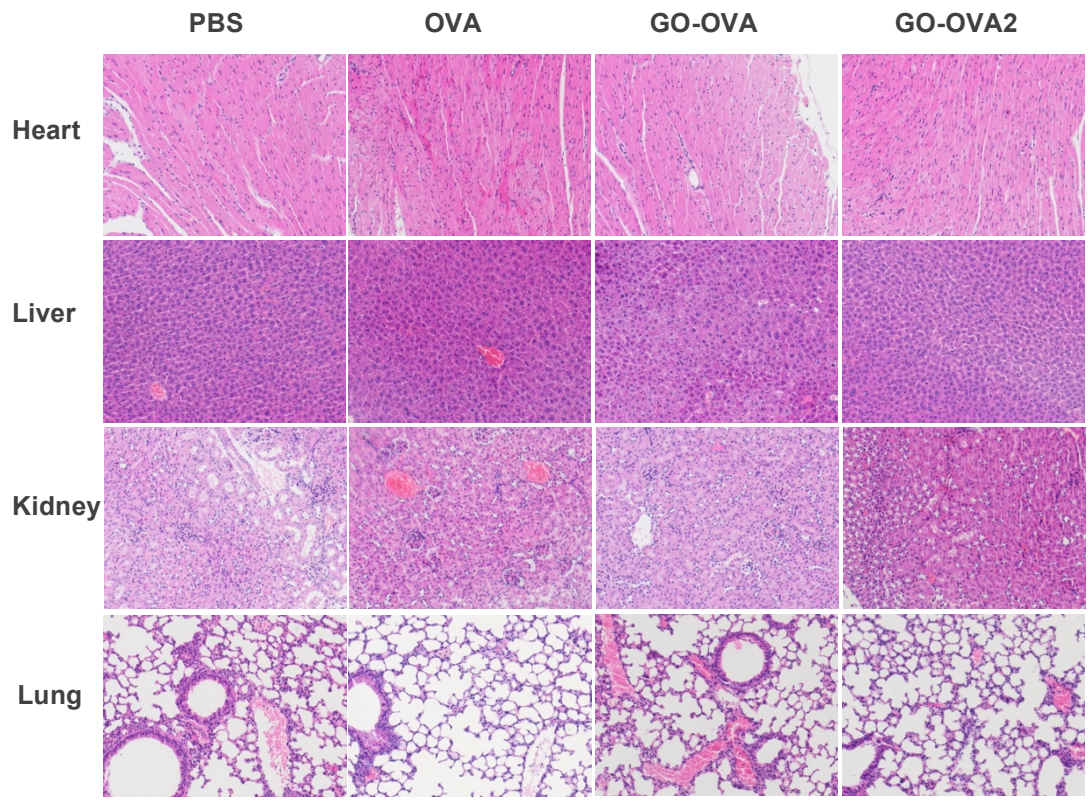

Fig. S10. H&E stained images of major organs. No obvious toxic effect was observed for vaccine treated mice. Mice subcutaneously injected with antigen formulations were sacrificed at day 24. No evident abnormality was observed in heart, liver, spleen, lung, kidney, and lymph nodes for GO-OVA group.

Table S1 Biochemical parameters of serum in tumor-bearing mice

	Marker	PBS	OVA	GO-OVA	GO-OVA2	Normal scale
Hepatic	**AST**	702±203	534±195	330±121	245±92	54~298
	ALT	128±68	133±77	111±49	76±38	17~77
Renal	**BUN**	13.82±3.89	14.40±4.03	13.91±2.90	14.33±3.65	8~33
Others	**LDH**	2541±389	2517±437	1060±121	890±112	215~1024
	ALP	93±14	80±10	75±11	78±13	60~209

Table S1. Determination of serum biochemical parameters in the tumor-bearing mice. To evaluate the health condition of tumor-bearing mice, blood samples were collected before scarification on day 24. Five serum biochemical parameters, including blood urea nitrogen (BUN), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and alkaline phosphatase (ALP) levels were determined. All these data together showed that mice received the GO based vaccine were in much better health condition and had a better prognosis. Data were represented as mean±s.d. with n=7.

Table S2 Biochemical parameters of serum in normal mice

	Marker	PBS	OVA	GO-OVA	GO-OVA2	Normal scale
Hepatic	**AST**	112±42	94±35	82±26	90±34	54~298
	ALT	40±21	47±24	46±19	46±16	17~77
Renal	**BUN**	10.26±3.24	11.41±4.03	12.10±5.90	11.93±3.65	8~33
Others	**LDH**	814±168	820±134	804±125	763±116	215~1024
	ALP	140±17	152±14	131±17	138±13	60~209

Table S2. Determination of serum biochemical parameters in the normal mice 24 days after the first immunization. Among these serum biochemical parameters, AST and ALT are specific indicators for hepatic toxicity; and LDH is a parameter related to injury of major organ including liver, heart, and kidney. Data were represented as mean±s.d. with n=7.