Electronic Supplementary Information for: Rapid screening method to evaluate impact of nanoparticles on macrophages

Supplementary materials and methods

Supplementary NP Synthesis

Additional NP were synthesized for figure S4: Citrate-stabilized gold NP were coated with only one layer of Cy5-labeled NH_2PVA polymer (homo-NH_2PVA AuNP) instead of two layers (NH_2PVA AuNP), or with unlabeled polyethylene glycol (PEG), PVA or a mixture of PEG and PVA (PEG/PVA). Carboxyl (COOH) functionalization and amine (NH_2) functionalization were used. Synthesis and characterization for all formulations has been described in detail previously (L. Rodriguez-Lorenzo et al., *Small*, 2014, **10**, 1341–50; K. Fytianos et al., *Nanomedicine*, 2015, **11**, 633–44.)

Myeloid suppressor cell (MSC) line

The myeloid-derived suppressor cell line MSC-2 (E. Apolloni et al, *J. Immunol*, 2000, **15**, 6723-30) was cultivated in MSC-2 medium consisting of RPMI 1640 (Bio west, Nuaillé, France) supplemented with 1 % glutamine (PAA laboratories, Pashing, Austria), 1 nM sodium pyruvate, 1 % 2-mercaptoethanol (Life Technologies, Carlsbad, USA), 100 ng/ml IL-4 (Peprotech, Rocky Hill, USA), 10 % FBS and 100 IU/ml penicillin and 100 µg/ml streptomycin (100x preparation from Corning, New York, USA). Before each experiment, cells were concentrated to 10^6 cells/mL before adding 100 µl per well (10^5 cells/well) in flat-bottom 96-well plates (Corning, New York, USA). After overnight incubation, medium was replaced by 50 µl fresh MSC-2 medium. NP, polymers and other test substances were added as indicated at 2x concentration in 50 µl MSC-2 medium. For all NP, the final working concentration was 20 µg/ml gold (determined by UV-Vis at 400 nm). As positive control for immune activation, MSC-2 macrophages were cultured with 0.5 µg/ml resiquimod (R848; Enzo life Sciences, Farmingdale, USA).

List of materials required to run one 96-well plate (up to 11 NP)

Instruments:

- Incubator (37°C, 5% CO_2)
- Inverted microscope
- (Automated) flow cytometer with at least 5 detectors
- Plate reader for ELISA (absorbance at 450 nm)

Plasticware:

- 2x 96-well flat bottom plates for macrophage exposure to NP
- 1x 96-well round bottom plate for flow cytometry analysis

Cell culture:

- J774.1 macrophage cell line
- T-75 Flask for J774.1 cell culture
- Lipopolysaccharide (positive control)
- Staurosporine (positive control)
- 12 mL of J774.1 Medium:
    - high glucose (4.5 g/l) DMEM
    - 1 % glutamine
    - 1nM sodium pyruvate
    - 10 % fetal bovine serum (FBS)
    - 0.5 % ciproxine

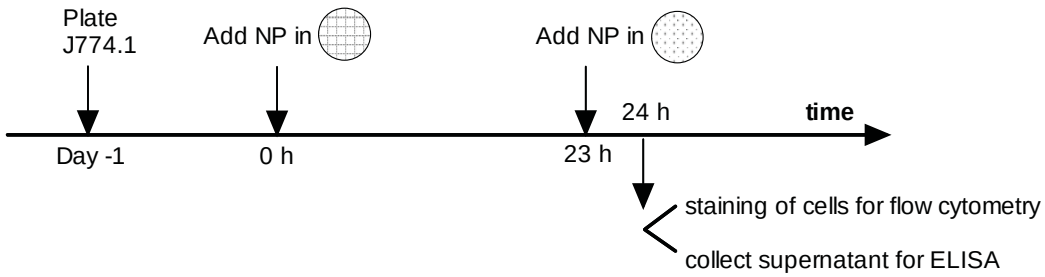
For flow cytometry staining:

- Labeled Annexin V (e.g. Pacific-Blue labeled Annexin V) and specific Annexin V buffer
- Propidium iodide or/and Zombie dye
- Labeled antibodies, one per marker (CD80, CD86, MHC-II, CD11b)
- Isotype controls for each marker
- unlabeled Fc-block (anti-CD16/32)
- 50 ml FACS buffer (PBS supplemented with 2 mM EDTA and 0.5 % BSA)

ELISA analysis:

- Kit for cytokine analysis (plate, buffer, antibodies…)

Supplementary Figures



Toxicity/uptake staining

	laser	filter	staining
V1	405	450/50	Annexin V
B3	488	655-730	Propidium iodide
R1	635	655-730	Cy5-labeled NP

Immune activation master mix

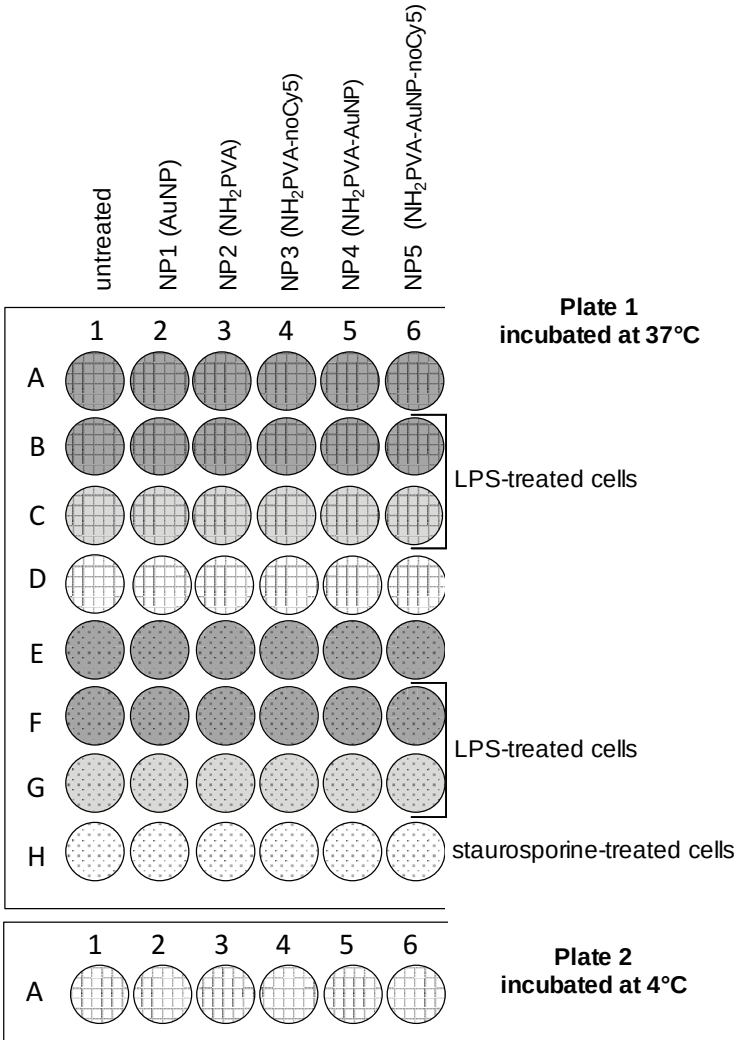
	laser	filter	staining
V1	405	450/50	CD80 - PB
B1	488	525/50	zombie green
B2	488	585/40	CD86 - PE
B4	488	750LP	CD11b-PE-Cy7
R1	635	655-730	Cy5-labeled NP
R2	635	750LP	MHCII-APC-Cy7

Isotype controls master mix

	laser	filter	staining
V1	405	450/50	isotype - PB
B1	488	525/50	zombie green
B2	488	585/40	isotype - PE
B4	488	750LP	isotype-PE-Cy7
R1	635	655-730	Cy5-labeled NP
R2	635	750LP	isotype-APC-Cy7

NP added at 0 h

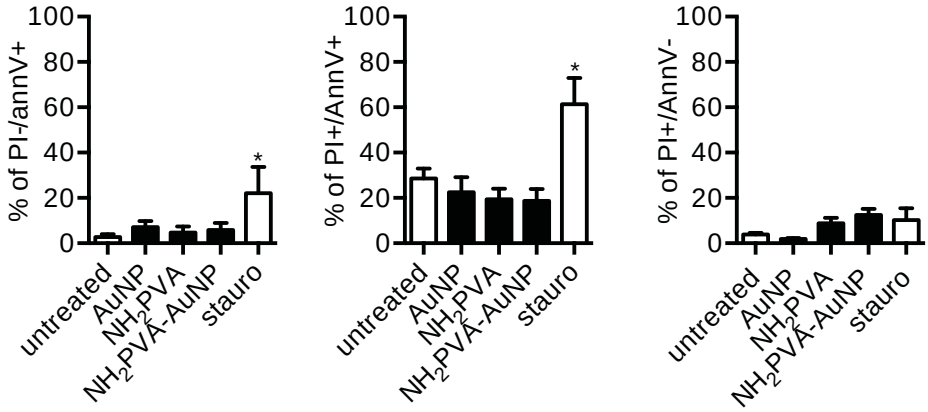
NP added after 23 h



Supplementary figure S1: plate organization and master mixes to perform the rapid screening method

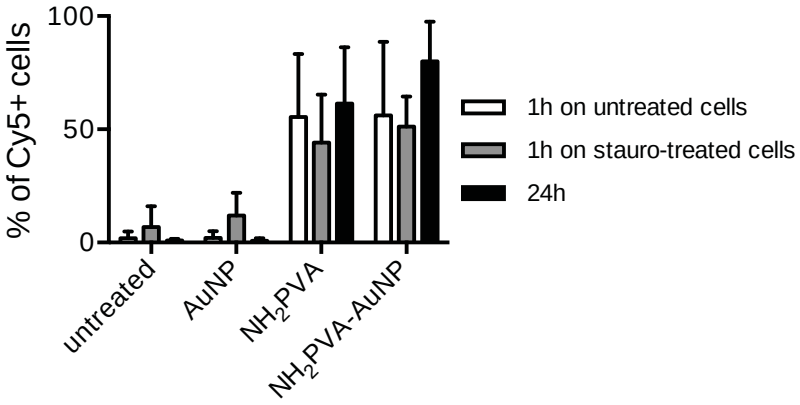
$5x10^4$ macrophages per well in 100 µl in 96-well flat-bottom plate were seeded in all the wells. After overnight incubation, medium was replaced with 100 µl fresh medium (squares) or 50 µl fresh medium and 50 µl NP-containing medium (dots). To inhibit active uptake, plate 2 was incubated at 4 °C. After 23 h incubation, 50 µl

medium were exchanged for 50 µl NP-containing medium in interference control wells (dots). One hour later, all the supernatants were harvested and stored at -20 °C for cytokine quantification by ELISA, and the cells were analysed with 3 different flow cytometry stainings. First, annexin V/PI staining allowed determination of the NP cytotoxicity (white). The same wells were used to determine the uptake of fluorescently-labeled NP in the live population. Secondly, we designed an immune activation master mix (dark grey) to determine the up-regulation of key activation markers. Thirdly, as control, we used a master mix containing the different isotype controls of the immune activation master mix (light grey). The different tables are based on the optical configuration of the MACSquant analyzer 10 equipped with 3 lasers: violet (405 nm), blue (488 nm) and red (635 nm).



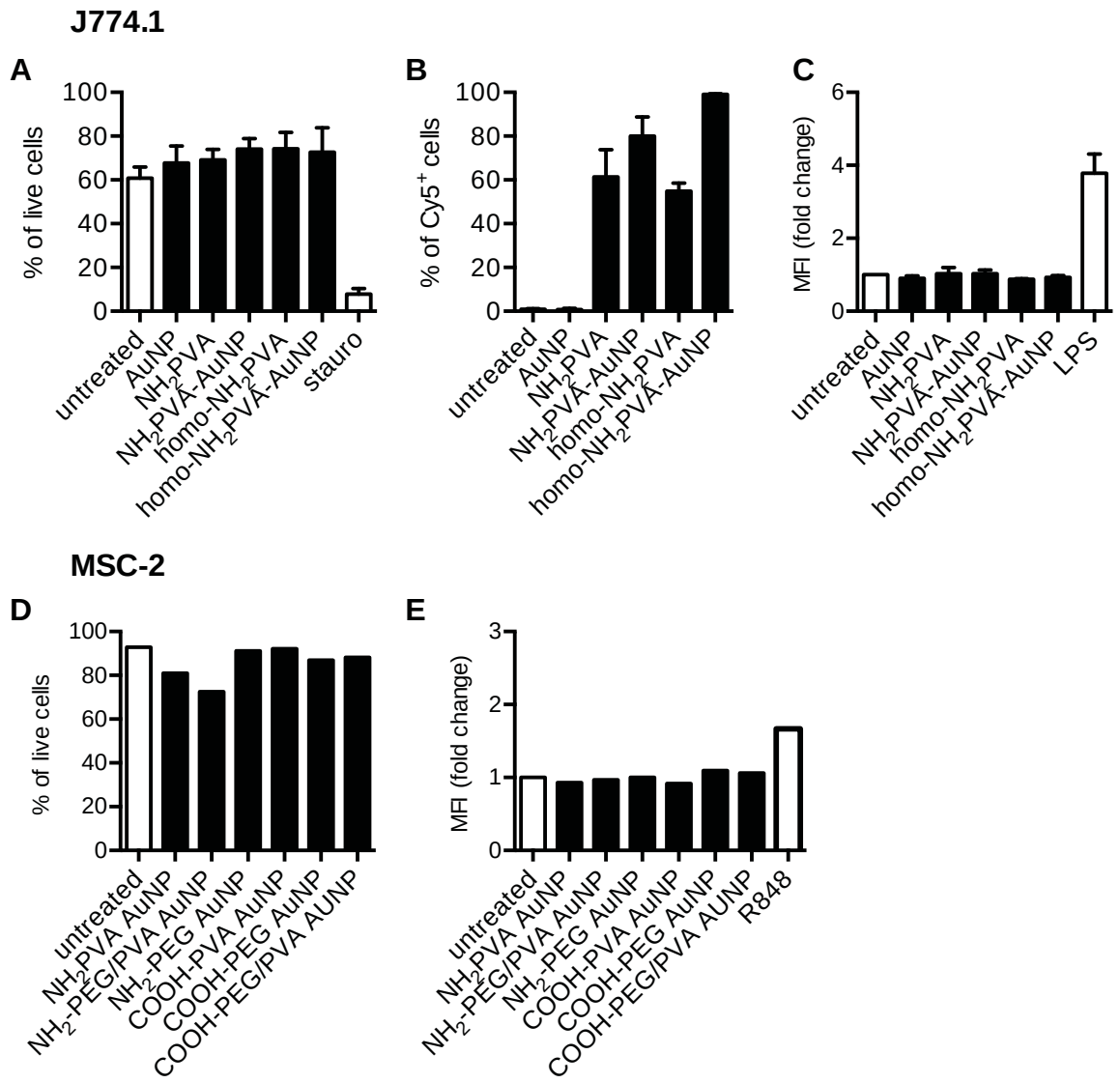
Supplementary figure S2: impact of NP on macrophage viability

As for figure 3, J774.1 macrophages were incubated for 24 h with NP, NH_2PVA polymer or staurosporine (stauro) before flow cytometry analysis with annexin V and PI. Each bar represents mean ± SEM of 5 independent experiments. Asterisks (*, P<0.05) indicate significant differences with untreated group using one-way ANOVA followed by Dunnett's multiple comparison test.



Supplementary figure S3: Uptake of Cy5-labeled NP after 1 hour by untreated and staurosporine cells

J774.1 macrophages were incubated for 24 h without (white) or with (grey) staurosporine before adding NP or NH_2PVA polymer for the last hour of incubation. As positive control, macrophages were incubated for 24 h with NP or NH_2PVA polymer (black). Uptake was analysed by flow cytometry after gating on live (annexin V/PI double-negative) cells. Percentage of Cy5+ cells is depicted. Each bar represents mean ± SEM of at least 4 independent experiments.



Supplementary figure S4: Cytotoxicity, uptake and immune activation of different formulations of AuNP with different cell types.

J774.1 (A-C) macrophage or MSC-2 myeloid suppressor cell lines (D,E) were incubated for 24 h at 37 °C with different formulations of AuNP or polymers before flow cytometry analysis. **(A,D)** Percentage of live (annexin V/PI double-negative) cells. **(B)** percentage of $Cy5^+$ cells and **(C,E)** MHC-II median fluorescence intensity (MFI; fold change compared to untreated sample). A-C: Each bar represents mean ± SEM of at least 3 independent experiments. D, E: Each bar represents mean of triplicates of one experiment.