Galactomannan-*graft*-poly(methyl methacrylate) nanoparticles induce an anti-inflammatory phenotype in human macrophages

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Methods

Materials

GM (from locust bean gum) was supplied by Glentham Life Sciences (Corsham, UK) and hydrolyzed in trifluoroacetic acid (1M, TFA, Fluka, Buchs, Switzerland) at 80 °C for 1 h to produce hGM and dialyzed against distilled water (regenerated cellulose dialysis membranes; molecular weight cut off 3500 Da; nominal flat width of 46 mm, diameter of 29.3 mm, wall thickness; Cellu·Sep, Membrane Filtration Products, Inc., Membrane Filtration Products, Inc., Seguin, TX, USA). Methyl methacrylate (MMA, Alfa Aesar, Heysham, UK) was distilled under vacuum at 35−40 °C to remove the radical inhibitor. Cerium(IV) ammonium nitrate (CAN, Sigma-Aldrich, St. Louis, MO, USA), nitric acid 70% (Bio-Lab Ltd., Jerusalem, Israel) and tetramethylethylenediamine (TEMED, Alfa Aesar) were used without further purification. All the solvents were of spectroscopic or analytical grade, purchased from Bio-Lab Ltd. or Gadot (Netanya, Israel), and used without additional purification. Dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) were supplied by Carlo Erba Reagents (Val de Reuil, France) and dried with activated molecular sieves 3A (Sigma-Aldrich) for at least 48 h before use.

Synthesis and characterization of hGM-*g*-PMMA nanoparticles

The grafting of MMA blocks on the backbone of hGM to produce a graft copolymer containing 30% w/w of PMMA (hGM-*g*-PMMA30) was conducted by the free radical graft polymerization of MMA in nitric acid solution in water by utilizing CAN as initiator and TEMED as activator, as described elsewhere [1,2]. Briefly, hGM (0.4 g)

was dissolved in 150 mL of a nitric acid aqueous solution (0.05 M) and mixed with 180 µL of TEMED and 213 µL of MMA. The mixture was degassed and purged with N_2 gas to remove oxygen. The reaction was initiated by adding CAN solution (0.66 g in 2 mL degassed water) and allowed to proceed at 35°C under N_2 atmosphere for 3 h. The reaction was terminated by adding hydroquinone powder (0.132 g) to produce a hGM-*g*-PMMA30 copolymer. For biological assays, the copolymer was labeled with fluorescein isothiocyanate (FITC, Sigma-Aldrich) to obtain fluorescently-labeled hGM-*g*-PMMA30 copolymer. For this, hGM-*g*-PMMA30 copolymer (0.1 g) was dissolved in DMF (2 mL). FITC (10 mg in 200 µL dry DMF) was added to the copolymer solution, and the reaction was allowed to proceed for 16 h at 32 °C under stirring. The reaction crude was dialyzed and freeze-dried (Labconco Free Zone 4.5 plus, Labconco, Corp., Kansas City, MO, USA) and stored at 4 °C until use. The percentage of PMMA in the copolymer (expressed as % w/w) was quantified by proton-nuclear magnetic resonance (^{1}H-NMR) in a 400 MHz Bruker Avance III 400 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). Data were analyzed with MestReNova software (Mestrelab Research, Santiago de Compostela, Spain). Solutions (5% w/v) were prepared in dimethyl sulfoxide (DMSO)-*d6* (D content of 99.9%, Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA). Chemical shifts were established in ppm, setting the signal of DMSO (2.5 ppm) as the internal standard. NP suspensions were prepared by direct dissolution of the copolymer at the desired concentration and medium. Fluorescently-labeled NPs were produced by direct dissolution of 85% and 15% w/w of unlabeled and FITC-labeled copolymer.

The size of hGM-*g*-PMMA30 NPs expressed as hydrodynamic diameter (D_h) and the polydispersity index (PDI, an estimation of the size distribution) were measured by dynamic light scattering (DLS) in a Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK) at a scattering angle of 173° and, 25 and 37 °C by utilizing 0.1% w/v samples. Data was analyzed using CONTIN algorithms (Malvern Instruments). Each value is expressed as mean ± S.D. of at least three independent samples and each measurement is an average of at least seven runs. For physical stability studies, samples were prepared in different media, stored at 25 or -20 ^{o}C and the D_h and the PDI measured by DLS at different time points.

Nanoparticle compatibility and uptake in a human macrophage cell line

The human monocyte cell line THP-1 (ATCC® TIB-202™, American Type Culture Collection, Manassas, VA, USA) was generously donated by Dr. David Meiri (Faculty

of Biology, Technion – Israel Institute of Technology) and cultured in RPMI-1640 (Life Technologies Corp., Carlsbad, CA, USA) supplemented with L-glutamine, 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich) and a penicillin/streptomycin antibiotic mixture (5 mL of a commercial mixture of 100 U/mL penicillin + 100 μg/mL streptomycin per 500 mL medium, Sigma-Aldrich) at 37 °C and humidified 5% CO_2. Cells were split every 3-4 days and maintained at a cell density up to 1.5 x 10^6 viable cells/mL.

To evaluate the compatibility of the NPs, THP-1 monocytes were initially differentiated into macrophages. For this, cells were cultured in 96-well plates (20 x 10^3 cells/well). Next, phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) was added at a concentration of 50 ng/mL in a serum-free RPMI-1640 medium and cells allowed to attach to the surface for 48 h at 37 °C and 5% CO_2. After 24 h, the cells were washed twice with PBS, and the medium was replaced with RPMI-1640 containing 10% FBS for further analysis. Then, the medium was replaced by fresh medium containing 10% FBS, and the sterile NPs sample (1% w/v in PBS of pH 7.4, Sigma-Aldrich) was added to a final NP concentration of 0.01%-0.1% w/v. For these assays, hGM-*g*-PMMA30 NPs were sterilized by filtration (sterile 0.22 μm syringe filters, Merck Millipore Ltd., Cork, Ireland) under the biological hood. After 24-72 h incubation, the medium was replaced by new medium (100 μL) and sterile 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 25 μL, 5 mg/mL, Sigma-Aldrich) was added. Following 4 h incubation at 37 °C and 5% CO_2, formazan crystals were dissolved in 100 μL DMSO and the absorbance measured at 530 nm (with reference to the absorbance at 670 nm) in a spectrophotometer (Multiskan GO™, Thermo Fisher Scientific Oy, Vantaa, Finland). Cells treated only with culture medium were considered as 100% viable.

THP-1 cell uptake of 0.1% w/v NPs in PBS at 37 °C was characterized by confocal laser scanning microscopy (CLSM). For this, THP-1-derived macrophages were cultured with 0.4 mL of RPMI-1640 medium containing FITC-labeled NPs (0.1% w/v in PBS) or without NPs (control) in an 8-well chamber (60 x 10^3 cells/well) with a glass bottom (Ibidi, Gräfelfing, Germany). Cells were incubated for 1 and 24 h at 37 °C and for 1 h at 4 °C. Subsequently, the medium was removed, and the cells were washed twice with PBS and Hoechst 33342 (0.2 μg/mL, Sigma-Aldrich) in RPMI-1640 was added. Then, samples were visualized under a CLSFM LSM 700 (Carl Zeiss Inc., Oberkochen, Germany).

NP uptake by THP-1 cells was quantified by imaging flow cytometry. For this, THP-1-derived macrophages were cultured with 1.5 mL of RPMI-1640 medium containing FITC-labeled NPs (0.1% w/v in PBS) or without NPs (control). The cells were subjected to incubation at 37 °C for 1 and 24 h. Following this, the medium was removed, and the cells were washed twice with PBS. The cells were harvested using a sterile plastic scraper, centrifuged at 250g for 5 min, and resuspended in 30 µL of culture medium. To quantify cell uptake, live cells were analyzed using an Amnis® ImageStream®X Mark II imaging flow cytometer (Luminex, Corp., Austin, TX, USA) equipped with 405, 488, 560, and 642 nm lasers, at a magnification of 40×. The instrument was calibrated using SpeedBeads™ (GE Healthcare, Chicago, IL, USA). In each experiment, 5000 events per sample were acquired. The acquired images were analyzed with IDEAS® analysis Software (Luminex, Corp.). We employed a four-step gating technique to identify exclusively the living cells that had taken up the NPs [2]. Initially, we utilized a gradient root mean square analysis on the image sharpness histogram to identify the cells that were in focus. Next, we used a scatter plot of aspect ratio/area to filter for single cells. Then, we applied the Haralick texture feature to eliminate the non-living cells. Lastly, we performed image analysis on the remaining living, single cells that were in focus to determine the presence of NPs in the cytosol and to rule out NPs that underwent adsorption onto the cell membrane. To achieve this, we used masks that exclude the membrane region. The percentage of cells that had taken up NPs (%Uptake) was calculated by averaging at least three separate experiments using Equation 1

$$\%Uptake = \frac{S_{NP}}{Total\ cells}\ x\ 100 \quad (1)$$

Where S_{NP} is the number FITC-positive THP-1 cells out of the Total cells.

Total RNA-sequencing and data processing

To investigate the effect of hGM-*g*-PMMA30 NPs on THP-1 gene expression, we conducted RNA-seq analysis. In this study, THP-1-derived macrophages were cultured in 6-well plates (1.5 x 10^6 cells/well) with either sterile hGM-*g*-PMMA30 NPs (at a concentration of 0.1% w/v in PBS) or without (control). The cells were incubated for 24 h at 37 °C, and the experiments were performed in quadruplicate (n = 4 for each experimental group). After removing the cell medium, the cells were washed with PBS,

and TRIzol (700 µL, Invitrogen, Carlsbad, CA, USA) was added to each well. The RNA was extracted according to the manufacturer's protocol and stored at -80 °C until further use. RNA sequencing was performed using the CEL-seq method on an Illumina HiSeq 2500 (Illumina, Inc., San Diego, CA, USA) at the "Technion Genome Center". Quality control was performed using FASTQC (version 0.11.5, Babraham Bioinformatics, The Babraham Institute, Cambridge, UK), and the reads were mapped to the genome assembly using the Tophat2 version 2.1.0 algorithm [3]. The reads were counted using HTseq-count version 0.11.2 [4], and the raw counts were normalized and analyzed for differential expression using DESeq2 in the R platform version 1.24.0 [5], with Padj values calculated using the Benjamini and Hochberg correction for false discovery.

Bioinformatics analysis of RNA-seq data

The DESEQ2 package in the R platform was used to generate a 2D principal component analysis (PCA) plot, which visualizes the samples based on their first two principal components [5]. To evaluate pathway enrichment in the RNA-seq data, gene set enrichment analysis (GSEA) was employed on all genes ranked by their fragments per kilobase of exon per million (FPKM) values in the RNA-seq data (http://www.gsea-msigdb.org/gsea/login.jsp; version 4.3.2) [6]. The Hallmarks (h.all.v2022.1.Hs.symbols.gmt) and c2.KEGG (c2.cp.kegg.v2022.1.Hs.symbols.gmt) gene set collections from the Molecular Signature Database (MSigDB) [7,8] were investigated in this study. To map Ensemble IDs to gene symbols for all genes in the MSigDB collection, the Array Annotations database (Human_Ensembl_Gene_ID_MSigDB.v2022.1.Hs.chip) was used. The false discovery rate (FDR) < 0.25 and normal $p < 0.05$ were set as significant cutoff values.

Gene expression

To study the expression of key genes, we conducted real time-quantitative polymerase chain reaction (RT-qPCR) analysis. For this, THP-1-derived macrophages were grown in 6-well plates (1.5×10^6 cells/well) in 1.5 mL of RPMI-1640 medium. After resting for 24 h, cells were exposed to two different treatments: (i) sterile NP suspensions (0.1% w/v) for 24 h, (ii) pre-treatment with LPS (50 ng/mL, Sigma-Aldrich) and IFN-γ (20 ng/mL, Biolegend) for 24 h and incubation with sterile NP suspensions (0.1% w/v) for 24 h. Untreated cells were used as control. Total RNA was extracted by using TRIzol (Invitrogen, Carlsbad, CA, USA) according to a standard protocol. Sample quality was assessed by a spectrophotometer (NanoDrop Technologies, Wilmington,

DE, USA). The 260/280 ratios in all the samples were >1.8, and the 260/230 ratios were >1.7. Complementary DNA (cDNA) was synthesized from 1 µg of RNA with the qScript™ cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, USA) according to the manufacturer's instructions. The mRNA expression levels of *IL-6* (Hs00174131_m1), *CXCL8* (Hs00174103_m1), *IL-10* (Hs00961622_m1), *TNF-α* (Hs00174128_m1), *MRC1* (Hs00267207_m1), and *β-actin* (Hs99999148_m1) were quantified using TaqMan® Gene Expression assays (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) and a RT-qPCR 7300 system (Applied Biosystems). The relative quantity (RQ) values were calculated according to the $2^{-\Delta\Delta Ct}$ method, which reflects the differences in the threshold for each target gene relative to β-actin and untreated control cells.

Cytokine release

To assess phenotypic changes, we assessed the effect of hGM-g-PMMA30 NPs on cytokine production by THP-1-derived macrophages. To quantify the levels of IL-6, IL-1β, INF-g, IL-8, IL-10, IL-4 and IL-17A, commercial enzyme-linked immunosorbent assays (ELISA) kits (Biolegend) were used, while the concentration of TNF-α was measured using an Invitrogen kit. For this, THP-1-derived macrophages were grown in RPMI-1640 medium in 24-well plates (2×10^5 cells/well) and exposed to two treatments: (i) sterile hGM-*g*-PMMA30 NPs (0.1% w/v) for 24 h, and (ii) LPS (50 ng/mL, Sigma-Aldrich) and INF-g (20 ng/mL, Biolegend) for 24 h followed by incubation with sterile hGM-*g*-PMMA30 NPs (0.1% w/v) for 24 h. Untreated cells were used as control. For quantification of INF-g release, cells were polarized only with LPS or left unpolarized. Following 24- or 72-h treatment, the medium was collected, centrifuged, and stored at -20 °C until analysis. The absorbance was measured at 450 nm (reference at 570 nm) using a spectrophotometer (Multiskan GOTM) to quantify the different cytokines.

Fibroblast migration

The migration of the murine fibroblast cell line NIH/3T3 (ATCC® CRL-1658™) in the presence of THP-1-derived macrophages exposed or not to the NPs was assessed in a co-culture model assay [28]. The NIH/3T3 was kindly supplied by Prof. Boaz Mizrahi (Department of Biotechnology and Food Engineering, Technion – Israel Institute of Technology) and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies, Corp.) supplemented with L-glutamine, 10% heat-inactivated FBS, and

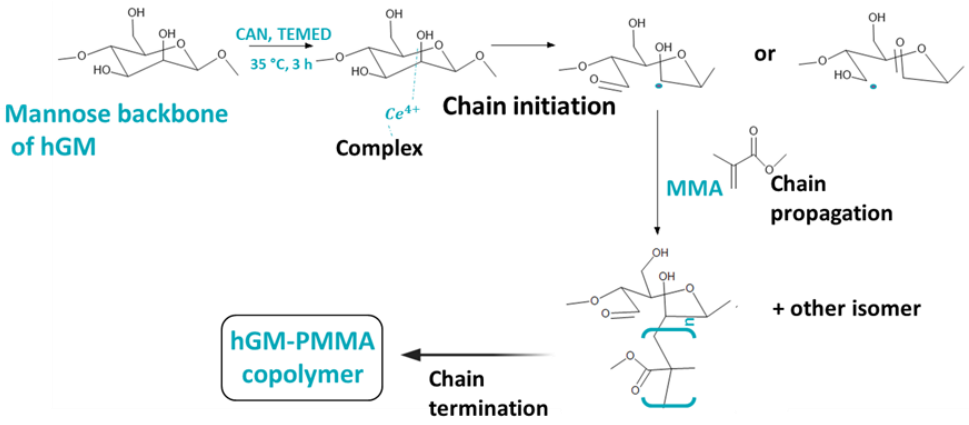
a penicillin/streptomycin antibiotic mixture (5 mL of a commercial mixture of 100 U/mL penicillin + 100 μg/mL streptomycin per 500 mL medium). Cells were trypsinized (trypsin-EDTA 0.25% w/v, Sigma-Aldrich) every 3-4 days, and the quantification of the living cells was carried out by using the trypan blue (0.4% w/v) exclusion assay.

For the co-culture migration assay, THP-1-derived macrophages (2×10^5 cells/well) were cultured in the bottom of 24-wells plate of chemotaxis chambers (membrane pore size of 8.0 μm, Corning® Costar®, Corning, NY, USA) at 37 °C in RPMI-1640 overnight. Then, cells were treated with NPs (0.1% and 0.5% w/v) for 24 h, the medium was washed out, cells were washed twice with PBS and 600 μL of RPMI-1640 was added. To conduct these experiments, NIH/3T3 cells were cultured in the upper wells of the chemotaxis chambers at a density of 1×10^4 cells/insert, in DMEM-high glucose containing 10% FBS. The medium was replaced with DMEM-high glucose containing 0.5% FBS, and the inserts placed above THP-1 cell cultures pre-treated with the NPs or left untreated. The co-culture system was incubated for 48 or 72 h, the fibroblasts in the upper side of the insert were removed using a sterile cotton swab, and the fibroblasts that crossed the membrane were stained and counted. For the 48-h migration assays, fibroblasts were stained with CellTracker™ Green (1:500, Abcam, Cambridge, UK) for 30 min at 37 °C, washed twice with PBS, and imaged using an Eclipse TS100 inverted fluorescent microscope (Nikon Instruments, Tokyo, Japan) equipped with a 1-FM Epi-Fluorescence Attachment. For the 72-h migration assays, cells were fixed with 700 μL ethanol (70% in water) for 10 min, and crystal violet (0.2% w/v in 95% ethanol, 700 μL, Sigma-Aldrich) added for 5 min at RT. The crystal violet excess was gently washed out and the insert was allowed to dry. Cell migration was assessed by counting the number of migrated cells in three randomly selected microscopy fields per well at 10x magnification with ImageJ 1.52 software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

GraphPad Prism software version 7.04 (GraphPad Software, San Diego, CA, USA) was used to perform all statistical analyses. The results were presented as the Mean ± S.D. and were derived from a minimum of three independent experiments. In all tests, a value of $P \leq 0.05$ was deemed significant.

Supplementary Scheme, Tables and Figures



Scheme S1. Synthetic pathway of the hGM-*g*-PMMA30 copolymer.

Table S1. Hydrodynamic diameter (D_h) and polydispersity index (PDI) of 0.1% w/v hGM-*g*-PMMA30 NPs in PBS over time, as measured by DLS at 25 °C.

Incubation time (Days)	Dh ± S.D. (nm) – by Intensity	Dh ± S.D. (nm) – by Number	PDI ± S.D.
1	140 ± 10	76 ± 4	0.27 ± 0.01
14	160 ± 5	85 ± 8	0.15 ± 0.02
730	140 ± 5	68 ± 5	0.54 ± 0.03

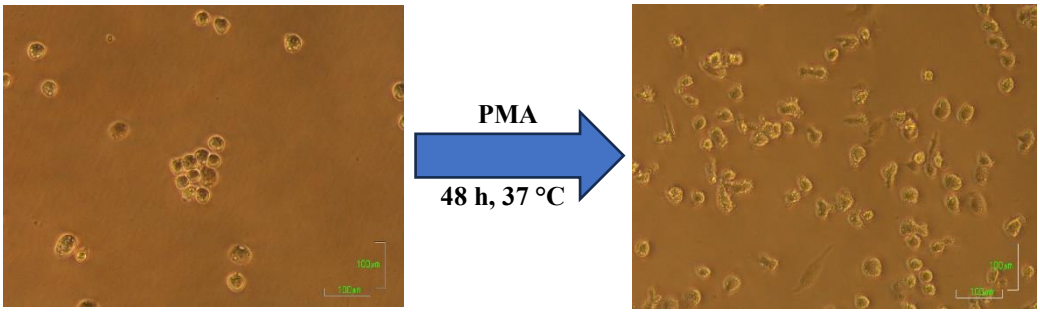


Figure S1. Morphological change in THP-1 monocytes upon differentiation into macrophages with PMA.

Table S2. Significantly upregulated and downregulated gene genes by THP-1-derived macrophages upon exposure to 0.1% w/v hGM-*g*-PMMA30 NPs for 24 h.

Gene symbol	Protein name	Biological function of the protein
Upregulated genes		
MT1L	Metallothionein-1L	Metal binding protein involved in metal homeostasis and detoxification
MT1G	Metallothionein-1G	Metal binding protein involved in metal homeostasis and detoxification
S100A8	S100 calcium-binding protein A8	Calcium-binding protein involved in immune response and inflammation
S100A9	S100 calcium-binding protein A9	Calcium-binding protein involved in immune response and inflammation
CILP	Cartilage intermediate layer protein	Involved in cartilage formation and maintenance
MT1F	Metallothionein-1F	Metal binding protein involved in metal homeostasis and detoxification
S100A12	S100 calcium-binding protein A12	Calcium-binding protein involved in immune response and inflammation
MT1X	Metallothionein-1X	Metal binding protein involved in metal homeostasis and detoxification
CCL22	C-C motif chemokine ligand 22	Chemokine involved in immune response and inflammation
MT2A	Metallothionein-2A	Metal binding protein involved in metal homeostasis and detoxification
G0S2	G0/G1 switch gene 2	Regulates adipogenesis and lipolysis
SAMD10	Sterile alpha motif domain containing 10	Protein of unknown function
APOC1P1	Apolipoprotein C1 pseudogene 1	Non-coding RNA
SPINT1-AS1	Serine peptidase inhibitor, Kunitz type 1 antisense RNA 1	Non-coding RNA
NPR1	Natriuretic peptide receptor 1	Receptor for natriuretic peptides involved in regulation of blood pressure and fluid balance
MT1E	Metallothionein-1E	Metal binding protein involved in metal homeostasis and detoxification
MCUB	Mitochondrial calcium uniporter	Transports calcium ions into mitochondria
MYO15B	Myosin XVb	Actin-based motor protein involved in vesicle transport
CORO1A	Coronin 1A	Actin-binding protein involved in cell motility and immune response
ASS1	Argininosuccinate synthase 1	Catalyzes the formation of argininosuccinate from citrulline and aspartate in the urea cycle
ZNF837	Zinc finger protein 837	Transcription factor involved in regulation of gene expression

KIFC2	Kinesin family member C2	Microtubule-based motor protein involved in intracellular transport
CA11	Carbonic anhydrase 11	Catalyzes the reversible hydration of carbon dioxide
APOE	Apolipoprotein E	Binds to and transports lipids, involved in cholesterol metabolism
IFT140	Intraflagellar transport protein 140 homolog	Involved in cilia formation and maintenance
PTGDS	Prostaglandin D2 synthase	Catalyzes the conversion of prostaglandin H2 to prostaglandin D2
ENKD1	Enkurin domain containing 1	Protein of unknown function
BEX1	Brain expressed X-linked protein 1	Involved in neuronal development and differentiation
CHIT1	Chitinase 1	Hydrolyzes chitin, a component of fungal cell walls
HMOX1	Heme oxygenase 1	HMOX1 is an enzyme that catalyzes the breakdown of heme into biliverdin, carbon monoxide (CO), and iron. It plays a role in regulating oxidative stress, inflammation, and cellular homeostasis. HMOX1 induction has been shown to confer protection against a variety of stresses, including ischemia-reperfusion injury, sepsis, and oxidative stress.
NXF3	NXF3	Nuclear RNA export factor 3
MGST1	MGST1	Glutathione S-transferase Mu 1
ALOX5AP	ALOX5AP	Arachidonate 5-lipoxygenase-activating protein
POU2F2	POU2F2	POU domain, class 2, transcription factor 2
YPEL3	YPEL3	Yippee-like protein 3
APOC1	APOC1	Apolipoprotein C1
SLC25A23	SLC25A23	Solute carrier family 25 member 23
ADD3	ADD3	Adducin 3
HSPB1	HSPB1	Heat shock protein beta-1
EPHX1	EPHX1	Epoxide hydrolase 1
LAIR1	LAIR1	Leukocyte-associated immunoglobulin-like receptor 1
FADS3	FADS3	Fatty acid desaturase 3
DGAT2	DGAT2	Diacylglycerol O-acyltransferase 2
SLC27A3	SLC27A3	Solute carrier family 27 member 3
CYP1B1	CYP1B1	Cytochrome P450 family 1 subfamily B member 1
SLC17A7	SLC17A7	Solute carrier family 17 member 7
TBL1XR1	TBL1XR1	Transducin beta-like 1X-linked receptor 1
MUTYH	MUTYH	MutY homolog
TIMP1	TIMP1	Tissue inhibitor of metalloproteinases 1
MMP7	MMP7	Matrix metallopeptidase 7
RPRD2	RPRD2	Regulation of nuclear pre-mRNA domain-containing protein 2

ARHGAP9	ARHGAP9	Rho GTPase-activating protein 9
PRXL2A	PRXL2A	Prospero homeobox-like 2
NRGN	NRGN	Neuronal regeneration-related protein
PDE1B	PDE1B	Phosphodiesterase 1B
MND1	MND1	Meiotic nuclear divisions protein 1 homolog
FTH1	FTH1	Ferritin heavy chain 1
NDUFS2	NDUFS2	NADH-ubiquinone oxidoreductase 75 kDa subunit
Downregulated genes		
MSR1	Macrophage scavenger receptor 1	Mediates the binding and uptake of oxidized low-density lipoproteins (oxLDL)
CD36	CD36 antigen	Binds multiple ligands, including lipids, apoptotic cells, and pathogens, and has roles in lipid metabolism, immune response, and angiogenesis
NRAP	Nebulin-related-anchoring protein	Anchors nebulin and alpha-actinin to the sarcoplasmic reticulum in skeletal muscle
FABP4	Fatty acid-binding protein 4	Binds fatty acids and other hydrophobic ligands, and is involved in the regulation of lipid metabolism and glucose homeostasis
CCL1	Chemokine (C-C motif) ligand 1	Chemoattractant for T cells and basophils, and has roles in inflammation and immune response
AC006967.3	Uncharacterized protein	Currently, there is no information available on the function of this protein
SSTR2	Somatostatin receptor type 2	Mediates the effects of somatostatin, a peptide hormone that regulates the endocrine and nervous systems
MAF	v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog	Transcription factor that regulates the expression of genes involved in cell differentiation, proliferation, and apoptosis
CAMK2N1	Calcium/calmodulin-dependent protein kinase II inhibitor 1	Inhibits the activity of calcium/calmodulin-dependent protein kinase II, which is involved in various cellular processes, including synaptic plasticity and gene expression
EPB41L2	Erythrocyte membrane protein band 4.1-like 2	Plays a role in the organization and stability of the cytoskeleton, and has been implicated in tumorigenesis
FAM131B	Family with sequence similarity 131 member B	Regulates the activity of AKT and mTOR, two protein kinases that play important roles in cell growth, survival, and metabolism
MEPE	Matrix extracellular phosphoglycoprotein	Involved in the regulation of mineralization in bones and teeth
AL109918.1	Uncharacterized protein	Unknown
CYP26B1	Cytochrome P450 family 26 subfamily B member 1	Catalyzes the metabolism of retinoic acid, a vitamin A derivative that plays important roles in embryonic development and tissue homeostasis

VAT1L	Vesicle amine transport protein 1 homolog-like	Involved in the transport and storage of neurotransmitters, including dopamine and serotonin
SECTM1	Secreted and transmembrane 1	Plays a role in immune regulation and tumor progression
INHBE	Inhibin beta E chain	Subunit of the activin/inhibin complex, which regulates the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH)
ZBTB7C	Zinc finger and BTB domain-containing protein 7C	Transcription factor that regulates the expression of genes involved in cell proliferation and differentiation
ANKRD29	Ankyrin repeat domain-containing protein 29	Plays a role in cell adhesion and migration, and has been implicated in cancer metastasis
SCG2	Secretogranin II	Involved in the processing and sorting of secreted proteins.
OR7E28P	Olfactory receptor 7E28 pseudogene	Unknown
TM4SF19	Transmembrane 4 L six family member 19	May be involved in the regulation of cell adhesion and migration.
WIPF3	WAS/WASL interacting protein family member 3	Involved in the regulation of actin polymerization and cytoskeletal organization.
DAPL1	Death associated protein like 1	May play a role in the regulation of cell death pathways.
SUCNR1	Succinate receptor 1	Functions as a G protein-coupled receptor for succinate, a metabolite involved in energy metabolism.
SCUBE1	Signal peptide, CUB domain, EGF-like 1	May be involved in the regulation of cell adhesion and migration.
CCL2	C-C motif chemokine ligand 2	Functions as a chemoattractant for monocytes and macrophages.
GPR34	G protein-coupled receptor 34	May play a role in immune regulation and inflammation.
LPAR6	Lysophosphatidic acid receptor 6	Functions as a G protein-coupled receptor for lysophosphatidic acid, a signaling molecule involved in various cellular processes.
FA2H	Fatty acid 2-hydroxylase	Catalyzes the hydroxylation of fatty acids, which may play a role in the regulation of cell signaling and metabolism.
KCNA2	Potassium voltage-gated channel subfamily A member 2	Forms a voltage-gated ion channel that regulates the flow of potassium ions across cell membranes.
SIAE	Sialic acid acetylesterase	Catalyzes the hydrolysis of acetyl groups from sialic acid residues, which may play a role in immune regulation and inflammation.
MFGE8	Milk fat globule-EGF factor 8 protein	Functions in the clearance of apoptotic cells and the regulation of inflammation.
LINC02154	Long intergenic non-protein coding RNA 2154	Unknown

GPC3	Glypican 3	Functions in the regulation of cell growth and differentiation.
SDK1	Sidekick cell adhesion molecule 1	May be involved in the regulation of cell adhesion and migration.
MTUS1	Microtubule associated tumor suppressor 1	May play a role in the regulation of microtubule dynamics and the cell cycle.
SLAMF6	SLAM family member 6	Functions as an activating receptor on natural killer cells and may play a role in immune regulation.
GDF15	Growth differentiation factor 15	Functions as a cytokine that regulates various cellular processes, including cell growth and differentiation, inflammation, and metabolism.
CD84	CD84 molecule	May be involved in the regulation of immune cell function and activation.
GPNMB	Glycoprotein non-metastatic melanoma protein B	May play a role in the regulation of cell growth and differentiation, and may be involved in the progression of certain types of cancer.
LRCH1	Leucine-rich repeats and calponin homology domain containing 1	May be involved in the regulation of cell adhesion and migration.
NLK	Nemo-like kinase	Plays a role in cell cycle regulation and apoptosis
CTSW	Cathepsin W	A lysosomal protease involved in protein degradation and antigen presentation
SPSB1	SplA/ryanodine receptor domain and SOCS box containing protein 1	A protein involved in regulating various signaling pathways
SERPINI1	Serpin family I member 1	A serine protease inhibitor involved in blood coagulation and fibrinolysis
FCRLA	Fc receptor-like A	Belongs to the immunoglobulin superfamily and is involved in immune responses
ITGB5	Integrin beta-5	A transmembrane protein that plays a role in cell adhesion and signaling
NMB	Neuromedin B	A peptide hormone involved in various physiological processes such as smooth muscle contraction and blood pressure regulation
CYBB	Cytochrome b-245 beta chain	A component of the phagocyte NADPH oxidase system involved in generating reactive oxygen species
GUCY1A2	Guanylate cyclase 1 soluble subunit alpha-2	An enzyme that catalyzes the production of cyclic GMP and is involved in various physiological processes such as smooth muscle relaxation
MCPH1	Microcephalin 1	Involved in brain development and cell cycle regulation
ISYNA1	Inositol-3-phosphate synthase A1	An enzyme involved in inositol synthesis

LIPA	Lysosomal acid lipase	A lysosomal enzyme involved in lipid metabolism
EMP1	Epithelial membrane protein 1	A membrane protein involved in cell adhesion and signaling
SLC15A3	Solute carrier family 15 member 3	A transporter protein involved in the transport of small peptides and drugs
UGCG	UDP-glucose ceramide glucosyltransferase	An enzyme involved in the synthesis of glycosphingolipids
DBI	Diazepam binding inhibitor	A protein involved in the regulation of the GABAergic system
A2M	Alpha-2-macroglobulin	A protease inhibitor involved in various physiological processes such as immune defense and wound healing
PNOC	Prepronociceptin	A precursor protein involved in pain modulation
CSF1	Colony stimulating factor 1	A cytokine that regulates the proliferation, differentiation, and survival of macrophages
DUSP2	Dual specificity phosphatase 2	A phosphatase involved in the regulation of various signaling pathways
ATP6V1H	ATPase H+ transporting V1 subunit H	A component of the V-ATPase complex involved in acidification of intracellular compartments
PCSK6	Proprotein convertase subtilisin/kexin type 6	A protease involved in the processing of various precursor proteins
LPL	Lipoprotein lipase	An enzyme involved in the hydrolysis of triglycerides in lipoproteins
LINC01010	Long intergenic non-protein coding RNA 1010	A non-coding RNA with an unknown function
PLD3	Phospholipase D family member 3	An enzyme involved in phospholipid metabolism
GINS3	GINS complex subunit 3	A component of the GINS complex involved in DNA replication
MGP	Matrix Gla protein	MGP is a vitamin K-dependent protein that acts as an inhibitor of calcification. It plays a role in regulating bone mineralization and the prevention of arterial calcification.
TGM2	Transglutaminase 2	Catalyzes the cross-linking of proteins and stabilizes extracellular matrix, involved in cell adhesion and apoptosis
RASD2	RASD family, member 2	Involved in the regulation of G protein signaling
AP3M2	Adaptor-related protein complex 3, mu 2 subunit	Component of the AP3 complex, involved in intracellular vesicle trafficking
DCSTAMP	Dendrocyte expressed seven transmembrane protein	Cell surface receptor involved in osteoclast differentiation
SGK1	Serum and glucocorticoid-regulated kinase 1	Involved in the regulation of ion transport, cell survival, and glucose homeostasis

DYSF	Dysferlin	Involved in the repair of damaged muscle membranes
FAM234B	Family with sequence similarity 234, member B	Function is currently unknown
PLXDC2	Plexin domain containing 2	Cell surface receptor involved in cell adhesion and migration
SAMD4A	Sterile alpha motif domain containing 4A	Involved in mRNA degradation and regulation of translation
ATP1B1	Sodium/potassium-transporting ATPase subunit beta-1	Component of the sodium-potassium ATPase, involved in the maintenance of ion homeostasis
ITGA11	Integrin alpha-11	Cell surface receptor involved in cell adhesion and migration
SPRED1	Sprouty-related EVH1 domain-containing protein 1	Involved in the regulation of cell growth and differentiation
RIOX1	Ribosomal oxygenase 1	Involved in the biosynthesis of modified nucleosides in ribosomal RNA
FCER1G	High affinity immunoglobulin E receptor gamma subunit	Component of the high-affinity IgE receptor, involved in allergic reactions
FCMR	Fc mu receptor	Cell surface receptor involved in the regulation of B cell activation and proliferation
AQP9	Aquaporin 9	Transmembrane protein involved in the transport of water and glycerol
GPC4	Glypican 4	Cell surface proteoglycan involved in the regulation of growth factor signaling
MAFF	Transcription factor MAFF	Involved in the regulation of gene expression and the response to oxidative stress
KLF6	Krüppel-like factor 6	Transcription factor involved in the regulation of cell proliferation and differentiation
FLRT2	Fibronectin leucine-rich transmembrane protein 2	Cell surface receptor involved in cell adhesion and migration
RBMX	RNA binding motif protein, X-linked	Involved in the regulation of RNA splicing and translation
RABGAP1L	RAB GTPase activating protein 1-like	Involved in the regulation of intracellular vesicle trafficking
CTSH	Cathepsin H	Lysosomal protease involved in protein degradation
ZFP36L1	Zinc finger protein 36, C3H type-like 1	Involved in the regulation of mRNA stability and the immune response
SAMHD1	SAM and HD domain-containing protein 1	Involved in the regulation of nucleotide metabolism and immune response
TKTL1	Transketolase-like protein 1	Involved in the regulation of glucose metabolism
CD109	CD109 antigen	Cell surface receptor involved in the regulation of TGF-beta signaling
KIAA0930	Unknown	Unknown

SCARB2	Scavenger receptor class B member 2	Involved in the uptake of lipids such as cholesterol and regulation of lipid metabolism
ATF3	Activating transcription factor 3	Transcription factor involved in stress response and inflammation
HSD17B4	Hydroxysteroid 17-beta dehydrogenase 4	Enzyme involved in fatty acid beta-oxidation
SUSD1	Sushi domain-containing protein 1	Involved in cell adhesion and signaling
DAB2	Disabled homolog 2	Adaptor protein involved in endocytosis and signal transduction
ARHGEF3	Rho guanine nucleotide exchange factor 3	Activates Rho GTPases involved in cytoskeletal organization and cell motility
SLC17A5	Solute carrier family 17 member 5	Transports sulfate and thiosulfate ions across the cell membrane
ERMP1	Endoplasmic reticulum metallopeptidase 1	Protease involved in processing of secretory proteins
OXR1	Oxidation resistance 1	Involved in antioxidant defense and protection against oxidative stress
C5AR2	Complement component 5a receptor 2	Receptor for complement component 5a, involved in inflammatory response
TNS3	Tensin-3	Adaptor protein involved in cell adhesion and migration
NES	Nestin	Intermediate filament protein involved in neural stem cell differentiation
ANKRD10	Ankyrin repeat domain-containing protein 10	Function unknown
RPLP0P2	Ribosomal protein lateral stalk subunit P0 pseudogene 2	Non-functional pseudogene of ribosomal protein
WARS	Tryptophan--tRNA ligase, cytoplasmic	Enzyme involved in protein synthesis
GNS	N-acetylglucosamine-6-sulfatase	Enzyme involved in the catabolism of glycosaminoglycans
VPS37C	Vacuolar protein sorting-associated protein 37C	Involved in endosomal protein sorting
NAP1L1	Nucleosome assembly protein 1-like 1	Involved in histone assembly and chromatin remodeling
HPCAL1	Hippocalcin-like protein 1	Calcium-binding protein involved in signal transduction
FGL2	Fibrinogen-like protein 2	Involved in blood coagulation and immune regulation
SNX9	Sorting nexin-9	Involved in endocytosis and intracellular trafficking
MSMO1	Methylsterol monooxygenase 1	Enzyme involved in cholesterol biosynthesis
RGS2	Regulator of G-protein signaling 2	Modulator of G protein-coupled receptor signaling
CTSS	Cathepsin S	Protease involved in antigen processing and presentation

SNX4	Sorting nexin-4	Involved in intracellular trafficking and membrane dynamics
ITGB1	Integrin beta-1	Cell adhesion protein involved in cytoskeleton organization and signaling
WDR1	WD repeat-containing protein 1	Regulator of actin cytoskeleton dynamics
ITGA5	Integrin alpha-5	Cell adhesion protein involved in cell migration and signaling
PI4K2A	Phosphatidylinositol 4-kinase alpha	Catalyzes the phosphorylation of phosphatidylinositol to form phosphatidylinositol 4-phosphate, which plays a role in cell signaling and membrane trafficking
ASAH1	Acid ceramidase	Hydrolyzes the sphingolipid ceramide to generate sphingosine, which is involved in cell signaling and apoptosis
RGS1	Regulator of G protein signaling 1	Inactivates G protein-coupled receptors (GPCRs) by accelerating the GTPase activity of the G alpha subunit
NCOA3	Nuclear receptor coactivator 3	Enhances the transcriptional activity of nuclear hormone receptors by binding to them and recruiting other transcriptional coactivators
LRRC8A	Leucine-rich repeat-containing protein 8A	Component of a volume-regulated anion channel that plays a role in cell volume regulation and apoptosis
PRAG1	PR/SET domain-containing protein 1	Regulator of histone methylation that plays a role in transcriptional regulation and DNA repair
IRS1	Insulin receptor substrate 1	Mediator of insulin signaling that plays a role in glucose homeostasis
SUN2	Sad1 and UNC84 domain-containing protein 2	Component of the linker of nucleoskeleton and cytoskeleton (LINC) complex that plays a role in nuclear positioning and migration
RBM3	RNA-binding motif protein 3	RNA-binding protein that plays a role in mRNA splicing and stability
ITGB7	Integrin beta-7	Cell adhesion molecule that plays a role in leukocyte trafficking and homing
STOM	Stomatin	Membrane protein that plays a role in the regulation of ion channels and transporters
LCP2	Lymphocyte cytosolic protein 2	Adaptor protein that plays a role in T cell activation and signaling
BCL2A1	B-cell lymphoma 2-related protein A1	Anti-apoptotic protein that plays a role in cell survival and differentiation
M6PR	Cation-dependent mannose-6-phosphate receptor	Mediates the transport of lysosomal enzymes from the Golgi to lysosomes
UBALD1	Ubiquitin-associated and SH3 domain-containing protein A	Adapter protein that plays a role in protein degradation and trafficking

SYT7	Synaptotagmin-7	Calcium-binding protein that plays a role in synaptic vesicle exocytosis and neurotransmitter release
HSD3B7	3-beta-hydroxysteroid dehydrogenase type 7	Enzyme involved in bile acid synthesis
TOP1	DNA topoisomerase 1	Enzyme that relieves DNA supercoiling by introducing reversible single-strand breaks
KRR1	KRR1 small subunit processome component homolog	Component of the small subunit (SSU) processome that plays a role in ribosome biogenesis
MMP14	Matrix metalloproteinase-14	Zinc-dependent endopeptidase that plays a role in extracellular matrix remodeling and cell signaling
MYO1E	Myosin IE	Actin-based motor protein that plays a role in intracellular transport and membrane dynamics
ESYT2	Extended Synaptotagmin-2	Binds to and transfers phospholipids between two bilayer membranes during vesicle fusion
HS2ST1	Heparan sulfate 2-O-sulfotransferase 1	Catalyzes the transfer of sulfate to position 2 of uronic acid residues of heparan sulfate proteoglycans
SLC16A3	Monocarboxylate transporter 4	Facilitates the transport of monocarboxylates, such as lactate and pyruvate, across the plasma membrane
PLEKHO1	Pleckstrin homology domain-containing family O member 1	Involved in cell signaling pathways that regulate the actin cytoskeleton and cell adhesion
PSEN2	Presenilin-2	Component of the gamma-secretase complex, which cleaves transmembrane proteins such as amyloid precursor protein and Notch
CNIH3	Cornichon family AMPA receptor auxiliary protein 3	Modulates AMPA receptor gating and trafficking
LMO4	LIM domain only 4	Transcription factor that plays a role in embryonic development and cell differentiation
KCNAB2	Potassium voltage-gated channel subfamily A member accessory beta subunit 2	Modulates the function and gating properties of voltage-gated potassium channels
TNFAIP8L3	Tumor necrosis factor alpha-induced protein 8-like 3	Involved in regulating cell proliferation and apoptosis, as well as inflammation and immune responses
SPP1	Secreted phosphoprotein 1	Mediates cell adhesion, migration, and signaling, and is involved in bone mineralization and immune responses
COL6A1	Collagen type VI alpha 1 chain	A component of extracellular matrix that provides structural support to tissues and organs

COPA	Coatomer subunit alpha	Forms a complex with other proteins that coats vesicles and facilitates the transport of proteins between different cellular compartments
SEPT9	Septin-9	Plays a role in cytokinesis, cell division, and cell migration
GLCCI1	Glucocorticoid-induced transcript 1 protein	Involved in the regulation of glucocorticoid receptor signaling and steroid hormone metabolism
FLNA	Filamin A	A cytoskeletal protein that crosslinks actin filaments and anchors transmembrane proteins to the cytoskeleton
CIZ1	CDKN1A-interacting zinc finger protein 1	Regulates DNA replication and cell cycle progression
CYFIP1	Cytoplasmic FMR1-interacting protein 1	Involved in the regulation of cytoskeletal dynamics and cell migration
LIMS1	LIM and senescent cell antigen-like-containing domain protein 1	Regulates cell adhesion and migration, and plays a role in the development of the nervous system
PTBP1	Polypyrimidine tract-binding protein 1	Involved in pre-mRNA splicing and mRNA stability and transport
CAB39	Calcium-binding protein 39	Activates the protein kinase activity of LKB1, which is involved in regulating cell growth and metabolism
MYL12A	Myosin regulatory light chain 12A	Involved in regulation of smooth muscle contraction and cell migration
FAM129B	Family with sequence similarity 129, member B	Functions in cell adhesion, migration, and proliferation
TPT1	Tumor protein, translationally-controlled 1	Regulates various cellular processes including proliferation, differentiation, and apoptosis
MBP	Myelin basic protein	Structural component of the myelin sheath in the central nervous system
IRAK1	Interleukin-1 receptor-associated kinase 1	Mediates signaling downstream of Toll-like receptors and interleukin-1 receptors
ZBTB7A	Zinc finger and BTB domain-containing protein 7A	Transcriptional regulator involved in cellular differentiation and proliferation
CTSB	Cathepsin B	Lysosomal cysteine protease involved in protein degradation and processing
TLN1	Talin-1	Adaptor protein that links integrins to the actin cytoskeleton and plays a role in cell adhesion and migration
CELF1	CUGBP, Elav-like family member 1	RNA-binding protein that regulates alternative splicing and translation of target mRNAs
ZYX	Zyxin	Acts as a scaffold protein at sites of cell adhesion and regulates actin cytoskeleton dynamics

CHST11	Carbohydrate sulfotransferase 11	Catalyzes the transfer of sulfate to carbohydrate residues and plays a role in the biosynthesis of sulfated glycosaminoglycans
ACTR3	Actin-related protein 3	Component of the Arp2/3 complex which mediates actin polymerization and cytoskeletal remodeling
PHLDA1	Pleckstrin homology-like domain family A member 1	Regulator of apoptosis and cellular proliferation, may also be involved in insulin signaling pathways

Table S3. Significantly upregulated and downregulated gene genes by THP-1-derived macrophages upon exposure to 0.1% w/v hGM-*g*-PMMA30 NPs for 72 h.

Gene symbol	Protein name	Biological function of the protein
Upregulated genes		
APOC1	Apolipoprotein C-I	Lipid metabolism
AC004241.3	Uncharacterized protein	Unknown
NMNAT3	Nicotinamide nucleotide adenylyltransferase 3	NAD+ biosynthesis
FBP1	Fructose-1,6-bisphosphatase 1	Glycolysis/gluconeogenesis
PMEPA1	Prostate transmembrane protein, androgen-induced 1	Transcription regulation
TAF15	TATA-box binding protein-associated factor 15	RNA splicing
APOE	Apolipoprotein E	Lipid metabolism
MTURN	Uncharacterized protein	Unknown
MID1IP1	Mid1-interacting protein 1	Microtubule organization
WBP4	WW domain-binding protein 4	RNA binding
MZT2B	Mitotic-spindle organizing protein 2B	Mitosis
SNHG8	Small nucleolar RNA host gene 8	Unknown
RPS15	40S ribosomal protein S15	Translation
RPL18	60S ribosomal protein L18	Translation
RPL37A	60S ribosomal protein L37a	Translation
PDLIM7	PDZ and LIM domain protein 7	Cytoskeleton organization
MMP7	Matrix metalloproteinase-7	Extracellular matrix remodeling
RPS19	40S ribosomal protein S19	Translation
RPL37	60S ribosomal protein L37	Translation
UQCRH	Ubiquinol-cytochrome c reductase hinge protein	Electron transport chain
RNF5	Ring finger protein 5	Protein ubiquitination
STMN1	Stathmin 1	Microtubule organization
CNPY3	Canopy FGF signaling regulator 3	Protein folding
CALM2	Calmodulin 2	Calcium binding
H3F3A	H3 histone family member 3A	Histone variant

Downregulated genes		
NCKAP1L	NCK-associated protein 1-like	Cytoskeleton organization
APLP2	Amyloid beta precursor protein-like protein 2	Neurotransmission
CANX	Calnexin	Protein folding
TM9SF3	Transmembrane 9 superfamily member 3	Vesicular trafficking
DCTN1	Dynactin subunit 1	Intracellular transport
SEC61A1	Sec61 translocon alpha subunit	Protein translocation
EMP1	Epithelial membrane protein 1	Cell adhesion
HM13	Hydroxymethylbilane synthase	Heme biosynthesis
MCL1	Myeloid cell leukemia 1	Apoptosis regulation
C5AR1	Complement component 5a receptor 1	Immune response
ARHGAP26	Rho GTPase-activating protein 26	Cytoskeleton organization
QSOX1	Quiescin sulfhydryl oxidase 1	Protein folding
YIPF6	Yip1 domain family member 6	Golgi vesicle trafficking
MRTFB	Myocardin-related transcription factor B	Transcription regulation
EHMT2	Euchromatic histone-lysine N-methyltransferase 2	Histone methylation
HYOU1	Hypoxia upregulated 1	Protein folding
HNRNPF	Heterogeneous nuclear ribonucleoprotein F	RNA splicing
RAB7B	Ras-related protein Rab-7b	Intracellular transport
LACC1	Laccase-like protein 1	Unknown
NDRG1	N-Myc downstream regulated 1	Cell cycle regulation
LARP4	La ribonucleoprotein domain family member 4	mRNA translation
SACM1L	SAC1 suppressor of actin mutations 1-like	Phospholipid metabolism
PLEKHO2	Pleckstrin homology domain-containing family O member 2	Signal transduction
ZNF35	Zinc finger protein 35	Transcription regulation
THBD	Thrombomodulin	Blood coagulation
SLC16A6	Monocarboxylate transporter 6	Metabolite transport

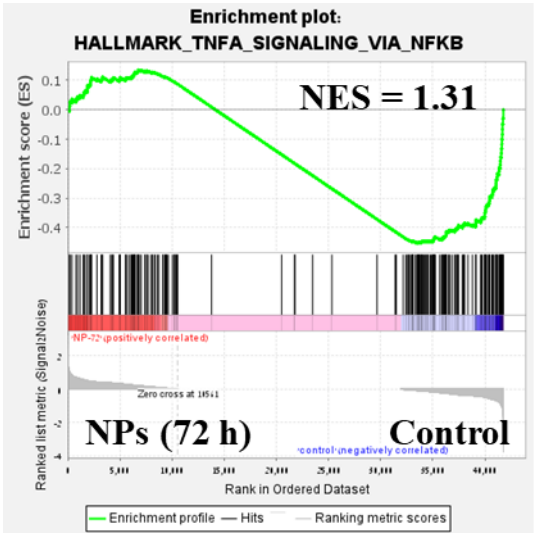


Figure S2. GSEA enrichment plots of the correlation of hGM-g-PMMA30 NPs (0.1% w/v, 72 h) treatment with TNF-α-signaling pathway via NfKb, NES value is applied in the plot.

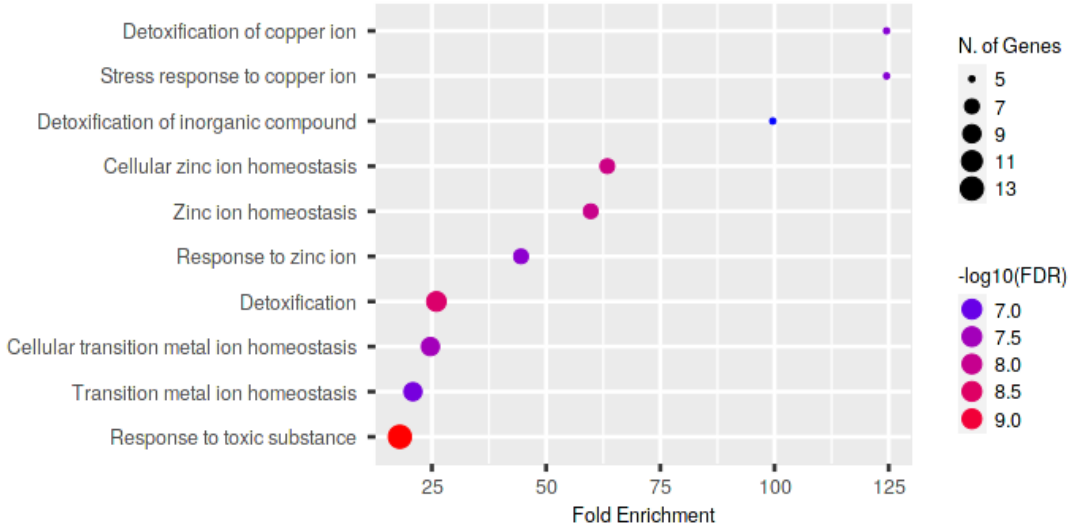


Figure S3. GO enrichment analysis of all significantly upregulated genes found in the RNA-seq data upon 24-h exposure to 0.1% w/v hGM-*g*-PMM30 NPs.

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