# Supporting Information

# Large-sized Graphene Oxide Synergistically Enhance Parenchymal Hepatocyte IL-6 Expression Monitored by Dynamic Imaging

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Keywords: Bioluminescence Imaging, Graphene Oxide, Hepatocyte IL-6, ROS, M1 Macrophage

## Experimental Section

### 1. Reagents

N-acetylcysteine (NAC), Pyrrolidinedithiocarbamate (PDTC), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, CAS: 25953-53-8) and N-Hydroxysuccinimide (NHS, CAS: 6066-82-6) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). FITC-mPEG-Amine (Mw=5000) was purchased from Shanghai Yare Biotech, Inc. CLI019 was purchased from Invitrogen. D-Luciferin was purchased from Promega (Madison, Wisconsin). Tissue protein extraction kit was purchased from Cwbiotech (Beijing, China). MDA, GSH-Px and SOD detection kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Fetal bovine serum (FBS) was purchased from PAN Biotech (America). DMEM cell media were purchased from Gibco. GOs of different lateral sizes were supplied by XFNANO (China).

### 2. Cell culture

Mouse macrophage cell line RAW264.7 and mouse hepatocyte cell line Hepa1-6 were purchased from the National Infrastructure of Cell Line Resource. Cells were individually cultured in DMEM (Hyclone), supplemented with 10% fetal bovine serum (FBS, PAN) and 100 U/mL penicillin/streptomycin (Gibco).

### 3. ROS assays in Kupffer cell, Hepa1-6 and RAW264.7

For ROS measurement, cells were cultured in 6-well plates, and subsequently treated with GOs. FACS analysis was performed 30 minutes after loading the DCF probe (Beyotime, S0033) into cells according to the instructions.

### 4. The detection of IL-1β, IL-6 and TNF-α by ELISA

Concentrations of IL-1β, IL-6 and TNF-α in serum, liver homogenate and cell supernatant were detected using an ELISA Kit (R&D) according to standard operation

protocol provided by the manufacturer. Serum was obtained by centrifuging samples at 2,000 rpm for 10 min at 4 ℃ after 15 min of incubation in 37 ℃. Mouse livers were removed and homogenized in 0.1 (g ml-1) of tissue protein extraction reagent (Cwbiotech) containing a protease inhibitor cocktail, according to the manufacturer's protocol. After the samples were centrifuged at 10,000 g for 15 min at 4 ℃, the supernatants were collected.

## 5. Preparation and Characterization of GO nanosheets

GO nanosheets were purchased from Nanjing XFNANO Materials Tech Co., Ltd., which were prepared from flake graphite by the modified Hummers' method[1]. After the oxidation step, the GOs were washed for at least three times and then filtrated. Low speed centrifugation was conducted to remove multi-layered GOs and increase the purity of the single-layered GO sheets. The supernatant was collected for freeze-drying overnight to obtain GO powder. Afterward, to acquire different-sized GOs, the GO powder was re-suspended in deionized water and then sonicated in a bath sonicator (Kunshan, 300W) for several h, and longer sonication time led to small lateral size.

The X-ray photoelectron spectroscopy (XPS) data were recorded using an Escalab 250Xi spectrometer with monochromated Al Kα (hυ = 1436.6 eV). Survey scans (0–1350 eV) were taken of each sample at a pass energy (constant analyzer energy mode) of 200 and 1 eV step size. Narrow scans for carbon 1S and and oxygen 1S were taken at pass energy (constant analyzer energy mode) of 50 eV and 0.1 eV step size. Fourier transform infrared (FT-IR) spectra were acquired on a Nicolet-460 FT-IR spectrophotometer, which were performed on powder GO samples combined with KBr and pressed into pellets. And data in the spectral region of 4000 to 500 cm-1 was recorded and analyzed. X-ray diffraction (XRD) patterns of GO sheets were collected using a Bruker D8 Phaser equipment with a Cu-Kα wisource (λ=1.5418Å, 40 KV, 40 mA). Raman measurements were carried out on a Thermo Fisher DXR Raman spectrometer. A He–Ne laser operating at= 532nm was used as the excitation source.

## 6. Labeling GOs with FITC

The method of labeling FITC onto graphene oxide is as follows, 0.06 g EDC and

0.12 g NHS were dissolved in water, dropped into 2 mL GO suspensions (0.5 mg/mL) under sonication for the first 10 minutes, and intensely stirred for 2 h at room temperature. Then, the GO sheets were collected by centrifugation and re-dispersed in 1mL deionized water. Finally, 1 mL suspension of FITC-mPEG-Amine at the concentration of 0.1 mg/mL was added to the EDC activated GOs system under constant stirring for 4 h before being subjected to the centrifugation process. The FITC-labeled GOs pellets were washed three times by repeating the centrifuge/water adding steps before storage for further use.

**7. Hydrodynamic gene delivery**

The pTA-attB was kindly provided by M. P. Calos (Department of Genetics, Stanford University, USA). Plasmid pattB-IL-6-Luc was generated by inserting attB, a 297-bp fragment in pTA-attB, into the Kpn I site of pIL-6-Luc[2]. A mouse codon-optimized PhiC31 recombinase (PhiC31o) expression vector was purchased from Addgene, Cambridge, USA (pPhiC31o). An Endotoxin-Free Maxi Kit (Qiagen, Hilden, Germany) was used for purifying the plasmid DNA. The hydrodynamic gene delivery technique has been detailed in previous studies[2]. In short, this procedure involves the rapid intravenous injection of the transfection agent within 5 seconds; the injection contains the plasmid DNA and a volume of saline equivalent to 10% of the mouse body weight.

**8. Confocal Raman imaging of GOs injected mouse tissue**

For Raman mapping, slides were focused in a DXR Raman microscope (Thermo Fisher) and excited with a 633 nm laser (10 mW). The laser spot size is ~ 1μm. Image was obtained by scanning a 100 μm × 100 μm area in 10 μm × 10 μm steps, and collecting the Raman spectrum at each spot (10 s integration time). G-peak signal at ~ 1600 $cm^{-1}$ was chose to generate the mapping image.

**9. Bioluminescence imaging**

Bioluminescence imaging was executed using an IVIS Spectrum system (PerkinElmer, Inc.). The mice were injected i.p. with D-luciferin (150 mg $kg^{-1}$ mouse

weight) 5 min prior to being imaged. Quantitation of luciferase activity and data analysis were performed with Living Image software 4.5.2 (Xenogen, Alameda, CA). Fluorescence imaging was executed using the same system. The data analysis was performed by Spectral Unmixing for the purpose of background subtraction.

Macrophage polarization assessment through Flow Cytometry

After treatment with 20 μg/mL S-GO or L-GO for 24 h, cells were collected and resuspended with cell stain buffer (Biolegend) at a concentration of $5.0\times10^5$ per 100 ul. Cells were subsequently incubated with APC anti-mouse CD86 (Biolegend, 105011) or FITC anti-mouse CD206 (Biolegend, 141703) at 4 ℃ for 30 min, and were washed for 3 times by PBS, then add 5μL of PI (BD. 556547) to each sample prior to FACS analysis.

**10. Flow Cytometry**

For Flow Cytometry analysis, cells were harvested by centrifugation at 1600 rpm for 5 min within cell stain buffer (Biolegend, 420201). Fc receptors were blocked by pre-incubating cells with 0.4 μg of TruStain FcX™ PLUS (anti-mouse CD16/32) Antibody per $10^6$ cells in a 100μl volume for 5-10 minutes on ice. After that, the corresponding antibodies were stained with for 30 min at 4℃. The reaction was stopped by washing twice with cell stain buffer and then re-suspend the cell with 200 μL of cell stain buffer. The positive population was gated by appropriate iso-type controls. For intracellular staining, the cells were harvested and fixed by the Fixation Buffer (Biolegend, 420801) for 20 min. After fixation, the cells were washed twice with Intracellular Staining Permeabilization Wash Buffer (Biolegend, 421002) and then stained with primary antibody in the washing buffer for 1 h followed by washing and fluorescent labeled secondary antibody for 30 min. Then wash the cell 2 times and re-suspend the cell with 200 μL of cell stain buffer for analysis. The antibodies used in this assay were listed as follows: FITC anti-mouse F4/80 (Biolegend,123107), FITC anti-mouse CD31 (Biolegend, 102405), FITC anti-mouse CD45 (Biolegend, 103108), IL-6 XP Rabbit mAb (CST, 12912), Cytokeratin 18 Rabbit Monoclonal Antibody (Beyotime, AF1285).

**11. Immunofluorescence staining**

Cells on cover slips or tissue sections were fixed in 4% paraformaldehyde at room temperature for 10 min, permeabilized with 0.1% Triton-100 (Solarbio, T8200) for 10 min, and blocked with 1.0% BSA (Solarbio, A8020) for 30 min before applying primary Ab at dilutions ranging from 1:50-1:500 overnight at 4℃ followed with secondary antibody for 1 h at room temperature. Nucleus was stained by DAPI (abcam). Images were taken by a NIKON A1RHD25 Laser Scaning Confocal Microscope. The antibodies used in this assay were listed as follows: IL-6 XP Rabbit mAb (CST, 12912), Anti-Cytokeratin 18 (Abcam, ab668), Anti-F4/80 (Abcam, ab6640).

**12. The generation of bone marrow-derived macrophages (BMDMs)**

The procedure of BMDM generation has been described previously[3]. BALB/c mice (6-8 weeks) were sacrificed for femur collection, and the femurs were immediately placed into a tube containing sterile PBS on ice. Bone marrow was flushed out by a syringe filled with 1640 medium; the marrow was then gently dispersed by pipet. Cell suspensions were centrifuged at 300 g for 8 min. The cell pellets were then re-suspended and differentiated in 1640 medium with 10% endotoxin-free fetal bovine serum (FBS) and 50 ng $ml^{-1}$ macrophage colony-stimulating factor (M-CSF, PeproTech, 315-02-10) for 7 days. Then, the cells were seeded in 24-well plates ($2 \times 10^5$ cells $well^{-1}$) and cultured in 1640 medium plus 10% FBS.

**13. Western blotting**

Post treatment, cells were collected and washed with cold PBS. Cells were then lysed using the Cell lysis buffer for Western and IP (Beyotime Inc., China). The concentrations of protein extracts were analyzed with the Enhanced BCA protein assay kit (Beyotime). The same amount of proteins was subjected to 10% SDS-PAGE, and then transferred onto PVDF (Merck millipore) membranes. Western blot analysis was thus preformed, as described previously[3]. The antibodies used in the Western blot assay were listed as follows: anti-NFκB p65 antibody (Arigo, ARG65677), anti-NFκB p65 phospho (Ser536) antibody (Arigo, ARG51518), HRP-labeled Goat Anti-Rabbit IgG (Beyotime, A0208), HRP-labeled Goat Anti-Mouse IgG (Beyotime, A0216), Actin

antibody (Beyotime, AA128).

**14. RT-PCR**

Total RNA was isolated from mouse livers using an EZgene Tissue RNA Kit (Biomiga), according to the manufacturer's protocol. RNA (1 μg) was reverse transcribed into cDNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO) following the manufacturer's protocol. Amplification was performed for 40 cycles using THUNDERBIRD SYBR qPCR Mix (TOYOBO). Relative expression levels of target genes were normalized to that of mouse actin and reported as relative fold changes over expression level of the control group. The primers involved in this study are listed in Table S1 in the Supporting Information.

**Table S1**: Sequences of primers for RT-PCR.

| | Forward | Reverse |
|---|---|---|
| Luciferase | 5'-ATACCGGGA AAACGCTGGGC-3' | 5'-TCAAGGCGTTGGTCGCTTCC-3 |
| IL-1β | 5'-CTCCACCTCAATGGACAGAA-3' | 5'-GCCGTCTTTCATTACACAGG-3' |
| TNF-α | 5'-AATGGCCTCCCTCTCATCAGTT-3' | 5'-CCACTTGGTGGTTTGCTACGA-3' |
| NOS2 | 5'-AATCTTGGAGCGAGTTGTGG-3' | 5'-CAGGAAGTAGGTGAGGGCTTG-3' |
| Arg1 | 5'-CTCCAAGCCAAAGTCCTTAGAG-3' | 5'-AGGAGCTGTCATTAGGGACATC-3' |
| Mgl1 | 5'-TGGCCTGAAGCTGACAAGTA-3' | 5'-AGGCCGATCCAACTAACCACAT-3' |
| Mrc2 | 5'-TGCAAGCAATGCATCCAAGCCT-3' | 5'-ACGGCTTTCCGTGTGAGTTT-3' |
| Actin | 5'-GCTTCTTTGCAGCTCCTTCGT-3' | 5'-GACCCATTCCCACCATCACA-3' |

**15. Preparation of Primary liver cells**

Hepatocytes were isolated from mice by the modified collagenase method as previously described[4, 5]. Mice were anesthetized and livers were perfused with 37 ℃ preheating in 20 mL buffer solution (10mM Hepes, 3 mM KCl, 130 mM NaCl, 1mM

NaH2PO4,10 mM glucose) containing 0.5 mM EDTA (pH 7.4) at a rate of 5 ml/min, and then with 37 ℃ preheating in buffer solution containing 5 mM CaCl2 and 100 U/mL collagenase type IV (Sigma) at a rate of 2 mL/min. The perfusate buffer solution enters the liver through portal vein (PV) and flows out through the inferior vena cava (IVC). The actual duration of 2ml/min digestion with collagenase type IV was within 6 minutes with periodically applying pressure to the IVC (4-5 seconds each time). The liver would expand and shrink with the repeated pressure to the IVC which could improve the efficiency of digestion. It should be noted that we recommend a slight liver expansion by IVC pressing. If the expansion was severe, it might cause mechanical damages to the hepatocytes which would influence the cell survival rate. The primary hepatocyte was purified by repeated low speed centrifugation (4 ℃, 40g×2min, 4 times). After purification, hepatocytes were plated at 6-well culture plates which were pro-coated with rat tail tendon collagen type I. The culture media we used was commercialization mouse hepatocytes complete DMEM medium (Procell, China, CM-M033). Remove unattached dead cells by gentle pipetting 2 h later, and the cell was maintained at 37 ℃ in a humidified incubator under a 5% CO2 atmosphere.

Primary Kupffer cells were isolated as previously described[5-7]. After perfusion of the liver with collagenase type IV, the non-parenchymal cells were separated from hepatocyte by repeated low speed centrifugation, and then the cells were re-suspended by PBS and located on the upper layer of 11.5% and 20% OptiPrep, respectively. After centrifugation (4 ℃, 15min, 400g), the upper layer of 20% OptiPrep contained the majority of Kupffer cell and other non-parenchymal cells. We purified Kupffer cell by selective adherence that removed unattached cells thoroughly after 1 h incubation in DMEM containing 10% FBS and 1% P/S at 37 ℃ in a humidified atmosphere containing 5% CO2. As determined by trypan blue exclusion, cell viabilities of the primary hepatocyte and Kupffer cell were more than 80% and 90%, respectively, in all experiments.

**16. Statistical analysis**

The data are presented as the mean ± SD. Statistical comparisons between different

treatments were assessed by t-tests, one-way ANOVA as introduced in legend respectively.

**Table S2 :** XPS, Raman characterization and Zeta potentials of the three GOs.

| | Zeta potential (mV) | $I_D/I_G$ | XPS | | | | |
|---|---|---|---|---|---|---|---|
| | | | C-C/C=C | C-OH | C=O | O=C-H | C/O ratio |
| **S-GO** | -32.87±1.12 | 1.00±0.02 | 31.80% | 18.89% | 42.71% | 6.60% | 1.94 |
| **M-GO** | -31.65±1.37 | 1.00±0.03 | 29.03% | 13.16% | 54.76% | 3.05% | 1.99 |
| **L-GO** | -30.98±0.87 | 0.98±0.02 | 29.99% | 11.61% | 55.29% | 3.11% | 1.98 |

**Figure S1**

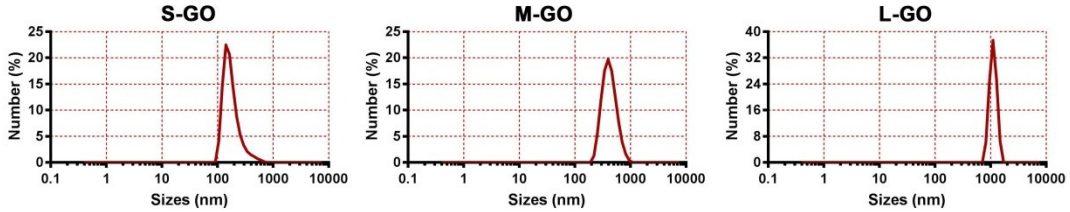


Figure S1. DLS measurements of S-GO, M-GO and L-GO, repectively. The data represent three independent experiments.

**Figure S2.**

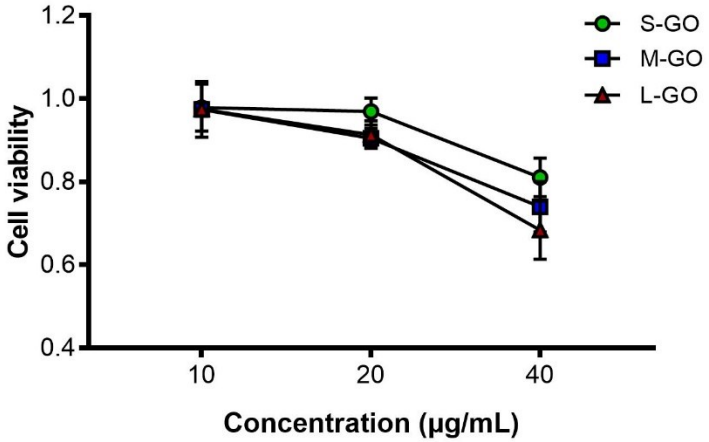


**Figure S2.** The cell viability of Hepa1-6 after the 24 hours co-incubation with GOs. Hepa1-6 cells were plated in 96-well plates at a density of $5\times10^3$ cells per well.10 ug/mL, 20 ug/mL, and 40ug/mL GOs were added and the procedure was followed by the instruction of 'MTT Cell Proliferation and Cytotoxicity Assay Kit (Beyotime,

China)'.

**Figure S3**

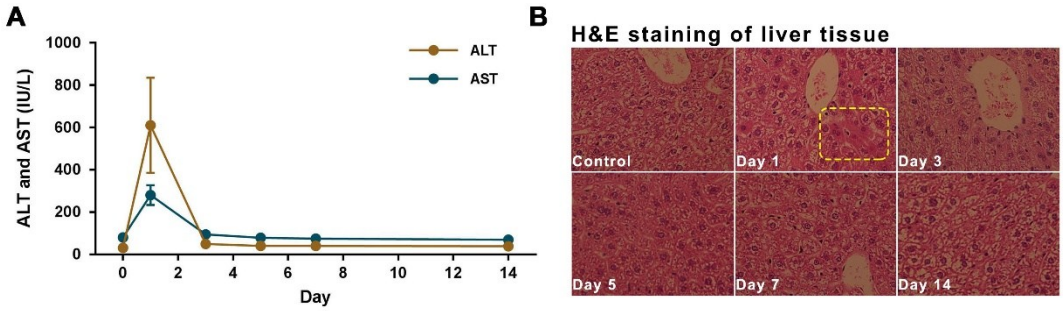


**Figure S3.** (A) ALT and AST levels in serum after hydrodynamic delivery. (B) H&E staining of liver tissue after hydrodynamic delivery. Yellow dotted frame indicates the location of liver damage. The data represent three independent experiments.

**Figure S4**

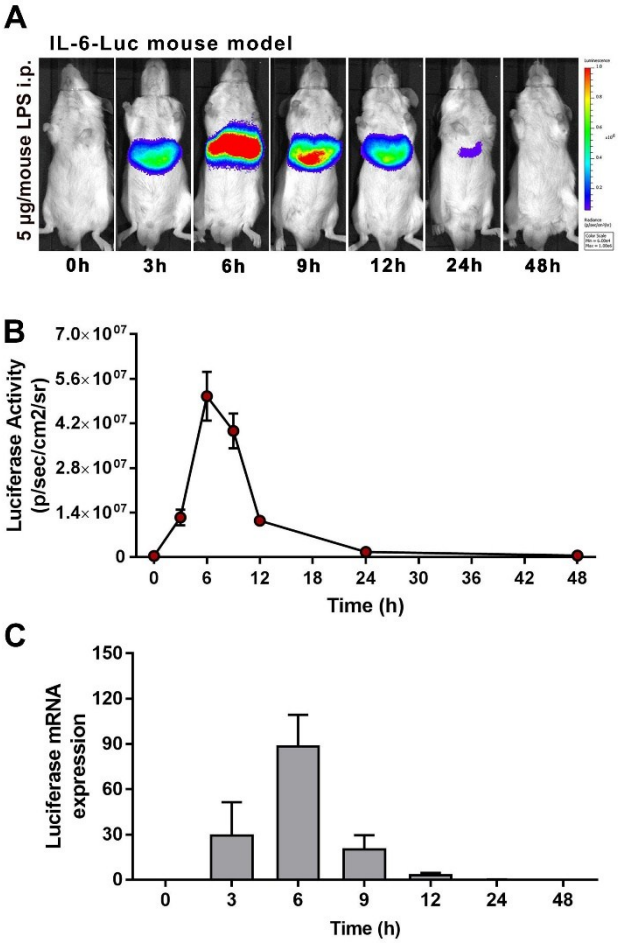


**Figure S4.** Establishment of visualized hepatocyte IL-6-Luc mouse model. (A) LPS was injected i.p. into the model mice at a dose of 10 μg/mouse. Then, hepatocyte IL-6 expression was monitored using BLI. (B) The luciferase signal intensity in (B) was quantified using Living Image software. (C) Luciferase mRNA was measured by RT-

PCR at the corresponding time points of BLI imaging. (E) The level of luciferase protein in IL-6-Luc mouse 6 h after LPS treatment by Western Blot. Data are represented as mean ± SD (n = 4). The results are representative of two independent experiments.

**Figure S5**

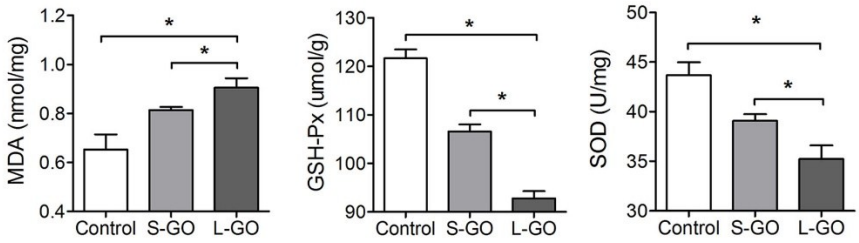


**Figure S5.** MDA, GSH-Px and SOD detection upon 5 mg/kg S-GO and L-GO exposure, respectively.

**Figure S6**

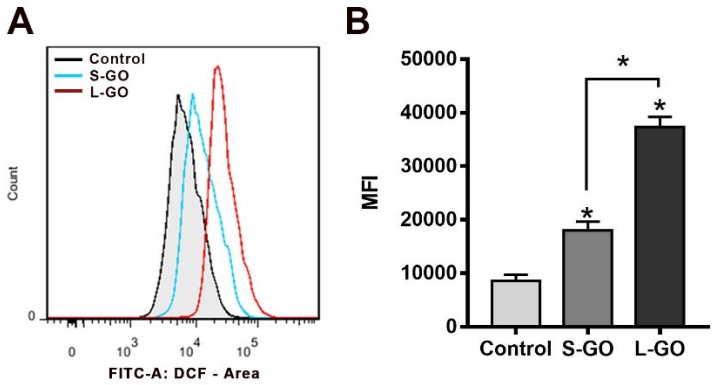


**Figure S6**. ROS generation in Kupffer cells after co-incubation with S-GO and L-GO, respectively. (A) FACS analysis of ROS generation after staining with fluorescent probe (DCF-DA). (B) Quantitative statistical analysis of MFI. *$p < 0.05$ as determined by one-way ANOVA followed by Turkey post hoc test. The data represent three independent experiments.

**Figure S7**

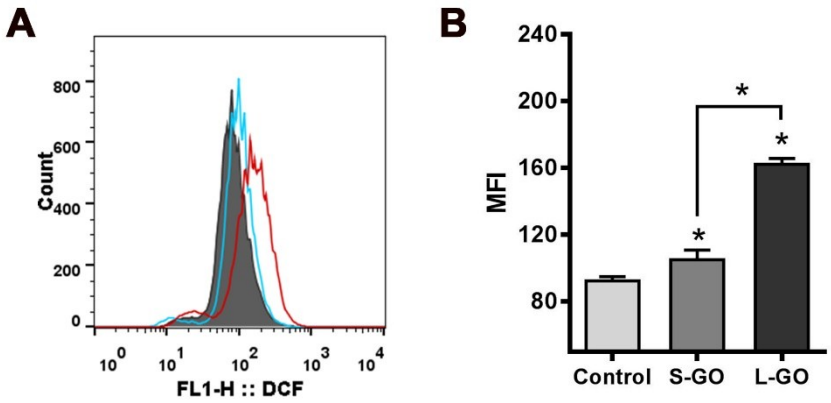


**Figure S7**. ROS generation in RAW264.7 after co-incubation with S-GO and L-GO, respectively. (A) FACS analysis of ROS generation after staining with fluorescent probe (DCF-DA). (B) Quantitative statistical analysis of MFI. *p < 0.05 as determined by one-way ANOVA followed by Turkey post hoc test. The data represent three independent experiments.

**Figure S8**

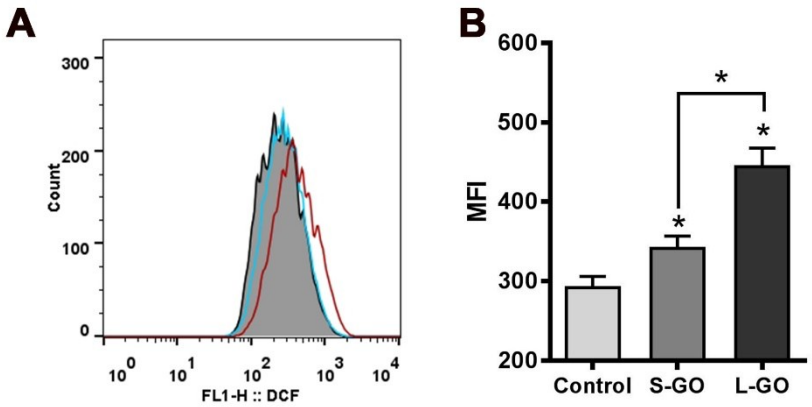


**Figure S8.** ROS generation in Hepa1-6 after co-incubation with S-GO and L-GO, respectively. (A) FACS analysis of ROS generation after staining with fluorescent probe (DCF-DA). (B). Quantitative statistical analysis of MFI. *p < 0.05 as determined by one-way ANOVA followed by Turkey post hoc test. The data represent three independent experiments.

**Figure S9.**

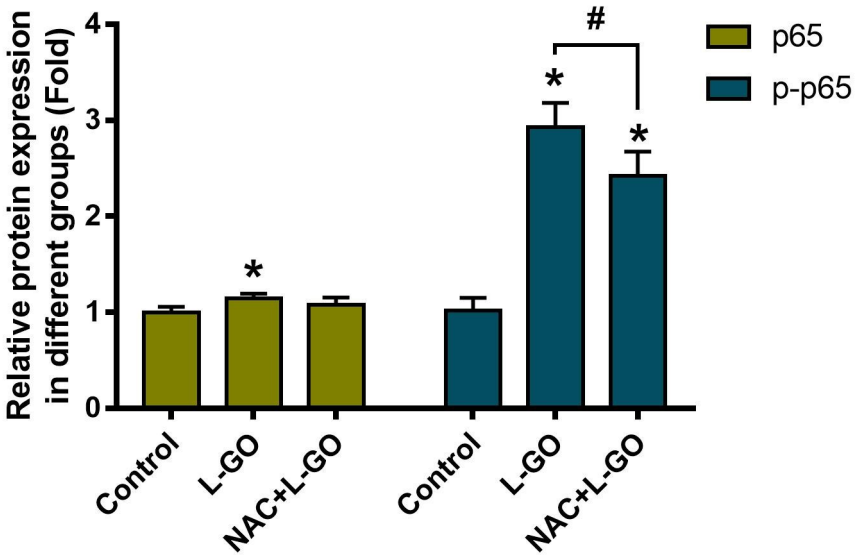


**Figure S9.** Statistic bar graph of the Western Blot data in Figure 4C. All data are represented as mean ± SD (n = 4). * represents the comparison with the control group. $p < 0.05$ as determined by one-way ANOVA followed by Turkey post hoc test.

**Figure S10**

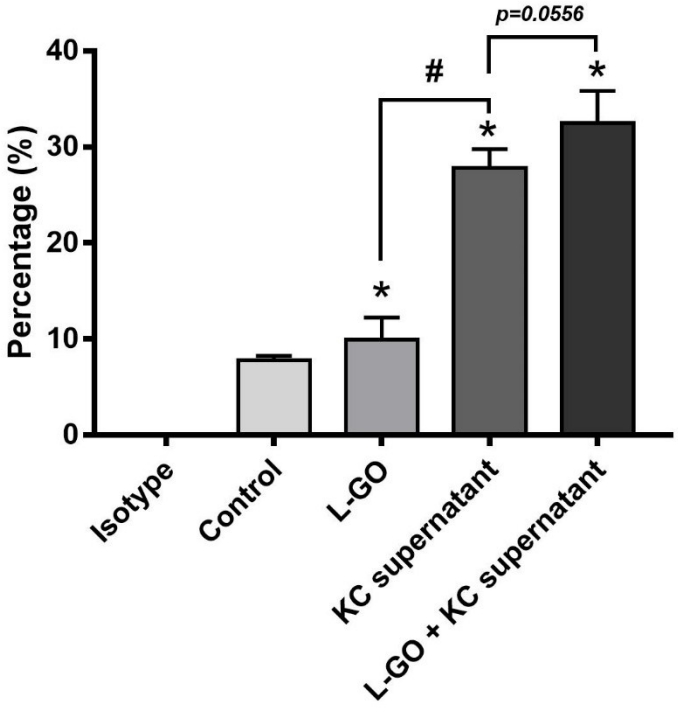


**Figure S10.** Statistic bar graph of the FACS in Figure 5A. All data are represented as mean ± SD (n = 4). * represents the comparison with the control group. $p < 0.05$ as

determined by one-way ANOVA followed by Turkey post hoc test.

**Reference:**


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