Electronic Supplementary Information

In Vivo Immunological Response of PEGylated Graphene Oxide *via* Intraperitoneal Injection

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Keywords: PEGylation, graphene oxide, immunological response, cytokine secretion, inflammation

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Part of the experimental details

Materials and chemicals

GO powder was provided by Yongjun Gao's Group of Hebei University. Methoxypolyethylene glycol amine (mPEG-NH_2, M_w=2000) was purchased from Beijing JenKem Technology. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was purchased from Beijing J&K China Chemical Ltd. Sodium chloroacetate was purchased from Shanghai Sigma-Addiction. Dulbecco's modified Eagle's medium (DMEM), Penicillin/Streptomycin Solution, Trypsin-EDTA Solution and phosphate buffer saline were all acquired from Invitrogen. BD™ Cytometric Bead Array (CBA) Mouse Inflammation Kits was purchased from BD Biosciences. Anti-Mouse CD19 FITC, Anti-Mouse F4/80 Antigen PE eflouor®610, Anti-Mouse Ly-6G (Gr-1) eFluor®450 and Anti-Mouse CD11b PerCP Cyanine5.5 were all purchased from eBioscience. The chemicals above were all analytical grade.

Preparation of GO

GO was made by a modified Hummers method. Concentrated H_2SO_4 (69 mL) was added to a mixture of graphite flakes (3.0 g) and $NaNO_3$ (1.5 g), and this mixture was cooled to 0°C by an ice bath. Next, $KMnO_4$ (9.0 g) was added in small portions to keep the reaction temperature below 20°C. The reaction was warmed to 35°C and stirred for 7 h. Additional $KMnO_4$ (9.0 g) was added to the mixture. After 12 h stirring at 35°C, the reaction mixture was cooled to room temperature. Ice (400 mL) was poured onto the mixture with 30% H_2O_2 (3 mL). The mixture was washed and collected by centrifugation.

To avoid heavy metal (Mn) contamination in GO product, 3% H_2O_2 was used for reduction of residual $KMnO_4$ and MnO_2 to manganese sulphate salts. These sulphate salts were removed by rinsing with 5% HCl, repeatedly washing with deionized water by centrifugation. Through the above purification process, neutral GO was finally allowed to be used into animals and cells.

Synthesis of GOP

The single layered GO with size of 200-300 nm was achieved by sonication for 2 h in the ultrasonic tank and separated from GO solution by centrifugation with 90000-110000 g for 20 min. Next, NaOH (8 g) and chloroacetic acid (11.658 g) were added to GO aqueous suspension (5 mL, with concentration of 100 μg/mL) for conversion hydroxyl and epoxide groups to carboxyl groups. After 70 min stirring at room temperature, the resulting solution was neutralized with 6 N HCl, purified by repeating rinsing and centrifugation, and diluted to 500 μg/mL with deionized water. EDC was then added to reach the concentration of 20mM, and the mixture was sonicated for 15 min, followed by addition of mPEG-NH_2 to reach 10 mg/mL and stirred for 12 h to react. The GOP was finally obtained by repeating rinsing and centrifugation.

Characterizations of GO and GOP

The carboxyl content was determined by conductometric titration as previous method. A conductivity titration curve was drawn using the value of conductivity as the Y-axis and the corresponding volume of the added NaOH as the X-axis. Based on this titration curve, the carboxyl group was valued by following equation. The carboxyl groups intensity (mmol/g) = $M \times (V_2 - V_1)/W$, where M (mol/L) is the concentration of NaOH, $(V_2 - V_1)$ (mL) is the linear fitting volume of NaOH, and W (g) is the GO quality.

The concentration of GO was quantified by standard absorption curve. GO products at certain concentration were scanned at Infinite M200 (Tecan) to observe absorption situation at 230 nm. Absorption curve was then drawn using the value of absorption as Y-axis and the corresponding concentration of GO samples as the X-axis. According to this curve, the equation for concentration determination was finally established by linear regression.

The zeta potential analysis of the GO/GOP in an aqueous dispersion were performed on Malvern Instruments.

The Fourier transform infra-red (FTIR) spectra was measured with chromatographically pure KBr as background. The GO powder was mixed with spectral pure boronic acid and then pressed into tablet. The tablet was detected on X-ray fluorescence (PANalytical B.V., AXIOS) to conduct the element analysis and determine the heavy metal content of GO before/after washing.

GO/GOP solution was dropped onto the mica slice for AFM. By using AFM (Bruker) at a FastScan mode, the surface morphology of GO/GOP was imaged. In addition, the surface roughness and apparent thickness of GO/GOP (n = 20) were measured and analyzed *via* software NanoScope Analysis 1.8 (Bruker).

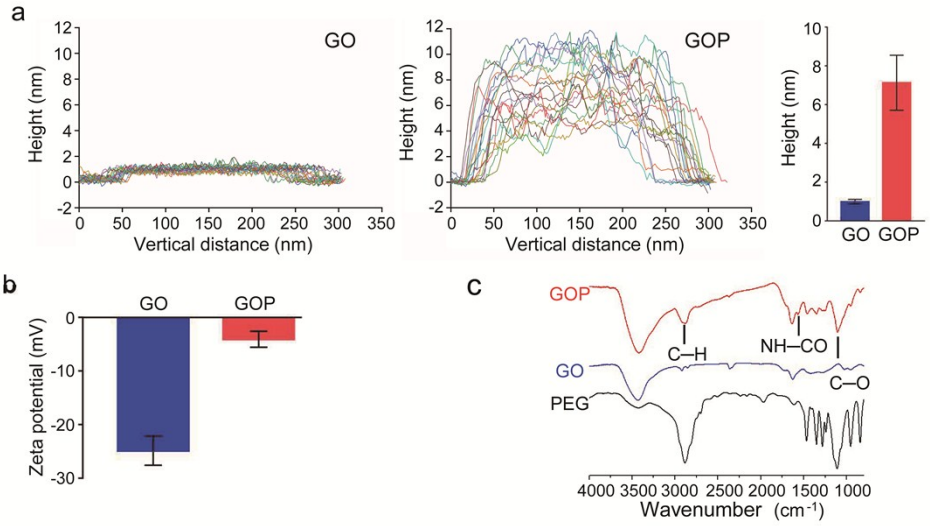

Fig. S1 Characterizations of GO and GOP.

(a) AFM height analysis of GO and GOP. (n = 20). After PEGylation, the height of GO increased from ～1 nm to about 5-10 nm.

(b) Surface zeta potential of GO and GOP. After PEGylation, the surface zeta potential of GO changed from -25 mV to -4 mV. Data represent the mean±s.d. (n = 3).

(c) Fourier transform infrared spectroscopy (FTIR) of PEG, GO and GOP. Decoration of methoxypolyethylene glycol (mPEG-NH_2) to GO was confirmed by C–H (～2900 cm^{-1}) vibrations and C–O (～1100 cm^{-1}) stretching vibration due to functional groups of mPEG-NH_2 molecules, and a characteristic NH–CO stretching vibration (～1640 cm^{-1}), indicating the achievement of PEGylation.

Table.S1 Heavy metal content of GO samples before/after washing.

GO	Mn Conc.%	Fe Conc.%
Before washing	1.06843	0.66581
After washing	0.00310	ND

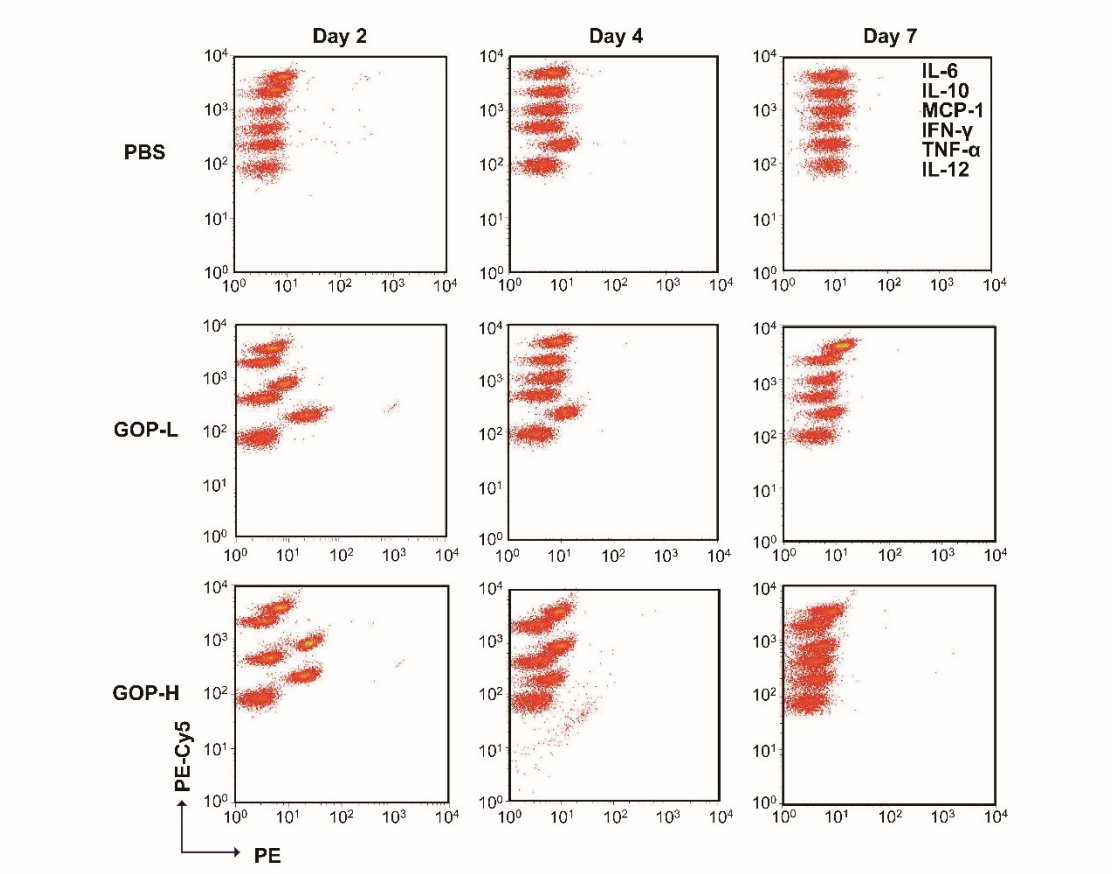

Fig. S2 Flow cytometry analysis of cytokine secretion after single injection at day 2, 4 and 7. Red clusters indicated cytokines of IL-6, IL-10, MCP-1, IFN-γ, TNF-α and IL-12 in order from top to bottom. Compared with PBS group, the single exposure of GOP in the peritoneal cavity induced higher production of inflammatory cytokines. Compared with the low dose (12 mg/kg) of GOP (GOP-L), treatment with the high dose (100 mg/kg) of GOP (GOP-H) elevated the cytokine levels at day 2 and gradually declined during 7 days.

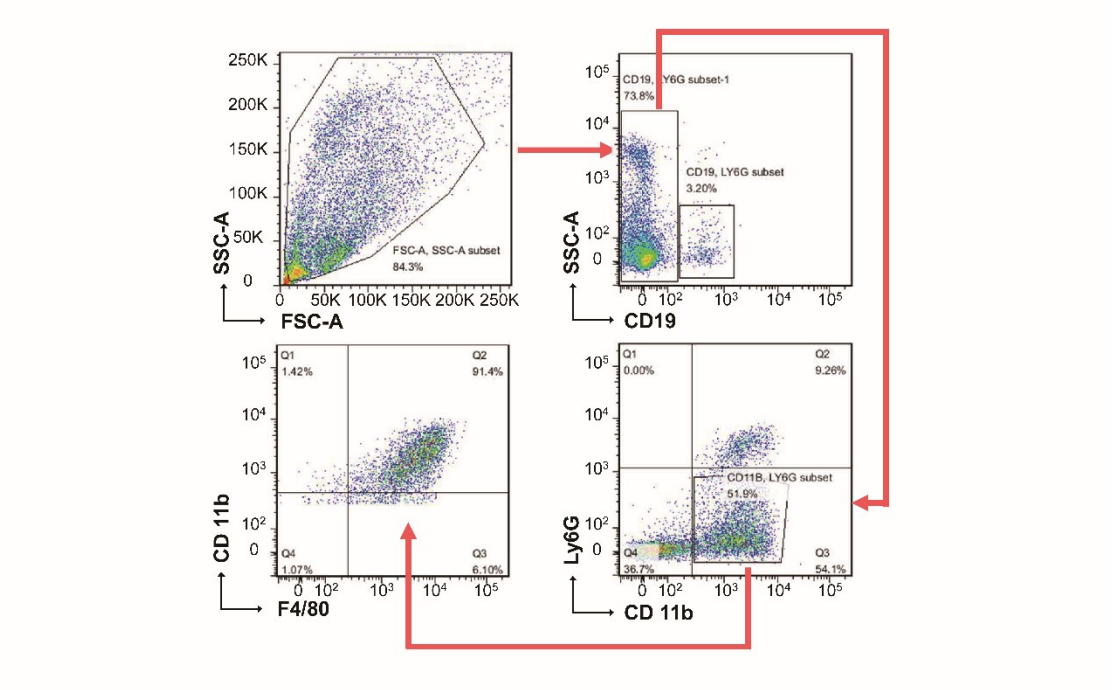

Fig. S3 Gating strategy for identification of different immune cell subsets in peritoneal cavity (CD19, B cell marker; Ly6G, neutrophil marker; CD11b and F4/80, macrophage marker).

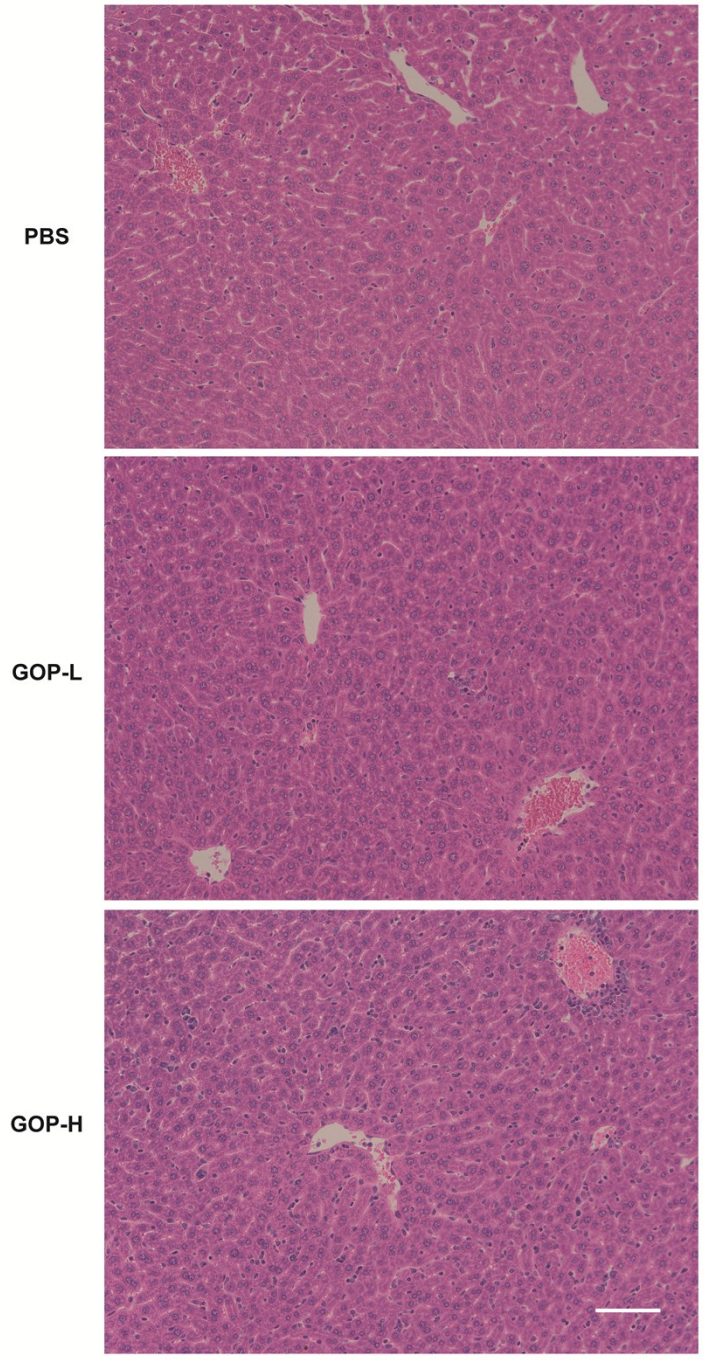

Fig. S4 High-resolution H&E stained images of liver. Scale bar: 100 μm.

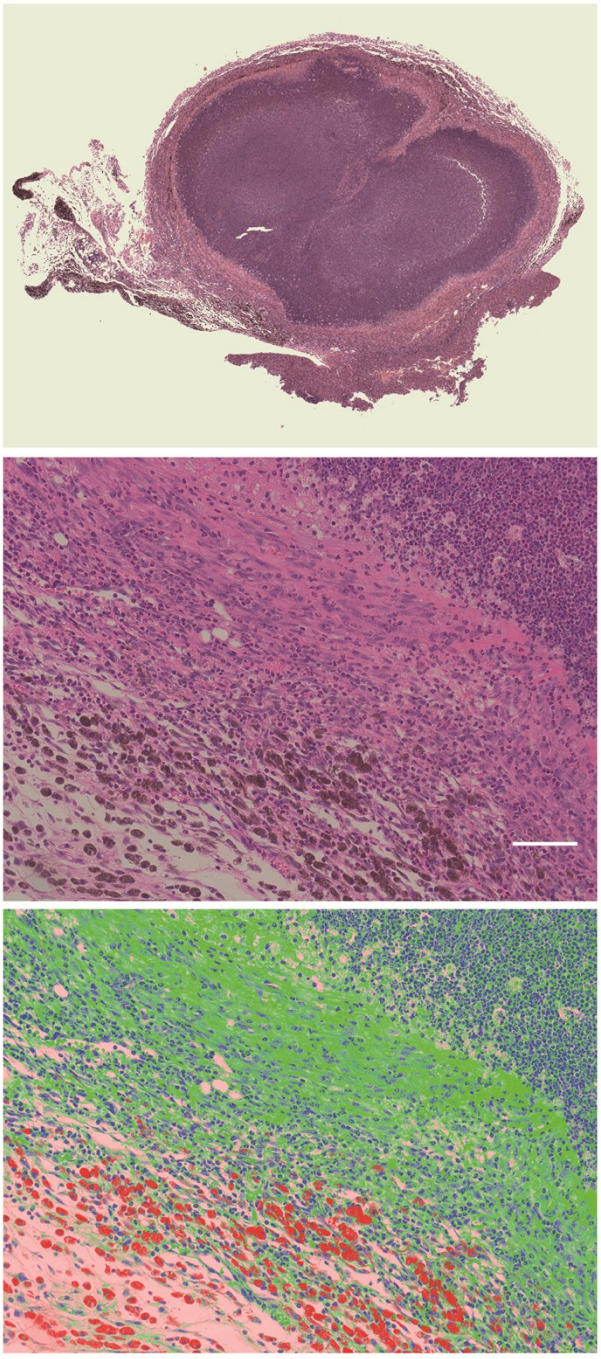

Fig. S5 High-resolution images of liver with GOP infiltration. Full scan (top), local section (middle), manually computed pure spectra and spectral unmixing (bottom). Scale bar: 100 µm.

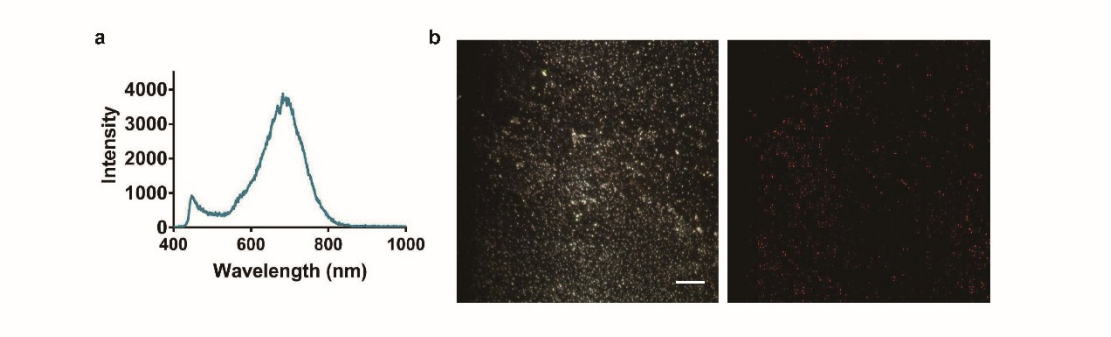

Fig. S6 Identification of brown signals in H&E sample.

(a) The reflected spectra from one of the GOP particles in liver sample. Similar to the reflected spectra of pure GOP in Fig. 1c, the peak at ~690 nm represented GOP. Another peak at ~450 nm represented liver tissue.

(b) Hyperspectral imaging of H&E sample of GOP infiltration in liver and the corresponding spectral mapping of GOP (marked in red). Scale bar, 100 μm. In the reference of left reflected spectra, the map spectral similarities in the hyperspectral image which matched GOP particles in liver tissue were highlighted in red.

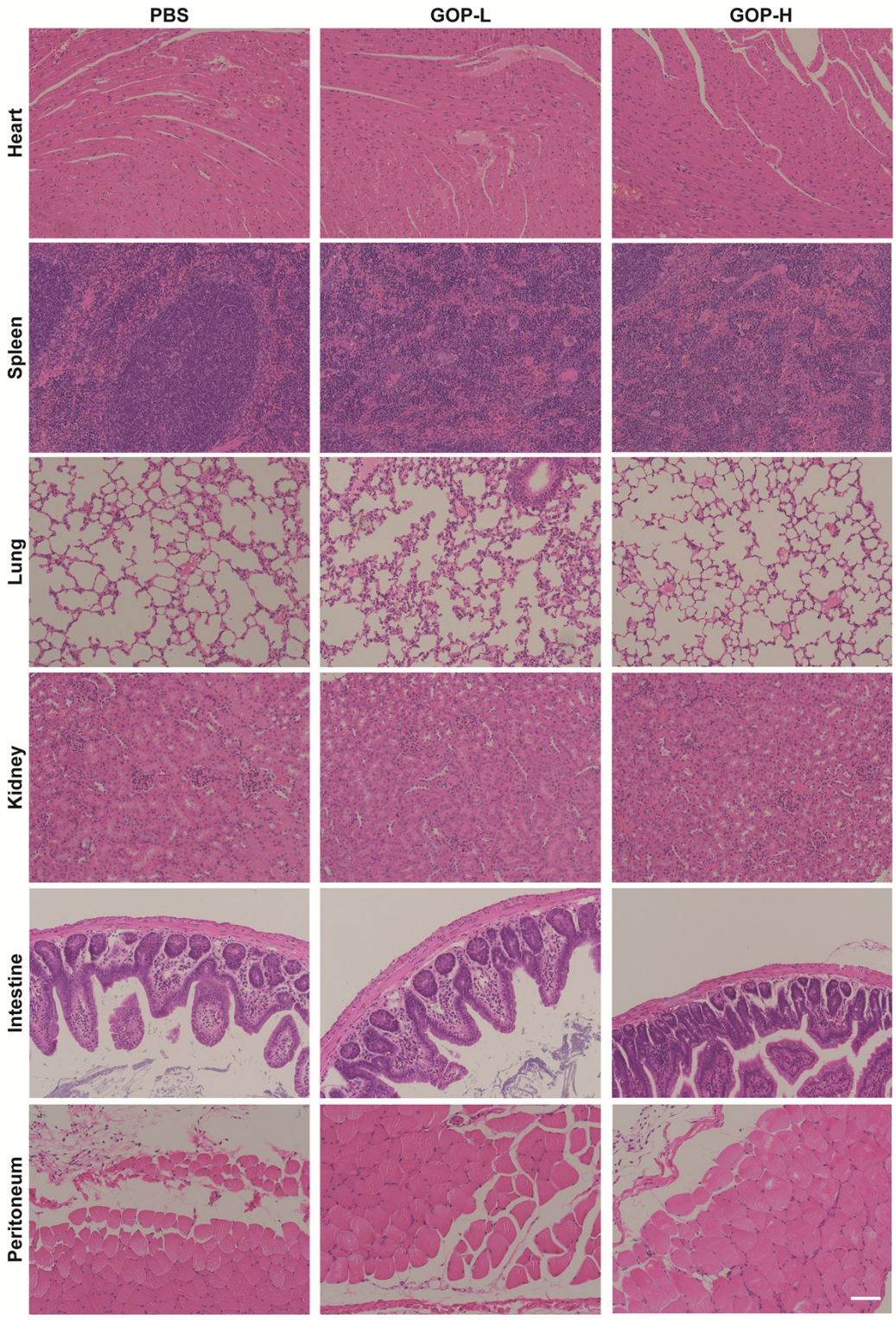

Fig. S7 High-resolution H&E stained images of major organs including heart, spleen, lung, kidney, intestine and peritoneum in different groups at day 7. Scale bar: 100 μm.

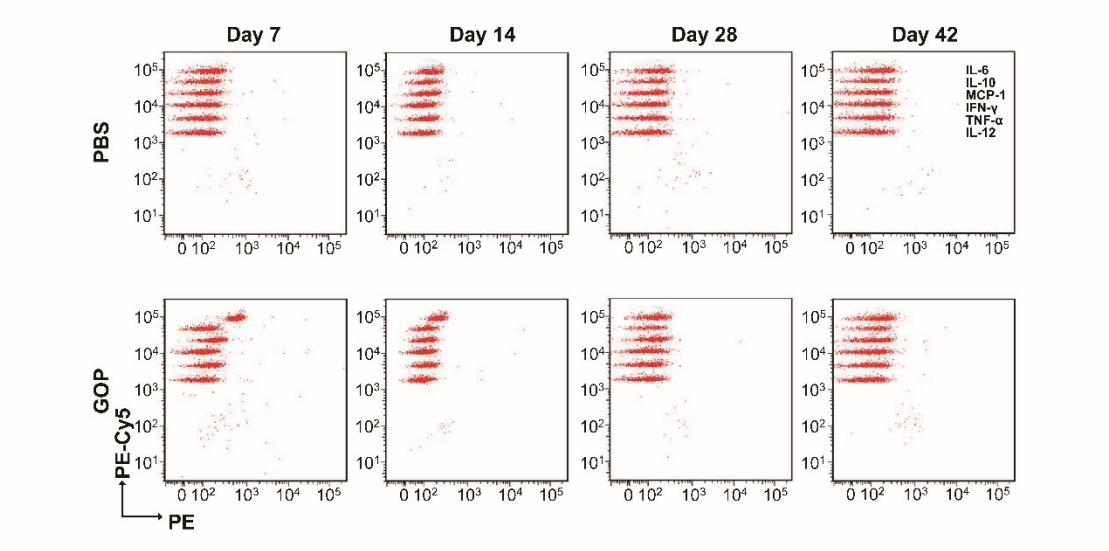

Fig. S8 Flow cytometry analysis of cytokine secretion after multiple injections at day 7, 14, 28 and 42. Red clusters indicated cytokines of IL-6, IL-10, MCP-1, IFN-γ, TNF-α and IL-12 in order from top to bottom. Compared with PBS group, repeated exposure of GOP at dose of 12 mg/kg showed an improvement on the cytokine productions and gradually declined to that of PBS group.

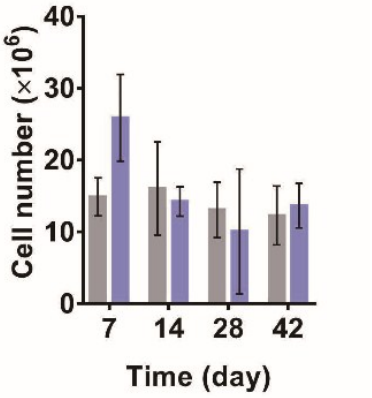

Fig. S9 Total cell counting in peritoneal cavity after multiple GOP injections at day 7, 14, 28 and 42. Data represent the mean±s.d. (n = 5).

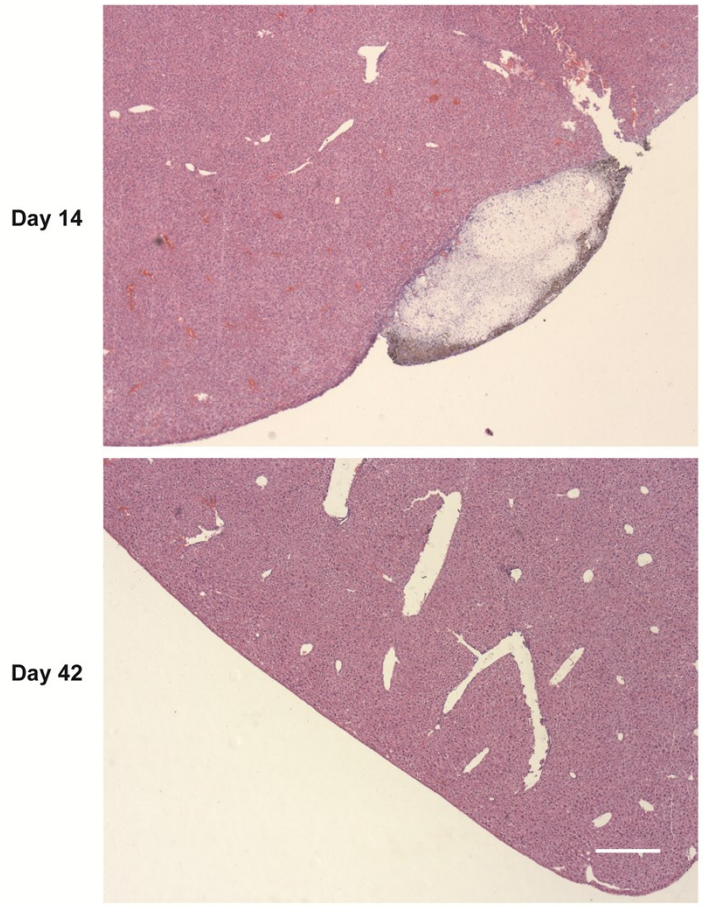

Fig. S10 High-resolution H&E stained images of liver with GOP infiltration at day 14 and 42, respectively. Scale bar: 100 μm.

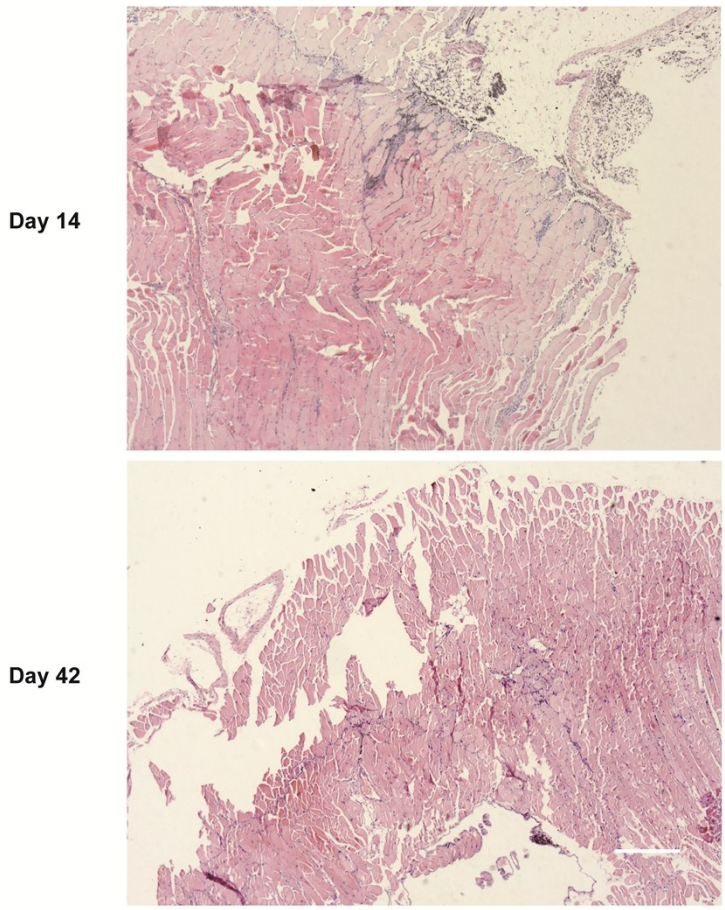

Fig. S11 High-resolution H&E stained images of peritoneum with GOP infiltration at day 14 and 42, respectively. Scale bar: 100 μm.

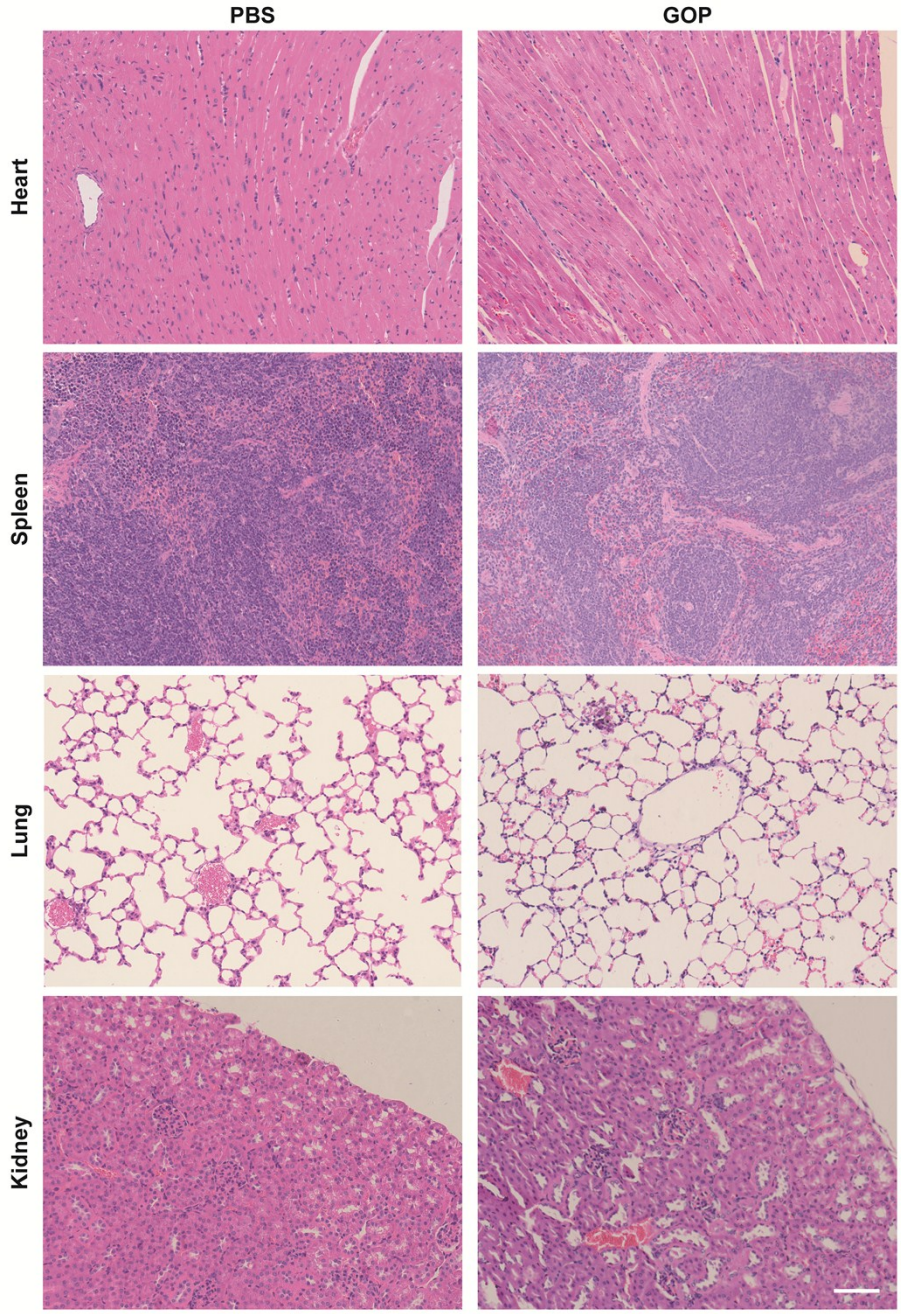

Fig. S12 High-resolution H&E stained images of major organs including heart, spleen, lung, and kidney at day 42. Scale bar: 100 μm.