Supporting Information

Moderating cellular inflammation using 2-dimensional titanium carbide MXene and graphene variants

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Experimental Section

Materials

GNP was obtained from XG Sciences, USA. Ti_3AlC_2 MAX phase powder was from Gogotsi's lab at Drexel University, USA. Graphite, silver nitrate ($AgNO_3$), sodium borohydride ($NaBH_4$), sulphuric acid (H_2SO_4), dialysis membrane, Live/Dead mammalian cell viability kit, Live/Dead bacterial viability kit (BacLight) and Pierce Chromogenic Endotoxin Quant Kit were supplied by Thermo Fisher Scientific, UK. Potassium permanganate ($KMnO_4$) was supplied by BDH Chemicals, England. Silver nanoparticles (AgNP), lithium fluoride, hydrochloric acid (HCl), hydrogen peroxide (H_2O_2), fetal bovine serum (FBS), Triton X-100, camptothecin, 2', 7'-Dichlorofluorescein diacetate (DCFH-DA), bovine serine albumin (BSA), phorbol 12-myristate 13-acetate (PMA), haemolysis assay kit and lipopolysaccharide (LPS) were supplied by Sigma-Aldrich, UK. RPMI-1640 Medium and Dulbecco's Modified Eagle Medium (DMEM) were obtained from Gibco, UK. Phosphate buffered saline (PBS), tryptone soya broth and tryptone soya agar were supplied by Oxoid, UK. Mouse embryo fibroblast (NIH-3T3), human peripheral blood monocyte (THP-1) and human peripheral blood T lymphocyte (Jurkat E6-1) cell lines were obtained from LGC Standards, UK. Fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit I was supplied by BD Biosciences, UK. Sodium citrate (3.2%) tubes and ethylenediaminetetraacetic acid (EDTA) tubes were supplied by Greiner Bio-One, Germany. Coagulometry assay reagents were purchased from Diagnostica Stago, France. Platelet activation assay reagents and cytokines (IL-8, TNF-α and IL-6) were supplied by BD

Pharmingen, UK. *Escherichia coli* (NCTC 8196) was obtained from Public Health England, UK. MTS, LDH and BacTiter-Glo Microbial Cell Viability assay kits were purchased from Promega Corporation, UK. Fresh frozen human plasma was supplied by Plasma Biological Services Incorporated, USA. Enzyme-linked immunosorbent assay (ELISA) kit reagents for IL-8, TNF-α and IL-6 quantification were obtained from BD OptEIA, UK. Only analytical grade chemicals and deionised water (H_2O; ~15 MΩ cm^{-1}) were used in this study.

Nanomaterial synthesis

GO synthesis

GO was synthesised from graphite following a modified Hummers method.[1] 1 g of graphite was slowly added to 23 mL of concentrated sulphuric acid in an ice bath. The mixture was stirred for 5 minutes whilst keeping the temperature below 20 °C. 3 g of potassium permanganate was added, the temperature was raised to 35 °C and the mixture was stirred for 30 minutes. 46 mL of deionised water was added after which the temperature was increased to 98 °C and kept at this temperature for 15 minutes. 140 mL of deionised water and 10 mL of 30% (v/v) hydrogen peroxide were added slowly to stop the reaction. The suspension was centrifuged at 3500 rpm for 10 minutes. The resulting pellet was washed three times with 5% (v/v) hydrochloric acid in deionised water followed by three washes with deionised water. The GO pellet was resuspended in deionised water in 50 mL Falcon tubes, freeze dried and stored at room temperature.

GO-Ag synthesis

GO-Ag was synthesised using the method of Faria *et al*.[2] 12.5 mg of GO was dispersed in 43.75 mL of deionised water and sonicated for 30 minutes. 5 mL of a 5 mM silver nitrate solution was added to the GO suspension. The suspension was sonicated for 30 minutes and then kept on ice for 30 minutes. 2.25 mL of a 1 mM sodium borohydride solution was slowly added to the suspension whilst stirring and the reaction was kept at room temperature overnight (16 hours). The suspension was dialysed for 24 hours in deionised water with a dialysis membrane. The purified suspension was stored in 50 mL Falcon tubes, freeze dried and stored at room temperature.

ML-MXene synthesis

ML-MXene was synthesised using MILD method.[3] 2.5 mL of deionised water and 7.5 mL of hydrochloric acid were combined and added to 1 g of lithium fluoride in a plastic vial. The suspension was stirred at 60 °C for 1 hour to dissolve lithium fluoride. 1 g of MAX phase powder was slowly added (in 1 minute) and the dispersion was stirred at 35 °C for 24 hours. A hole was bored in the vial lid to allow hydrogen release during etching. The suspension was centrifuged at 3500 rpm for 5 minutes and the pellet was washed repeatedly with deionised water until the pH was 7. The ML-MXene pellet was vacuum dried in a desiccator. DL-MXene was synthesised by resuspending the ML-MXene pellet in deionized H_2O followed by bath sonication under helium bubbling for 1 hour. Following centrifugation of the suspension at 3500 rpm for 1 hour, the supernatant was freeze dried to yield DL-MXene. ML-MXene and DL-MXene were stored in a vacuum desiccator at 4 °C in the dark.

Physical characterisation

Scanning electron microscopy (SEM) images were captured using a field emission gun scanning electron microscope (Carl Zeiss SIGMA, UK) at an accelerating voltage of 5 kV**.** Prior to imaging, the nanomaterials were mounted on aluminium stubs and coated with a 4 nm layer of platinum using a sputter coater (Quorum Technologies QT150ES, UK). Elemental composition of the nanomaterials was measured with energy dispersive x-ray spectroscopy (EDS) using an 80 mm^2 X-Max detector (Oxford Instruments, UK) at the same accelerating voltage used for SEM. Transmission electron microscopy (TEM) images were acquired using a transmission electron microscope (JEOL JEM1400-Plus, Germany) at an accelerating voltage of 120 kV. Before imaging, aqueous nanomaterial suspensions (1 mg/mL) were sonicated for 30 minutes. Next, 5 µL of each suspension was dropped onto the matte side of a formvar/carbon coated 400 mesh copper TEM grid and excess suspension blotted off. The mean diameter of silver nanoparticles on GOAg sheets was calculated with ImageJ software (National Institutes of Health, version 1.49) following the ImageJ particle analysis method.[4] Fourier transform infrared (FTIR) spectra were acquired over 4000 to 650 cm^{-1} using a spectrometer (Perkin Elmer Spectrum 65, UK). Dynamic light scattering (DLS) measured sheet size and surface charge of aqueous nanomaterial suspensions (1 mL) using a zetasizer (Malvern Nano ZS90, UK) at 25 °C. Using the Brunauer, Emmett and Teller (BET) method and

Quantachrome data analysis tools (Quantachrome ASiQwin), the surface area of nanomaterials was measured using an Autosorb-1 gas sorption analyser (Quantachrome Instruments, USA) with nitrogen (N_2) gas as adsorbate at liquid nitrogen temperature of 77.3 K. X-ray photoelectron spectroscopy (XPS) analyses were carried out using a spectrometer (Thermo Fisher Scientific ESCALAB 250 Xi) connected to an aluminium Kα x-ray source ($h\upsilon$ = 1436 eV).

Cell interaction studies

Induction of cytotoxicity

Nanomaterial impact on 3T3 mouse fibroblast viability was assessed using the MTS, LDH and Live/Dead assays. 3T3 cells were seeded in 96-well plates in DMEM supplemented with 10% (v/v) FBS at a concentration of 1 x 10^5 cells mL^{-1} and incubated for 24 hours at 37 °C, 5% CO_2. Spent medium was removed and fresh medium (100 µL) was added to each well. Nanomaterial suspensions in medium were each added to triplicate wells over a concentration range of 6.25 to 200 µg mL^{-1} and the plate was incubated for 24 hours. Untreated cells served as a negative control while the positive control was dibutyltin maleate (MTS assay)/Triton X (0.8% v/v) (LDH and Live/Dead assays).

Induction of apoptosis

Nanomaterial induction of apoptosis in Jurkat T lymphocytes was measured using the FITC Annexin V Apoptosis Detection Kit. Jurkat cells were seeded in 12-well plates in RPMI 1640 medium supplemented with 10% (v/v) FBS at a concentration of 1 x 10^6 cells mL^{-1}. Cells were incubated with nanomaterial suspensions in medium (200 µg mL^{-1}) at 37 °C for 1 and 4 hours. Untreated cells served as a negative control while the positive control was camptothecin (4 µM). Cells were analysed with a flow cytometer (BD Accuri C6, UK) at a flow rate of 35 µL/minute. Unstained cells and single stained cells were included as compensation controls. The forward scatter threshold was set to 200,000 and >10,000 events were collected for each sample. Early apoptotic cells (Annexin V+, propidium iodide-) were expressed as a fraction of the total cell number.

Induction of oxidative stress

Nanomaterial stimulation of reactive oxygen species (ROS) production in THP-1 monocytes was investigated using a DCFH-DA assay protocol adapted from literature.[5] THP-1 cells were incubated with 2 mL of a 50 µM DCFH-DA solution in serum-free RPMI 1640 medium at 37 °C for 40 minutes. Cells were washed 2 times with 3 mL of warm PBS to remove excess probe. Next, cells were seeded in black 96-well plates in RPMI 1640 medium supplemented with 10% (v/v) FBS at a concentration of 3 x 10^5 cells mL^{-1}. Nanomaterial suspensions in medium were each added to triplicate wells over a concentration range of 6.25 to 200 µg mL^{-1} and the plate was incubated for 24 hours. Untreated cells served as a negative control while the positive control was cells treated with H_2O_2 (400 µM). Fold increase in ROS levels was expressed as the ratio of the fluorescence intensity of treated cells to that of untreated cells. Nanomaterial interference with fluorescence intensity was investigated using a method adapted from literature.[6]

Haemocompatibility studies

Haemocompatibility was assessed using healthy human donor blood following ethical approval by the University of Brighton Research Ethics Committee. Blood samples for platelet activation and coagulometry assays were collected into sodium citrate anticoagulated tubes while blood samples for the haemolysis assay were collected with EDTA anticoagulated tubes. GNP, GO, ML-MXene and DL-MXene (50 mg) were pre-wetted in PBS (1 mL) overnight at room temperature with shaking at 120 rpm. Each suspension was centrifuged at 14000 rpm for 10 minutes, PBS was removed and the nanomaterial pellet was resuspended in 1 mL of healthy donor blood. Time 0 samples were taken and the suspensions were then incubated at room temperature for 1 hour while shaking at 120 rpm. Blood samples lysed with Triton X-100 (1% v/v) were included as a positive control for the haemolysis assay. Nanomaterial impact on the extrinsic and intrinsic plasma coagulation pathways was investigated using the prothrombin time and activated partial thromboplastin time (APTT) assays respectively. Normal and abnormal human plasma were included as negative and positive controls for the coagulation assays. PMA (100 nM) was included as a positive control for the platelet activation assay.

Antibacterial studies

Escherichia coli suspensions (0.25 mL; 10^6 CFU mL^{-1}) were incubated with GNP, GO, GO-Ag, ML-MXene and DL-MXene suspensions (0.25 mL; 125 µg mL^{-1}) at 37 °C, 120 rpm for 4 hours. The negative control was untreated bacteria while bacteria treated with silver nanoparticles (AgNP) were included as the positive control. The antibacterial activity of GO and GO-Ag was further investigated over a concentration range of 6.25 to 200 µg mL^{-1} and a bacterial concentration of 10^8 CFU mL^{-1}. Bacterial viability was measured using colony counts, ATP and Live/Dead staining assays.

Suppression of inflammatory stimulus

Cytokine adsorption from spiked human plasma

GNP, GO, ML-MXene and DL-MXene (5 – 50 mg) were pre-wetted in PBS (1 mL) overnight at room temperature with shaking at 120 rpm. Fresh frozen human plasma was spiked with the pro-inflammatory cytokines (IL-6, IL-8 and TNF-α) at a concentration of 1000 pg mL^{-1} to simulate physiological levels of cytokines in sepsis. Following centrifugation and supernatant removal, the pre-wetted nanomaterials were incubated with cytokine spiked plasma (1 mL) at 37 °C with shaking at 120 rpm. Spiked plasma without adsorbent was included as a control. An end point of 4 hours was selected to simulate haemodialysis durations. Residual cytokine concentrations in plasma supernatants were measured using ELISA.

Repression of LPS induced cytokine production in THP-1 monocytes

Nanomaterial impact on cellular cytokine production was investigated using an *in vitro* model of LPS-stimulated THP-1 monocytes. THP-1 monocytes were seeded in 12-well plates in RPMI 1640 medium supplemented with 10% (v/v) FBS (0.5 mL) at a concentration of 1 x 10^6 cells mL^{-1}, stimulated with LPS (2000 ng) and incubated at 37 °C for 1 hour. GNP, GO, ML-MXene and DL-MXene suspensions in media (0.5 mL) were then added to the cells at a final concentration of 12.5 mg mL^{-1} and the plate was incubated at 37 °C. Cytokine concentrations in media supernatants, collected after total incubation times of 8 (TNF-α) and 24 hours (IL-6 and IL-8), were measured using ELISA. Prior to setting up this experiment, cytokine production by THP-1 cells in response to LPS stimulation was measured as a function of concentration

and time in order to select optimal end points (Figure S5).

LPS adsorption by the nanomaterials

Pre-wetted GNP, GO, ML-MXene and DL-MXene (12.5 mg) were incubated with LPS spiked cell culture media (2000 ng mL^{-1}) for 24 hours at 37 °C with shaking at 120 rpm. Residual LPS concentrations in media supernatants were measured with the limulus amebocyte lysate (LAL) assay. All materials used were pyrogen-free to prevent endotoxin contamination.

Statistical analysis

Data was expressed as the mean ± SEM of 3 independent experiments. Images were representative of the experimental group. Statistical analyses were performed using GraphPad Prism version 7.0e software (GraphPad Software Inc., USA). Differences between means were evaluated using one- or two-way ANOVA, where applicable, and Tukey or Dunnett post hoc tests. P values below 0.05 were considered statistically significant.

Supporting Experimental Results

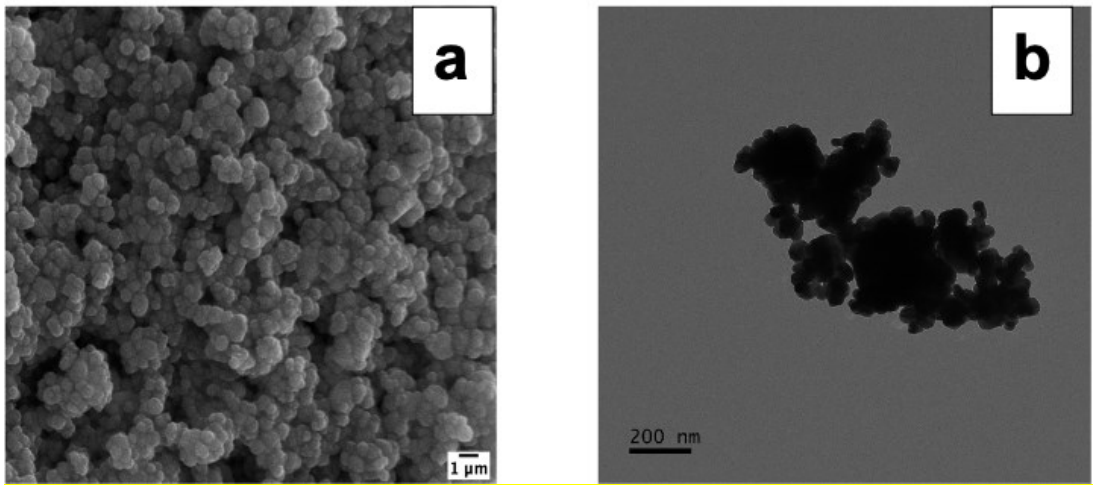

Figure S1 SEM (a) and TEM images (b) of AgNP

MTS, LDH and Live/Dead assay results in Figure S2 showed that AgNP at concentrations up to 200 µg/mL did not impact 3T3 cell viability. MTS assay results in Figure S2b indicated that higher concentrations of AgNP above 250 µg/mL are required for any impact on cell viability.

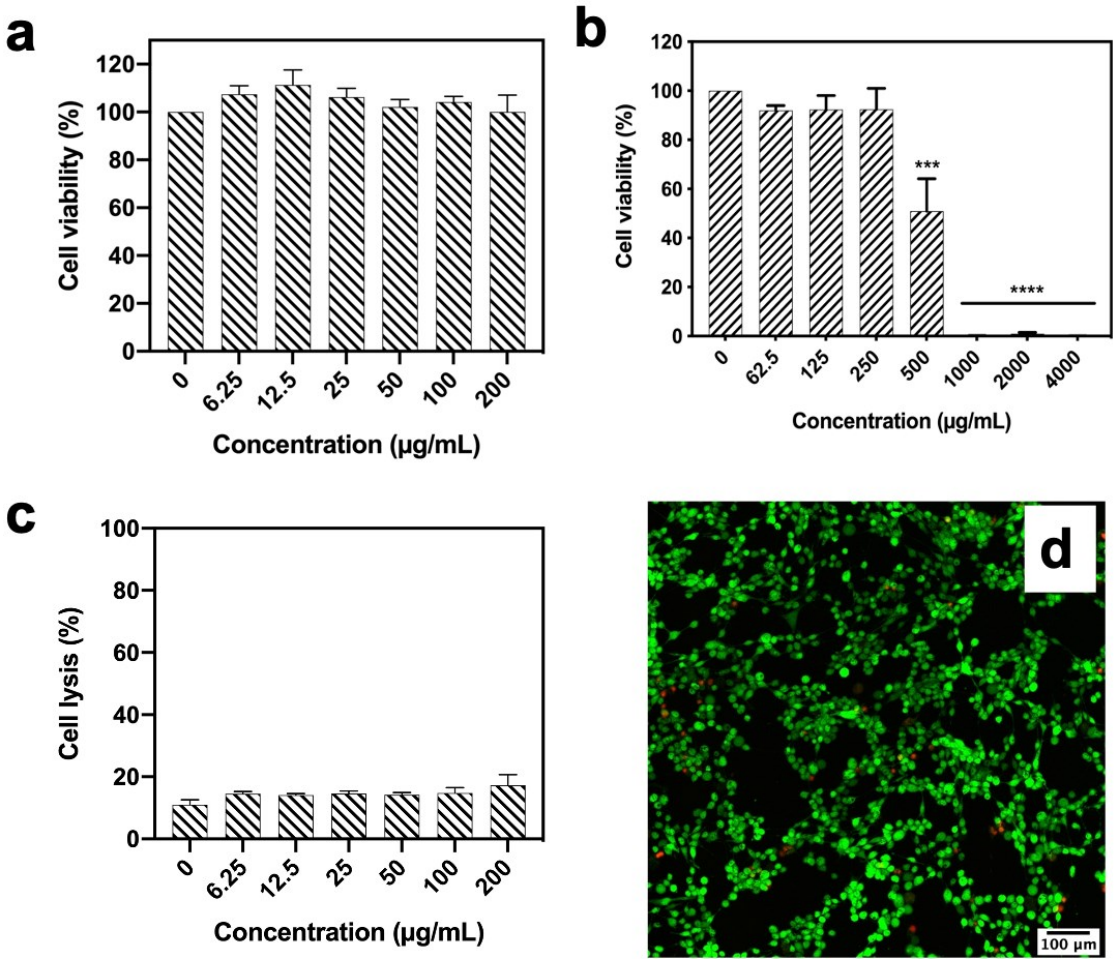

Figure S2 3T3 cell viability from the MTS assay after 24-hour incubation with AgNP at concentration ranges of 12.5 – 200 µg/mL (a) and 62.5 – 4000 µg/mL (b). 3T3 cell viability from the LDH assay after 24-hour incubation with AgNP (12.5 – 200 µg/mL) (c). Data were analysed using one-way ANOVA and a Dunnett post hoc test by comparing treated cells to untreated cells (***$p<0.001$, ****$p<0.0001$). Confocal microscopy images showing 3T3 cell viability after 24-hour incubation with AgNP at 200 µg/mL (d). Cells were stained with calcein AM and ethidium homodimer-1 - the green and red spots represent live and dead cells respectively.

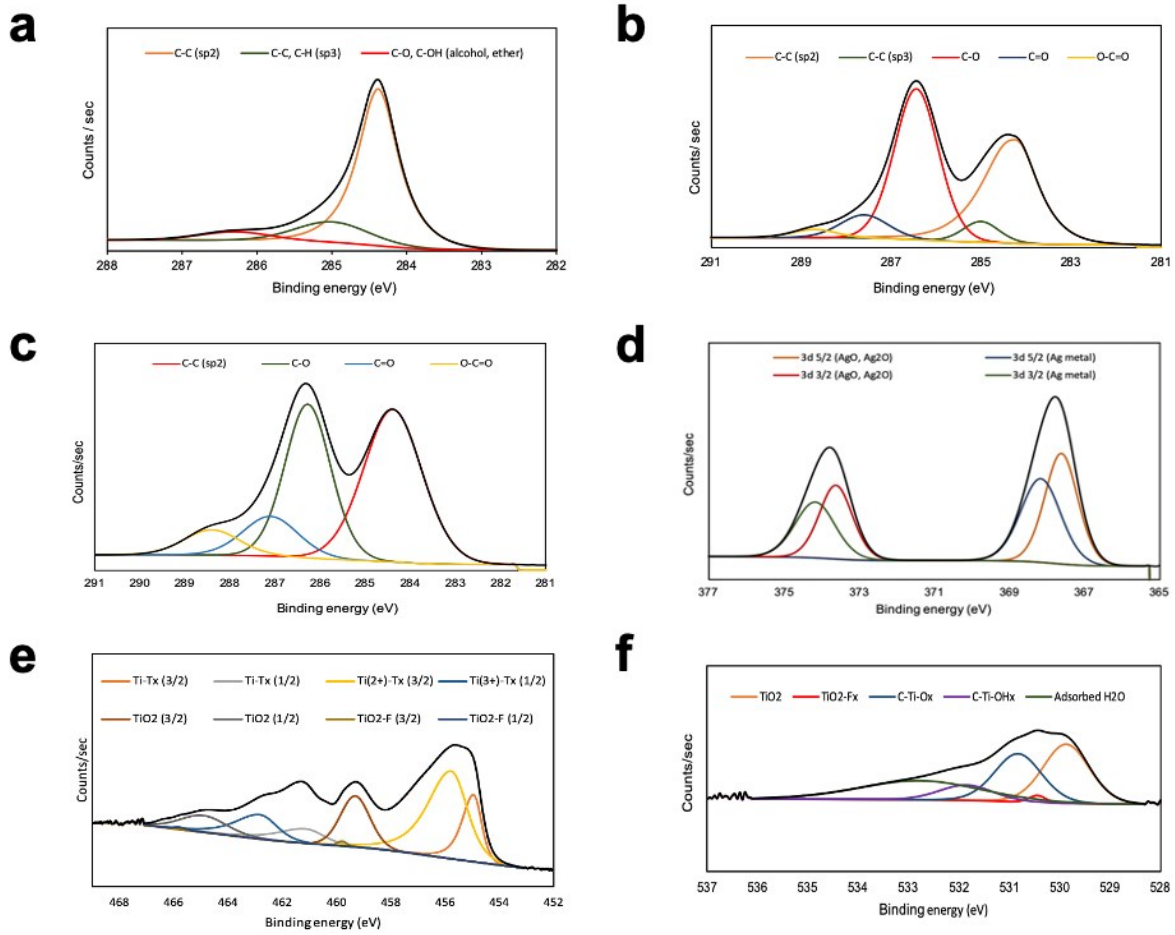

Figure S3 GNP C1s (a), GO C1s (b), GO-Ag C1s (c), GO-Ag 3d (d), ML-MXene Ti2p (e) and ML-MXene O1s (f) XPS narrow scan spectra highlighting characteristic chemical bonds. Doublet peaks at 368.1 and 374.2 eV in (d) are attributed to metallic silver in the $3d_{5/2}$ and $3d_{3/2}$ orbitals respectively. This, coupled with the ~6.0 eV slitting between both peaks, evidenced the formation of metallic silver nanoparticles on GO sheets.[7, 8] XPS narrow scan spectra was acquired using an aluminium Kα x-ray source (*h*υ = 1436 eV) and peak fitted using a combination of Gaussian and Lorentzian functions (70:30).

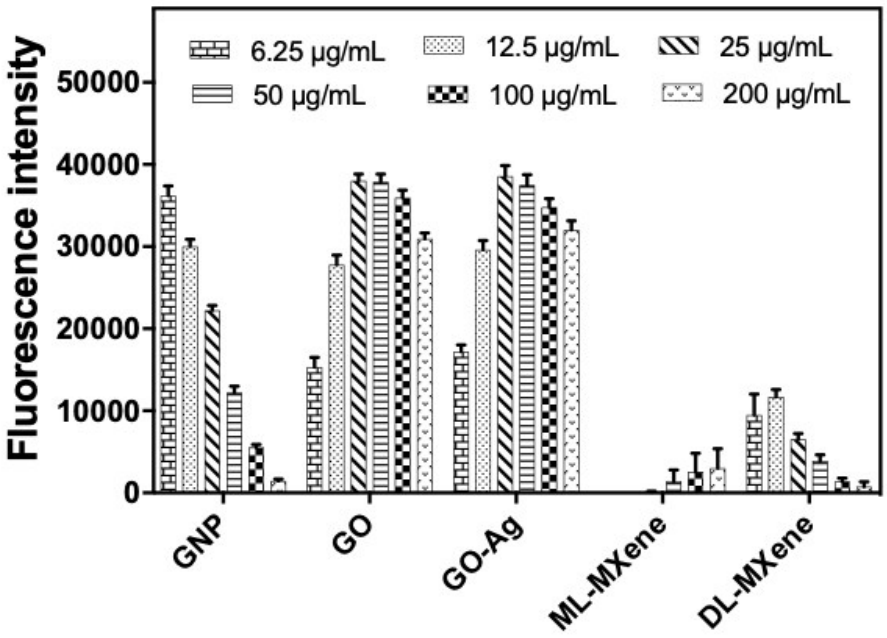

Figure S4 DCF fluorescence intensity after 24-hour incubation of DCFH (2.5 µM) with nanomaterial suspensions in PBS (6.25 – 200 µg/mL). Data represent mean ± SEM (n = 3). GNP, GO and GO-Ag induced DCFH oxidation to DCF. Zhao *et al*[9] similarly demonstrated that GO catalysed oxidation of the coenzyme, NADH to NAD^+ by acting as an electron acceptor. Although GO and GO-Ag increased DCF fluorescence intensity, this effect was not translated to elevated ROS levels after incubation with THP-1 cells due to differences in experimental setup. To investigate nanomaterial impact on ROS levels, the THP-1 cells were first labelled with DCFH-DA - the probe passively diffused into cells and underwent deacetylation to the non-fluorescent DCFH by cellular esterases. Next, the nanomaterial suspensions were introduced to the labelled cells for 24-hour incubation. This setup limited direct interaction between nanomaterials and DCFH, the active substrate, to minimise the risk of assay interference. Since the interference experiments were conducted in a cell-free medium (PBS), DCFH-DA had to be chemically deacetylated to DCFH before introduction of nanomaterial suspensions. This meant that the nanomaterials were in direct contact with DCFH during incubation which potentially enhanced DCFH oxidation to DCF as opposed to the ROS induction experiment in Figure 5b where the DCFH molecules were located within the cells.

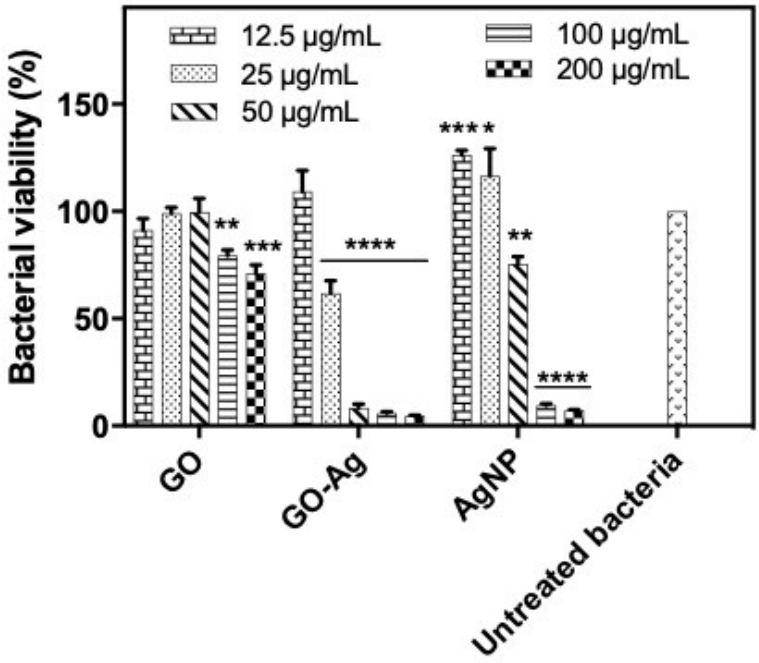

Figure S5 ATP assays conducted over a concentration range of 12.5 – 200 µg/mL showed that GO-Ag and AgNP significantly reduced *E. coli* viability. Data were analysed using two-way ANOVA and Dunnett post hoc tests by comparing treated bacteria to untreated bacteria (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Cytokine production by THP-1 cells was significantly impacted by LPS concentration (Figure S6a-c) and time (Figure S6d-f).

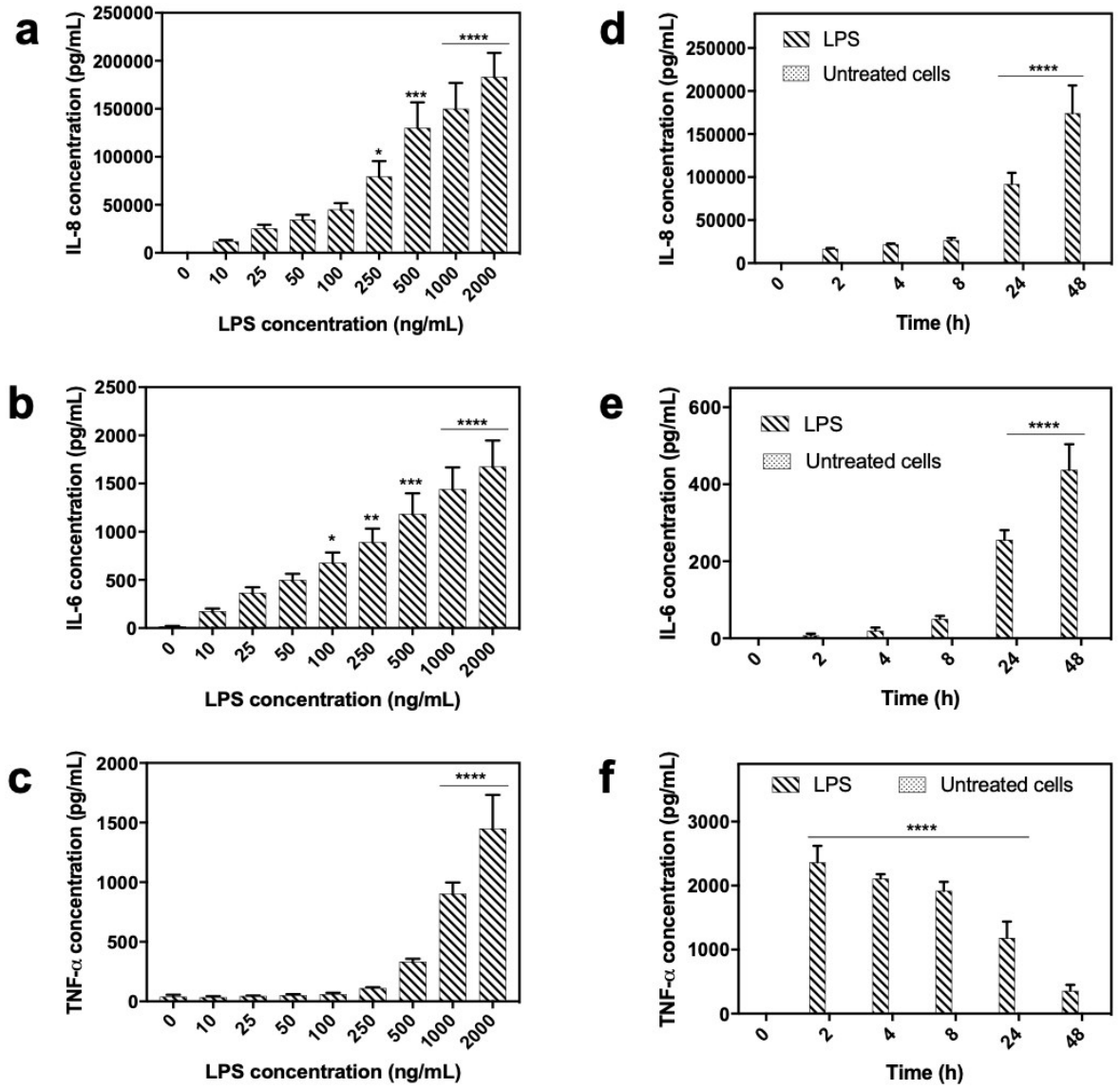

Figure S6 IL-8, IL-6 and TNF-α levels in THP-1 cells after 24-hour stimulation with LPS (10 - 2000 ng/mL) (a-c) and over 48-hour stimulation with LPS (2000 ng/mL) (d-f) (n = 3, mean ± SEM). For a-c, data were analysed using one-way ANOVA and a Dunnett post hoc test while for d-f, data were analysed using two-way ANOVA and a Sidak post hoc test, by comparing treated cells to untreated cells (*p<0.5, **p<0.01, ***p<0.001, ****p<0.0001).

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