Electronic Supplementary Material (ESI) for RSC Advances.

Nanoparticle size influences the proliferative responses of lymphocyte subpopulations

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Experimental section:

All chemical products were purchased from Sigma Aldrich (Spain). Ultra-pure Milli-Q water was used for the synthesis.{Hötzer, 2012 #5}

Synthesis.

NC synthesis was slightly modified from the preparation previously described[1]. Briefly, 326 µL of $HAuCl_4 \bullet 3H_20$ (50 mM) was added to 5 mL of glutathione (GSH) (1 mg/mL) and stirred at 70 °C. After 10 min, 50 µL of freshly prepared $AgNO_3$ (10 mg/mL) was added to the solution, changing colour from pale yellow to white and then black. The solution was left stirring for another 5 hours at 70 °C and then cooled down to room temperature. Afterwards, the solution was centrifuged at 10,000 rpm for 10 min to remove the big aggregates. The yellowish supernatant containing the fluorescent nanoclusters was filtered twice with 3 kDa cut-off Amicon filters to remove free glutathione and the pH was adjusted to 7. NC solution was kept refrigerated until use and was stable for months.

Gold nanoparticles coated with GSH (NPs) were prepared using the following protocol: 40 µL of $HAuCl_4 \bullet 3H_20$ (50 mM) was added to 10 mL of water under rapid stirring. Afterwards, 200 µL of $NaBH_4$ (6 mg/mL) was added dropwise and stirred for 30 min. Following this, 2 mg of GSH was added and stirred for another 90 min. The solution was then centrifuged at 13,600 rpm for 20 min to remove excess GSH and resuspended in water after adjusting the pH to 7.

Particle characterisation.

Particle size was determined by dynamic light scattering (DLS) using a Malvern instrument Nanosizer ZS. Thermal analysis of the samples were performed by Thermogravimetry (TG), and differential scanning calorimetry (DSC) using a METTLER TOLEDO model TGA/DSC 1 between 30 and 850°C at 10 °C/min with an air flux of 50 mL/min.

Electron Microscopy analysis. High-resolution transmission electron microscopy (HRTEM) measurements of the NCs was carried out with a JEOL 2200FS double aberration corrected FEG microscope, operated at 200 kV. TEM images of NPs were obtained on a FEI Tecnai G2 Twin TEM at 200 kV.

Optical measurement. Absorption spectra were collected on a UV-visible spectrophotometer Cary 100Bio (Varian) in the 190 nm - 900 nm range. Steady-state fluorescence measurements were obtained with a diluted solution of Au NCs using a Perkin Elmer LS45 Fluorescence Spectrometer.

Generation of Monocyte-Derived DCs. Fresh peripheral blood mononuclear cells (PBMC) obtained from 40 mL of blood from each individual were used for monocyte purification by means of anti-CD14 microbeads following the manufacturer's protocol (Miltenyi Biotec, Germany). The $CD14^-$ fraction was placed in 10% dimethyl sulfoxide and frozen for subsequent lymphocyte proliferation testing. To generate DCs, $CD14^+$ monocyte cells were incubated in complete medium (CM) containing Roswell Park Memorial Institute 1640 medium (Life Technologies, Invitrogen, USA) supplemented with 10% Fetal Calf Serum (FCS; Life Technologies, Invitrogen, USA), streptomycin (100 µg/ml), gentamicin (1.25 U/mL) as well as recombinant human rhGM-CSF (200 ng/mL) and rhIL-4 (100 ng/mL) (both from R&D Systems Inc., USA) for 5 days at 37°C and 5% CO_ENREF_3$_2$.

Cell toxicity analyses. The cytotoxic effects of the NCs and NPs on DCs were analysed by flow cytometry. Typically, 1×10^5 DCs were incubated with the NCs, NPs or free ligand GSH at 1, 5, 10 and 25 µg/mL concentrations in CM for 48 h at 37 °C and 5% CO_2. Untreated DCs were used as controls. After incubation, cells were stained with the Live/Dead NearIR kit (Life Technologies-Invitrogen, USA) for 15–20 min. Cells were then analysed using a flow cytometer (FACSCanto II flow cytometer, BD Biosciences, USA) according to the kit's instructions.

Dendritic Cell Maturation. DCs were incubated at 1×10^5 cells/well in 96-well plates (Nunc, Roskilde, Denmark) with NCs, NPs or the corresponding amount of free GSH at gold concentrations of 1, 5, 10 and 25 µg/mL in CM for 48 h at 37 °C and 5% CO_2. Between 7 and 15 experiments were performed with the different particle concentrations in order to obtain statistically-valid results. Treated DCs were then labelled with CD80 and CD86-specific monoclonal antibodies (BD Pharmigen, CA), analysed using a FACSCanto II flow cytometer (BD Biosciences, USA), and data processed with FLOWJO software (Tree Star, Inc, USA). Results are expressed as a maturation index (MI) calculated as the ratio between the percentage of stimulated and non-stimulated cells expressing the aforementioned

markers. Culture supernatants were collected and stored at −20°C for subsequent secreted cytokine analysis.

Lymphocyte Proliferation. $CD14^-$ PBMCs were labelled with 2 μM CFSE using the CellTrace CFSE proliferation kit (Life Technologies, Invitrogen, USA) at room temperature and darkness for 10 min. After washing, labelled PBMC (1.5×10^5) were co-cultured with 1.5×10^4 autologous DCs in 96-well plates (Nunc, Roskilde, Denmark) for 6 days at 37 °C and 5% CO2. DCs were pre-incubated with NCs or NPs at different concentrations as described above and washed several times (primed DCs). Between 5 and 10 experiments were performed with the different particle concentrations in order to obtain statistically-valid results . Co-cultured PBMCs were labelled for CD3, CD4, CD8 (to identify T lymphocyte subpopulations), CD56 (to identify NK cells), CD127, CD25, ROR-γt, Fox p3, and IL-17 monoclonal antibodies (BD Pharmigen, CA, USA) in order to detect the different lymphocyte subpopulations. Th17 cells were defined as $CD4^+$ ROR-γt^+ IL-17^+; and Treg as $CD4^+$ $CD127^{low}$ $CD25^{high}$ Fox $p3^+$. The percentage of proliferating cells with lower levels of CFSE fluorescence ($CFSE^{dim}$) was assessed by flow cytometry. Results are expressed as a proliferation index (PI), calculated as:

$$\frac{\left[\%CD3^+CFSE^{dim}\ stimulated\ (lymphocytes + DCs)\right] - \left[\%CD3^+CFSE^{dim}\ unstimulated\ (lymphocytes + DCs)\right]}{\%CD3^+CFSE^{dim}\ unstimulated\ (lymphocytes)}$$

PI was considered positive when greater than 2. Culture supernatants were collected and stored at −20 °C for subsequent secreted cytokine analysis.

Cytokine Secretion. Th1/Th2/Th17 cytokine and Granzyme-B production were determined in culture supernatant using the BD Cytometric Bead Array (CBA) human soluble protein Kit (BD Biosciences, CA, USA) following the manufacturer's protocol. Culture supernatants were obtained after incubating DCs primed with NCs or NPs at 10 or 25 μg/mL concentrations, alone for 48 h or mixed with PBMCs for 6 days. Cytokine measurements were conducted in 5 independent experiments. The results are expressed in pg/mL.

Ethical Statement. This study was approved by the institutional review board "*Comisión de Ética y de Investigación del Hospital Regional Universitario de Málaga*", and the

experiments were carried out in accordance with the Declaration of Helsinki. Oral and written informed consents for all the procedures were obtained from subjects included in the study.

Statistical Analysis. In this study, nonparametric analysis comparing obtained results with the different stimuli of the quantitative variables was performed using the Wilcoxon test for related samples. Maturation measurements were analysed using 17 patients; Lymphocyte proliferation was analysed for 8 patients and cytokine secretion for 5 patients. All reported *P* values represent 2-sided tests, with the level of significance set at the probabilities of $*P < 0.05$ or $** P < 0.01$. The Bonferroni correction was applied for comparison of three groups as two independent experiments and then statistical differences were considered significant when $p < 0.025$.

NC/NP quantification in DCs. Quantification of NCs or NPs in DCs was estimated by inductively coupled plasma high-resolution mass spectrometry (ICP-HRMS) using an ELEMENT XS (Thermo Fisher) system after digesting cells (an average of 70,000 cells per sample) with strong acid. Results are expressed as particles/cell calculated assuming average NC and NP sizes of 2 nm and 12 nm, respectively, as described by Lewis et al[3]. Briefly, the number of gold atoms per particle was estimated using the following formula:

$N_{atom} = (R_{particle} / R_{atom})^3$ with $R_{atom} = 0.l37$ nm (R= radius)

and the number of gold atoms from ICP: N_{ICP}= mass of gold measured /M_{gold} *N_A

with $N_A=6.02x10^{23}$; $M_{gold}= 197$ g.mol^{-1}

Finally particle/cell = $N_{ICP.}/(N_{atom}*cell_{numb.})$ with $cell_{numb.}= 70,000$.

Confocal Microsopy

Au NCs and Au NPs stabilised with GSH ligand were analysed using a Leica DM6000 inverted microscope connected to a Leica SP5 confocal laser scanning and multiphoton system. Sub-membrane cortical actin in treated DCs, washed and fixed with 4% paraformaldehyde, was stained using fluorochrome-conjugated Phalloidin. NC-incubated

DCs were stained for ~20 minutes with 10 μM Atto488-conjugated Phalloidin (Sigma) with Atto488 and NC fluorescence visualised by confocal microscopy simultaneously with ~500-520 nm and ~570-650 nm emission detection windows, respectively. NP-treated DCs were stained with Alexa 647-conjugated Phalloidin and visualised by confocal microscopy using 633 nm laser excitation and a ~650-700 nm detection window. NP photoluminescence was detected sequentially using 720 nm two photon excitation and a ~550-650 nm detection window. Images were captured using 25x NA 0.95 water or 63x NA 1.4 oil immersion objectives.

Characterisations

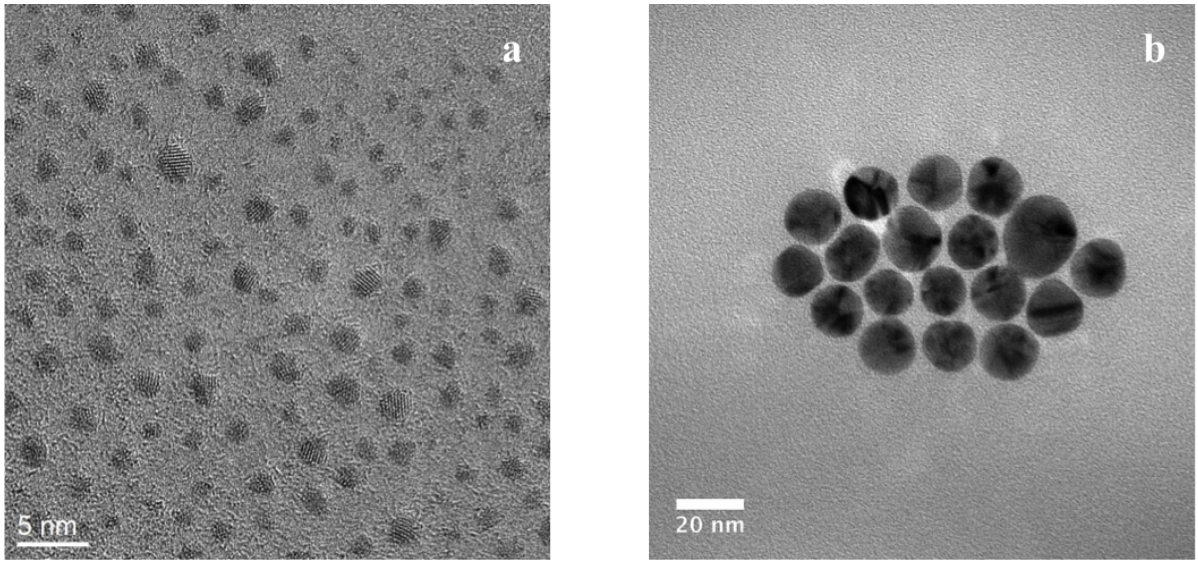

Figure S1. Transmission electron microscopy images of (a) NCs and (b) NPs.

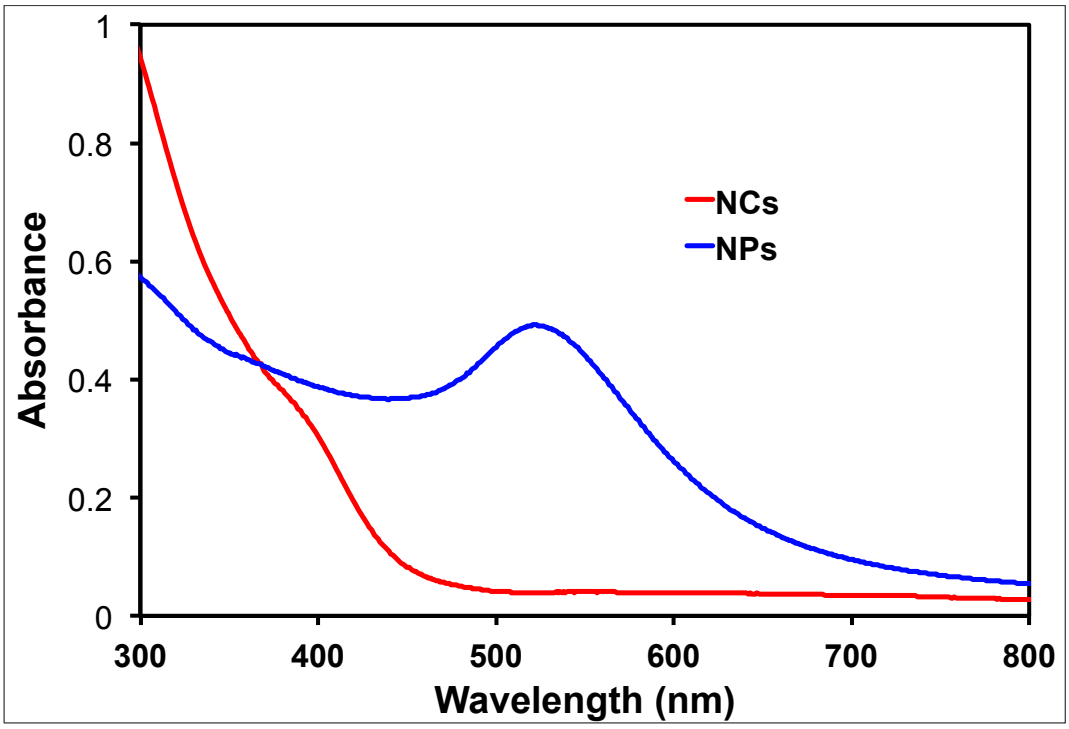

Figure S2. Absorption spectra of the NC (red line and NP (blue line) solutions. Measurements indicated strong UV absorption for the NCs and the plasmon band at λ= 520 nm for the NPs.

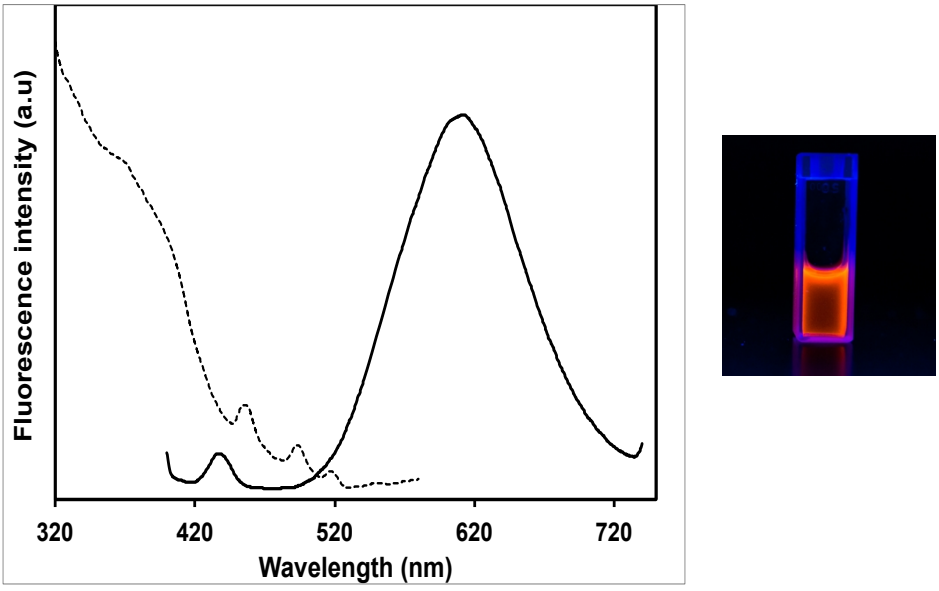

Figure S3. Excitation (dashed line) and emission (solid line) spectra of a diluted aqueous NC solution. NCs exhibited intense orange fluorescence under UV illumination (picture on the right).

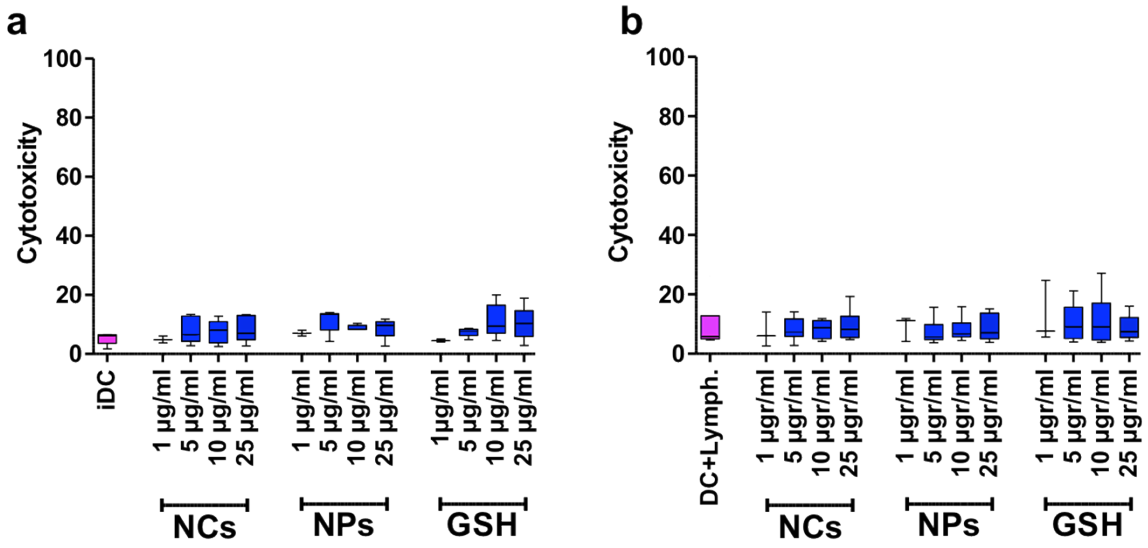

Figure S4. Cytotoxicity tests of (a) DCs for 48 hours in presence of NCs, NPs (1-25 µg/mL in RPMI1640 + 10% FCS) and GSH, and (b) for the lymphocyte proliferation experiments.

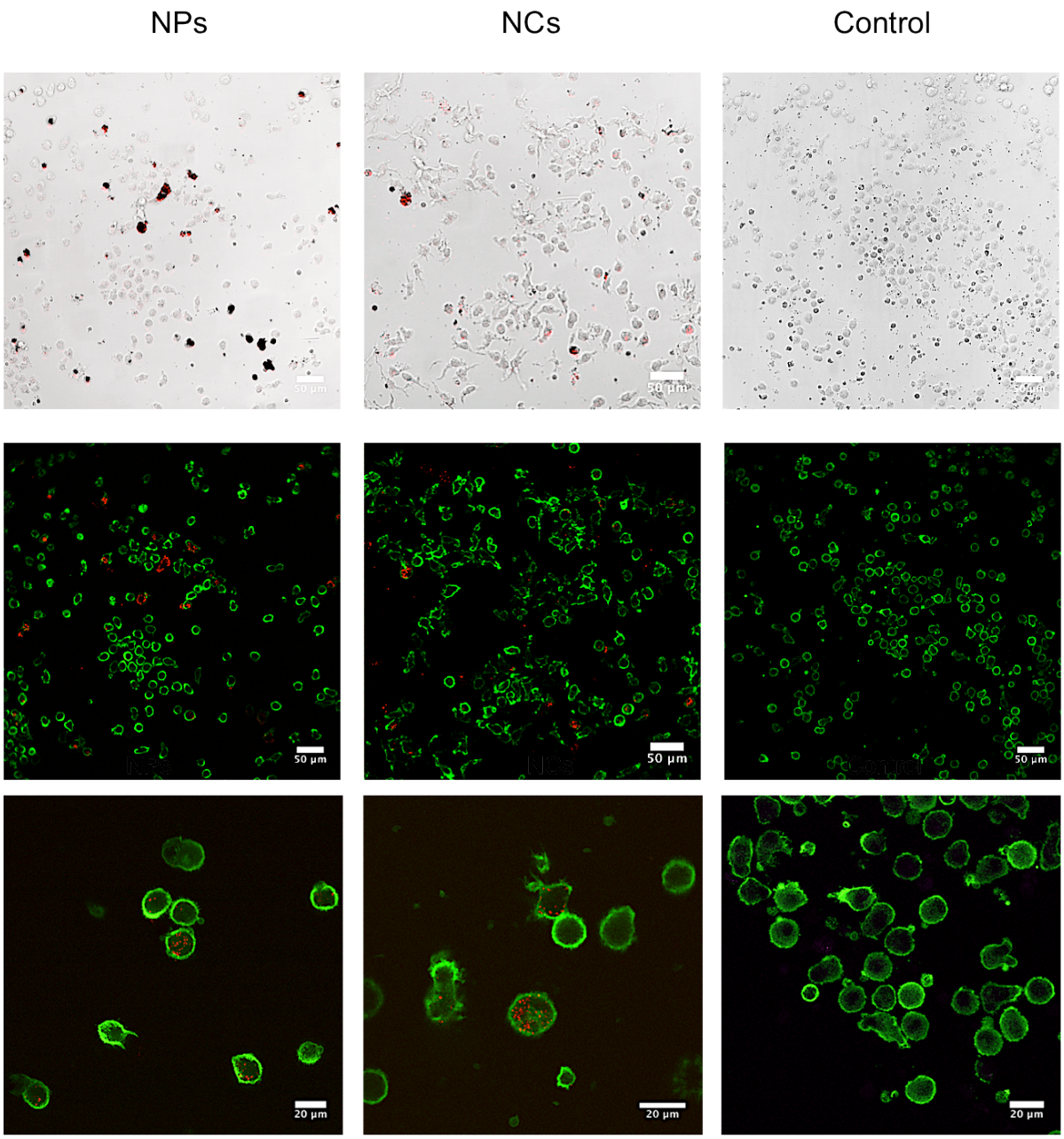

Figure S5. Dendritic cells (DCs) incubated with NCs, NPs (10 µg/mL in RPMI+10%FCS) and without particles (control) for 48 hours. Brightfield and fluorescence microscopy images show the internalisation of NCs and NPs (red) in DCs. Sub-membrane actin was stained (green) with Phalloidin-Alexa 647 (NPs) or Phalloidin-Atto 488 (NCs).

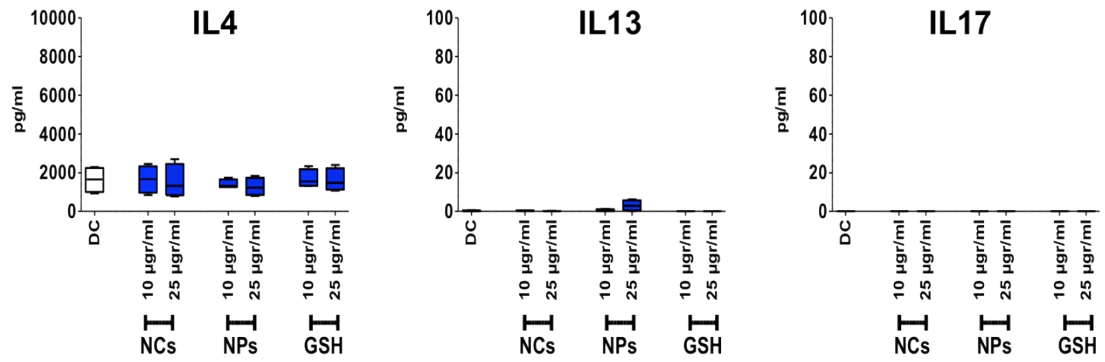

Figure S6. Cytokine secretion (IL4, IL13, IL17) after DCs were incubated with NCs, NPs (1-25 µg/mL in RPMI+10%FCS) and GSH for 48 hours.

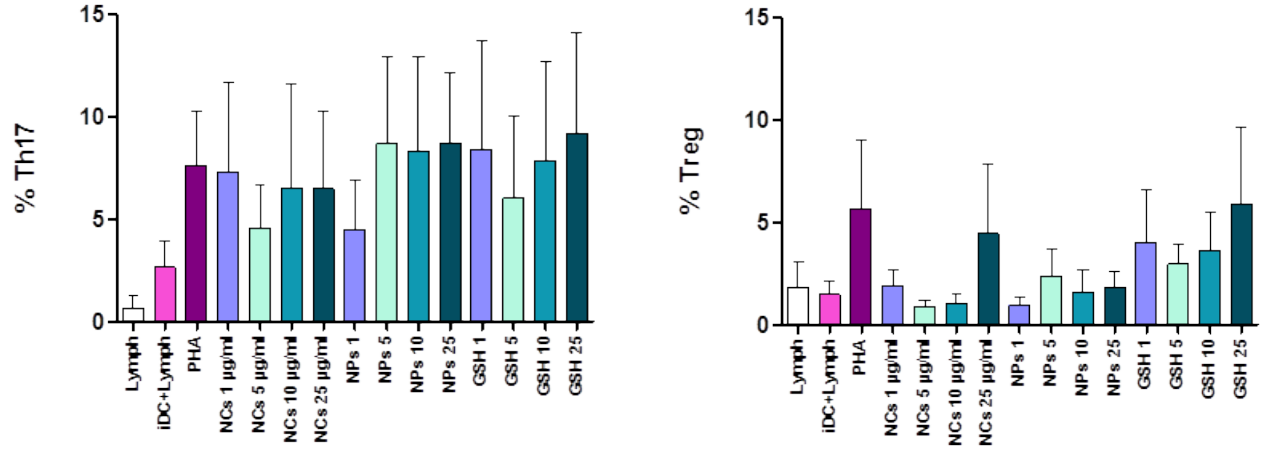

Figure S7. Percentage of different lymphocyte subpopulations as judged by the expression of Th17 or Treg markers in proliferating $CD4^{+}$T cells following a 6 day incubation of PBMCs with DCs pre-treated with NCs, NPs or free GSH. Statistical comparisons have been performed using the non-parametric test for related samples (Wilcoxon test). The Bonferroni correction was applied for comparison of three groups. Statistical differences were considered significant when $p < 0.025$.

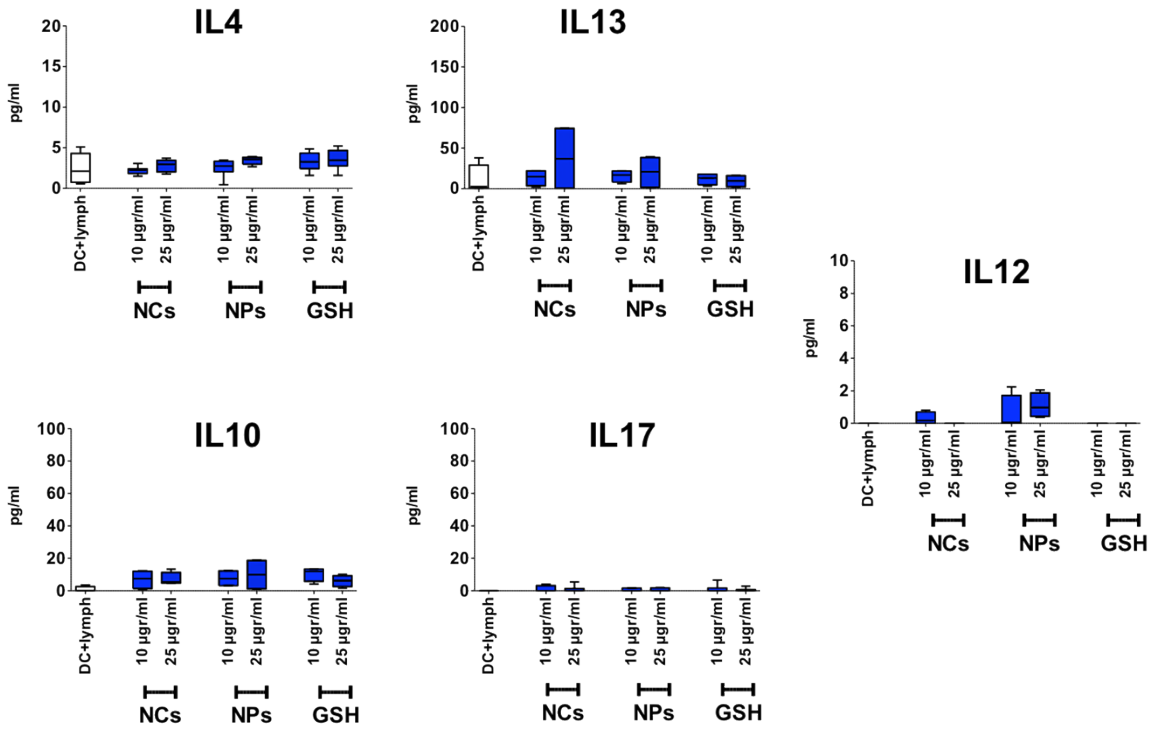

Figure S8. Cytokine production (IL4, IL10, IL12, IL13, IL17) following the pre-treatment of DC with NCs, NPs or free GSH and after several washing step incubated with peripheral blood lymphocytes for 6 days.

REFERENCES

1. X. Le Guevel, V. Trouillet, C. Spies, K. Li, T. Laaksonen, D. Auerbach, G. Jung and M. Schneider, *Nanoscale*, 2012, 4, 7624-7631.
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