

1 **Supplemental material:**

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3 **Lateral size of graphene oxide determines differential cellular**
4 **uptake and cell death pathways in Kupffer cells, LSECs, and**
5 **hepatocytes**

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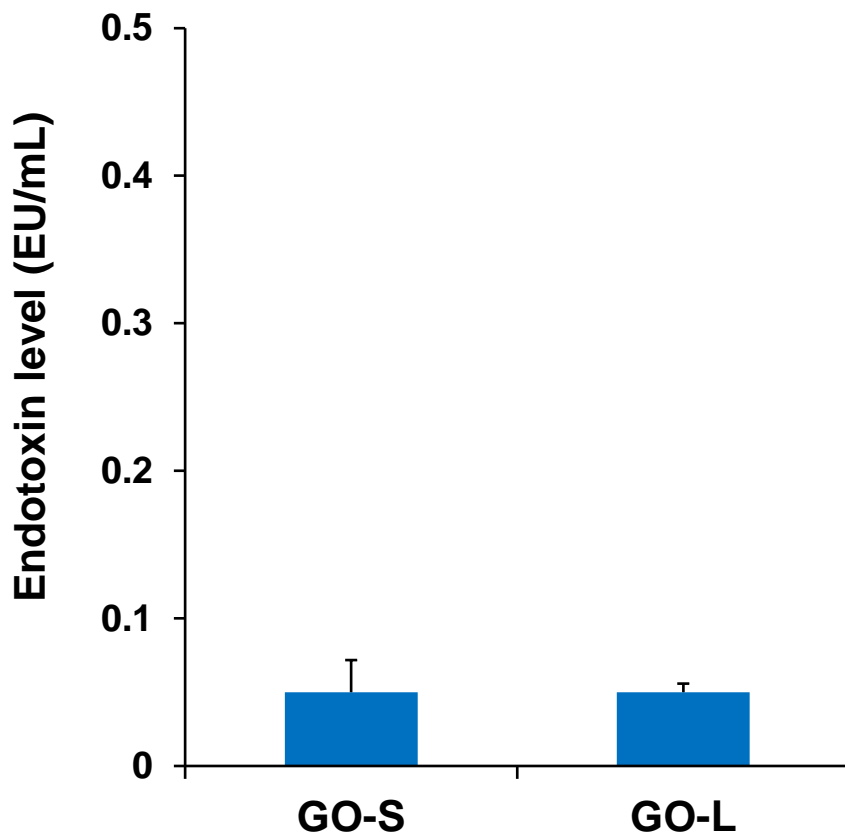
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26 **Figures**

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29 **Fig. S1.** The endotoxin level of GOs suspended at 50 $\mu\text{g}/\text{mL}$ in DI water was determined using
30 a Limulus Amebocyte Lysate assay kit (Lonza, Walkersville, MD). The endotoxin limit (for
31 medical devices) recommended by the FDA is 0.5 EU/mL.

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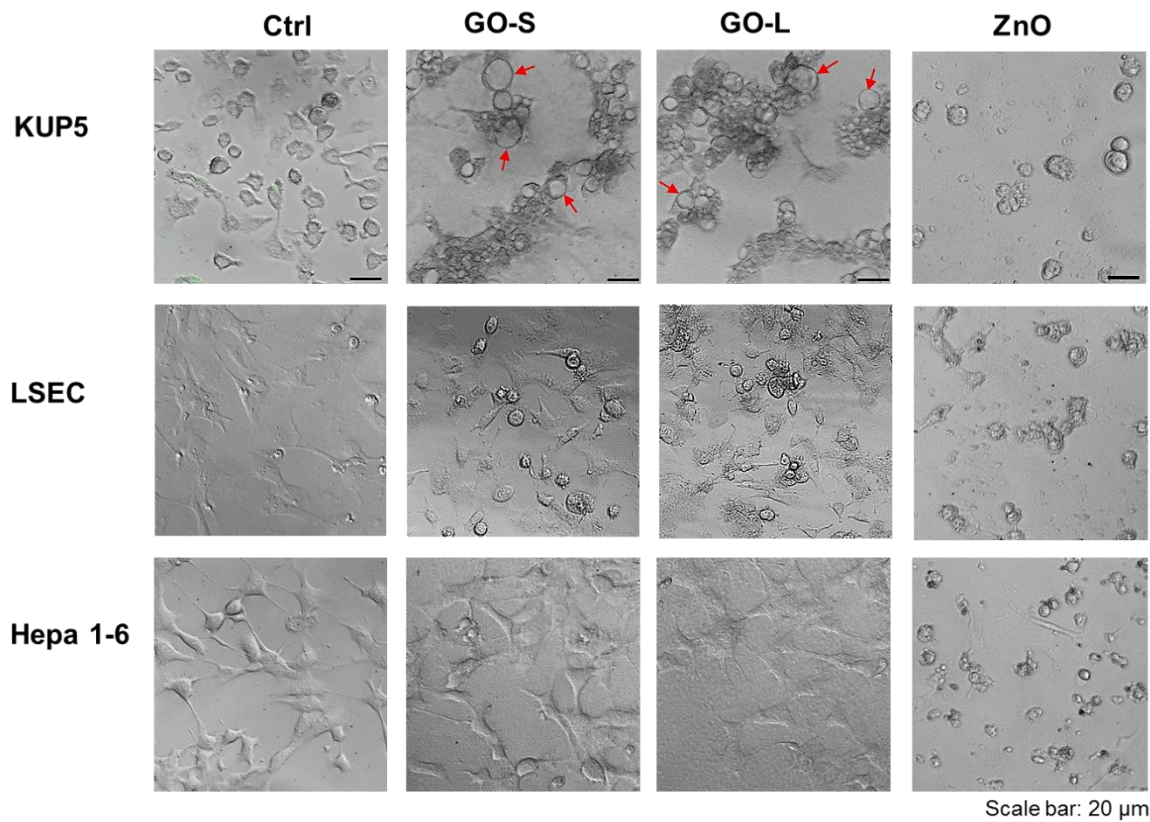
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45 **Fig. S2.** Optical microscope images to compare the KUP5, LSEC, and Hepa 1-6 cell
46 morphological changes by GO. The cells were exposed to 12.5 $\mu\text{g}/\text{mL}$ GO for 16 h at 37 $^{\circ}\text{C}$
47 and 5% CO_2 in a 12-well plate, respectively. The morphology of the cell was monitored using
48 a Zeiss Optical Microscope (Carl Zeiss Microscopy LLC, White Plains, NY, USA). Red arrows
49 indicate significant morphological changes by GO sheets in cells. The scale bar in the image is
50 20 μm .

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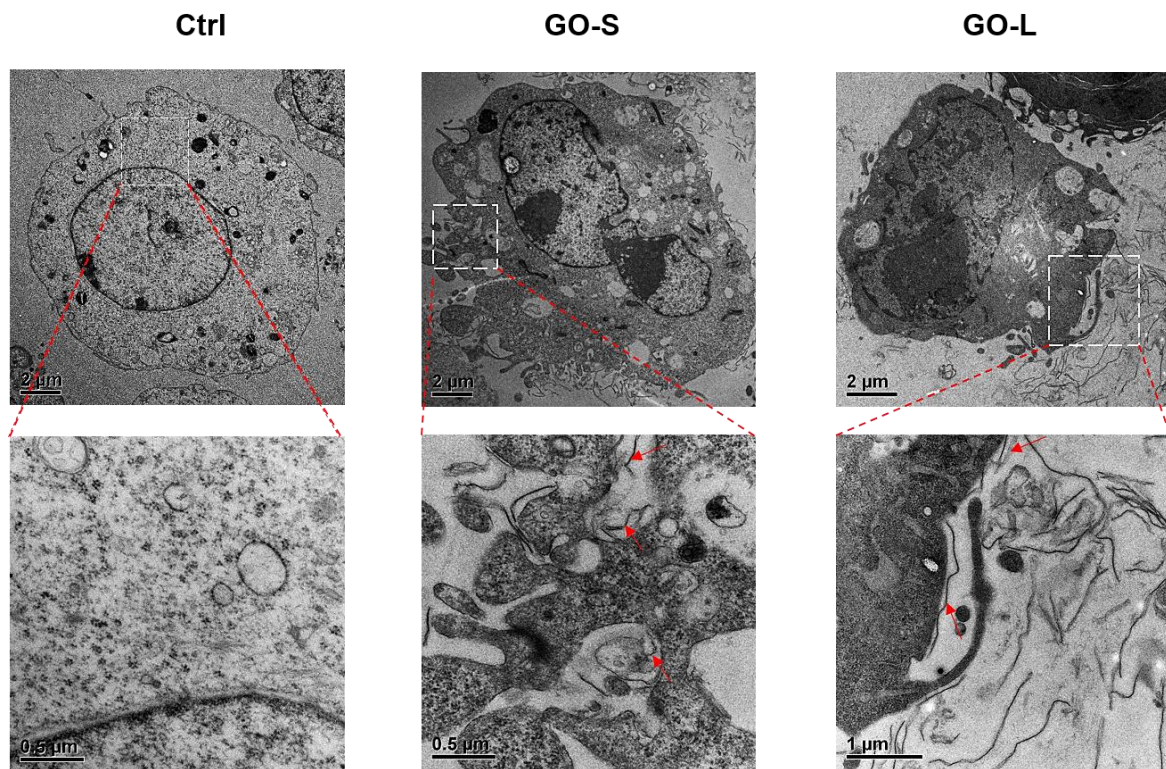
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61 **Fig. S3.** Visualizing the interactions of GO with LSECs by TEM. After exposure to 50 μg/mL
62 of GO for 16 h, the cells were washed, fixed, and stained for TEM viewing under a JEOL 1200-
63 EX TEM with an accelerating voltage of 80K eV. Red arrows indicate GO sheets within the
64 cytosol or on the plasma membrane. TEM images for untreated cells were also shown. Scale
65 bar in the upper row is 2 μm; Scale bar in the lower row is 0.5 or 1 μm.

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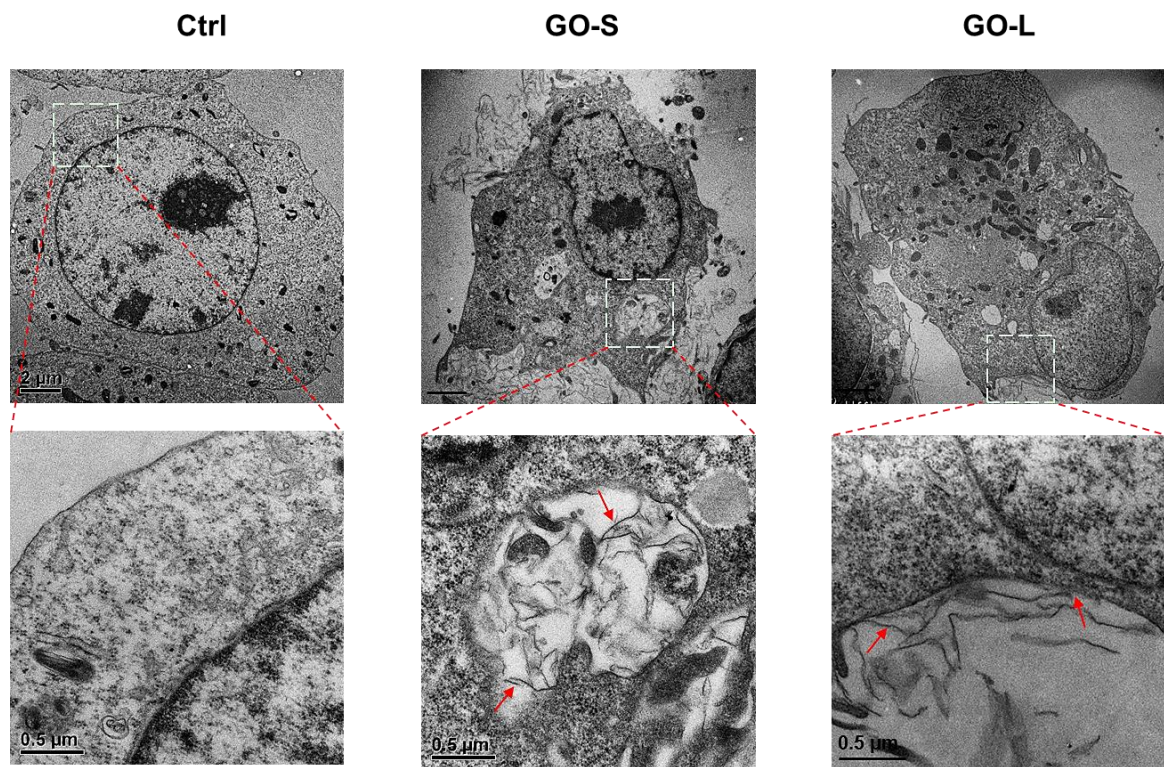
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78 **Fig.S4.** Visualizing the localization of GO in Hepa 1-6 cells by TEM. After exposure to 50
79 μg/mL of GO for 16 h, the cells were washed, fixed, and stained for TEM viewing. Red arrows
80 indicate GO sheets within the cytosol or on the plasma membrane. TEM images for untreated
81 cells were also shown. Scale bar in the upper row is 2 μm; Scale bar in the lower row is 0.5 μm.

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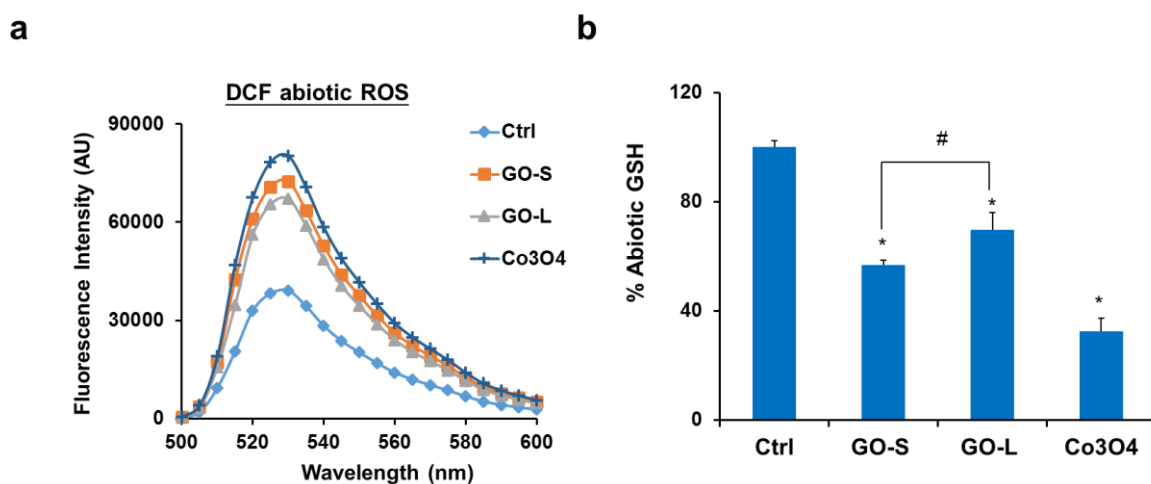
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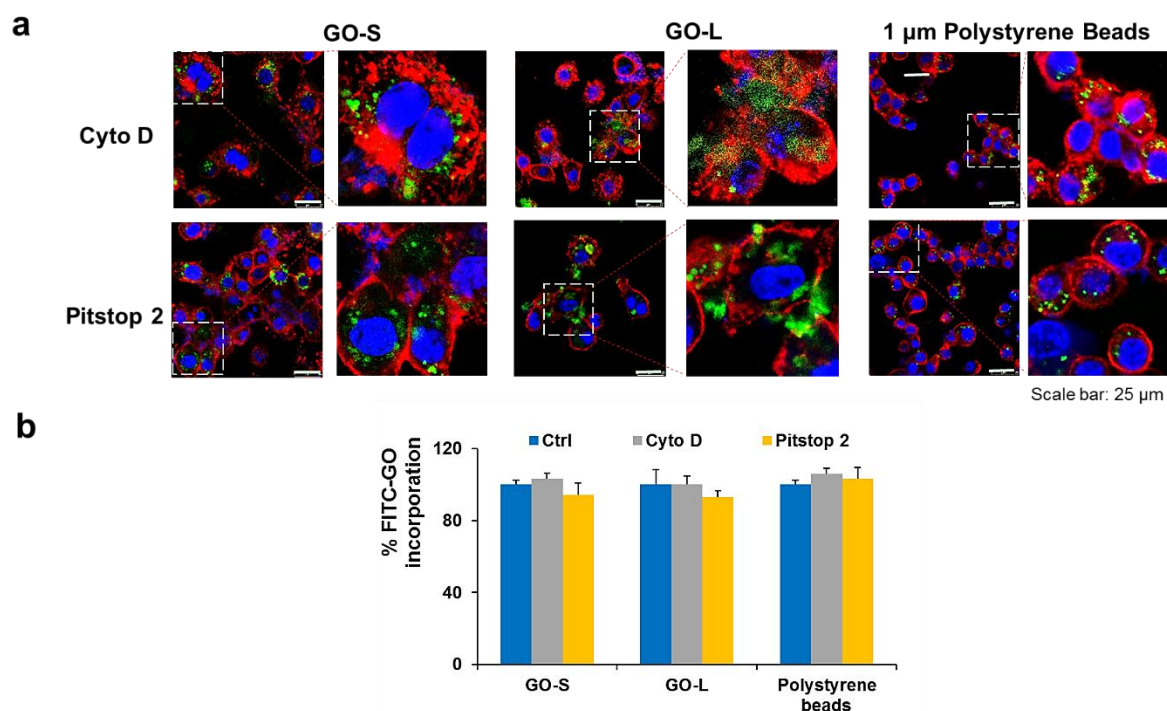


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Fig. S5. Determination of intrinsic oxidative stress of GOs. (a) The abiotic ROS generation by 50 $\mu\text{g/mL}$ of GO was determined by H_2DCFDA fluorescence. DCF fluorescence emission spectra in the range of 500–600 nm were collected with an excitation wavelength of 490 nm. The treatment of Co_3O_4 was set as a positive control. (b) Assessment of the abiotic GSH content was performed by adding 10 μL of GOs at 50 $\mu\text{g/mL}$ to a 96-well plate together with 90 μL of GSH-Glo agent for 30 min. The luminescence was detected by a SpectraMax M5e microplate reader. The treatment of Co_3O_4 was set as a positive control. Asterisk (*) means $P < 0.05$, compared to the control, and #, $P < 0.05$, indicates significance between GO-S and GO-L treatments.

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116 **Fig. S6.** Effects of cellular uptake inhibitor on FITC-GOs incorporation in KUP5 cells. (a)

117 Confocal images to determine the cellular localization of FITC-GO in KUP5 cells under the

118 cellular uptake inhibitor treatments. Before exposure to GO, KUP5 cells were treated with 5

119 μg/mL of cytochalasin D for 1 h and 20 μM of pitstop 2 for 0.5 h, respectively. After staining

120 with Hoechst 33342 dye and Alexa Fluor 594-labeled WGA antibody, the cells were visualized

121 under a Leica Confocal SP8-SMD confocal microscope. The scale bar in the left panel image

122 is 25 μm. (b) Quantification for the effects of cellular uptake inhibitor on FITC-GOs

123 incorporation in KUP5 cells by a fluorescence microplate reader.

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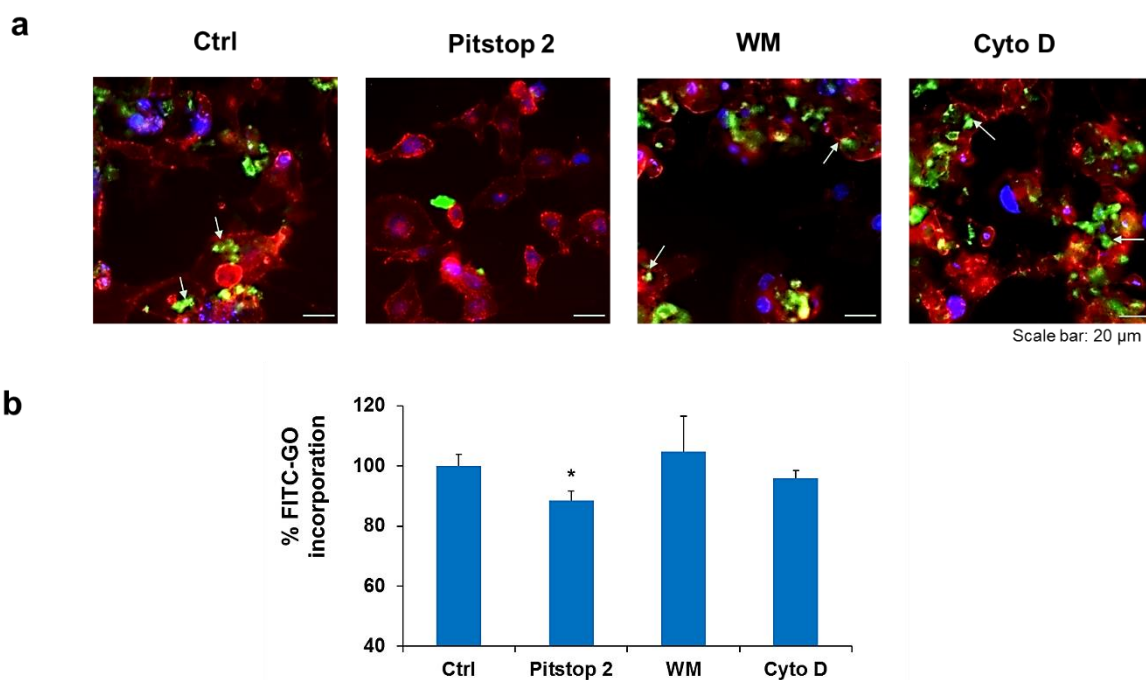
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134 **Fig. S7.** Effects of cellular uptake inhibitor on FITC-GOs incorporation in LSECs. (a)

135 Representative fluorescence images to determine the cellular localization of FITC-GO in

136 LSECs under the cellular uptake inhibitor treatments. Before exposure to GO, cells were treated

137 with 20 μ M of pitstop 2 for 0.5 h, 1 μ M of wortmannin for 0.5 h, and 5 μ g/mL of cytochalasin

138 D for 1 h, respectively. White arrows indicated the uptake GO inside cells. The scale bar is 20

139 μ m. (b) Quantification for the effects of cellular uptake inhibitor on FITC-GOs incorporation

140 in LSECs by a microplate reader. *, $P < 0.05$, indicates significance compared to the control.

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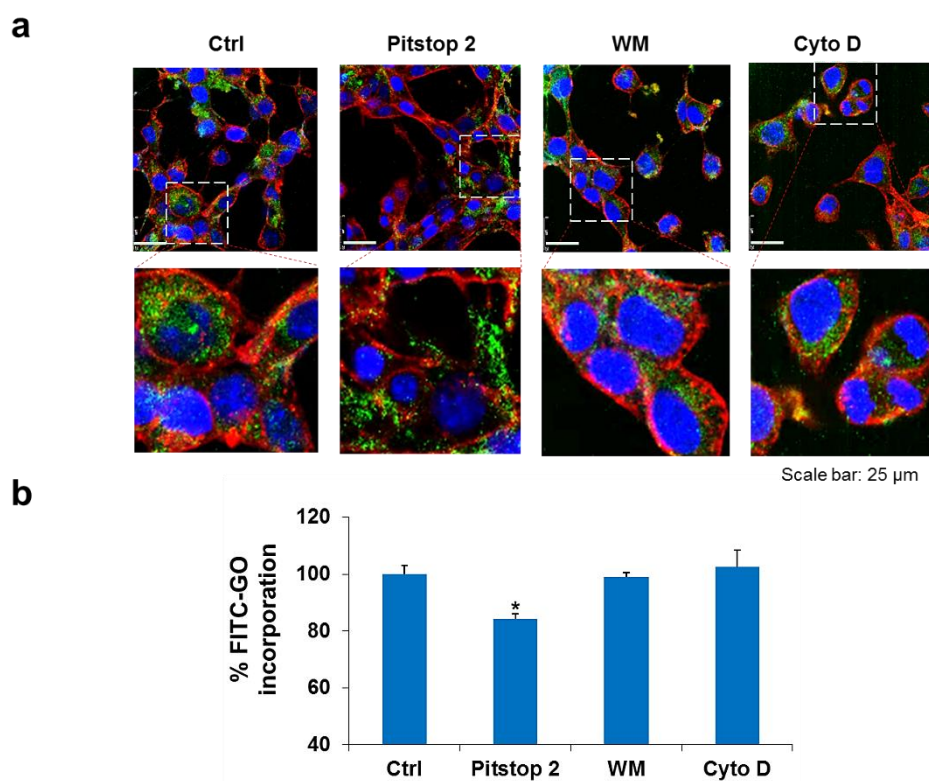
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153 **Fig. S8.** Effects of cellular uptake inhibitor on FITC-GOs incorporation in Hepa 1-6 cells. (a)

154 Representative confocal images to determine the cellular localization of FITC-GO in Hepa 1-

155 6 cells under the cellular uptake inhibitor treatments. Before exposure to GO, Hepa 1-6 cells

156 were treated with 20 μ M of pitstop 2 for 0.5 h, 1 μ M of wortmannin for 0.5 h, and 5 μ g/mL of

157 cytochalasin D for 1 h, respectively. The scale bar in the left panel image is 25 μ m. (b)

158 Quantification for the effects of cellular uptake inhibitor on FITC-GOs incorporation in Hepa

159 1-6 cells by a microplate reader. *, $P < 0.05$, indicates significance compared to the control.

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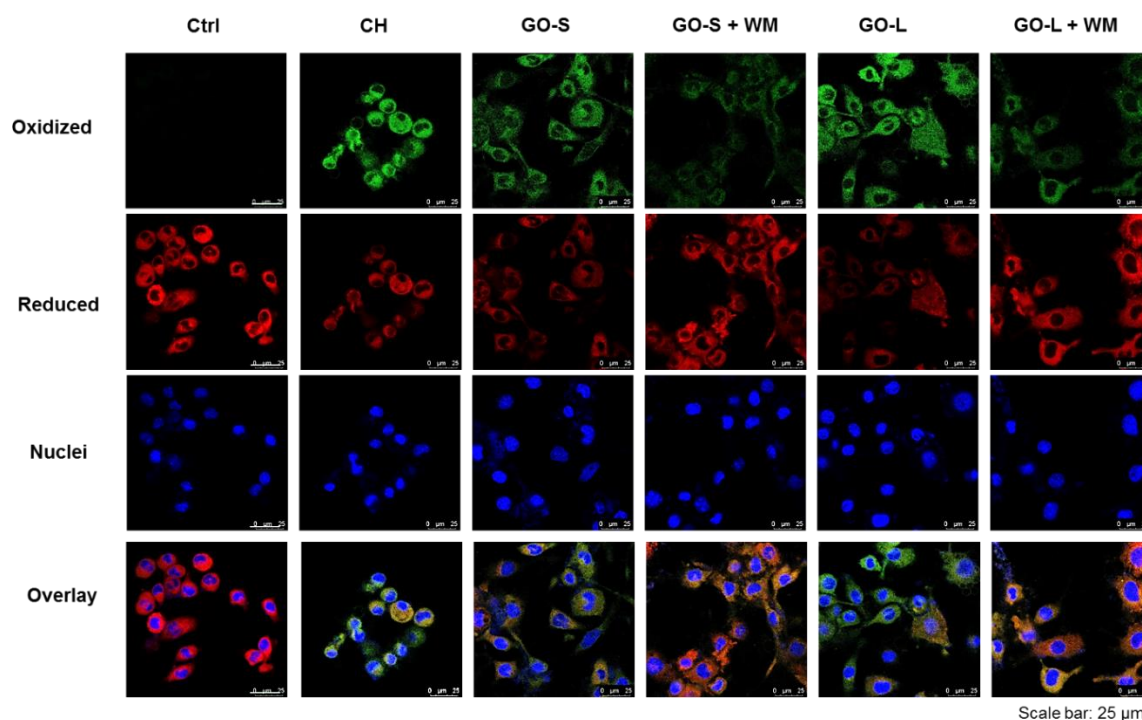
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170 **Fig. S9.** Assessment of the plasma membrane lipid peroxidation production by GO in KUP5
171 cells pretreated with WM. Confocal images to demonstrate the reduction of lipid peroxidation
172 production in KUP5 cells. The images were acquired to visualize the reduced (red) and
173 oxidized (green) fluorescent dye at excitation/emission wavelengths of 581/591 nm and
174 488/510 nm. The scale bar is 25 μm.

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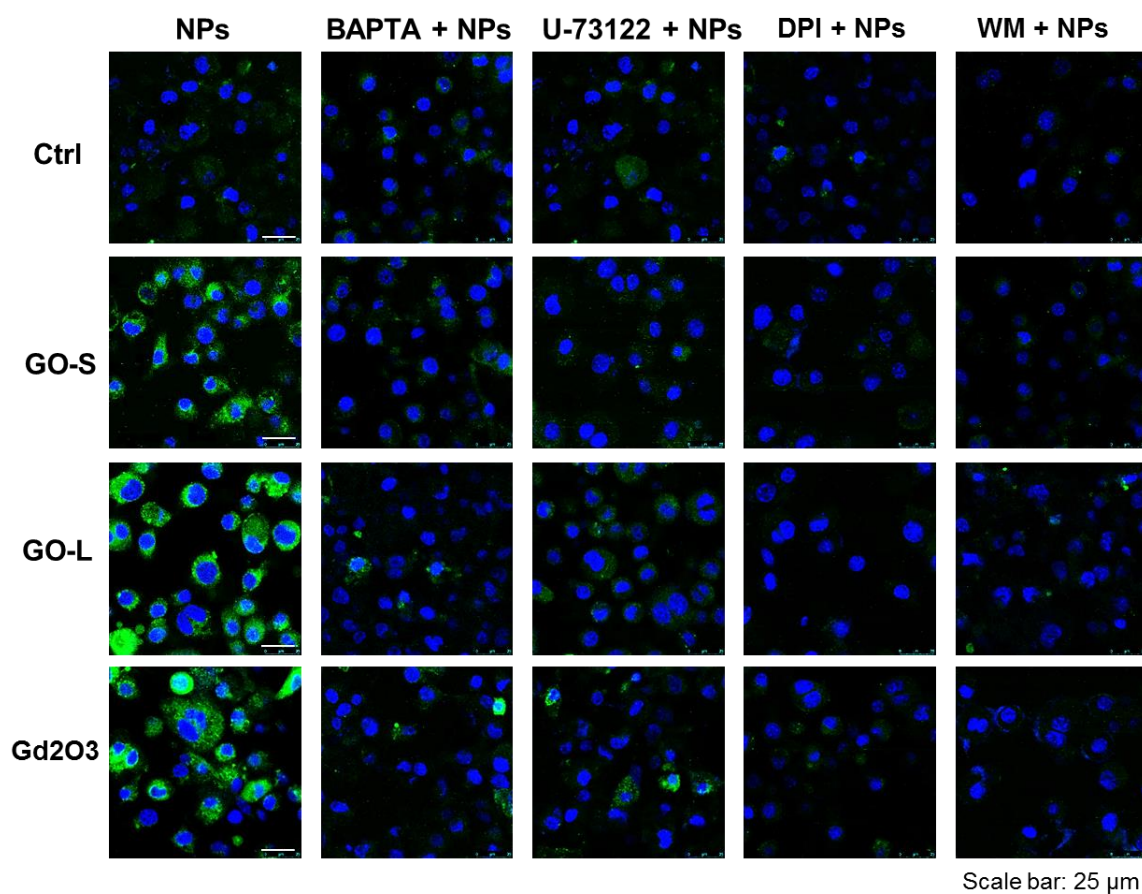
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188 **Fig. S10.** Intercellular calcium accumulation assessed by confocal microscopy. KUP5 cells
189 were pretreated with or without inhibitors (BAPTA as calcium chelator and U-73122 used as
190 PLC inhibitor, DPI used as NADPH oxidase, WM used as phagocytosis inhibitor) before
191 treatment of 12.5 μg/mL of GO or Gd₂O₃ for 16 h. Cells were stained with 5 μM of Fluo-4 AM
192 ester (green) for 60 min and Hoechst 33342 (blue) for 15 min. The scale bar is 25 μm.

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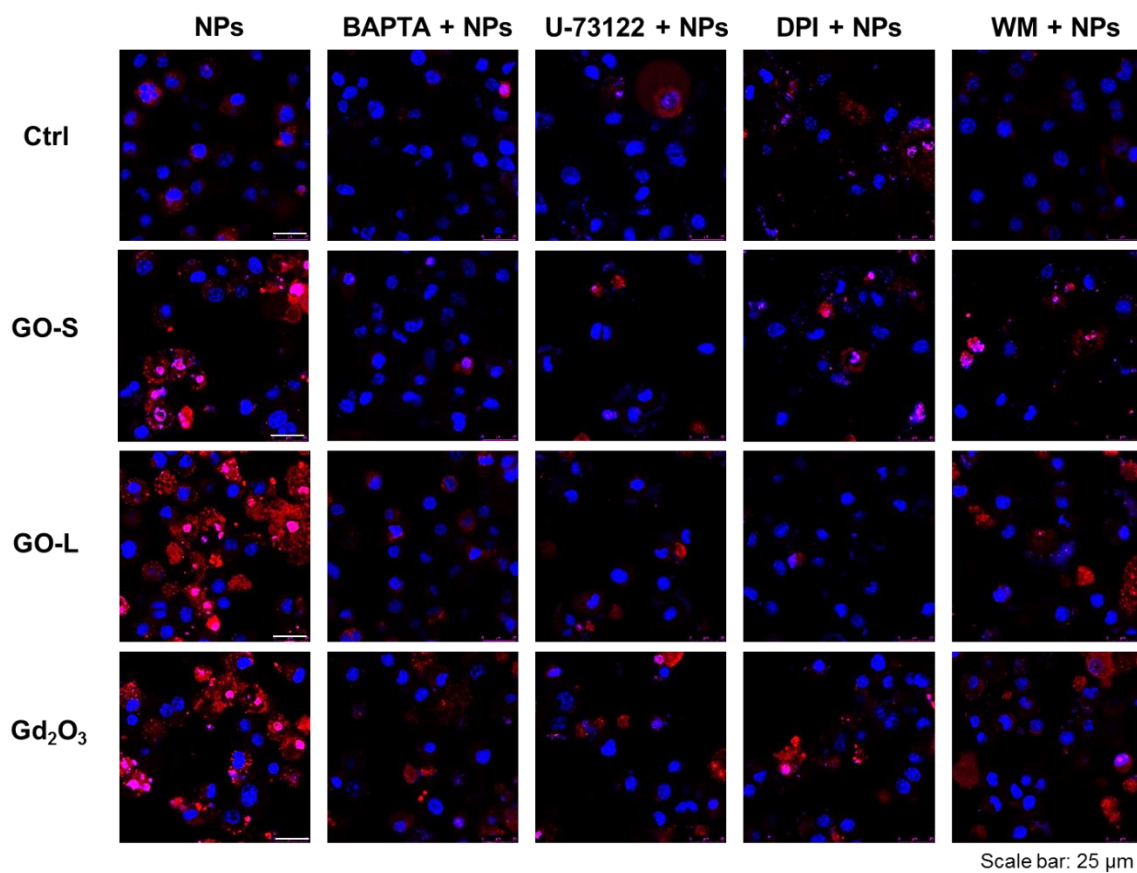
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203 **Fig. S11.** Cellular mitochondrial ROS generation in KUP5 cells post-exposed to GO. KUP5
204 cells were pretreated with or without inhibitors BAPTA, U-73122, DPI, and WM before
205 exposure to 12.5 $\mu\text{g}/\text{mL}$ of GO or Gd_2O_3 (positive control) for 16 h. Cells were stained with 5
206 μM of red MitoSOX and Hoechst 33342 for 15 min, respectively. The scale bar is 25 μm .

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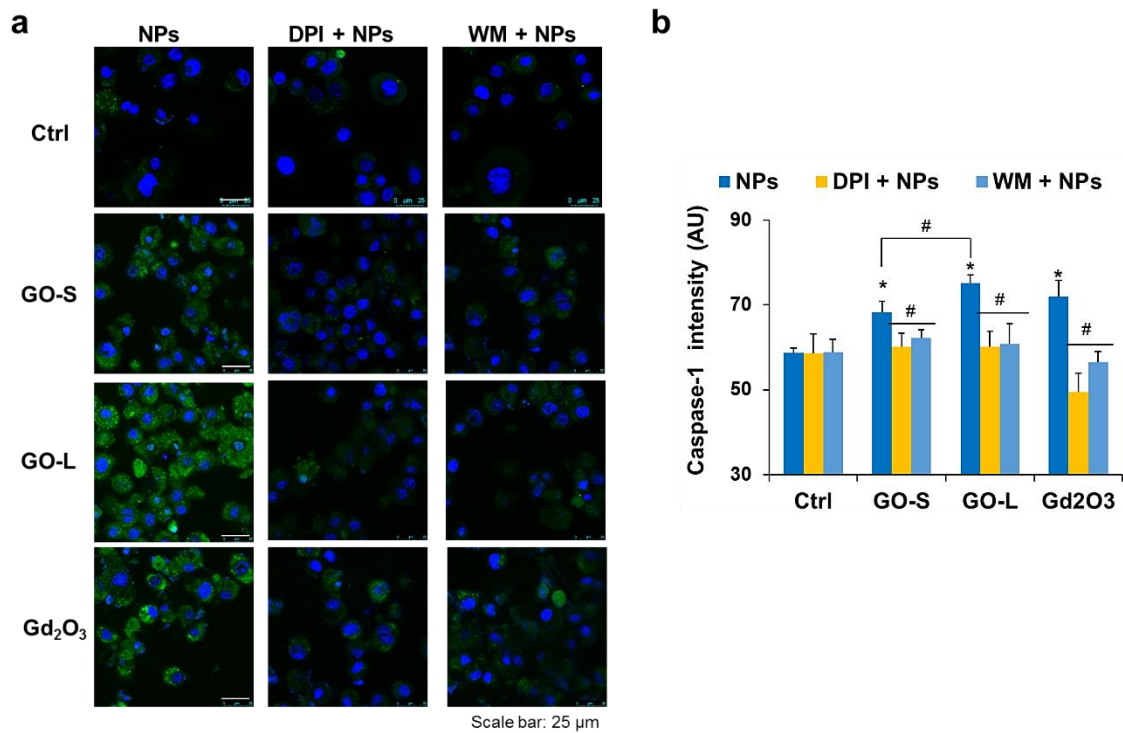
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218 **Fig. S12.** Effect of various inhibitors on caspase-1 activation in GO-treated KUP5 cells. (a)
219 The LPS-primed KUP5 cells were incubated with various inhibitors (DPI used as NADPH
220 oxidase inhibitor and WM used as phagocytosis inhibitor) were exposed to 12.5 μg/mL GO or
221 Gd₂O₃ (positive control) for 16 h. Cells washed with PBS were stained with FAM-FLICA
222 caspase substrate (green) for 1 h, stained with Hoechst 33342 for 15 min, and imaged using
223 Leica Confocal SP8-SMD microscope. The scale bar is 25 μm. (b) Quantification for caspase
224 1 activation in KUP5 cells under GO treatments with inhibitors. The fluorescence intensity was
225 monitored at excitation/emission wavelengths of 492/520 nm. *, $P < 0.05$, indicates
226 significance compared with the control; #, $P < 0.05$, indicates significance compared to NP
227 treatment alone.

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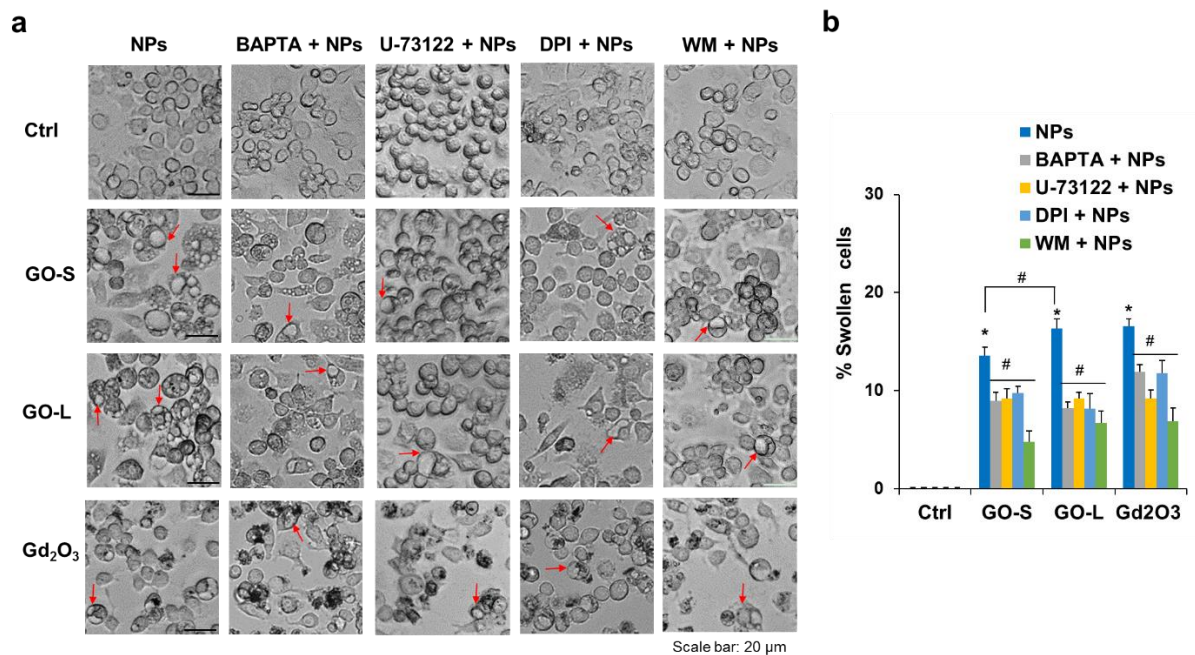
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235 **Fig. S13.** Effect of various inhibitors on pyroptosis in KUP5 cells. (a) Optical microscope
236 images to compare the GO-treated KUP5 cell morphological changes by various inhibitors
237 (BAPTA used as calcium chelator, and U-73122 used as PLC inhibitor, DPI, and WM). Red
238 arrows indicate the swollen cells. The scale bar is 20 μm. (b) The percentage of swollen cells
239 was calculated based on the cells expressing at least two giant blebs in KUP5 cells treated with
240 various inhibitors before 12.5 μg/mL of GO or Gd₂O₃ exposure for 16 h. *, $P < 0.05$, indicates
241 significance compared with the control; #, $P < 0.05$, indicates significance compared to NP
242 treatment alone.

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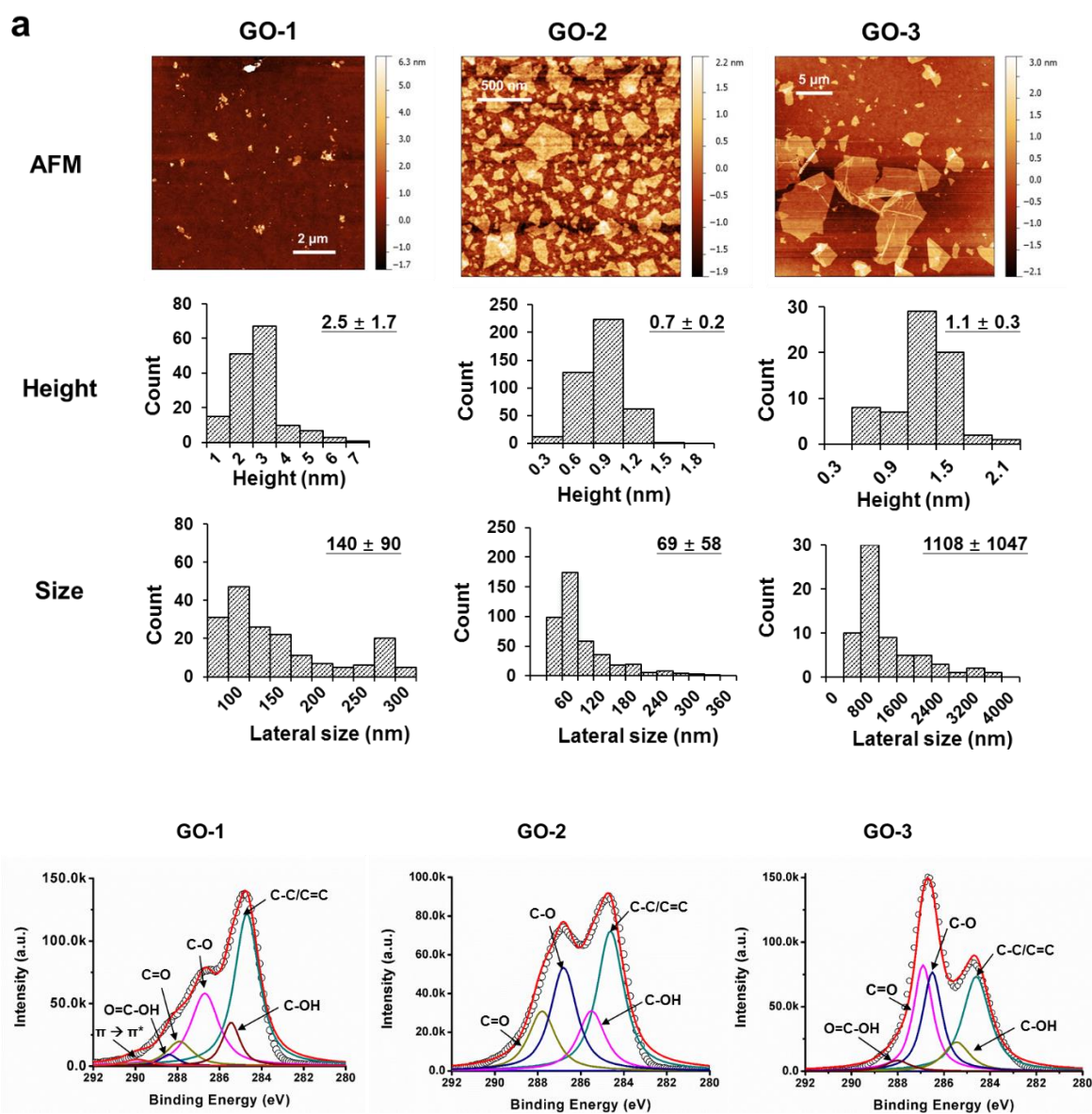
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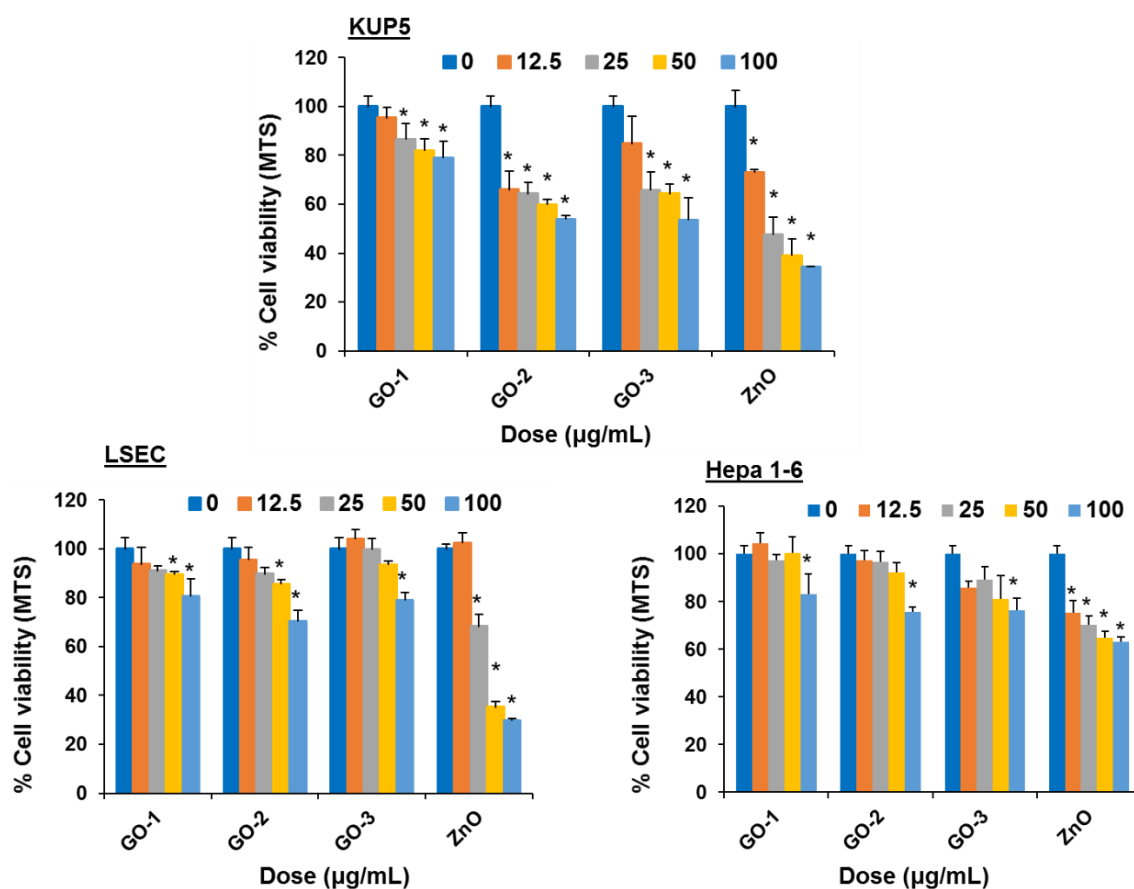
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255 **Fig. S14.** Characterization for differential physicochemical properties among GO-1, GO-2, and
 256 GO-3 samples. (a) Characterization of GO-1, GO-2, and GO-3 through AFM analysis. The
 257 panels in the top row show the histograms of GO height distributions. The average height of
 258 GO-1 is 2.5 ± 1.7 nm, the average height of GO-2 is 0.7 ± 0.2 nm, and the average height of
 259 GO-3 is 1.1 ± 0.3 nm. The panels in the bottom row show the histograms of GO lateral size
 260 distributions. The average lateral size of GO-1, GO-2, and GO-3 is 140 ± 90 , 69 ± 58 , and 1108
 261 ± 1047 nm, respectively. (b) C 1s XPS spectra of GO-1, GO-2, and GO-3.

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266 **Fig. S15.** Cell viability of KUP5, LSEC, and Hepa 1-6 cells on GOs exposure for 24 h at a dose
267 range of 0–100 $\mu\text{g/mL}$ determined by MTS assay. The viability of untreated control cells was
268 set to 100%. Asterisk (*) means $P < 0.05$, compared to the control. KUP5 cells were more
269 sensitive to the cytotoxic effects of GOs than LSECs and Hepa 1–6 cells.

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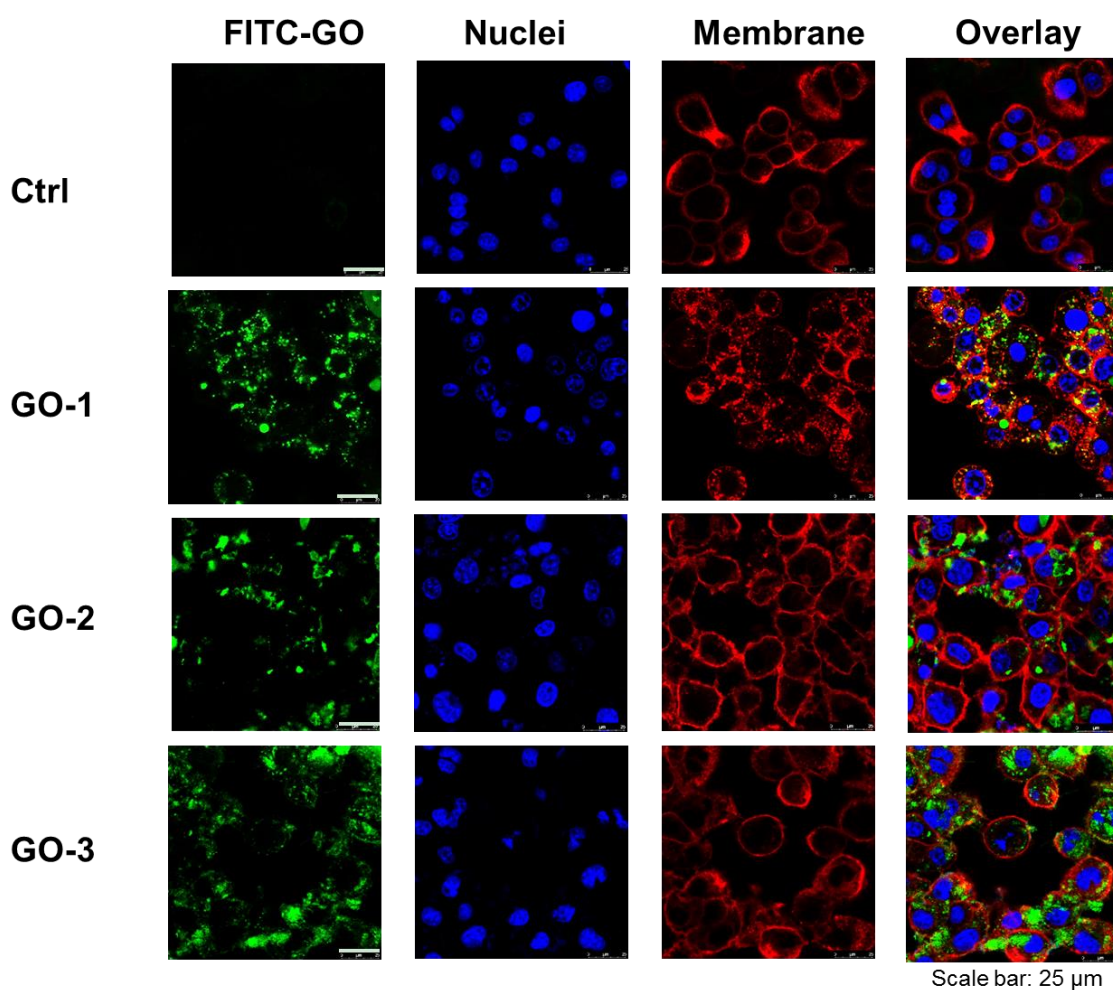
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281 **Fig. S16.** Determination of cellular localization of GO-1, GO-2, and GO-3 in KUP5 cells using
282 confocal imaging. KUP5 cells were incubated with 12.5 μ g/mL of FITC-labeled GOs (green)
283 for 16 h, followed by staining with Hoechst 33342 dye (blue) and Alexa Fluor 594-labeled
284 WGA antibody (red). The scale bar is 25 μ m.

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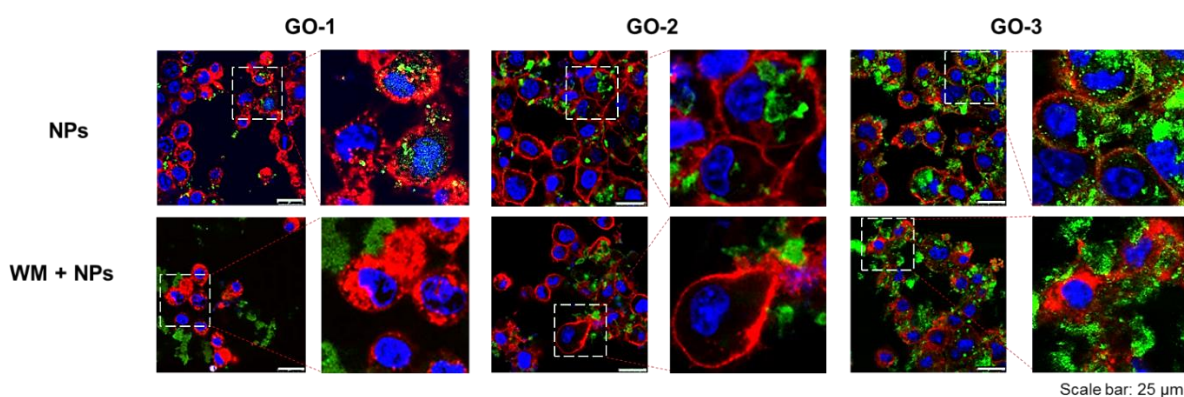
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294 **Fig. S17.** Representative confocal images to determine the phagocytosis of GO-1, GO-2, and
295 GO-3 by KUP5 cells. Before exposure to FITC-GOs, KUP5 cells were pretreated with 1 μ M
296 WM for 0.5 h. After staining with Hoechst 33342 dye and Alexa Fluor 594-labeled WGA
297 antibody, the cells were visualized under a confocal microscope. The scale bar in the left panel
298 is 25 μ m.

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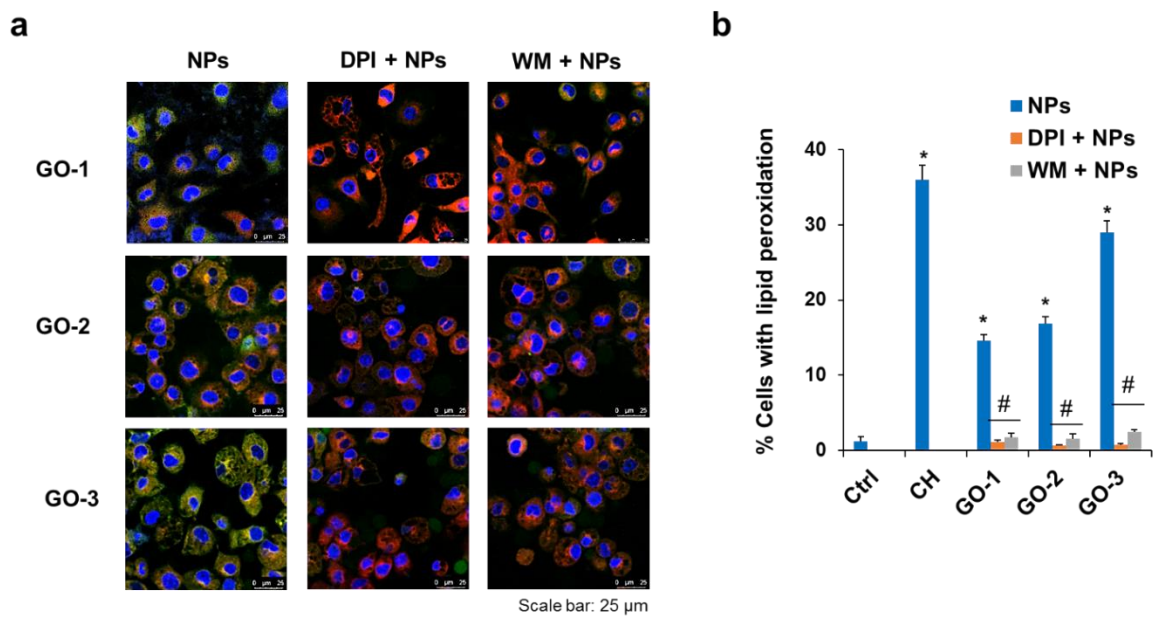
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317 **Fig. S18.** Assessment of the plasma membrane lipid peroxidation production by GO-1, GO-2,
318 and GO-3. (a) Confocal images to demonstrate the reduction of lipid peroxidation production
319 in KUP5 cells pretreated with an NADPH oxidase inhibitor DPI and a phagocytosis inhibitor
320 WM, respectively. The images were acquired to visualize the reduced (red) and oxidized (green)
321 fluorescent dye at excitation/emission wavelengths of 581/591 nm and 488/510 nm. The scale
322 bar is 25 μm. (b) Quantification for the percentage of cells with lipid peroxidation induced by
323 GO-1, GO-2, and GO-3 by flow cytometry. *, $P < 0.05$, indicates significance compared with
324 the control; #, $P < 0.05$, indicates significance compared to GO treatment alone.

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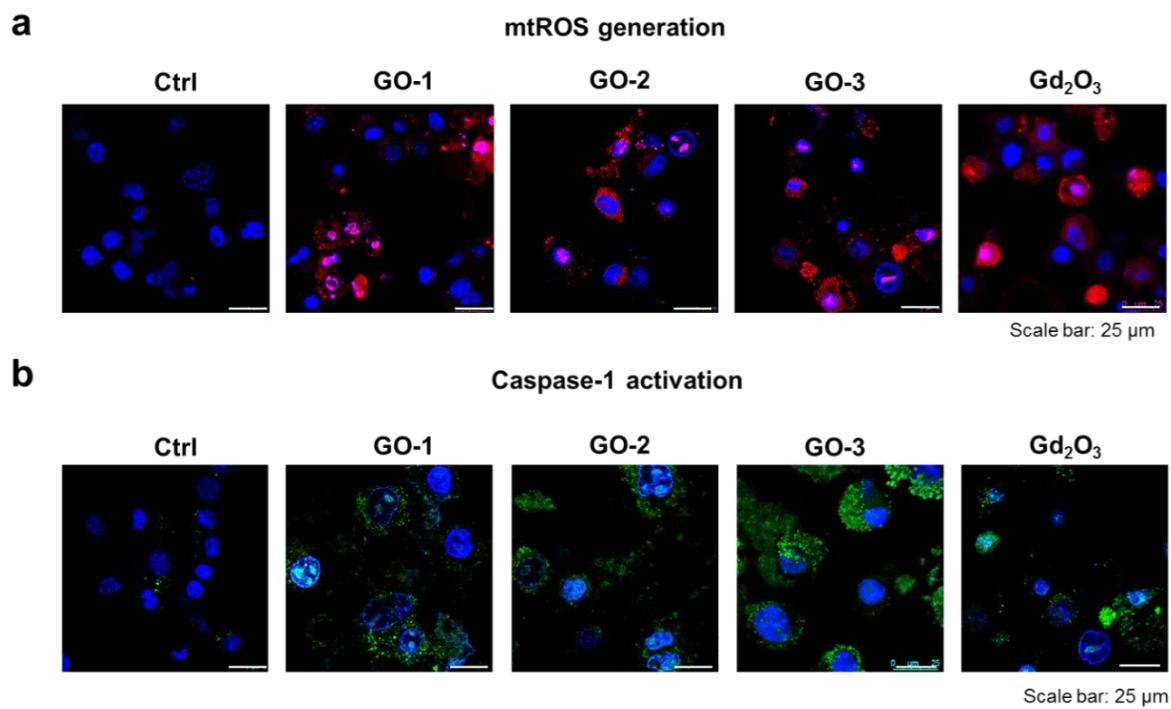
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336 **Fig. S19.** Determination of the mtROS generation and caspase-1 activation in KUP5 cells
337 treated with GO-1, GO-2, and GO-3. (a) MtROS generation was assessed by confocal
338 microscopy. KUP5 cells exposed to 12.5 μg/mL of GOs or Gd₂O₃ (positive control) for 16 h
339 were stained with 5 μM of red MitoSOX and Hoechst 33342 for 15 min, respectively. (b)
340 Assessment of caspase-1 activation in GO-treated KUP5 cells. The LPS-primed KUP5 cells
341 were washed with PBS, stained with FAM-FLICA caspase substrate (green) for 1 h and
342 Hoechst 33342 for 15 min, and imaged using Leica Confocal SP8-SMD microscope. The scale
343 bar is 25 μm.

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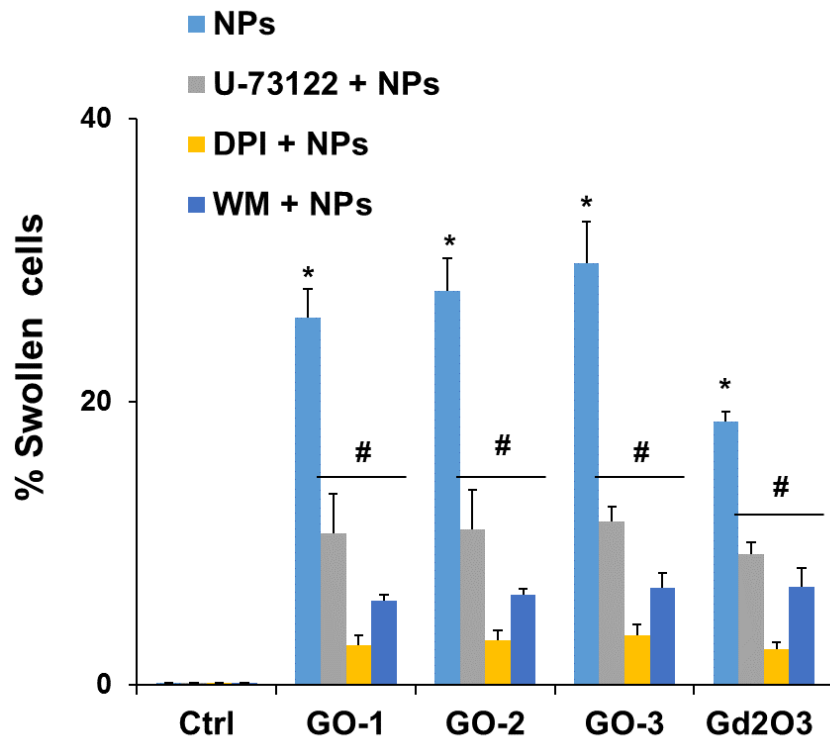
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354 **Fig. S20.** Effect of various inhibitors on cells swelling by GO-1, GO-2, and GO-3. The
355 percentage of swollen cells was calculated based on the cells expressing at least two giant blebs
356 in KUP5 cells pretreated with various inhibitors, including U-73122 used as a PLC inhibitor,
357 DPI used as an NADPH oxidase inhibitor, and WM used as an inhibitor on cellular uptake of
358 GO. *, $P < 0.05$, indicates significance compared with the control; #, $P < 0.05$, indicates
359 significance between GO treatments without and with inhibitors.

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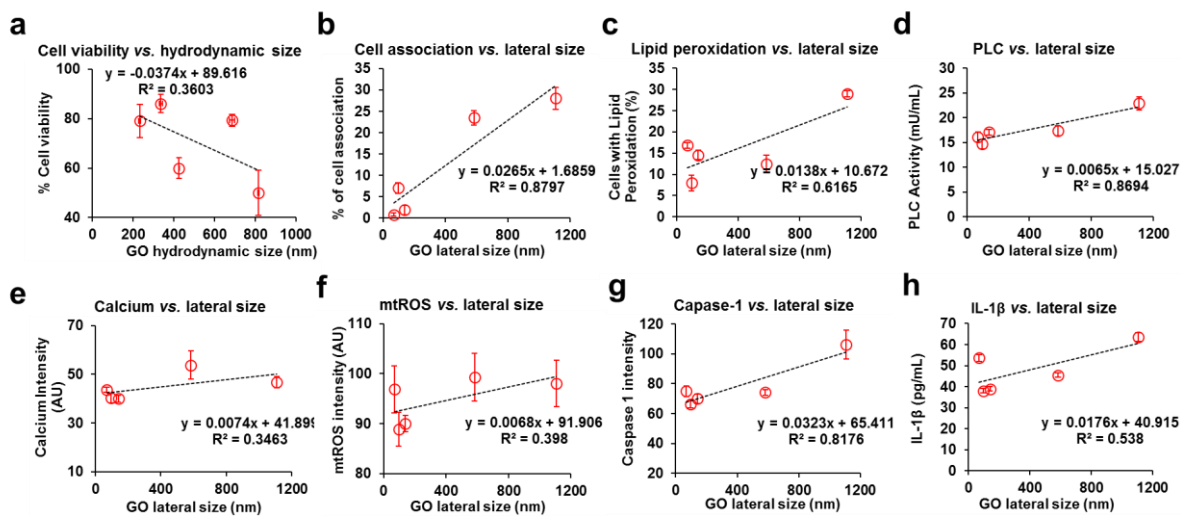
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371 **Fig. S21.** The correlation plots of the GO in KUP5 cells. (a) The plotting of the GO
372 hydrodynamic size showed correlation coefficients with cell viability. (b-h) Plotting of the GO
373 lateral size (AFM) showed excellent correlation coefficients with cell association (b), lipid
374 peroxidation (c), PLC activation (d), calcium flux (e), mtROS generation (f), caspase-1
375 activation (g), as well as IL-1 β release (h).

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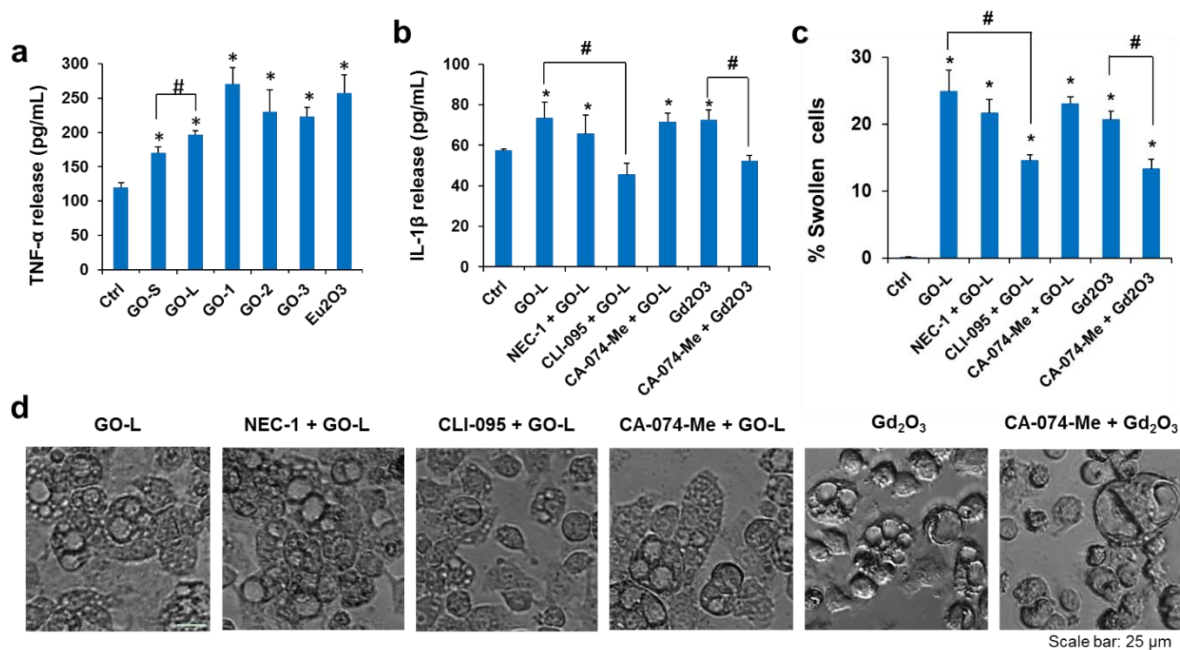
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392 **Fig. S22.** TNF- α production, IL-1 β release, and cell swelling in GO-treated KUP5. LPS-primed

393 (1 μ g/mL, 4 h) KUP5 cells were pretreated with 30 nM of necroptosis inhibitor NEC-1

394 (necrostatin-1) overnight, 1 μ g/mL of CLI-095 (TLR4 inhibitor) overnight, and 30 μ M of the

395 cathepsin B inhibitor CA-074-Me for 6 h, respectively, before GO exposure. Supernatants were

396 collected to measure TNF- α (a) and IL-1 β (b) release by ELISA, respectively. The percentage

397 of swollen cells (c) was calculated based on the cells expressing at least two giant blebs in

398 KUP5 cells (d) pretreated with various inhibitors. The scale bar is 20 μ m. *, $P < 0.05$, indicates

399 significance compared with the control; #, $P < 0.05$, indicates significance between GO

400 treatments

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408 **Table**

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410 **Tab S1.** Hydrodynamic size, polydispersity index, and zeta potential of GO in different media.

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Media	Sample	Hydrodynamic size (nm)	Polydispersity index (PDI)	Z-potential (mV)
DI H2O	GO-1	148.0 ± 1.1	0.2	-40.7 ± 2.0
	GO-2	260.3 ± 0.6	0.3	-59.86 ± 1.3
	GO-3	417.8 ± 14.3	0.3	-51.57 ± 2.4
DMEM (KUP5)	GO-1	233.8 ± 4.2	0.2	-7.5 ± 1.8
	GO-2	426.4 ± 0.5	0.3	-10.2 ± 0.6
	GO-3	815.5 ± 2.8	0.3	-11.4 ± 1.9
Prigrow medium (LSEC)	GO-1	172.6 ± 5.2	0.2	-11.1 ± 1.7
	GO-2	327.1 ± 4.1	0.3	-11.4 ± 3.6
	GO-3	529.4 ± 14.2	0.3	-12.3 ± 2.4
DMEM (Hepa 1-6)	GO-1	217.6 ± 1.9	0.2	-10.9 ± 1.9
	GO-2	345.3 ± 15.1	0.3	-9.7 ± 2.5
	GO-3	816.8 ± 29.3	0.3	-7.5 ± 0.6

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