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Synergistic immunomodulatory effect in macrophages mediated by magnetic nanoparticles modified with miRNAs

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Experimental Section/Methods

Materials for magnetic nanoparticles synthesis, surface modification and characterization: Ultrapure reagent grade water MiliQ (18.2 MΩ, Wasserkab) was employed in all experiments. $FeCl_2 \cdot 4H_2O$, $FeCl_3 \cdot 6H_2O$, NH_4OH 25%, $Fe(NO_3)_3 \cdot 9H_2O$, HNO_3 65%, NaOH, Dextran 40 KDa, Carboxymethyldextran, *meso*-2,3-dimercaptosuccinic acid and dialysis tubing cellulose membranes were purchased in Sigma Aldrich.

Transmission electron microscopy (TEM, JEOL JEM 1010 operating at 80 kV) images were examined to determine particle shape and size distribution through manual analysis of over 300 particles for each sample. Samples were prepared by adding one drop of a diluted suspension on a carbon-coated copper grid and leaving it to dry overnight. Simultaneous thermogravimetric/differential thermal analyses (TGA/DTA) were done in a TA Instruments TGA 500, with a heating rate of 10 °C min^{-1}, in air atmosphere from room temperature to 800 °C. Inductively coupled plasma optical emission spectrometry (ICP-OES, Perkin Elmer Optima 2100 DV at ICMM-CSIC) was used to determine the iron concentration in the nanoparticle's dispersions. X-ray diffraction patterns of the dried samples (XRD, SmartLab SE, Rigaku) were acquired though Cu Kα radiation, scan angle $2\theta = 20°\text{-}80°$ at a 0.04 scan step, using a D D/tex Ultra 250 as detector.

The hydrodynamic diameter and zeta potential were obtained in a Zetasizer (DLS, Nano-ZS device, Malvern Instruments) using a laser at 633 nm and an angle of 173° between the detector and the sample. Dilute aqueous suspensions at pH 7.4 of the nanoparticles (0.05 mg Fe per mL) were dispersed in a disposable sizing cuvette or in a zeta potential cell, respectively, and both measurements were carried out at 25 °C. Moreover, the stability of the magnetic nanoparticles was evaluated in three different media (PBS pH 7.4, DMEM supplemented with 10% FBS and RPMI supplemented with 10% of FBS) by

measuring the hydrodynamic size after 0, 1, 5, 10, 24, 48 and 72 h of incubation under stirring conditions.

For CMD-MNP functionalization, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS), cysteamine hydrochloride (Cist HCl), sodium hydroxide (NaOH), aldrithiol, tris(2-carboxyethyl)phosphine hydrochloride solution 0.5 M (TCEP) and NAP-5 columns were obtained from Sigma Aldrich. Dimethylformamide (DMF) was obtained from Scharlab. The oligonucleotides polyT-Cy5, miRNA-155 (pass and guide), miRNA-125b (pass and guide) and miRNA-146a (pass and guide) were synthetized in a H DNA/RNA synthesizer (K&A Laborgeraete) (**Table S1**). Acetonitrile (MeCN) DNA synthesis grade was obtained from Sharlab. Oxidizer - 0.02M Iodine in THF/Pyridine/H_2O (70:20:10), Cap A - THF/Pyridine/Acetic anhydride (8:1:1), Cap B - 10% NMI/THF, Deblock 3% TCA/DCM, Activator 0.3M BTT/MeCN, rA (Bz) CE-Phosphoramidite, rG (dmf) CE-Phosphoramidite, rC (Ac) CE-Phosphoramidite, U CE-Phosphoramidite, 3'-Cyanine 5 CPG, 3'-Thiol Modifier C3 S-S CPG and 5'-Thiol Modifier C6 S-S CE-Phosphoramidite were obtained from Link Technologies. dT CE-Phosphoramidite was obtained from Wuhu Huaren.

Table S1. Oligonucleotides sequences synthetized in a H8 DNA/RNA synthesizer.

	Sequence (5'→3')
PolyTCy5	Thiol-C6-TTTTTTTTTTTTTTTTTTTTTTTTT-Cy5
miRNA155	Guide: UUAAUGCUAAUCGUGAUAGGGGUU
	Pass-thiol: AACCCCUAUCACGAUUAGCAUUAAAAAAA-C6-Thiol
miRNA125b	Guide: UCCCUGAGACCCUAACUUGUGA
	Pass-thiol: AACCCAUGGAAUUCAGUUCUCAAAAAA-C6-Thiol
miRNA146a	Guide: UGAGAACUGAAUUCCAUGGGUU
	Pass-thiol: AACCCAUGGAAUUCAGUUCUCAAAAAA-C6-thiol

The purity and concentration of the oligonucleotides were determined by UV–Vis spectra (λ= 260 nm) in a Cary 5000 Spectrophotometer (Agilent Technologies). miRNAs were prepared as duplexes, each comprising a mature miRNA (guide) and its passenger strand (pass), to protect them from degradation. For their preparation, Sigma protocol was followed.[1]

Materials for cell culture studies: Modified Eagle's medium (DMEM), streptomycin-penicilin, Roswell Park Memorial Institute medium (RPMI 1640), fetal bovine serum (FBS), L-glutamine, trypsin, phosphate-buffered saline (PBS), ethylenediamine tetraacetic acid (EDTA), non-essential aminoacids (NEAA) were obtained from Hyclone. Cell culture plasticware were purchased from Corning. CellTiter-Glo luminescent cell viability assay was obtained from Promega. Propidium iodide (PI), RNAase A, potassium ferrocyanide trihydrate, hydrochloric acid (HCl), neutral red, ferrozine, ascorbic acid, ammonium acetate, neocuproine, potassium permanganate ($KMnO_4$), chlorpromazine, genistein, filipin III, cytochalasin D, phorbol 12-myristate 13-acetate (PMA), diacetylated 2,7-dichlorofluorescein (DCF-DA) were purchased in Sigma Aldrich. For immunomodulation studies, lipopolysaccharides from *Echerichia coli* O111:B4 (LPS) and Interferon-γ human (hIFN-γ) were obtained from Sigma Aldrich. Interferon-γ mouse (mIFN-γ), and human and mouse FcR Blocking reagent were obtained from Miltenyi Biotec. For flow cytometry studies, PE Mouse Anti-human CD80 antibody and APC Mouse Anti-Human CD86 antibody were obtained from Biosciences, and PE anti-mouse CD80 antibody and APC anti-mouse CD86 antibody from Biolegend. For RT-qPCR studies, qPCR plates, optical adhesive films, SYBR Green Master Mix, TaqMan Advanced miRNA cDNA Synthesis Kit, TaqMan Fast Advanced Master Mix, TaqMan Advanced miRNA Assay for hsa-miR-423-5p, hsa-miR155-5p and hsa-125b-5p were

obtained from Thermofisher. NucleoSpin miRNA for miRNA and RNA purification was purchased in Cultek. ProtoScript II First Strand cDNA Synthesis Kit was obtained from New England Biolabs. The primers sequences were obtained from IDT.

RAW 264.7 murine macrophages cells (ATCC® TIB-71™) were cultured in DMEM high glucose (4.5 g · L^{-1}) and THP-1 human monocytic leukemia cells (ATCC® TIB-202™) were cultured in RPMI 1640. All cell culture mediums were supplemented with 10% of FBS, 1% of L-glutamine, 1% of NEAA, and 1% penicillin-streptomycin. Cells were cultured in the 5% CO_2 incubator at 37 ºC.

For THP-1 cell differentiation into macrophages, cells were cultured for 48 h with 50 ng mL^{-1} PMA and allowed to rest 24 h without PMA.

Synthesis of γ-Fe_2O_3 magnetic nanoparticles (MNP): The γ-Fe_2O_3 cores (MNP) were synthesized by the coprecipitation method described by Massart[2] followed by an optimized acid treatment, as described elsewhere.[3] Briefly, 75 mL of NH_4OH 25% were added at 0.1 mL s^{-1} to 425 mL of an aqueous solution of $FeCl_3 \cdot 6H_2O$ (0.212 M) and $FeCl_2 \cdot 4H_2O$ (0.126 M) under vigorous stirring at room temperature. After 5 min, the reaction was heated to 90 °C for 3 h, washed with water three times by magnetic decantation and stirred overnight at room temperature. Then the precipitate was isolated by magnetic decantation, 300 mL of HNO_3 (2 M) were added, and the stirring was maintained for 15 min. Then, HNO_3 was removed by magnetic decantation, and 75 mL of $Fe(NO_3)_3$ (1 M) and 130 mL of distilled water were added. The mixture was boiled and stirred for 30 min. After that time, the solution was cooled down to room temperature and the supernatant was removed by magnetic decantation. Finally, 300 mL of HNO_3 (2 M) were added and stirred for 15 min. The γ-Fe_2O_3 particles obtained were washed with water through magnetic decantation to remove the excess of acid and concentrated in the rotary evaporator.

Surface modification of γ-Fe_2O_3 magnetic nanoparticles (MNP) with dextran (D), carboxymethyldextran (CMD) and meso-2,3-dimercaptosuccinic acid (DMSA):

Dextran coated magnetic nanoparticles (D-MNP): a published procedure with slight modifications was used.[4] NaOH (200 mg, 5 mmol) was dissolved in 2 mL of miliQ water and added to a dispersion of 200 mg γ-Fe_2O_3. Then, a solution of dextran 40KDa (800 mg) in water (10 mL) was added to the previous dispersion with MNP. The final mixture was sonicated for 10 h under refrigeration. The final D-MNP nanoparticles were centrifuged using amicon ultra centrifugal filters to remove impurities. Finally, the pH was adjusted to 7.

Carboxymethyldextran coated magnetic nanoparticles (CMD-MNP): a previous described protocol was used with some modifications.[5] 800 mg of γ-Fe_2O_3 (MNP) were dispersed in 2 mL of miliQ water. Then, a solution of 800 mg of carboxymethyl-dextran in 10 mL of water was added and a solution of HNO_3 (65%) was used to adjust the pH at 3. The obtained mixture was sonicated for 10 h under refrigeration. The final CMD-MNP nanoparticles were centrifuged using amicon ultra centrifugal filters to remove impurities and the pH was adjusted to 7.

Meso-2,3-dimercaptosuccinic acid magnetic nanoparticles (DMSA-MNP): a slightly modified published procedure was used.[6] 10 mg of *meso*-2,3 dimercaptosuccinic acid (DMSA) were added to a dispersion containing 86 mg of γ-Fe_2O_3 in 0.910 mL of water and diluted to a final volume of 20 mL. Then, the mixture was sonicated for 2 h and the pH was adjusted with KOH 1M to 11. After that, the sample was dialyzed in water during 4 days to remove impurities and the pH adjusted to 7.

Covalent attachment of polyTCy5, miRNA-155, miRNA-125b and miRNA-146a via disulfide bonds on CMD-MNP: Firstly, to CMD-MNP at 2 mg Fe per mL (1 mL) in water

were added 600 μmol of EDC per g of Fe (20 μL 120 mM in water) and 300 μmol of NHS per g of Fe (20 μL 60 mM in water), and the mixture was stirred overnight. Then, the CMD-MNP were washed by 3 cycles of centrifugation and redispersion (60 min, 12,000×*g*). After that, 200 μmol of cysteamine hydrochloride per g of Fe (20 μL 40 mM in water) previously neutralized with 200 μmol of NaOH per g of Fe (20 μL 40 mM in water) were added and the solution was maintained in continuous stirring overnight. Then, the cells were washed again by cycles of centrifugation and redispersion as mentioned before and mixed with 50 μmol aldrithiol per g of Fe (200 μL 500 μM in DMF). After 16 h of continuous stirring, CMD-MNP were washed again and mixed for 16 h with the corresponding miRNA (miRNA-155, miRNA-125 b or miRNA-146a; 2.5 μmol/g Fe, 100 μL 50 μM) previously deprotected with TCEP for 2 h and purified by NAP-5 column, following the manufacturer's instructions. The following day, the samples were centrifuged for 20 min at 8,000×*g*. From the collected supernatants, the polyTCy5 or miRNA incorporated was determined by quantification of the 2-pyridinethione released (λ_{max} 343 nm, ε_{343nm} 8,080 $L^{-1} \cdot mol^{-1} \cdot cm^{-1}$). Finally, CMD-MNP-miRNA were resuspended in 1 mL of miliQ water.

Hemolysis assay and study of nanoparticles-red blood cells (RBCs) membrane interactions: Fresh RBCs from human blood were obtained from anonymous donors from the Finnish Red Cross Blood Service. The experiments were conducted in compliance with laws and with the permission of anonymous subjects. For the hemolysis studies, an already described protocol was used with slight modifications.[7,8] Briefly, the RBCs (1 mL) were washed 3 times by cycles of centrifugation (4 min, 835×*g*) and redispersion with a 150 mM sodium chloride solution to remove protective reagents. Nanoparticles' suspension were prepared at the concentration 10 mg mL^{-1} in miliQ water and further

diluted in PBS 1X (pH 7.4) to specific concentrations. Then, 2×10^6 RBCs were added into 200 µL of each sample solution in triplicates and incubated at 37 °C with gentle shaking for 1, 4 and 24 h. After that, all samples were centrifuged at 1485×g for 4 min, the pellet with the intact RBC was discarded and the supernatant was centrifuged again at 4000×g for 10 min to force the precipitation of all possible nanoparticles present. Then, 100 µL of the supernatant were transferred to a 96-well plate to measure the absorbance values of hemoglobin at 540 nm using a Varioskan LUX multimode microplate reader (Thermo Scientific, USA). RBCs incubated in PBS 1X were used as negative controls and defined as 0% hemolysis, while RBCs incubated with MiliQ water were used as positive controls and defined as 100%. The final hemolysis for each nanoformulation was calculated as shown in Eq. (1):

$$\text{Relative Hemolysis (\%)} = \frac{Abs\ (sample) - Abs\ (negative\ control)}{Abs\ (positive\ control) - Abs\ (negative\ control)} x\ 100 \quad (1)$$

The morphological changes and nanoparticles-RBCs interactions were also investigated following a described method with sligh modifications[9]. Briefly, 500 µL of nanoparticles suspension at 0.01 and 0.1 mg of Fe per mL in PBS 1X were incubated for 1×10^6 of RBCs at 37 °C. Then, the samples were centrifuged at 1485×g for 4 min. The pellet was resuspended, fixed with 2.5% of glutaraldehyde in PBS 1X for 1 h at 37ºC and followed by post-fixation using 0.5% osmium tetroxide in PBS for 1.5 h. Then, RBCs were dehydrated in increasing concentrations of 50, 70, 96 and 100 % of ethanol for 5, 10, 20 and 15 min, respectively. Finally, RBCs suspensions were dropped onto plastic coverslips, dried and sputter-coated with platinum before being observed under SEM (Zeiss DSM 962).

Evaluation of protein corona formation: the protein corona formation was evaluated with human serum (human male AB plasma, USA origin, sterile filtered, Sigma Aldrich). The particles in PBS 1X (0.5 mg Fe per mL) were incubated with different concentrations of human serum (HS 0, 5, 10, 25 y 50%) for 24 h at 37º C in a final volume of 600 µL in low binding protein eppendorfs. After completion of the incubation period, the dispersions were centrifuged twice (16,000×*g*, 15 min) to separate the unbound human serum proteins (supernatant) to the particles with the protein corona. The resulting pellet (MNP@HS) was resuspended in 600 µL of PBS 1X. The formation of the protein corona with the nanoparticles in MNP@HS was evaluated by two different techniques:

- Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis: 10 µL of each MNP@HS sample were mixed with an equal volume of 2X SDS sample buffer (VWR) and the samples were heated for 5 min at 95 ºC to denature the proteins. Then, the bound HS protein samples were separated in a 10% SDS-PAGE in an electric field using electrophoresis at a constant voltage of 120 V for 60 min. The obtained gels were stained with Coomassie Brilliant Blue R-250 Dye (ThermoFisher) and the images were scanned using a Chemidoc Imaging Instrument (Bio-Rad Laboratories). A calibration line of HS (0.5-10%) was included in each gel to quantify the amount of plasma proteins attached to the MNP.
- Atomic Force Microscopy (AFM): For AFM visualization, 15 µL of MNP@HS50 sample diluted in water (0.05 mg of Fe per mL) were added to the cleaved mica and incubated for 10 min at room temperature. Then, the surface was rinsed twice with miliQ water and dried with N_2. After that, AFM measurements of the samples were performed in air. Samples of HS (0.5 %) and the corresponding MNP (0.05 mg of Fe per mL) were used as controls. For HS sample preparation, the same

procedure mentioned before was used. In the case of MNP preparation, an additional step was needed. Firstly, 15 μL of 10 mM $MgCl_2$ were droped onto cleaved mica substrate and left for 3 min. Then, the surface was rinsed with miliQ water and dried with N_2 before adding the MNP sample (0.05 mg of Fe per mL). Topography images were obtained in intermittent contact mode in air using JPK Nanowizard 2 microscope and HQXSC11-D (Mikromash) cantilevers (nominal spring constant of 42 N/m and resonance frequency of 350 kHz). These experiments were performed in the laboratory of Atomic Force Microscopy at IMDEA Nanociencia.

Cytocompatibility Studies: the cytocompatibility of coated MNP was assessed on RAW 264.7 and THP-1 cells using a CellTiter-Glo luminescent cell viability assay.

For RAW 264.7, 10,000 cells per well were seeded on a 96-well plate in cell culture medium. The following day, coated MNP were added at the concentrations indicated in the figures in quadruplicates and incubated for 24, 48 or 72 h.

For THP-1 cells, 20,000 cells per well were seeded on 96-well plate in the presence of PMA 50 ng mL^{-1} for 48 h and left to rest for 24 h in complete media. Then, coated MNP were added at the concentrations indicated in the figures in quadruplicates and incubated for 24, 48 or 72 h.

After that time, cells were washed twice with PBS 1X and CellTiter-Glo was added, according to manufacturer's instructions. The resulting luminescence was read in a Varioskan™ LUX multimode microplate reader and it represents the amount of ATP produced by the viable cells. The results were represented as % of cell viability and calculated, according to Eq. (2):

$$\%\ Cell\ viability = \frac{Sample\ data - Negative\ Control}{Positive\ Control - Negative\ Control} \times 100 \quad (2)$$

The positive control corresponds with untreated cells, and the negative control was a solution in complete medium without cells.

Cell cycle analysis: cells were seeded in P6 plates. For RAW 264.7 cells, 200,000 cells per well were seeded overnight and treated with coated MNP the following day. In the case of THP-1 cells, 400,000 cells per well were seeded in the presence of PMA 50 ng mL^{-1} for 48 h and left to rest for 24 h. Then, they were treated with the corresponding coated MNP. The particles were incubated for 24, 48 and 72 h. Then, cells were washed with PBS 1X to remove the not internalized coated MNP and detached with PBS-EDTA 1 mM. The recollected cells were centrifuged at 177×*g* for 5 min, washed with PBS and fixed in cold ethanol 70% for 15 min. After that, cells were centrifuged at 177×*g* for 15 min to remove the ethanol and they were resuspended in PBS 1X. Then, each sample was treated with 10 µg of RNAase A and 20 µg PI for a total volume of 500 µL. Cell cycle analysis was performed in a Beckman Coulter Cytomics 500 Flow Cytometer using 20,000 cells. The acquired data was analyzed with FlowJoe software. These experiments were performed in the Flow Cytometry Service at the CNB-CSIC

Nanoparticle-cells interactions and uptake studies: Three different methods were employed.

- Colorimetric ferrozine assay:[10] This assay was used to estimate iron concentration in RAW 264.7 and THP-1 cells. In the case of RAW 264.7 cells, they were seeded in P12 wells (100,000 cells per well) overnight and treated with coated MNP at different concentrations in triplicates the following day.

  In the case of THP-1 cells, cells were seeded in P12 wells (200,000 cells per well) in the presence of PMA 50 ng mL^{-1} for 48 h and left to rest for 24 h in complete medium. Then, cells were treated with coated MNP in triplicates at different concentrations.

The ferrozine assay was done after 24, 48 and 72 h of incubation. The cells were washed twice with PBS 1X, and lysate in 50 mM NaOH. Aliquots of cell lysates in 50 mM NaOH (100 μL) were mixed with equal volumes of 10 mM HCl and iron-releasing agent (1.4 M HCl and 4.5% w/w $KMnO_4$ in water). The mixtures were incubated for 2 hours at 60 °C and cooled to room temperature. Then, the iron-detection reagent (30 μL) was added (6.5 mM ferrozine, 6.5 mM neocuproine, 2.5 M ammonium acetate, and 1 M ascorbic acid in water). After 30 min, the absorbance at 565 nm was measured Varioskan™ LUX multimode microplate reader into a well of a 96-well plate. The experiments were performed in triplicates. A similar procedure was done to elaborate the calibration line with our MNP.

- Prussian blue staining:[11] RAW 264.7 and THP-1 cells were seeded in coverslips in P24 wells. In the case of RAW 264.7, 50,000 cells per well were seeded and the following day they were treated with the corresponding MNP (0.1 mg Fe per mL) and incubated for 48 h. For THP-1 cells, 100,000 cells per well were incubated with PMA 50 ng mL^{-1} for 48 h and left to rest for 24 h in complete media. Then, cells were treated with the corresponding MNP (0.1 mg Fe per mL) and incubated for 48 h. After that time, cells were fixed in ice-cold methanol for 5 min and dried. Afterwards, cells were stained with an equal volume of 2% potassium ferrocyanide trihydrate and 2% HCl for 15 min and counterstained with 0.5 % neutral red for 3 min. Finally, the samples were mounted in DePeX and visualized in a LeicaDMI300 B optical microscope.
- TEM images in cell culture: RAW 264.7 cells were harvested in coverslips in 24-well plate (50,000 cells per well) overnight and incubated the following day with MNP at 0.1 mg of Fe per ml for 48 h. Then, the medium was removed, and cells were fixed with 2% glutaraldehyde in 0.1 M NaCac buffer (pH 7.4) for 30 min. Afterwards, cells

were washed for 3 min twice with 0.1 M NaCac buffer. The samples were provided to the Electron Microscopy Service at University of Helsinki for further staining and fixation. Samples were analyzed using TEM (Jeol JEM-1400, Jeol Ltd., Japan).

To elucidate the internalization pathways of the nanoparticles, ferrozine-based assay was performed after the incubation of coated MNP in cells previously treated with endocytosis inhibitors (**Table S2**). In the case of RAW 264.7, cells were seeded in P12 wells (100,000 cells per well) overnight and treated with the endocytosis inhibitors for 1 h. In the case of THP-1, cells were also seeded in P12 wells (250,000 cells per well) and incubated with 50 ng mL^{-1} PMA for 48 h and left to rest for 24 h in complete media. Next, cells were treated with the inhibitors for 1 h. After that time, RAW 264.7 and THP-1 cells were washed with PBS 1X twice, and nanoparticles were incubated for 4 h. The data obtained was normalized *vs.* the control (cells treated with coated MNP without inhibitors) and represented as % of internalized Fe.

Table S2. Endocytosis inhibitors employed to inhibit the uptake of coated MNP in RAW 264.7 and THP-1 cells.

	RAW 264.7	**THP-1**
Chlorpromazine	5 µg/mL	10 µg/mL
Cytochalasin D	5 µg/mL	2.5 µg/mL
Filipin II	12 µg/mL	9 µg/mL
Genistein	40.5 µg/mL	27 µg/mL

Measurement of intracellular ROS: RAW 264.7 and THP-1 were seeded in P96 wells as indicated in the cytocompatibility studies section. After 24, 48, and 72 h of incubation with coated MNP (0.01-0.1 mg Fe per mL) or LPS (10, 50, 100 ng per mL), cells were

washed twice with PBS 1X and incubated with 30 μM diacetylated 2',7'-dichlorofluorescein (DCF-DA) probe for 30 min at 37ºC. Then, cells were washed again, and DCF fluorescence (λ_{exc} 485 nm, λ_{em} 535 nm) was measured in the Synergy H4 Hybrid multimode plate reader. The obtained values were normalized on CellTiter-Glo luminescent cell viability assay.

Immunostimulation assay: The immunostimulative effect of D-MNP, CMD-MNP and DMSA-MNP was evaluated over RAW 264.7 and THP-1 by flow cytometry and qRT-PCR. RAW 264.7 cells were seeded in P12 wells (100,000 cells per well) and incubated overnight before adding the coated MNP. In the case of THP-1 cells, cells were seeded in P12 wells (200,000 cells per well), incubated with PMA 50 ng/mL for 48 h and left to rest for 24 h in complete media. Then, cells were treated for 48 h with 100 ng mL^{-1} of LPS and 0.5 ng mL^{-1} of IFN-γ and used as the M1 phenotype control, or 0.1 mg Fe/mL of D-MNP, CMD-MNP or DMSA-MNP. Untreated cells were used as the M0 phenotype control. Then, the effects were evaluated by the analysis of surface markers CD80 and CD86 using flow cytometry, and the mRNA detection of target sequences IL-6 and TNF-α using RT-qPCR. This time point was selected since the stimulation with LPS and IFN-ϒ to produce M1 macrophages usually takes 24-72 h. [12–14]

- Flow cytometry: After the incubation time, cells were washed with PBS 1X twice, detached from plate's surface with PBS-EDTA 1 mM and transferred to FACS crystal-clear polystyrene tubes. The cells were centrifuged at 317×*g* for 5 min, washed with PBS 1X three times, and incubated for 10 min at room temperature with a solution with FcR blocking reagent. Afterwards, cells were immunostained with the corresponding APC-anti CD86 and PE-anti CD80. In all the cases, antibodies were incubated at 1 μg mL^{-1} in FACS buffer (PBS 1X 1% BSA) at 4 ºC for 30 min. Then, the cells were washed again with PBS 1X three times, and

subsequently analyzed by flow cytometry in triplicates. Cells without antibody staining were used as negative controls. The expression of CD80 or CD86 was normalized against M0 phenotype control.

- qPCR for mRNA TNF-α and IL-6 quantification: After the incubation time, cells were washed with PBS 1X twice, followed by RNA extraction (RNA isolation NucleoSpin RNA Plus, Cultek), cDNA synthesis (ProtoScript® II First Strand cDNA Synthesis Kit, New England Biolabs) and RT-qPCR (PowerUp™ SYBR™ Green Master Mix, Thermofisher). The list of primers used are listed in **Table S3**. The experiments were run in triplicates in a Qtower^3 qPCRsoft (Cultek). Expression data were analysed according to the Livak method. The data of TNF-α and IL-6 was firstly normalized against β-actin and then it is represented as $2^{-\Delta\Delta Ct}$ using an untreated well as control.

Table S3. Primers sequences employed for RT-qPCR assays in RAW 264.7 and THP-1 cells

Gene	**Forward (5'→3')**	**Reverse (5'→3')**	**Reference**
Mouse			
TNF-α	CTATGTCTCAGCCTCTTCTC	CATTTGGGAACTTCTCATCC	15
IL-6	AAGAAATGATGGATGCTACC	GAGTTTCTGTATCTCTCTGAAG	15
β-actin	GATGTATGAAGGCTTTGGTC	TGTGCACTTTTATTGGTCTC	15
Human			
TNF-α	AGGTTCTCTTCCTCTCACATAC	ATCATGCTTTCAGTGCTCATG	16
IL-6	TTCAATGAGGAGACTTGCCTG	ACAACAACAATCTGAGGTGCC	17
β-actin	GACGACATGGAGAAAATCTG	ATGATCTGGGTCATCTTCTC	15

Additionally, to evaluate miRNA155 and miRNA125b expression in THP-1 cells, RT-PCR was used. Firstly, THP-1 cells were seeded in P6 wells (400,000 cells per well) in the presence of PMA 50 ng mL^{-1} for 48 h and left to rest for 24 h. Then, cells were incubated with the corresponding treatments for 48 h. After that time, total miRNAs were extracted (NucleoSpin miRNA, Mini kit for miRNA and RNA purification, Cultek) and cDNA synthesis was performed (TaqMan™ Advanced miRNA cDNA Synthesis Kit, ThermoFisher) and RT-qPCR run (TaqMan™ Fast Advanced Master Mix and TaqMan™ Advanced miRNA Assay probes for hsa-miRNA-155-5p, hsa-miRNA 125b-5p and hsa-miRNA-423-5p, ThermoFisher). The experiments were run in triplicates in a Qtower3 qPCRsoft (Cultek). Expression data were analysed according to the Livak method.[18] The data of miRNA155 and miRNA125b was firstly normalized against miRNA-423-5p and then it is represented as $2^{-\Delta\Delta Ct}$ using an untreated well as control.

Supplementary Tables and Figures:

Table S4. Hydrodynamic size (diameter size; PDI: polydispersity index) and zeta (ζ)-potential of magnetic nanoparticles MNP, D-MNP, CMD-MNP and DMSA-MNP in water measured by DLS (mean ± SD, n=3).

	Hydrodynamic size		ζ-potential (mV)
	Diameter (d.nm)	PDI	
MNP	140.50 ± 2.87	0.20 ± 0.03	15.00 ± 0.28
D-MNP	91.19 ± 0.34	0.12 ± 0.02	-9.58 ± 0.05
CMD-MNP	103.60 ± 1.65	0.13 ± 0.01	-22.00 ± 0.11
DMSA-MNP	82.65 ± 0.80	0.15 ± 0.01	-25.90 ± 0.14

Table S5. Hydrodynamic size (diameter size; PDI: polydispersity index) and ζ-potential of modified CMD-MNP in water measured by DLS (mean ± SD, n=3).

	Hydrodynamic size		Z-potential (mV)
	Diameter (nm)	PDI	
CMD-MNP-PolyTCy5	95.10 ± 0.98	0.11 ± 0.01	-29.40 ± 0.45
CMD-MNP-miRNA155	160.20 ± 2.39	0.20 ± 0.02	-22.60 ± 0.23
CMD-MNP-miRNA125b	123.30 ± 1.76	0.13 ± 0.02	-23.80 ± 0.06
CMD-MNP-miRNA146a	144.50 ± 1.51	0.16 ± 0.02	-26.00 ± 0.61

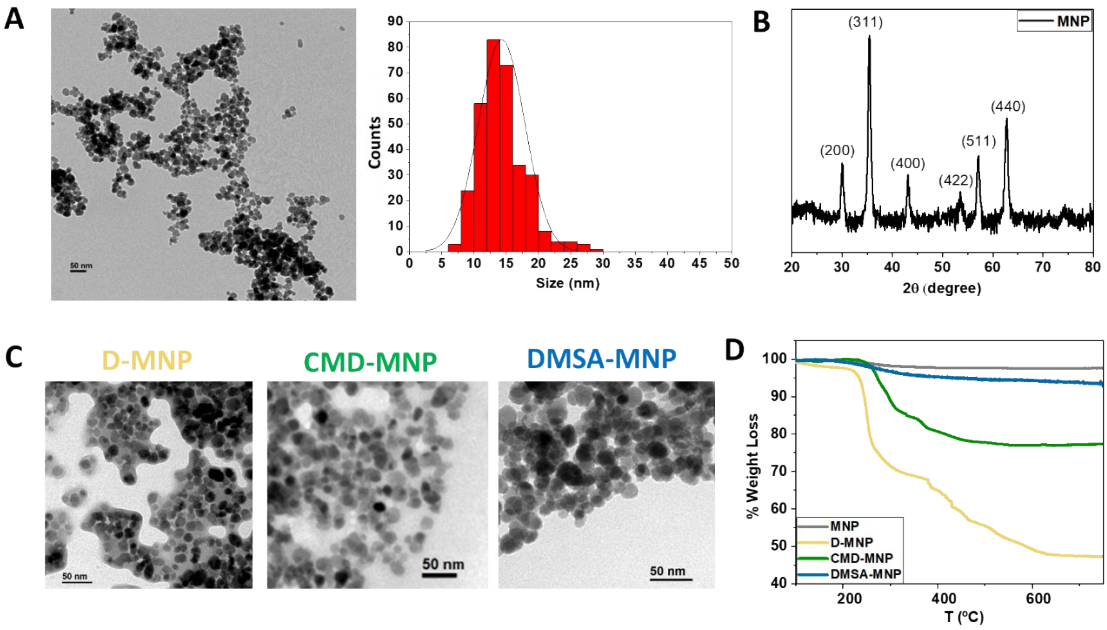

Fig. S1. (A) TEM micrographs (left) and size distributions (right) of 14 nm Fe_2O_3 cores (MNP). Scale bar: 50 nm. (B) X-ray diffraction pattern of MNP including (hkl) indices corresponding to a maghemite phase. (C) TEM micrographs of coated MNP (D-MNP, CMD-MNP and DMSA-MNP). Scale bar: 50 nm. (D) Thermogravimetric analysis of MNP, D-MNP, CMD-MNP and DMSA-MNP.

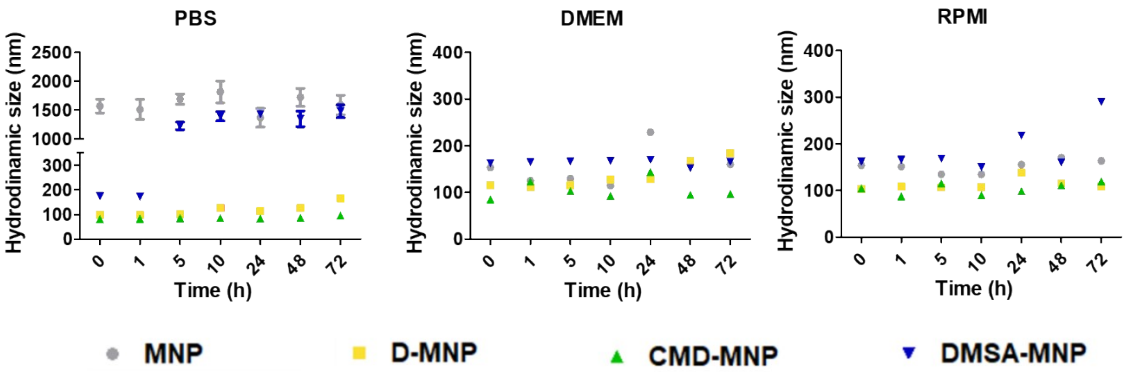

Fig. S2. Hydrodynamic size evaluation of MNP, D-MNP, CMD-MNP and DMSA-MNP in PBS, complete DMEM and complete RPMI for 72 h (mean ± SD, n=3).

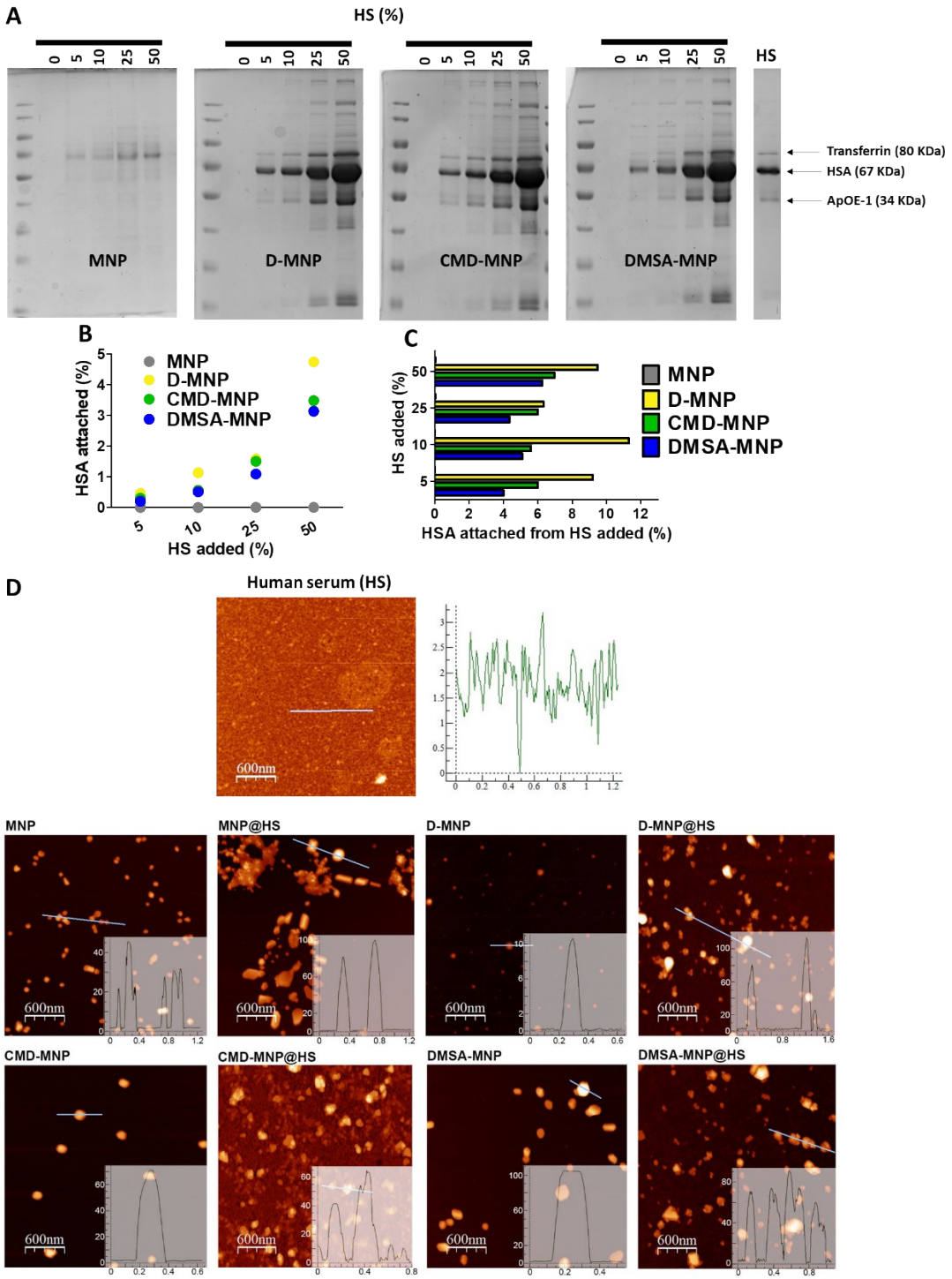

Figure S3. A) SDS-PAGE protein-nanoparticles (MNP, D-MNP, CMD-MNP, DMSA-MNP) complexes after incubation with human serum (HS) at different concentrations (0, 5, 10, 25 and 50 %). B) Quantification by SDS-PAGE of the percentage of HSA attached to nanoparticles *versus* the concentration of HS added. C) Quantification by SDS-PAGE of the percentage corresponding to human serum albumin (HSA) attached to the nanoparticles relative to the percentage of total plasma proteins added at different concentrations of HS.D) AFM images of the human serum (HS), nanoparticles before incubation with HS (MNP, D-MNP, CMD-MNP, DMSA-MNP) in PBS 1X and after incubation with HS (MNP@HS, D-MNP@HS, CMD-MNP@HS, DMSA-MNP@HS). Graphic represents standard profile of the nanoparticles (Y, nm; X, µm).

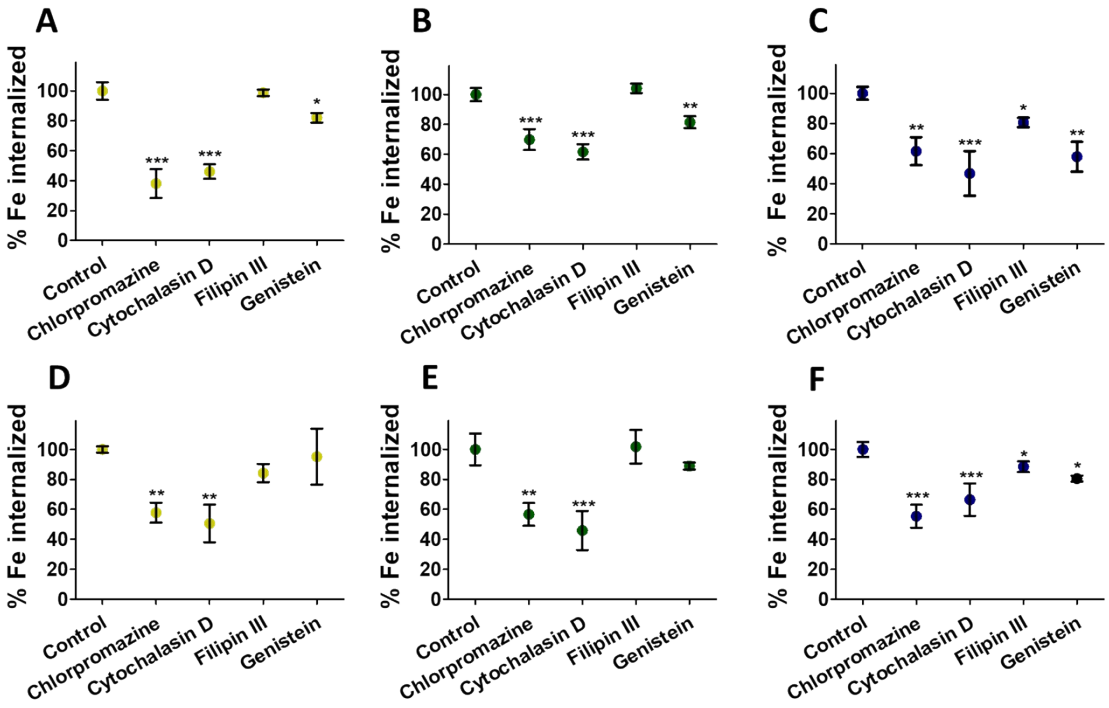

Fig. S4. Ferrozine assay of D-MNP (A, D), CMD-MNP (B, E), and DMSA-MNP (C, F) in RAW 264.7 (A-C) and THP-1 (D-F) cells after using inhibitors of endocytic pathways. Control represents the cells treated with the corresponding magnetic nanoparticles without inhibitors and considered 100% of Fe internalized. Data represent means ± SD (n=3). Statistical analysis was performed using one-way ANOVA test (control *vs* each inhibitor). * $p < 0.05$, ** $p < 0.001$, *** $p < 0.001$.

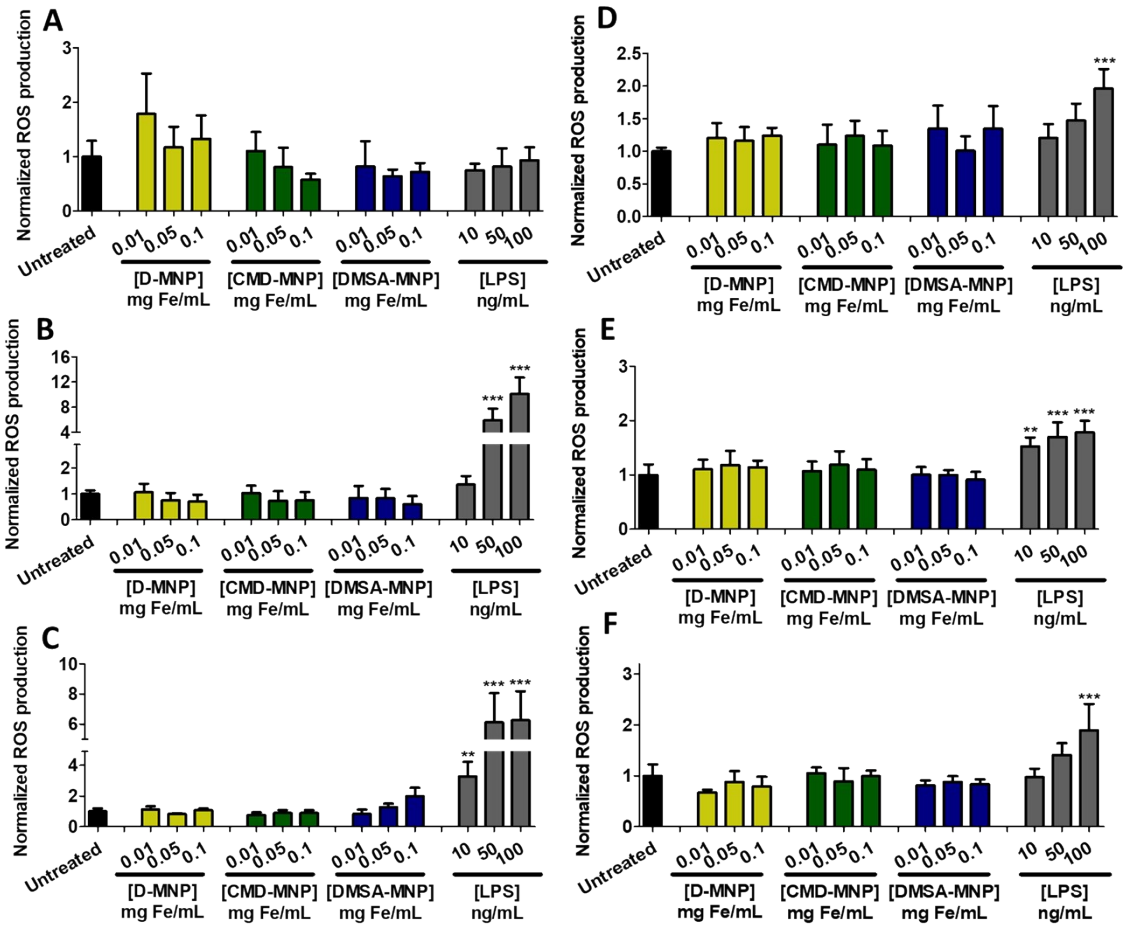

Fig. S5. Quantification of ROS levels in RAW 264.7 (A-C) and THP-1 (D-F) cells by the detection of oxidized DCF-DA 24 (A, D), 48 (B, E), and 72 (C, F) hours after treatment. Data represent means ± SD (n=6). Statistical analysis was performed using one-way ANOVA test (untreated *vs* each treatment). * $p < 0.05$, ** $p < 0.001$, *** $p < 0.001$.

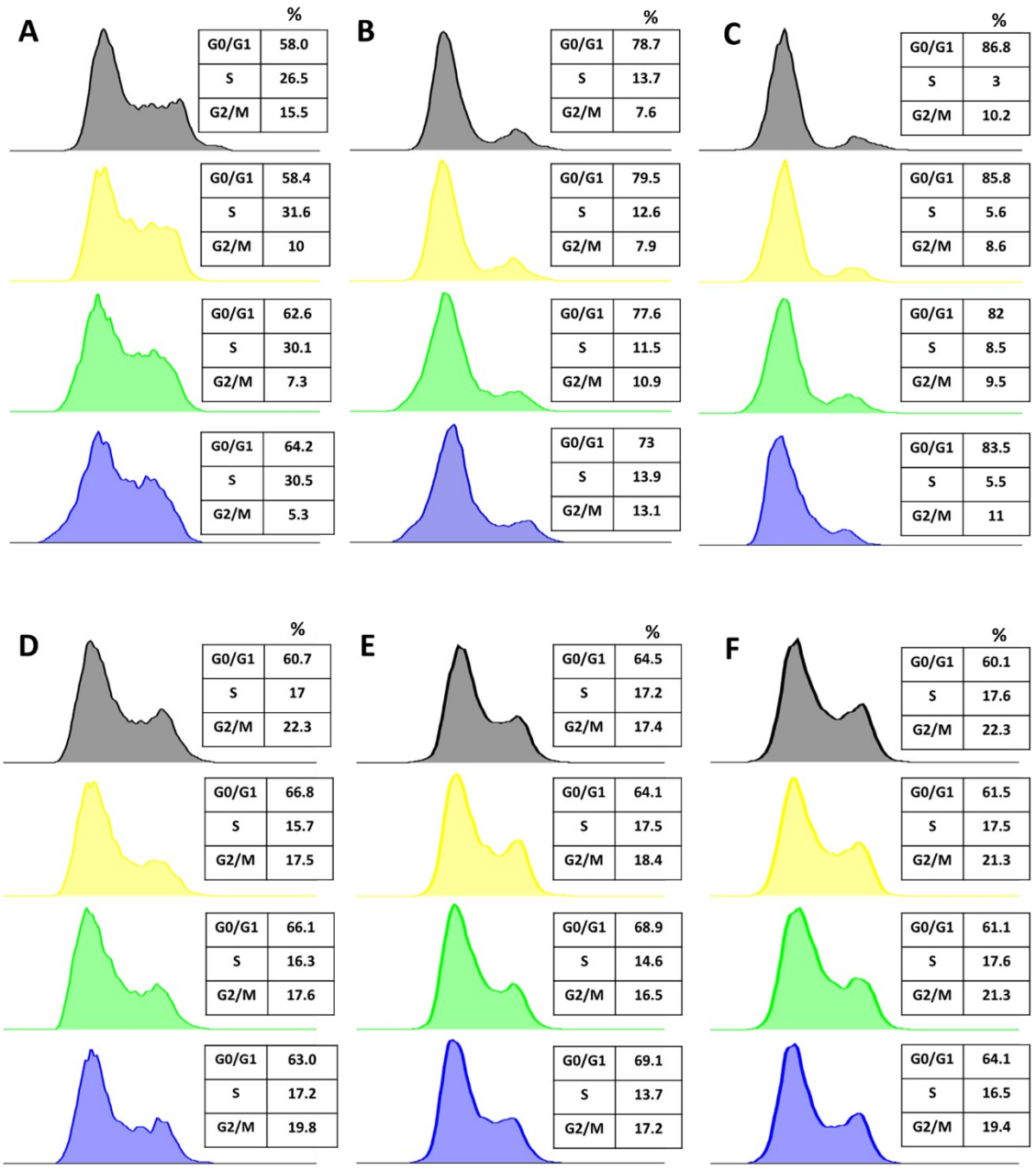

Fig. S6. Flow cytometry analysis of the cell cycle in RAW 264.7 (A-C) and THP-1 (D-F) after 24 (A, D), 48 (B, E) and 72 (C, F) hours of treatment with D-MNP (yellow), CMD-MNP (green), and DMSA-MNP (blue). The cell cycle of untreated cells is represented in grey color.

References

1 Annealing Oligonucleotides Protocol, https://www.sigmaaldrich.com/US/en/technical-documents/protocol/genomics/pcr/annealing-oligos, (accessed 5 October 2021).

2 R. Massart, *IEEE Trans. Magn.*, 1981, **17**, 1247–1248.

3 R. Costo, V. Bello, C. Robic, M. Port, J. F. Marco, M. P. Morales and S. Veintemillas-Verdaguer, *Langmuir*, 2012, **28**, 178–185.

4 M. C. Bautista, O. Bomati-Miguel, X. Zhao, M. P. Morales, T. González-Carreño, R. Pérez de Alejo, J. Ruiz-Cabello and S. Veintemillas-Verdaguer, *Nanotechnology*, 2004, **15**, S154-S159.

5 S. Dutz, J. H. Clement, D. Eberbeck, T. Gelbrich, R. Hergt, R. Müller, J. Wotschadlo and M. Zeisberger, *J. Magn. Magn. Mater.*, 2009, **321**, 1501–1504.

6 Y. Luengo, S. Nardecchia, M. P. Morales and M. C. Serrano, *Nanoscale*, 2013, **5**, 11428–11437.

7 R. Chen, S. Khormaee, M. E. Eccleston and N. K. H. Slater, *Biomaterials*, 2009, **30**, 1954–1961.

8 S. Wang, S. Wannasarit, P. Figueiredo, G. Molinaro, Y. Ding, A. Correia, L. Casettari, R. Wiwattanapatapee, J. Hirvonen, D. Liu, W. Li and H. A. Santos, *Adv. Ther.*, 2020, 2000058.

9 M. A. Shahbazi, M. Hamidi, E. M. Mäkilä, H. Zhang, P. V. Almeida, M. Kaasalainen, J. J. Salonen, J. T. Hirvonen and H. A. Santos, *Biomaterials*, 2013, **34**, 7776–7789.

10 J. Riemer, H. H. Hoepken, H. Czerwinska, S. R. Robinson and R. Dringen, *Anal. Biochem.*, 2004, **331**, 370–375.

11 A. Villanueva, M. Cañete, A. G. Roca, M. Calero, S. Veintemillas-Verdaguer, C. J. Serna, M. P. Morales and R. Miranda, *Nanotechnology*, 2009, **20**, 115103.

12 X. Huang, Y. Li, M. Fu and H. B. Xin, *Methods Mol. Biol.*, 2018, **1784**, 119–126.

13 S. Wang, S. Wannasarit, P. Figueiredo, G. Molinaro, Y. Ding, A. Correia, L. Casettari, R. Wiwattanapatapee, J. Hirvonen, D. Liu, W. Li and H. A. Santos, *Adv. Ther.*, 2021, **4**, 2000058.

14 F. Fontana, M. A. Shahbazi, D. Liu, H. Zhang, E. Mäkilä, J. Salonen, J. T. Hirvonen and H. A. Santos, *Adv. Mater.*, 2017, **29**, 1603239.

15 J. M. Rojas, L. Sanz-Ortega, V. Mulens-Arias, L. Gutiérrez, S. Pérez-Yagüe and D. F. Barber, *Nanomedicine*, 2016, **12**, 1127–1138.

16 T. Brzicova, E. Javorkova, K. Vrbova, A. Zajicova, V. Holan, D. Pinkas, V. Philimonenko, J. Sikorova, J. Klema, J. Topinka, P. Rossner and Jr., *Nanomaterials*, 2019, **9**, 687.

17 A. V Sudhakaran, H. Panwar, R. Chauhan, R. K. Duary, R. K. Rathore, V. K. Batish and S. Grover, *Genes Nutr.*, 2013, **8**, 637–648.

18 K. J. Livak and T. D. Schmittgen, *Methods*, 2001, **25**, 402–408.