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In vitro assessment of silver nanoparticles immunotoxicity

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ABSTRACT

This study aimed to characterize unwanted immune effects of nanoparticles (NP) using THP-1 cells, human whole blood and enriched peripheral blood monocytes. Commercially available silver NP (AgNP < 100 nm, also confirmed by Single Particle Extinction and Scattering) were used as prototypical NP. Cells were treated with AgNP alone or in combination with classical immune stimuli (i.e. LPS, PHA, PWM) and cytokine assessed; in addition, CD54 and CD86 expression was evaluated in THP-1 cells. AgNP alone induced dose-related IL-8 production in all models, with higher response observed in THP-1 cells, possibly connected to different protein corona formation in bovine versus human serum. AgNP potentiated LPS-induced IL-8 and TNF-α, but not LPS-induced IL-10. AgNP alone induced slight increase in IL-4, and no change in IFN-γ production. While responses to PHA in term of IL-4 and IFN-γ production were not affected, increased PWM-induced IL-4 and IFN-γ production were observed, suggesting potentiation of humoral response. Reduction in PHA-induced IL-10 was observed. Overall, results indicate immunostimulatory effects. THP-1 cells work as well as primary cells, representing a useful and practical alternative, with the awareness that from a physiological point of view the whole blood assay is the one that comes closest to reality.

1. Introduction

In the field of nanomaterials, the number of discoveries, innovations, and potential bioapplications is tremendous. Although opening new opportunities for the advancement of technology and medicine, NP could also lead to unforeseen adverse health effects in exposed human subjects (Piperigkou et al., 2016; Li et al., 2016a, 2016b). With the field of nanotoxicology rapidly growing (Shvedova et al., 2016), toxicology is unable to provide rapid responses to the potential toxicity of NP, thus struggling beyond progress and leading, following approval, to market withdrawn due to hypersensitivity and toxicity as in the case of iron oxide NP (Anselmo and Mitragotri, 2015). Alternatives to traditional animal testing are clearly necessary, and in vitro methods may offer important opportunities. In addition, in vitro assays may offer the possibility to screen NP toxicity, limiting the number of NP that must be tested in vivo.

The immune system can be the wanted or unwanted target of NP. An incredible progress has been made in the understanding of the basis of NP immunocompatibility and potential immunotoxicity (Dobrovolskaia et al., 2009; Engin et al., 2017; Pallardy et al., 2017), but our understanding of the interactions between NP and the immune system remains incomplete. Characterizing the effects of NP on the immune system is central to the design of safe and effective NP-based drugs and consumer products (Dobrovolskaia et al., 2016; Giannakou et al., 2016). Published data indicate that immunotoxicity of NP is mainly associated with inflammation in healthy animals and aggravation of pathologies in disease models (reviewed by Fröhlich, 2015). Unwanted effects on the immune system include immunosuppression, with increased risk of infections and cancer, and inappropriate immunostimulation, which may lead to autoimmune disorders as well as hypersensitivity reactions.

Due to their anti-bacterial properties, AgNP are used in a variety of consumer and medical products (Ge et al., 2014; Tulve et al., 2015), and in the current study they were used as a prototypical NP. To mention some of the immunotoxic effect of AgNP, in a 28 days repeated dose toxicity study in rats following AgNP iv injection, the immune

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system was identified as the most sensitive compartment (De Jong et al., 2013). Intravenous administration of 20 nm up to 6 mg/kg body weight resulted in almost complete suppression of natural killer cell activity at the highest doses, decreased concanavallin A-induced IFN-γ and IL-10 production in spleen cells, increased LPS-induced IL-1β and decreased IL-6, IL-10 and TNF-α in spleen cells, increased spleen weight and cellularity, increased serum IgM and IgE, and increase in blood neutrophils. Similarly, 100 nm AgNP 6 mg/kg b.w. decreased natural killer cells activity, decreased concanavallin A-induced IL-10 production in spleen cells, increased spleen weight and cellularity, increased serum IgM and IgE. In vitro studies demonstrated that AgNP are cytostatic at low concentrations and cytotoxic at high concentrations to human peripheral blood lymphocytes (Devanabanda et al., 2016; Ghosh et al., 2012), resulting in decreased proliferative response to T and B cells mitogens. A comprehensive review on the immunomodulatory effects of AgNP and other NP can be found in Fröhlich (2015).

Using the current knowledge about the relationship between NP and their effects on immune cells, the purpose of the present study was to improve the capacity to identify in vitro undesirable effects on the immune system of NP and to simplify the attending procedures. Comparative experiments were conducted using the human promyelocytic cell line THP-1, human whole blood and enriched peripheral blood monocytes. Silver NP were used as reference NP. If one just wants to evaluate the pro-inflammatory effect of NP, THP-1 cells are sufficient, while to evaluate immunomodulatory effects the diluted whole blood is better, as offering the possibility of measuring, using appropriate immune stimuli on the same model, monocyte and T or B cells responses as well as other immune parameters (i.e. proliferation, NK cell activity, etc.). From a physiological point of view the whole blood assay is the one that comes closest to reality. In parallel, to shed light on the differential cellular behavior we observed, biocorona formation following exposure of AgNP to bovine versus human serum was qualitatively investigated. Overall, the models we propose may help to design in a simple way NP targeting/non-targeting the immune system.

2. Materials and methods

Chemicals. Silver NP (CAS Number 7440-22-4) was obtained from Sigma Aldrich (St Louis, MO, USA); as certified by the supplier, the particle size was < 100 nm, with a surface area of 5.0 m^2/g, resistivity of 1.59 μΩ-cm at 20 °C, and 99.5% purity. AgNP was suspended in sterile endotoxin free phosphate-buffered saline (PBS, 10 mg/ml). Stock solutions were sonicated in a water bath for 20 min immediately before cell exposure. Lipopolysaccharide (LPS) from *Escherichia coli* serotype 0127:B8 and pokeweed mitogen (PWM) were obtained from Sigma, while phytohaemagglutinin (PHA) was from Invitrogen (Invitrogen, Paisley, UK). All reagents were purchased at the highest purity available.

Single Particle Extinction and Scattering (SPES). To confirm the size of AgNP, measurements were performed with the recently introduced Single Particle Extinction and Scattering (SPES) method (Potenza et al., 2015a). A 100 μg/ml AgNP suspension in PBS was used. SPES measures two different properties of each particle driven through a focused laser light by the surrounding liquid flown through a flow cell. The former property is related to the light power removed by the particle, the latter to its polarizability. Thus, it is possible to extract additional information besides the particle size, that enables to recognize particles of different materials with a good accuracy (Potenza et al., 2015b). The measurement of metallic NPs is advantageous (Potenza et al., 2017) because of the huge absorption occurring at the surface, making even simpler to distinguish the signals of interest from spurious signals coming, for example, from liquid impurities. Moreover, the absorption properties also increase the sensibility of the method, thus allowing to push the smallest measured diameter of a single particle well below 100 nm. Finally, thanks to the flow measurements, a huge number of particles can be measured in short times, thus overcoming the typical limited statistics of direct imaging methods.

Cells. The human monocytic THP-1 cell line was obtained from Istituto Zooprofilattico (Brescia, Italy). Cell culture media and all supplements were from Sigma. For experiments, THP-1 cells were diluted to 10^6 cells/mL in RPMI 1640 containing 2 mM L-glutamine, 0.1 mg/ml streptomycin, 100 IU/mL penicillin, 50 μM 2-mercaptoethanol, supplemented with 10% heated-inactivated fetal calf serum (media) and cultured at 37 °C in 5% CO_2 incubator.

Blood samples were taken by venous puncture with sodium citrate 0.5 M as anticoagulant. Healthy subjects (n = 6) were selected according to the guidelines of the Italian Health authorities and to the Declaration of Helsinki principles and signed an informed consent (average 40 y, min 25 max 53). Criteria for exclusion were the use of medication known to affect the immune system, i.e. steroids, or patients suffering from malignancies, inflammations and infections. Blood samples were diluted 1:10 in cell culture medium RPMI 1640 (Sigma, St Louis, USA) containing 2 mM L-glutamine, 0.1 mg/ml streptomycin, 100 IU/ml penicillin, and cultured at 37 °C in 5% CO_2 incubator.

Human monocytes were enriched using RosetteSep™ from StemCell Technologies Inc. (Vancouver, BC, Canada) from whole blood. The RosetteSep™ consists of a cocktail of patented reagents complexed tetrameric antibody. The cocktail targets a variety of unwanted cells and crosslinks them to red blood cells present in the sample, forming rosettes. The desired (unrosetted) cells are then collected as a centrifuged population from a Ficoll gradient. With this method, an 80% enrichment in $CD14^+$ cells were obtained. Following enrichment, cells were brought to 0.25×10^6/ml in RPMI supplemented with 10% fetal calf serum and cultured at 37 °C in 5% CO_2 incubator.

Treatments and cell viability. Cells were treated as described in the legends. To evaluate IL-8 production, cells were treated with increasing concentrations of AgNP (1–100 μg/ml) for 24 h. To investigate the modulation on LPS-induced cytokine production, THP-1 cells or diluted whole blood samples were treated simultaneously with increasing concentrations of AgNP (1–100 μg/ml) and LPS (10 ng/ml) for 24 h. Conversely, to investigate the modulation of PHA- or PWM-induced cytokine production, diluted blood samples were treated simultaneously with increasing concentrations of AgNP (1–100 μg/ml) in the presence or absence of PHA 3 μg/ml and PWM 5 μg/ml and incubated for 48 h before the assessment of IL-4 and IFN-γ production. Owing to the variability in stimulation assays, the same lot of each reagent was used in all experimental cultures. The suboptimal concentration of LPS, PHA and PWM were chosen in preliminary dose-response experiments. The use of suboptimal concentrations allows to highlight any costimulatory effects, and at the same time to highlight reductions in the response.

Cell viability was assessed by lactate dehydrogenase (LDH) leakage from damaged cells. LDH is a well-known indicator of cell membrane integrity and cell viability. LDH activity was determined in cell-free supernatants using a commercially available kit (Takara Bio Inc., Japan). In addition, in THP-1 cells, cytotoxicity was assessed by flow cytometric evaluation of propidium iodide (PI)-stained cells following 24 h of treatment. After incubation, cells were centrifuged at 1500 rpm for 5 min and suspended in 0.5 ml PBS containing 1 μg/ml PI. The percentage of positive cells was analyzed using a FACSCalibur flow cytometer, and data were quantified using CellQuest software (Becton Dickinson). In both cases, results are expressed as % of viable cells.

To investigate the uptake of AgNP in THP-1 cells, the method described by Suzuki et al. (2007) was used, based on the measurement of the intensity of laser light scattered by particulates inside the cell. Briefly, THP-1 cells were treated for 24 h with increasing concentrations of AgNP (1–100 μg/ml). The amounts of particles taken up by the cells were analyzed using a flow cytometer (FACS) (FACScan, BD, Italy). The laser beam (488 nm) illuminates cells in the sample stream, which go through the sensing area. The laser light scattered at narrow angles to the axis of laser beam is called forward-scattered light (FSC). The laser light scattered at about a 90° angle to the axis of the laser beam is called

side-scattered light (SSC). The intensities of FSC and SSC are proportional to the size of cells and the granularity and particulates inside the cells, respectively. Results are expressed as percentage of gated cells.

To investigate the role of bovine or human sera and heat inactivation (HI) on AgNP-induced cellular uptake and IL-8 release, THP-1 cells were suspended in culture media supplemented with different sera (10% FCS or human serum, HI and not HI) and treated with AgNP (50 μg/ml) for 24 h.

To investigate a possible presence of endotoxin, AgNP (50 μg/ml final concentration) was pre-incubated with polymixin B sulfate (15 μg/ml final concentration) for 1 h at 37 °C and then added to THP-1 cell for 24 h. LPS was included as a positive control. LPS (10 ng/ml) was incubated with polymixin B as described for NP.

Cytokine production. Cytokine production was assessed in cell free supernatants by specific commercially available sandwich ELISA (R&D System, Minneapolis, MN, USA). Cell-free supernatants obtained by centrifugation at 2500 rpm for 5 min were stored at −20 °C until measurement. Results are expressed as Stimulation Index (SI) or as pg/ml. The SI was calculated by dividing the amount released following treatment with the one released in the control group. Spiking experiments using cell-free supernatants were performed to exclude potential interference of AgNP with ELISA: a known amount of human recombinant IL-8 (500 pg/ml) was used to spike directly culture supernatant from cells exposed to 100 μg/ml of AgNP. If the calculated value obtained from the ELISA assay differed from 500 pg/ml then the AgNP interfered with the test. No interference was observed.

Flow cytometric analysis of surface marker expression. CD86 and CD54 expressions were evaluated as previously described (27). Briefly, after 24 h of treatment, THP-1 cells were centrifuged, washed once with cold PBS and suspended in PBS supplemented with 1% FCS and 0.1% NaN_3. Aliquots of 10^5 cells were stained in the dark for 30 min for THP-1 cells with specific FITC-conjugates antibodies against CD86 or CD54 (BD Biosciences) or with an irrelevant control antibody of the same isotype at room temperature following supplier's instructions (BD Biosciences). An aliquot of 1 ml PBS was then added and the cells were centrifuged at 1200 rpm for 5 min then suspended in 0.5 ml of PBS supplemented with 1% FCS and 0.1% NaN_3. The intensity of fluorescence and the percentage of positive cells were analyzed using a FACSCalibur flow cytometer and data were quantified using CellQuest software (Becton Dickinson). Aliquots of 10,000 viable cells were analyzed for mean fluorescence intensity (MFI) and percentage of positive cells (PC). All experiments were performed in triplicate.

Changes in expression are reported as stimulation index (SI), calculated by the following equation:

$$SI = PCt \times MFIt / PCc \times MFIc$$

PCt and MFIt stand for chemical-treated cells, whereas PCc and MFIc for the untreated ones.

Real time RT-PCR. Total RNA was isolated using a commercial available kit (TriReagent from Sigma) following the supplier's instructions. For the synthesis of cDNA, 2.0 μg of total RNA was retro-transcribed using a high-capacity cDNA archive kit from Applied Biosystems (Foster City, CA, USA) following the supplier's instructions. IL-8 gene expression was evaluated by Real time reverse transcription-polymerase chain reaction (Real time-PCR). For PCR-analysis, TaqMan™-PCR technology was used. PCRs were performed in duplicate and according to the standard protocol suggested by the manufacturer. For each PCR reaction, 10 ng of total RNA were used. The 18S ribosomal RNA transcription was used as endogenous reference and the quantification of the transcripts was performed by the $2^{-\Delta\Delta CT}$ method.

Protein corona. 200 μg of AgNP in 2 mL of culture media containing 10% HI and not HI FCS or HI and not HI human serum were incubated for 60 min at 37 °C in 2.0 mL microtubes. Tubes were centrifuged at 5000 rpm for 5 min, pellets washed three times by resuspension in 1.0 mL of PBS with vortexing, followed by centrifugation and aspiration of the supernatant. Protein were desorbed from AgNP by sonication in 100 μl of Laemmli loading buffer (Laemmli, 1970) for 20 min followed by incubation in boiling water for 10 min. Samples were then centrifuged at 12,000 rpm for 5 min to sediment AgNP. Supernatants (40 μl) were separated by SDS-PAGE under reducing conditions on a 12.5% polyacrylamide gel. The proteins bands were visualized by silver staining (Heukeshoven and Dernick, 1988).

Transmission electron microscopy (TEM). THP-1 cells were collected by centrifugation (1500 rpm for 5 min at 4 °C) and immediately fixed in 3% glutaraldehyde diluted in 0.1 M.

Sörensen phosphate buffer, pH 7.4, overnight at 4 °C. Pellets were then washed with the same buffer (3 times, 30 min each at room temperature), post-fixed in 1% osmium tetroxide for 90 min at 4 °C in the dark. Ultrathin sections obtained with a Reichert Ultracut R-Ultramicrotome (Leica, Wien, Austria) were stained with uranyl acetate and lead citrate and examined by CX100 transmission electron microscope (Jeol, Tokyo, Japan).

Statistical analysis. All experiments using THP-1 were repeated at least three times, with representative results shown. Six donors were used for the whole blood assay and four for the human monocyte enrichment. Statistical analysis was performed using GraphPad InStat version 3.0a for Macintosh (GraphPad Software, San Diego, CA, USA). For multiple comparisons, analysis of variance was performed with Tukey post-hoc test. For blood samples, paired Student's *t*-test was used. Differences were considered significant at $p \leq .05$.

3. Results

3.1. Effects of AgNP on THP-1 cells

With this study, we wanted to compare the response to AgNP in different in vitro models, including the human cell line THP-1 and primary human cells (i.e. whole blood, and enriched peripheral blood monocytes) as a strategy to improve the in vitro assessment of NP immunotoxicity. THP-1 cells were chosen as surrogate of monocytes/macrophages, which, due to their ubiquitous body distribution and their role in innate and specific immune responses, are considered central to NP immunotoxicity (Fröhlich, 2015). Commercially available AgNP (< 100 nm) were used as prototypical NP. In the inset of Fig. 1A inset, the particle size distribution obtained with the SPES method is reported. SPES results show a distribution centered at 100 nm in diameter, as expected. Extinction and scattering from more than 3500 AgNP were characterized and easily distinguished by spurious signals from the surrounding medium (Potenza et al., 2017).

To investigate the in vitro effects of AgNP, first, we assessed the effect of increasing concentrations of AgNP (1–100 μg/ml) on cell viability as assessed by PI staining and LDH leakage (Fig. 1A). For these experiments, cells were cultured in RPMI + 10% HI FCS. A statistically significant decrease in cell viability was observed at the concentration of 100 μg/ml, with a maximum 25 ± 3% decrease as assessed by PI staining ($p < .01$).

Under the same experimental conditions (24 h treatment with increasing concentrations of AgNP), we then investigated AgNP uptake as described by Suzuki et al. (2007) by measuring changes in size and intracellular density of cells by FACS analysis. It is important to emphasize that the method does not distinguish particles that are internalized from particles attached to the external side of the cell membrane. Despite this limitation, as shown in Fig. 1B, a dose-related increase in AgNP uptake was observed, which reached statistical significance at concentrations ≥50 μg/ml, with an increase even if not statistically significant already at 10 μg/ml.

As signs of immune activation surface marker expression (i.e. CD86 and CD54) and IL-8 release were assessed. CD86 and CD54 were used as markers of cell maturation and activation, whereas IL-8 as indicator of inflammation. THP-1 cells were treated for 24 h with increasing concentrations of AgNP (1–100 μg/ml). As shown in Fig. 1C no statistically

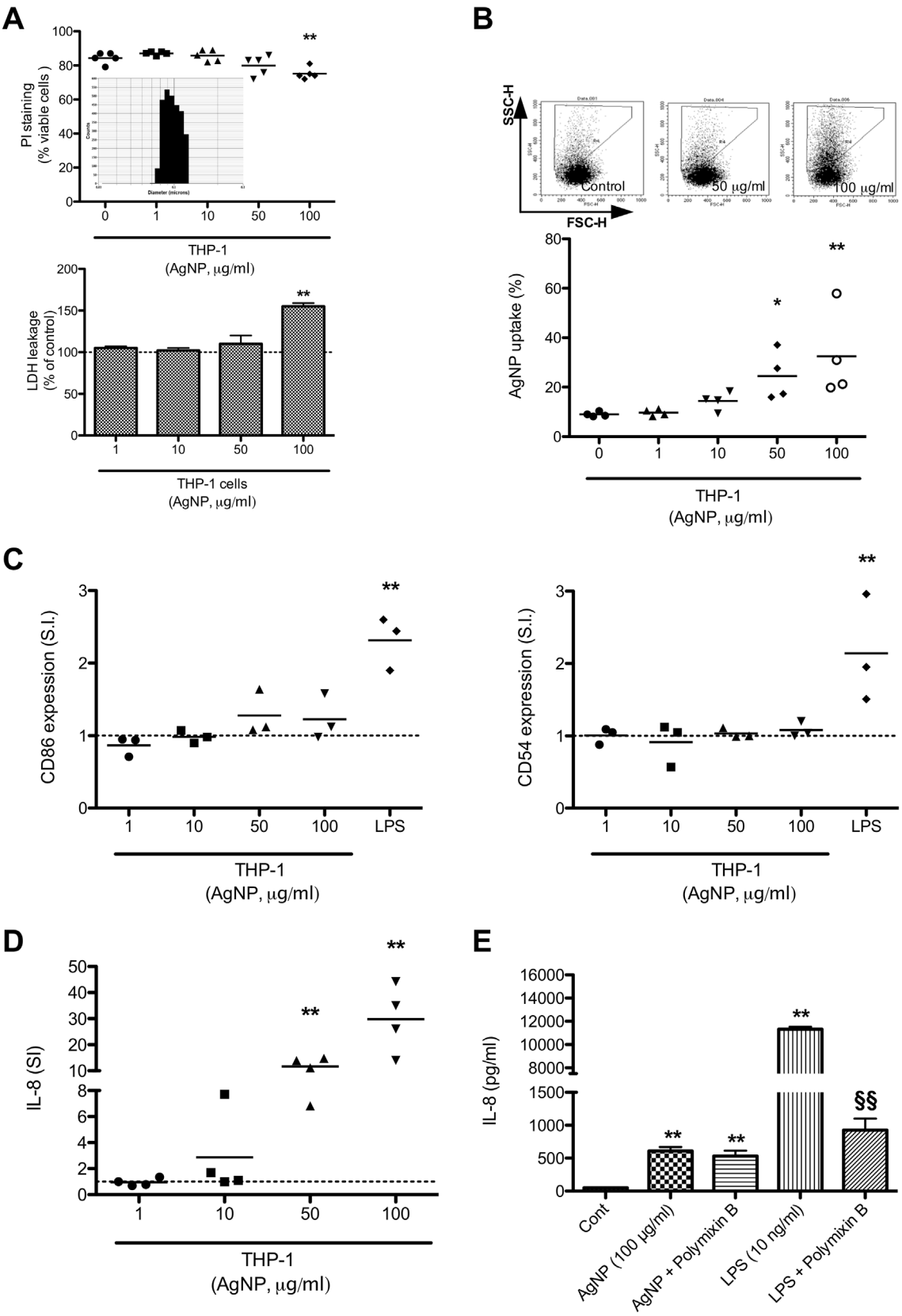

Fig. 1. Effects of AgNP on THP-1 cells.

A-D) THP-1 cells were treated for 24 h with increasing concentrations of AgNP (1–100 μg/ml) in RPMI + 10% HI FCS. The effect on cell viability was assessed by PI staining and LDH leakage, and in the inset the particle size distribution obtained with the SPES method are shown (A), AgNP uptake with representative dot blots shown (B) and surface markers expression (C) by FACS analysis, while IL-8 release (D) was evaluated by ELISA. LPS (10 ng/ml) was used as positive control. Each dot represents independent experiments, with mean value reported. Statistical analysis was performed by ANOVA followed by Dunnett's multiple comparison test with *$p < .05$ and **$p < .01$ versus control (0). E) to evaluate a possible endotoxin contamination polymixin B (15 μg/ml) was used. LPS (10 ng/ml) was used as positive control. Each value represents the mean ± SD, n = 3 representative of two independent experiments. Statistical analysis was performed by ANOVA followed by Tukey's multiple comparison test with **$p < .01$ versus control (Cont) and §§$p < .01$ vs LPS treated cells.

significant changes were observed in surface marker expression, while cells properly responded to LPS (10 ng/ml) with a statistically significant increase in both markers. Similarly, no induction in CD86 or CD54 expression was observed either after 48 h of treatment with AgNP (data not shown). While failing to upregulate surface markers, AgNP induced a dose-related increase in IL-8 release, which reached statistical significance at concentrations ≥50 μg/ml but with an increase even if not statistically significant already at 10 μg/ml, indicative of an inflammatory potential. The stimulatory effect was not due to an endotoxin contamination as assessed by lack of modulation in the presence of polymixin B (Fig. 1E). To confirm the effective ability of polymixin B to sequester endotoxin, LPS was used as control. In this case, the production of IL-8 induced by LPS was almost completely abrogated by polymixin B (§§$p < .01$ vs LPS alone). In addition, to evaluate whether free silver ions released from NP could play a role in the observed effects, particle-free supernatants were collected from AgNP incubated in culture medium. Specifically, AgNP (50 μg/ml) were incubated in RPMI 1640 containing 10% FCS for 24 h at 37 °C. NP-free supernatants were obtained by centrifugation under sterile conditions (14.500 rpm for 20 min). THP-1 cells were then cultured in the above mentioned supernatants or in culture medium containing AgNP (50 μg/ml), and IL-8 release was assessed. IL-8 release was not induced in AgNP supernatants at the investigated concentration: control 13 ± 3 pg/ml, AgNP 323 ± 18 pg/ml, and NP-free supernatants 9 ± 2 pg/ml (mean ± SD, n = 3). Same lack of effects was observed using supernatants obtained from AgNO incubated in RPMI + 10% human serum or in the absence of serum (data not shown).

Results suggest that if the endpoint is the assessment of the proinflammatory potential of NP, THP-1 works nicely, responding in a dose-related manner to NP and allowing the evaluation of a possible endotoxin contamination of NP.

3.2. Effects of AgNP on human primary cells

Next, we investigated the response by primary human cells. We performed a whole blood assay; to confirm the ability of AgNP to induce IL-8 production in monocytes, the test samples were enriched in peripheral blood monocytes. Whole blood samples obtained from 6 healthy donors were diluted 1:10 in culture media, while monocytes were enriched from peripheral blood obtained from 4 healthy donors. Primary cells were treated with increasing concentrations of AgNP (1–100 μg/ml). In whole blood samples, AgNP induced a dose- and time-related cytotoxic effect as assessed by LDH leakage at 24 and 48 h (Fig. 2A), which reached statistical significance at 100 μg/ml, with an overall pattern very similar to that observed in THP-1 cells.

Concerning IL-8 production, similarity to what observed in THP-1 cells, AgNP induced a dose-related increase in IL-8 release, which reached statistical significance at concentrations ≥50 μg/ml but with an increase even if not statistically significant appreciable at 10 μg/ml using both whole blood (Fig. 2B) and enriched monocytes (Fig. 2C).

Results suggest that also the whole blood assay works nicely in the identification of the pro-inflammatory role of NP.

3.3. Role of serum in AgNP-induced IL-8 production and protein corona

From a qualitative point of view, the response observed in term of IL-8 production was similar in all three models used. However, comparing the SI obtained in THP-1 cells with the one observed in the whole blood samples or in enriched monocytes, THP-1 seems to respond more to AgNP with higher SI. This could of course be easily explained by a different numbers of effector cells. Nevertheless, we wanted to investigate if the type of serum (bovine vs human) could have a role in the different response observed. In addition, considering that in the whole blood assay serum is not heat-inactivated, which reflects the real-life situation, THP-1 cells were cultured in 10% HI and not HI FCS or HI and not HI human serum in the presence or absence of

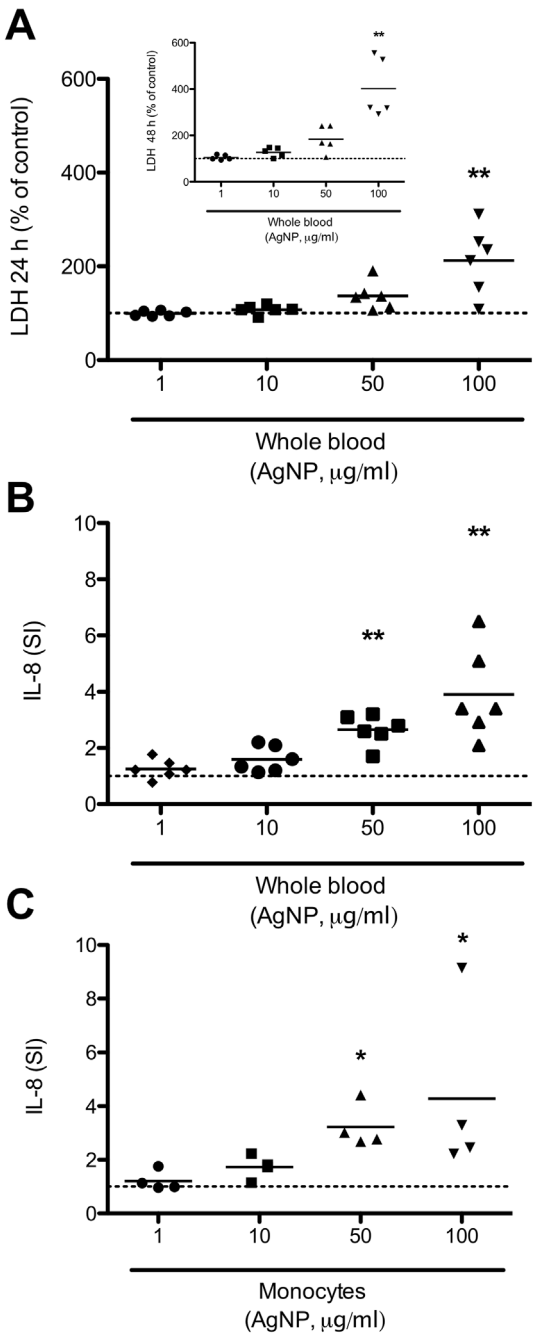

Fig. 2. Effects of AgNP on diluted whole blood and enriched monocytes.
A) Diluted whole samples were diluted 1:10 in culture media and treated with increasing concentrations of AgNP (1–100 μg/ml) for 24 h and 48 h (inset) to assess cell viability using LDH leakage. B) Diluted whole samples were diluted 1:10 in culture media and treated with increasing concentrations of AgNP (1–100 μg/ml) for 24 h to assess IL-8 release. Each dot represents independent donors, with mean value reported. C) Monocytes were enriched from peripheral blood leukocytes and treated with increasing concentrations of AgNP (1–100 μg/ml) for 24 h to assess IL-8 release. Each dot represents independent donors, with mean value reported. Statistical analysis was performed by ANOVA followed by Dunnett's multiple comparison test with *$p < .05$ and **$p < .01$ versus control.

AgNP (50 μg/ml). Commercially available sterile-filtered human male AB serum was used (Sigma Cat. N° 4522). Serum was heat inactivated at 56 °C for 30 min. After 24 h, IL-8 release and AgNP uptake were measured and, in parallel, protein corona was investigated with the working hypothesis that a different protein corona formation could also explain the different response observed possibly affecting AgNP uptake. As shown in Fig. 3A (IL-8 production), the highest response to AgNP was

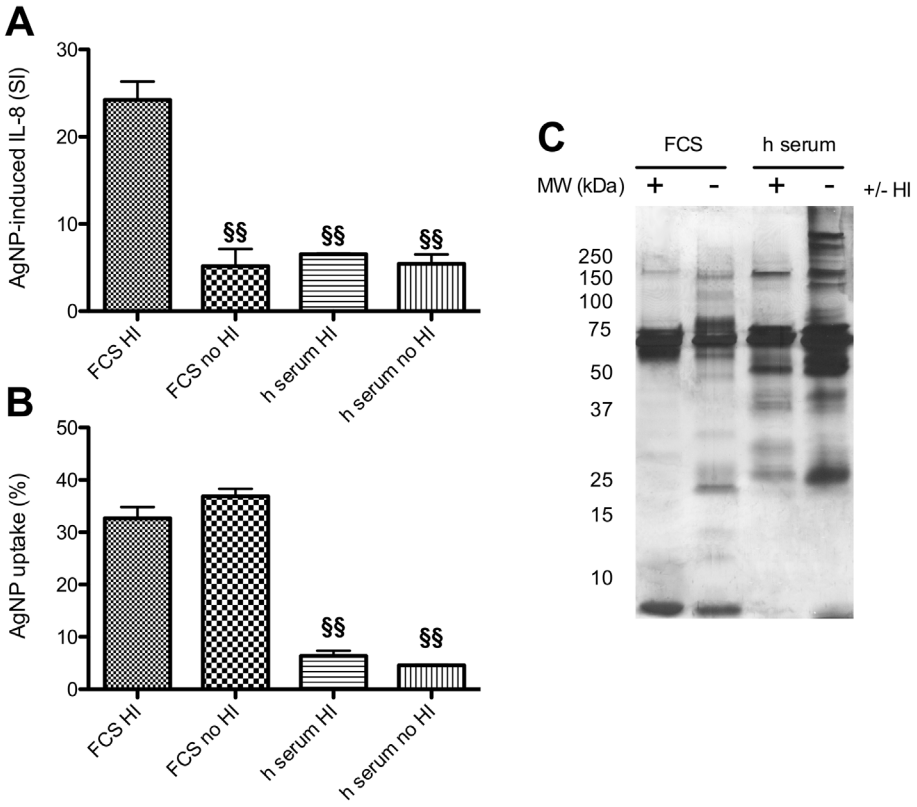

Fig. 3. Role of FCS vs human serum in the response to AgNP in THP-1 cells and protein corona formation.
THP-1 cells were treated for 24 h with AgNP (50 μg/ml) in 10% HI and not HI FCS or 10% HI and not HI human serum. After 24 h, AgNP uptake (A) and IL-8 release (B) were evaluated. Each value represents the mean ± SD, n = 3 representative of three independent experiments. Statistical analysis was performed by ANOVA followed by Tukey's multiple comparison test with **p < .01 versus HI FCS. C) Qualitative pattern of serum proteins absorbed to AgNP. AgNP were resuspended in culture media containing 10% HI and not HI FCS or HI and not HI human serum for 60 min as described under Materials and Methods. Desorbed proteins were analyzed by SDS-PAGE under reducing conditions on 12.5% PAA. Similar results were observed in three other independent experiments.

observed in cells treated in 10% HI FCS compared to the other sera, supporting the hypothesis that different serum samples may differently affect cellular response.

Independently of HI, a lower uptake was observed in cells cultured with human serum compared to FCS (Fig. 3B). Data do not suggest a direct correlation between cellular uptake and IL-8 production: in cells cultured in not HI FCS, IL-8 SI was lower than in cells cultured in HI FCS despite a similar AgNP uptake. The lower response in human serum was not due to a different kinetics, as in time course experiments the release of IL-8 induced by AgNP was always lower compared to experiments conducted in FCS (data not shown). To support AgNP uptake data, TEM analysis was conducted. AgNP were absent in control THP-1 cells grown with serum (Fig. 4A and B) or without serum (Fig. S1A). In absence of FCS but in presence of AgNP, nanoparticle cytoplasmic uptake occurred (Fig. S1B). Ultrastructural differences were not evident in control cells grown either in 10% HI or not HI FCS and HI or not HI human serum (data not shown). In 10% HI FCS cells, several AgNP aggregates were identified in the cytoplasm, but not in the nucleus (Fig. 4C). In HI human serum cells only scattered nanoparticles were present (Fig. 4D, arrow) and several lipid droplets occupied the cytoplasm (Fig. 4D, asterisks). Cells grown in 10% not HI FCS showed a comparable ultrastructure to cells grown in 10% HI FCS and abundant AgNP uptake occurred (Fig. 4E, compare to 4C). When cells were grown with human serum not HI in presence of AgNP, many mitochondria were evident and the nanoparticle uptake was similar to HI human serum condition (Fig. 4F, compare to Fig. 4D).

Interestingly, the protein absorption patterns (Fig. 3C) show remarkable differences both at the quantitative and at the qualitative level. Less proteins are absorbed to AgNP exposed to HI FCS in comparison with the other sera tested, which may result in greater biological response [the biological response to AgNP, in term of IL-8 production as assessed at the protein and mRNA level, is at least twice as high in the absence than in the presence of serum (Table 1)]. Even more striking are the differences between the two types of serum, with the not HI human serum able to bind the highest amount and largest assortment of proteins, whose identity and biological significance is to be investigated. Not surprisingly, in all the four test conditions, albumin (~68 kDa) appears as one of the most abundant bound proteins. Proteins with MW < 25 kDa were more absorbed, proteins with MW between 60 and 200 kDa less absorbed, from FCS than from human serum.

3.4. Modulation of the response to classical immune stimuli by AgNP

To investigate the immunomodulatory properties of AgNP, THP-1 and whole blood samples were treated with increasing concentrations of AgNP (1–100 μg/ml) in the presence of LPS (10 ng/ml) for 24 h, and whole blood samples also with PHA (3 μg/ml) or PWM (5 μg/ml) for 48 h. In response to LPS, the release of IL-8, TNF-α and IL-10 was assessed (Fig. 5), while the release of IL-4 and IFN-γ was assessed following exposure to PHA and PWM (Fig. 6), as indicator of modulation of Th2/Th1 response. Cytokines were measured by commercially available ELISA.

As shown in Fig. 5, AgNP increased the response to LPS in term of IL-8 (Fig. 5A and D) and TNF-α (Fig. 5B and B) release both in THP-1 cells and more remarkably in whole blood samples, while no appreciable changes were observed for the release of IL-10 (Fig. 5C; in THP-1 no IL-10 could be measured under the experimental condition used), further supporting a pro-inflammatory effect. The average release of the measured cytokines following whole blood samples LPS stimulation was of 2637 pg/ml (spontaneous release 170 pg/ml) for IL-8, 221 pg/ml (spontaneous release 12 pg/ml) for TNF-α and 119 pg/ml (spontaneous release 28 pg/ml) for IL-10.

Concerning the lymphocyte response, PHA was used to activate T cells, while PWM was used to address mainly B cells activation (Stevenson et al., 1983; Bekeredjian-Ding et al., 2012). In the absence of PHA or PWM, while no change occurred in spontaneous release of IFN-γ (Fig. 6A), an increase in IL-4 release was observed in samples treated with AgNP, which reached statistical significance at 100 μg/ml (Fig. 6B), and a slight not statistically significant decrease in the IFN-γ/IL-4 ratio (Fig. 6C), consistent with the increase in IL-4. In addition, a

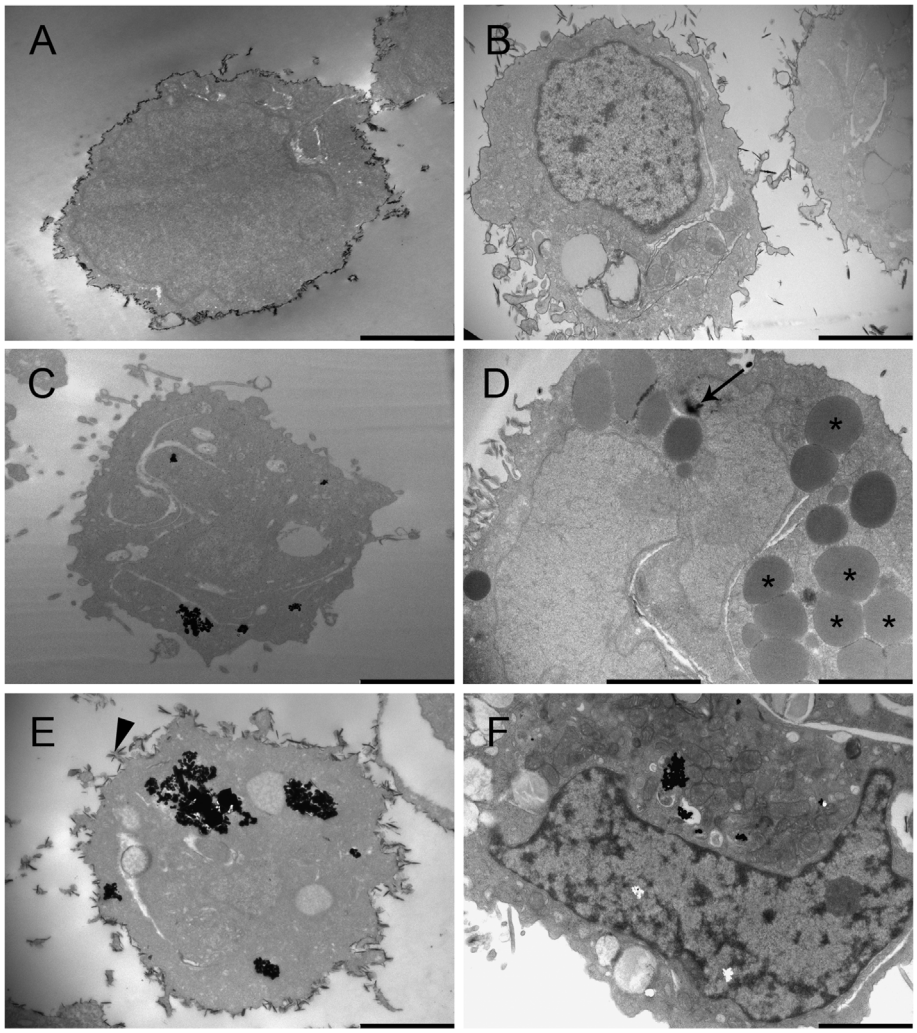

Fig. 4. Ultrastructure of THP-1 cells. A: THP-1 control cells grown in 10% HI FCS; B: THP-1 control cells grown in 10% HI human serum; C: THP-1 cells treated with AgNP 100 μg/ml for 24 h in 10% HI FCS; D: THP-1 cells treated with AgNP in 10% HI human serum; E: THP-1 cells treated with AgNP in 10% not HI FCS; F: THP-1 cells treated with AgNP in 10% not HI human serum. Black arrow indicates AgNP; asterisks indicate lipid droplets. Bars: 2 μm.

Table 1
The presence of serum reduces silver nanoparticles-induced interleukin-8 production.

TREATMENT	AgNP uptake (%)	IL-8 release (SI)	IL-8 mRNA ($2^{-\Delta\Delta Ct}$)
No serum	58.4 ± 3.8	32.0 ± 2.2	14.1 ± 1.3
10% FCS	43.1 ± 1.4**	17.3 ± 0.3**	6.1 ± 0.7**

THP-1 cells were cultured in the presence or absence of 10% FCS and treated in the presence or absence of AgNP 50 μg/ml for 24 h. Uptake was assessed by FACS analysis, IL-8 release by ELISA, and IL-8 mRNA by Real Time-PCR as described in the Materials and Methods section. Results are expressed as mean ± SD, n = 3 independent experiments. Student's *t*-test, with **p < .01 vs no serum group. The SI (Stimulation Index) was calculated by dividing the amount released following treatment with AgNP with the amount released in the control group.

slight not statistically significant decrease in IL-10 was observed (Fig. 6D). In the presence of T and B cells mitogens, no changes in the response to PHA were observed in term of IFN-γ and IL-4 release (Fig. 6E–G), while a reduction in IL-10 was observed (Fig. 6H), indicating a minor effect on T lymphocytes. On the contrary, in response to PWM an increase in the production of IFN-γ (Fig. 6I) and IL-4 (Fig. 6L) was seen, with no changes in the IFN-γ/IL-4 ratio (Fig. 6I), which may suggest B cells stimulation. No change in IL-10 release was found (Fig. 6N). The average release of the measured cytokines in the control groups (0) following PHA stimulation was of 1223 pg/ml (spontaneous release 48 pg/ml) for IFN-γ, 294 pg/ml (spontaneous release 40 pg/ml) for IL-4; while following PWM stimulation 6026 pg/ml for IFN-γ, and 109 pg/ml for IL-4 were measured.

Overall, results obtained are indicative of stimulatory effects on monocytes and B cells, with minor effects on T cells.

4. Discussion

The characterization of the effects of NP on the immune system is central to the design of safe and effective NP. In this study, we use AgNP to investigate its immunotoxic potential using in vitro methods. AgNP was selected as prototypical NP due to its wide use in many consumer products (Ge et al., 2014; Tulve et al., 2015), and human cells, both cultured and primary, were chosen as the experimental systems. From a toxicological perspective, any statistical significant deviation from controls must be considered as unwanted/immunotoxic effect independently from the physiological role of the parameters investigated (if a compound is inert it must behave exactly like the control). Following in vitro treatment with AgNP, proinflammatory cytokine productions (e.g. increased IL-8 production, amplified response to LPS), B cell activation (e.g. increased response to PWM), no effect on PHA-induced IL-4 and IFN-γ production in T cells and a reduction in IL-10 release, were observed, overall, indicative of immunomodulatory properties.

As a general strategy, before starting with in vitro tests, bioavailability should be considered (Gennari et al., 2005; Galbiati et al., 2010). If the compound does not have appreciable bioavailability, immunotoxicity is unlikely to occur. For the general population, the exposure to NP through contact with the skin, inhalation and oral uptake

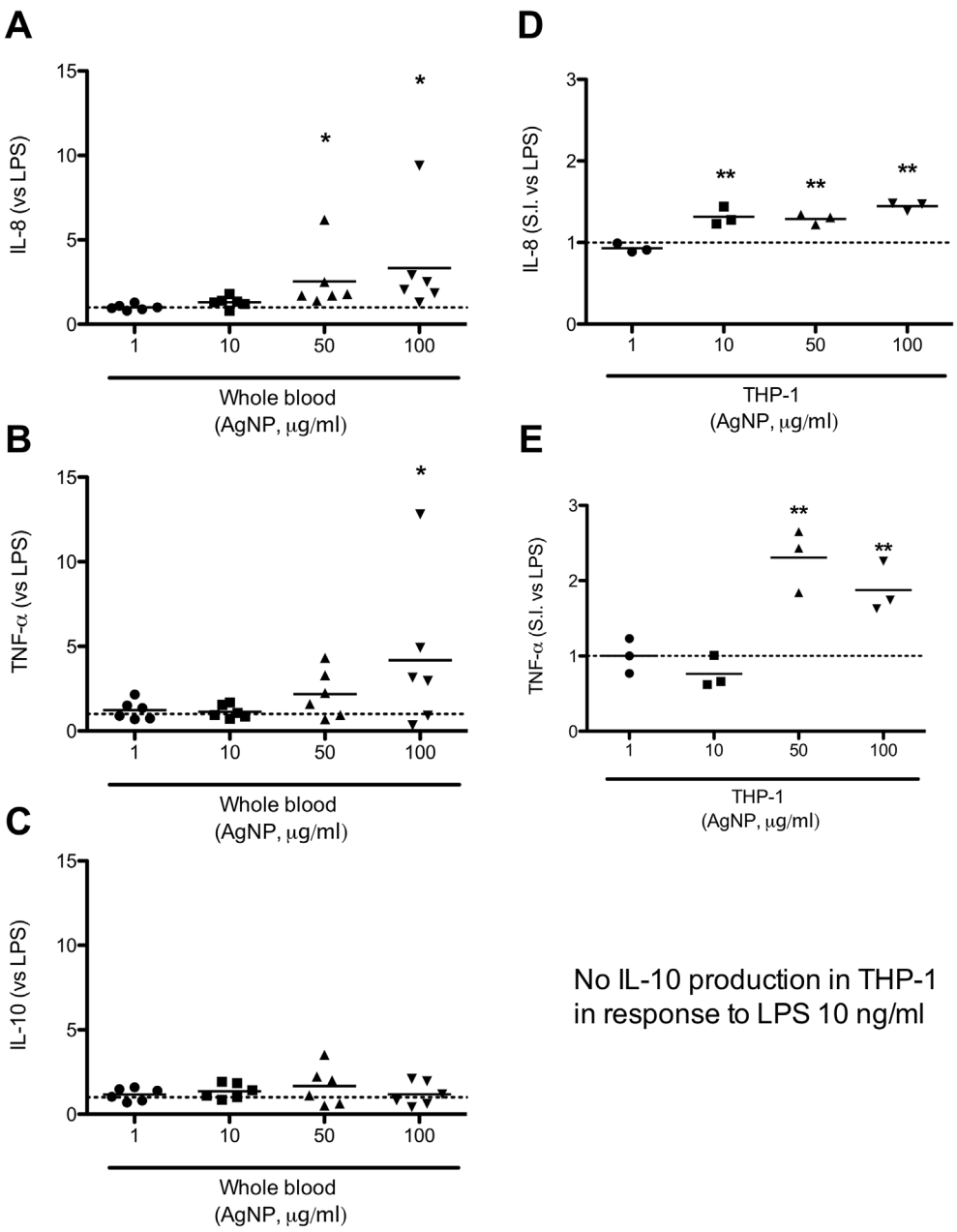

Fig. 5. Immunomodulatory effects of AgNP on LPS-induced cytokine production.
A-C) Diluted whole samples were diluted 1:10 in culture media and treated with increasing concentrations of AgNP (1–100 μg/ml) in the presence of LPS (10 ng/ml). After 24 h IL-8 (A), TNF-α (B) or IL-10 (C) were evaluated by ELISA. D, E) THP-1 cells were treated with increasing concentrations of AgNP (1–100 μg/ml) in the presence of LPS (10 ng/ml). After 24 h IL-8 (A), TNF-α (B) were evaluated by ELISA, under the experimental conditions used no IL-10 was detected. Each dot represents independent donors (n = 6) or experiments (n = 3), with mean value reported. Statistical analysis was performed by ANOVA followed by Dunnett's multiple comparison test with *p < .05 and **p < .01 versus control.

are most relevant (Fröhlich and Roblegg, 2016). In the case of drugs, parenteral administration will offer the maximal exposure, with size, shape and surface modification of NP significantly affecting adsorption (Salatin et al., 2015). Generally oral, dermal, or inhalational absorption of AgNP is considered low, but may increase with smaller sizes, negative charge, and appropriate coatings (Lin et al., 2015). Orally administered AgNP have been described to be absorbed in a range of 0.4–18% in mammals with a human value of 18% (Hadrup and Lam, 2014), which indicate bioavailability of AgNP.

A panel of in vivo, ex-vivo and in vitro methods at different levels of complexity is available to assess immunotoxicity (Corsini, 2006; Galbiati et al., 2010). Besides ethical concerns, standard in vivo animal models have several drawbacks, including low sensitivity, inter-species variability, high costs and relatively low throughput, which point out the need for reliable in vitro assays (Halamoda-Kenzaoui et al., 2015; Dobrovolskaia and McNeil, 2013; Oostingh et al., 2011).

Due to the central role of monocytes/macrophages/dendritic cells in innate immunity as well as in the activation of acquired immunity, the use of these cells to study immunotoxicity of NP is highly recommended (Fröhlich, 2015; Petrarca et al., 2015; Gustafson et al., 2015). In the proposed approach, as screening we used the human promyelocytic cell line THP-1 as surrogate of the monocyte lineage. Continuous availability, high reproducibility, independency from blood donors, and safety in use are among the advantages of using a cell line instead of primary cells. This cell line has been extensively used to study monocyte/macrophage functions, mechanisms, signaling pathways, and nutrient and drug transport, and it is the most widely used model for primary human monocytes/macrophages (Chanput et al., 2014 Genin et al., 2015; Bosshart and Heinzelmann, 2016). These cells offer the opportunity to reveal the inflammatory potential of NP, possibly their role in hypersensitivity, through the assessment of IL-8 release a cytokine selectively upregulated by chemical allergens (Corti et al., 2015; Mitjans et al., 2008, 2010; Toebak et al., 2006); to discover a possible endotoxin contamination and its contribution to the inflammatory effect through the use of polymixin B; and to investigate immunostimulatory or immunosuppressive effects through the assessment of their modulatory effect on LPS-induced cytokine production. In the case of AgNP, our results indicate proinflammatory cytokine

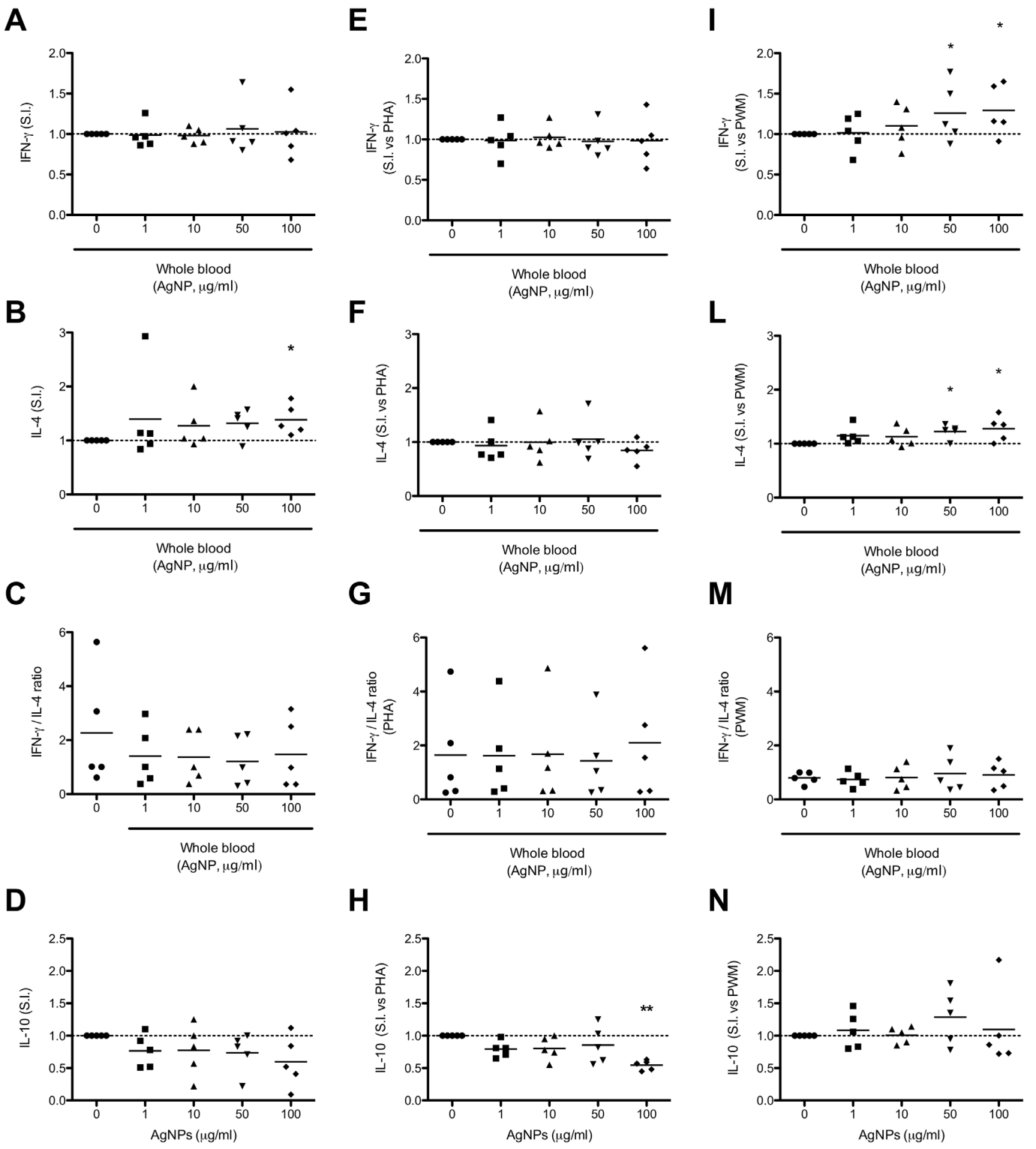

Fig. 6. Immunomodulatory effects of AgNP on PHA or PWM-induced IL-10 production.
A-C) Diluted whole samples were diluted 1:10 in culture media and treated with increasing concentrations of AgNP (1–100 μg/ml) in the presence or absence of PHA (3 μg/ml) or PWM (5 μg/ml). After 48 h IL-10 release was evaluated by ELISA. In panel A is reported the release in samples treated with AgNP alone, in panel B in the presence of PHA and in panel C in the presence of PWM. Each dot represents independent donors, with mean value reported. Statistical analysis was performed by ANOVA followed by Dunnett's multiple comparison test with **$p < .01$ versus control.

productions with increased IL-8 production not due to endotoxin contamination, and amplified response to LPS. In addition, our results emphasize how relevant culture conditions are in affecting the magnitude of the response, which is important for a correct interpretation of the results of in vitro toxicological assays.

To confirm results, the screening on THP-1 cells was followed by the whole blood assay, which allows to confirm the inflammatory potential as well as to point out other immunomodulatory effects of NP in a more realistic and appropriate scenario, in which interactions among different immune cells are contemplated. By using specific immune

activators (e.g. PHA, PWM), the effects of AgNP on T and B cells responses were also investigated. It is important to remember that LPS, contrary to rodents, is not a B cells mitogen. In the present study, 4 to 6 donors were used; while the absolute amount of cytokine released differs among donors, referring to the stimulation index (SI) a decrease in individual variability and better consistency of the results is obtained. Differences in the amount of cytokine released can be due to several factors, including actual number of leukocytes, sex, age, and genetic polymorphisms. The small number of donors is consistent with data reported by Langezaal et al. (2002) for the human whole blood cytokine release assay, where two-three donors have been reported to be sufficient to identify potentially immunotoxic compounds. The whole blood represents a reliable, reproducible, fast and feasible in vitro method to assess human cellular immune responses (Bailey et al., 2013; Silva et al., 2013; Laufer et al., 2008; Remick et al., 2000). Although the kinetics and absolute yields of cytokines differ between in vitro and in vivo, the whole blood assay may serve as a risk-assessment tool for in vivo NP- and chemical-induced cytokine release.

Even if not investigated in the current manuscript, primary monocytes or THP-1 cells can be used to study the role of NP on M1/M2 macrophage differentiation or the response of M1/M2 macrophages to NP. From literature, it is known that traditional polarization of macrophages to an M1 phenotype enhances NP uptake, while M2 polarization appears to reduce particle uptake (Herd et al., 2015; Gustafson et al., 2015). This will require a different experimental approach and represent a different goal, which could be investigated in follow up experiments.

A wide spectrum of cytokines can be used to assess the immunomodulatory potential of xenobiotics, and they have been indeed widely used both in vitro and in vivo to assess immunotoxicity. In the current investigation, we referred to IL-8 and TNF-α to assess pro-inflammatory responses, to IL-10 as anti-inflammatory cytokine and to IL-4 and IFN-γ as cytokines reflecting modulation of Th2/Th1 activation, with IL-4 deeply involved in immunoglobulin E synthesis, eosinophil activation, mucus secretion and airways remodeling. In our experiments, we used mainly ELISA to measure cytokine levels, but other methods, e.g. ELISpot, mRNA expression, etc. should work as well. These cytokines were selected as representative of the immune responses; needless to say many other cytokines can be evaluated and different techniques used (Corsini and House, 2010). In the case of AgNP, our results indicate proinflammatory cytokine productions (e.g. increased IL-8 production, amplified response to LPS), confirming the observation in THP-1 cells, B cell activation (e.g. increased response to PWM), no effect on PHA-induced IL-4 and IFN-γ production in T cells and a reduction in IL-10 release, with a potentially enhanced humoral immunity (e.g. increased IL-4 production), which is consistent with in vivo data following inhalation or intradermal exposure (reviewed by Fröhlich, 2015; Xu et al., 2013). The decrease in PHA-induced IL-10 production also agrees with data from animal models following iv injection of 100 nm AgNP (De Jong et al., 2013).

The use of human peripheral blood mononuclear cells to assess the immunosafety of engineered NP has been proposed also by other researchers (Shishatskaya et al., 2016; Li et al., 2016a, 2016b; Lappas, 2015; Mendoza et al., 2014; Shin et al., 2007), however, despite different cytokines, different experimental conditions and different NP sizes, the common observation is that of a pro-inflammatory effect in most cases.

The proinflammatory effect is common to many NP (Li et al., 2016a, 2016b), and has been related to the ability of monocytes to phagocytose external materials. Changes in cytokine production were also observed at non-cytotoxic concentrations, arguing against the hypothesis of cell death as the main driving force of inflammation. Internalization of NP and reactive oxygen species generation remain a tenable explanation of the observed pro-inflammatory effect. The emerging evidence is that different NP, including NP of natural origin, have many properties in common, which suggest that they can be viewed as a coherent class of particulate toxins with the potential to cause oxidative stress and, through oxidative stress-responsive signaling pathways, to affect responses such as inflammation as an integral part of their pathogenic mechanism (Donaldson et al., 2005; Li et al., 2016a, 2016b; Pallardy et al., 2017).

Our data do not support a direct correlation between AgNP cellular uptake and immunotoxicity, in agreement with published data (Nishijima et al., 2017; Prietl et al., 2014; Zhang et al., 2011). The characterization of AgNP protein corona provided interesting information on differentially absorbed serum proteins, which were dependent upon the source of serum, human vs bovine, and upon heat inactivation or lack thereof. It is suggested that the composition of protein corona influences the trafficking and biological effects of NP (Monopoli et al., 2012; Neagu et al., 2017; Rahman et al., 2013; Yan et al., 2013). In vivo protein corona can also influence organ distribution and clearance rate of NP (Karmali and Simberg, 2011; Yan et al., 2013). While the response to AgNP is greater in the absence of serum - a situation not reflecting real-life, as cells are always surrounded by proteins at high concentrations - the use of bovine vs human serum resulted in different responses, with higher activation in cells exposed to AgNP in 10% heat inactivated FCS than in 10% human serum. This suggests that higher protein absorption to AgNP correlates with decreased biological activity, which, together with the observation of the high response in the absence of serum, indicates that 'nude' AgNP have higher biological activity. The different response in the presence or absence of serum (no protein corona) has also been observed with other NP (Lesniak et al., 2012; Yan et al., 2013; Halamoda-Kenzaoui et al., 2015) The identification of the absorbed proteins and the evaluation of their biological significance are left to future investigations. Results, however, further supports the importance of understanding the NP protein coronas as a critical step toward manipulating their subsequent immune response and toxic effects, and indicate how different compositions of cell culture media can ultimately influence the effect on cellular response. The role of protein corona in the uptake and biological response is, however, highly dependent upon the NP considered, and generalization cannot be made: protein coronas formed on NP can either stimulate or mitigate the immune response (Dobrovolskaia et al., 2014; Lee et al., 2014; Kettler et al., 2016; Pallardy et al., 2017). In addition, Shannahan et al. (2016) demonstrated that also disease-induced variations in physiological environments have a significant impact on corona formation, cellular association, and cell response. Among the proteins absorbed, albumin (68 kDa), apolipoprotein A-I (28 kDa), immunoglobulins (ca. 25 and 50 kDa under reducing conditions), complement proteins (30–43 kDa) and fibrinogen (50–70 kDa depending on the chain) have been reported. The protein profile we observed in AgNP incubated with not heat inactivated human serum is overall similar to the ones reported for other NP (Monopoli et al., 2011). These findings support the idea that different culture conditions can lead to the formation of different protein coronas, which ultimately influences the cellular response. If the aim is to predict toxicity in humans, the use of human serum or simply the whole blood assay is perhaps the best choice. The differences in protein corona should be considered when extrapolating animal data to humans. The evaluation and the understanding of NP protein corona is also important for the manipulation of NP and their subsequent effect on the immune system.

If there is no need to track identified cytokines back to a defined cell population, the whole blood assay reduces ex vivo manipulation and better approximates the in vivo situation, representing the best choice for the in vitro identification of immunotoxic, immunomodulatory NP. However, for in vitro screening of pro-inflammatory effects of NP, THP-1 cells work as well as primary cells, representing a useful and practical alternative.

5. Conclusions

This was a pilot study aimed to characterize the immunotoxic/

immunomodulatory potential of NP, using AgNP as prototype. The model we propose can be used to assess unwanted or wanted immunomodulatory effects in relation to the intended use of NP. Additional NP of different compositions and sizes should be tested, but the characterization of the immunotoxic potential of NP as here described for AgNP might be extremely helpful for screening or design NP, providing also indication of potential in vivo immune targets. Understanding immunotoxicity of NP is the starting point for a more focused approach to safety assessment of NP, reducing their toxicity, and limiting the need of animals. The in vitro assessment of the immunotoxic potential of NP combined with data on realistic exposure levels may provide useful information on human health risk associated with NP. Biokinetic model may indeed provide indication of internal doses, which if comparable with in vitro active concentrations indicate a possible health risk.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fct.2017.12.023.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2017.12.023.

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