Supporting information

Assessing the Immunosafety of Engineered Nanoparticles with a Novel *in vitro* Model Based on Human Primary Monocytes

Yang Li [1*], *Paola Italiani* [1], *Eudald Casals* [2], *Dirk Valkenborg* [3,4], *Inge Mertens* [3,4], *Geert Baggerman* [3,4], *Inge Nelissen* [3], *Victor F. Puntes* [2,5,6] *Diana Boraschi* [1]

[1] Institute of Protein Biochemistry, National Research Council, Napoli, Italy

[2] Institut Català de Nanotecnologia, Campus of the UAB, Bellaterra, Spain

[3] Flemish Institute for Technological Research, Mol, Belgium

[4] Center for Proteomics, University of Antwerp, Antwerp, Belgium

[5] Vall d'Hebron Institute of Research, VHIR, Barcelona, Spain

[6] Institut Català de Recerca i Estudis Avançats, ICREA, Barcelona, Spain

* Corresponding author

Email: yang.li.nano@gmail.com; yang.3.li@ucdenver.edu

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Experimental details of the synthesis and characterization of NPs

Nanoparticle synthesis:

The wet chemistry method was used for synthesizing Au NPs, in conditions allowing us collecting stable and narrowly dispersed NPs (Figure 1). All reagents were from Sigma-Aldrich, Inc. (St. Louis, MO, USA). To obtain citrate-coated 10 nm Au NPs, 1 ml hydrogen tetrachloroaureate ($HAuCl_4$) 0.025 M is rapidly added to 150 ml of boiling trisodium citrate (SC) 2.2 x 10^{-3} M under vigorous stirring, as described by Turkevich *et al.*[1] After 3-5 min, the formation of colloidal gold becomes evident as the mixture develops a characteristic red color. The solution is left to cool to room temperature (RT). The Au NPs synthesized in this way are loosely coated with negatively charged citrate ions, which act as stabilizers. Synthesis of 14 nm Ag NPs is carried out by adding 5 ml of SC 100 mM to 50 ml of a boiling solution of $AgNO_3$ 1 mM under vigorous stirring,. The mixture is then cooled in another tube. Also Ag NPs synthesized in this way are loosely coated with the negative-charged citrate ions that act as stabilizers.

The NP solvents used as controls in the biological experiments are the aqueous solutions in which NPs are dispersed. To obtain NP solvents, NPs were removed from solution by high-speed centrifugation. It is important to underline the notion that citrate ions, which are present in the solvents of both Au and Ag NPs, had been proved biocompatible.[2] Stability of the colloids requires the presence of solvents, which therefore cannot be excluded from the NP formulations.

Nanoparticle characterization*:*

Transmission Electron Microscopy (TEM): NP images were acquired with a JEOL 1010 electron microscope (JEOL Ltd., Akishima, Tokyo, Japan), operating at an accelerating voltage of 80 kV. For sample preparation was carried out by drop casting on carbon-coated copper TEM grids, which were then dried at RT. Several areas of the grid were observed at different magnifications, and size distribution was calculated by computerized analysis of over 400 particles.

Z-Potential measurement and Dynamic Light Scattering (DLS): A Malvern ZetaSizer Nano ZS (Malvern Instruments Ltd., Malvern, Worcestershire, United Kingdom) was used for measurements on colloidal NPs (0.8 ml aliquots). The machine operated at a light source wavelength of 532 nm and a fixed scattering angle of 173°, with its software set with the specific parameters of refractive index and absorption coefficient of NP material and solvent viscosity.

UV-visible spectrophotometry (UV-VIS): A Shimadzu UV-2400 spectrophotometer (Shimadzu Corp., Nakagyo, Kyoto, Japan) was used for acquiring the UV-VIS spectra of NPs (1 ml aliquots), by performing the spectral analysis in the range 300-800 nm.

1. Turkevich, J.; Stevenson, P. C.; Hillier, J. A Study of the Nucleation and Growth Processes in the Synthesis of Colloidal Gold. *Discuss Faraday Soc.* **1955**, 11, 55-75.

2. Pfaller, T.; Puntes, V.; Casals, E.; Duschl, A.; Oostingh, G. J. In Vitro Investigation of Immunomodulatory Effects Caused by Engineered Inorganic Nanoparticles-the Impact of Experimental Design and Cell Choice. *Nanotoxicology* **2009**, 3, 46-59.

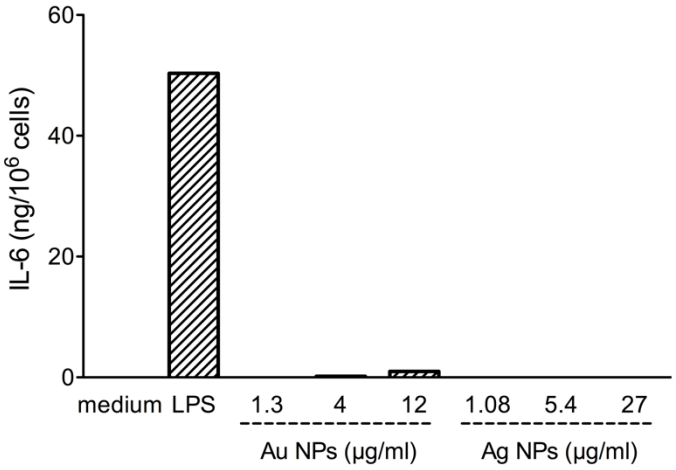

Figure S1. Au and Ag nanoparticles do not induce IL-6 production in human monocytes *in vitro*. Human primary monocytes were exposed to culture medium alone (negative control), or medium containing 1 ng/ml LPS (positive control) or increasing concentrations of Au and Ag NPs for 24 h. The production of IL-6 was measured by ELISA in the cell culture supernatants. Data are the mean ± SD of duplicates from one representative experiment.

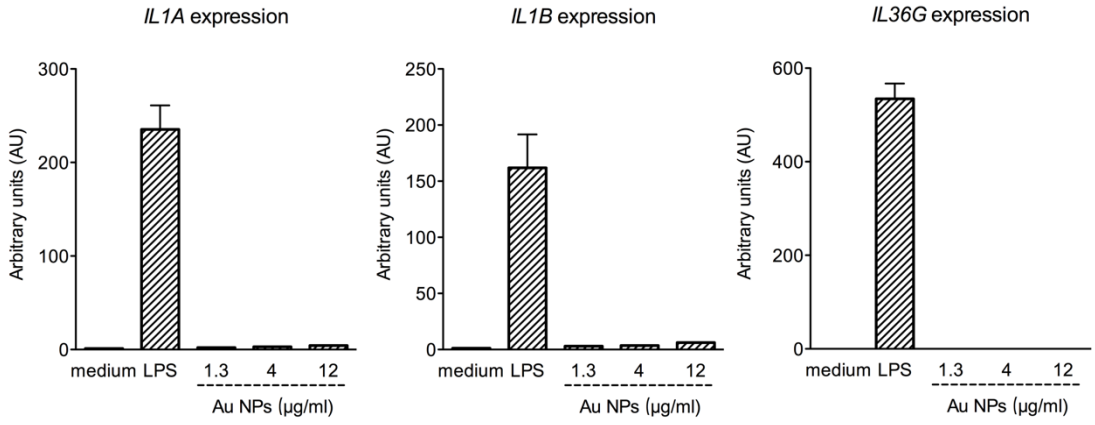

Figure S2. IL-1 family gene expression in primary human monocytes exposed to Au nanoparticles. The expression of the *IL1B* gene, coding for the prototypical inflammatory cytokine IL-1β, was assessed in parallel to expression of the *IL1A* and *IL36G* genes, coding for the inflammatory IL-1 family cytokines IL-1α and IL-36γ. Gene expression was measured by qPCR in human monocytes exposed to Au NPs for 24 h, in comparison to culture medium as negative control and LPS (1 ng/ml) as positive/inflammation-inducing stimulus. Data are the mean ± SD of duplicate values from one representative experiment.

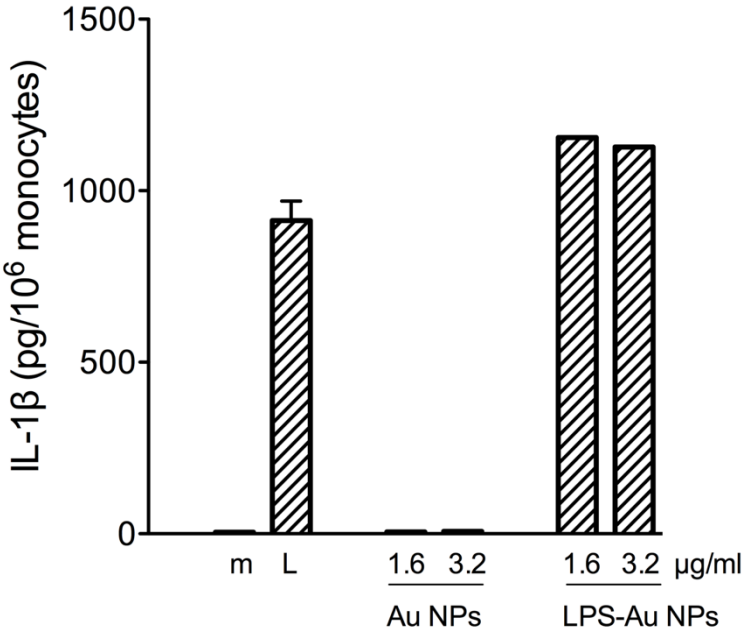

Figure S3. Endotoxin-contaminated Au nanoparticles induce inflammatory activation of monocytes. Human primary monocytes were exposed to culture medium alone (m, negative control) or containing 10 ng/ml LPS (L, positive control) for 24 h. Au NPs were deliberately contaminated with 1 µg/ml LPS for 1 h at room temperature and then thoroughly washed with endotoxin-free water to eliminate unbound LPS. Monocytes were exposed to either endotoxin-free and endotoxin-coated Au NPs for 24 h. The production of IL-1β in the culture supernatants was measured by ELISA. Data are the mean ± SD of duplicates from one representative experiment.

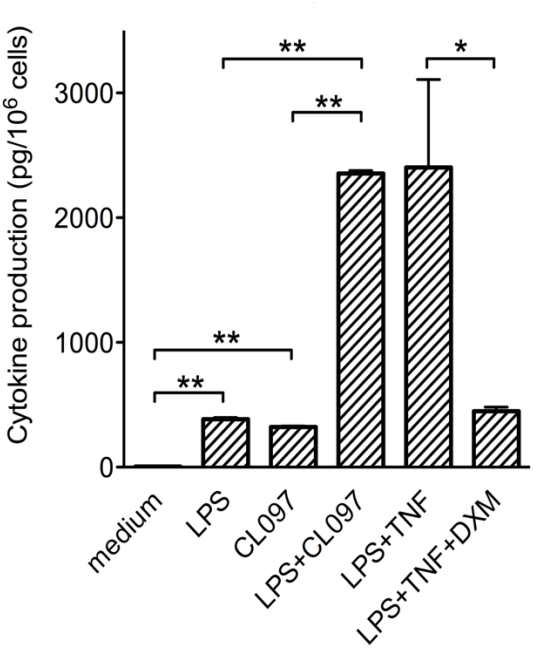

Figure S4. Modulation of IL-1β release in monocytes by immunostimulatory and immunosuppressive agents *in vitro*. Monocytes were exposed in culture for 18 h to LPS (5 ng/ml) in the absence or in the presence of the TLR7/8 agonist CL097 (100 ng/ml), or to LPS + TNF-α (5 ng/ml + 10 ng/ml) in the absence or in the presence of the immunosuppressive drug DXM (10 μM). The production of IL-1β was measured in the culture supernatants by ELISA. Data are the mean ± SD of 2-3 replicate values from one representative experiment or from 3-5 replicate independent experiments. * $p<0.05$, ** $p<0.01$

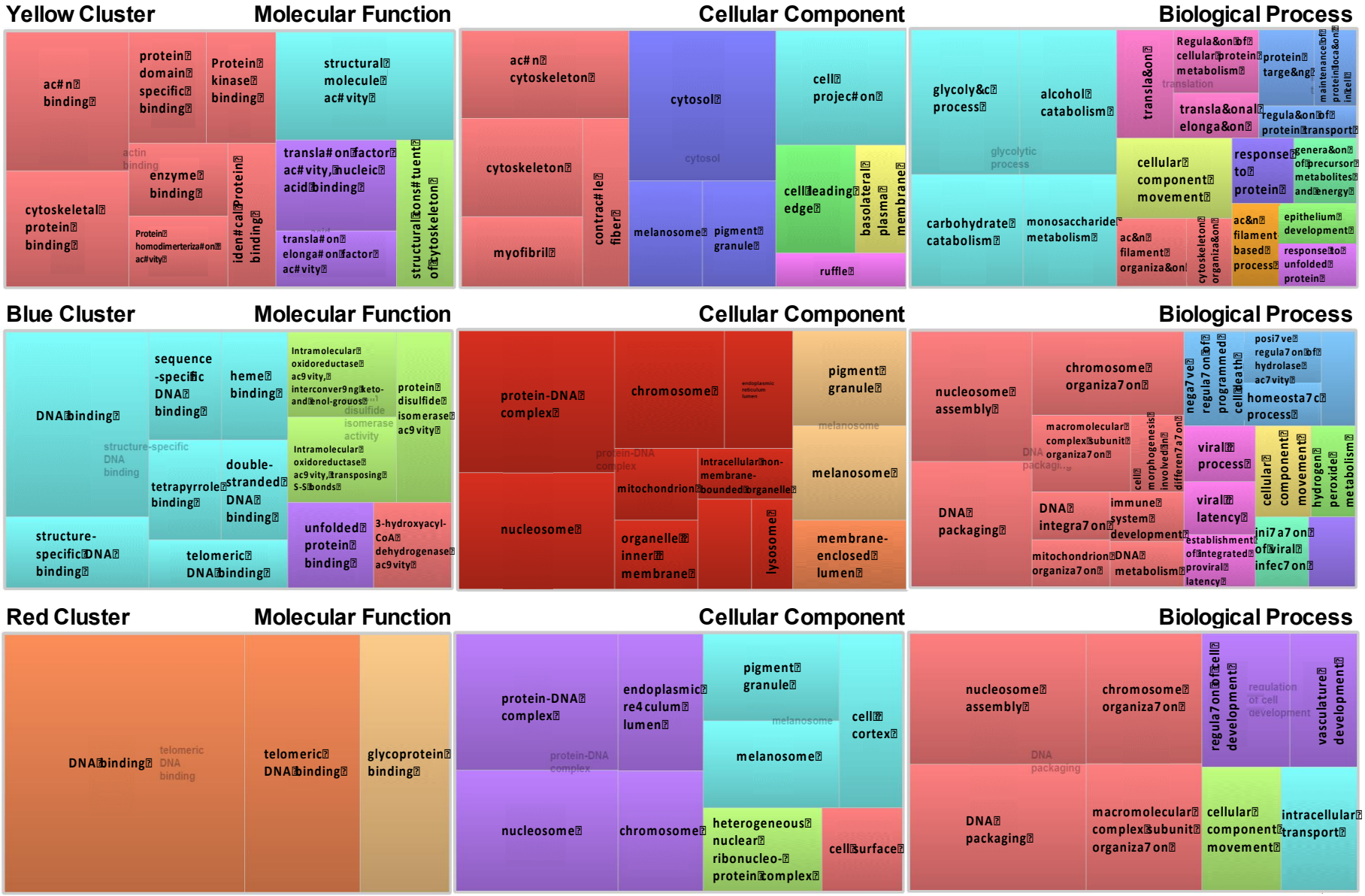
Yellow Cluster
Molecular Function
Cellular Component
Biological Process
Blue Cluster
Molecular Function
Cellular Component
Biological Process
Red Cluster
Molecular Function
Cellular Component
Biological Process

Figure S5. The molecular function, cellular component and biological process ontologies for the yellow, blue and red peptide clusters.
The enriched functional categories in the differential proteomic profile (see Figure 3) were analysed and visualized with ReVIGO webtool by finding a representative subset of ontologies using a simple clustering algorithm that relies on semantic similarity measures. Molecular function, cellular component and biological process are presented as tree maps. The terms in the tree map correspond to the representative subset of ontologies, whilst the size of the block indicates the enrichment of this term in the differential proteins. The colorful map is used to group the representative terms that were semantic similar, and the colorful groups were named after the most enriched term.

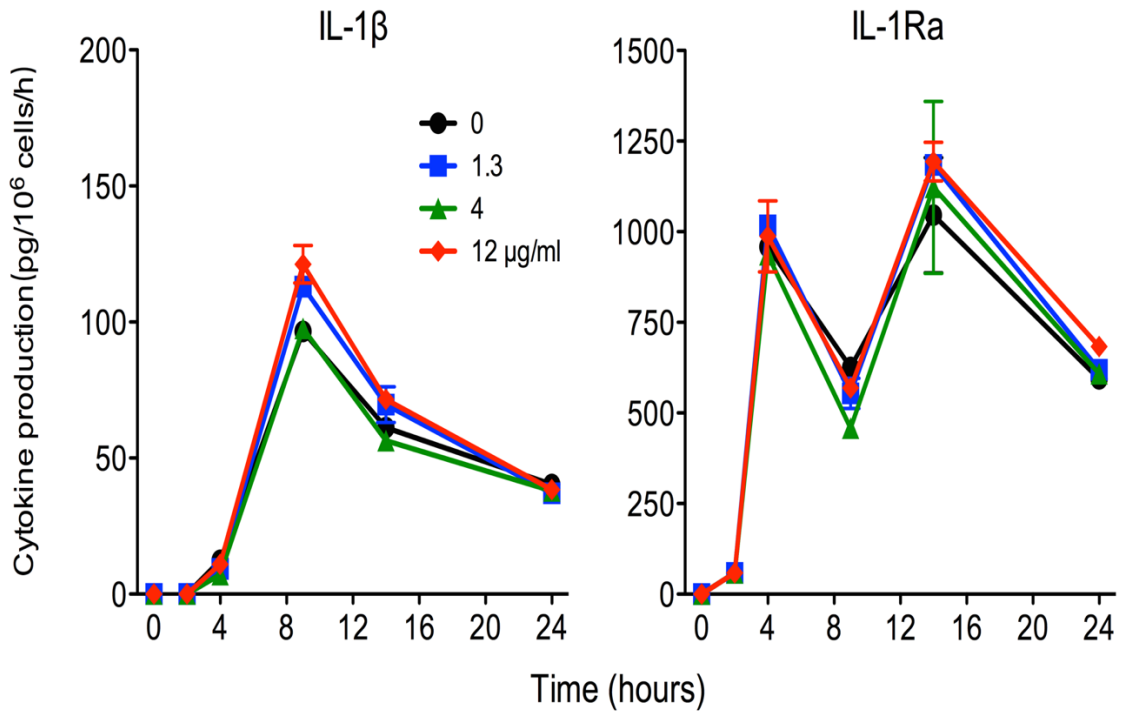

Figure S6. Dose-response analysis of the effect of Au nanoparticles on the course of the inflammatory reaction *in vitro*. Monocytes in the kinetic inflammation model were exposed to increasing Au NP concentrations. The production of IL-1β (left) and IL-1Ra (right) was measured by ELISA and expressed as rate of production/release. Data are the mean ± SD of duplicate values from one representative experiment.