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Silver and fullerene nanoparticles' effect on interleukin-2dependent proliferation of CD4 (+) T cells

- Guillaume Côté-Maurais,
- Jacques Bernier[,]

Abstract

Immunotoxicity studies of nanoparticles on T cells addressed their effects on activation by T antigen receptor, but have neglected the regulation of proliferation by IL-2. In this study, the IL-2-dependent T lymphoblastoid WE17/10 cell line was used to compare silver (Ag-NPs) and fullerene (C60-NPs) nanoparticles' toxicity and evaluate whether these NPs could interfere with IL-2-dependent proliferation. Results have shown that Ag-NPs are more toxic, as they reduced cell viability at the highest concentration tested (100 μ g/ml), while C₆₀-NPs have shown good biocompatibility. Characterization of NP suspensions by dynamic light scattering measured large aggregates for C₆₀-NPs, whereas Ag-NPs were relatively stable and well dispersed. This translated into a much larger uptake of Ag-NPs compared to C₆₀-NPs, as measured by flow cytometry. Proliferation measurements by CFSE following 72 h incubation have shown that Ag-NPs decrease cell proliferation and C₆₀-NPs slightly increase proliferation. CD25 expression was unchanged following exposure to C₆₀-NPs, but was significantly increased by Aq-NPs' presence for short and long-term incubations. Analyses of three key signaling proteins activated by IL-2 receptor (Stat5, JNK and ERK1/2) by western immunoblotting have shown no effects from either NPs on Stat5 and JNK phosphorylation. ERK1/2 was slightly activated following a short exposure to Ag-NPs, while C₆₀-NPs had no effect. Our results show that C₆₀-NPs have good biocompatibility and do not interfere with IL-2-dependent proliferation. A deeper investigation would be needed for the case of Ag-NPs, since the mechanism of their action is still unclear.

1. Introduction

Products containing nanomaterials (NMs) are already on the market and their numbers are rapidly increasing. With the growth of nanotechnologies, the importance accorded to toxicological research has also increased, since the requirement for ensuring safe usage of this technology is becoming a central issue. Two interesting nanoparticles are silver- (Ag) and fullerene-based (C_{60}) NMs. Presently, silver is the most commercialized (<u>Stensberg et al., 2011</u>). Their utilization is mostly due to their antimicrobial properties, which explains their addition to creams, dressings, surgical instruments and bone prostheses (<u>Arora et al., 2009</u>). Fullerene NPs are also an interesting type of NM because of their remarkable stability and the presence of 30 double bonds to its structure, which can react with free radicals (<u>Aschberger et al., 2010</u>). These NPs are used for their antioxidant properties (<u>Aschberger et al., 2010</u>). C₆₀-NPs are presently used in cosmetics, but could also have medical applications for treatment of diseases associated with free radical formation, such has neurodegenerative disorders and some types of cancers (<u>Aschberger et al., 2010</u>, <u>Dugan et al., 2001</u>, <u>Krug and Wick, 2011</u> and <u>Markovic and Trajkovic, 2008</u>).

NPs may enter the human body through inhalation, ingestion, injection for therapeutic purposes and dermal contact (Oberdorster, 2010). Ag-NPs are considered to be among the most toxic types of NPs, since an exposure to these NPs can induce apoptosis at much lower concentrations than with other types of NPs. C₆₀-NPs, on the contrary, were shown to have a protective effect against reactive oxygen species (ROS) toxicity in certain conditions (Gharbi et al., 2005, Xiao et al., 2006 and Yin et al., 2009). Presently, the need in nanotoxicology is to understand the interactions of NPs with biological processes at the protein level to investigate whether it has an influence on signaling pathways. The identification and characterization of these interactions permit not only to evaluate potential harmful effects, but are also essential to many of the biomedical applications, especially at the level of the immune system. Because of their utilization in preventing wound infections, action of Ag-NPs on the immune system has been extensively evaluated. Results on its effects are sometimes contradictory, but it is generally considered that they have immunosuppressive effects. Their presence in vitro with lymphocytes or macrophages is usually associated with a reduced pro-inflammatory cytokine production (<u>Shin et al., 2007</u> and <u>Wong et al., 2009</u>). *In vivo* studies with mice, using nanosilver-coated dressings or topical nanocrystalline silver creams for injuries or contact dermatitis, have shown anti-inflammatory effects also via reduction of pro-inflammatory cytokine production (Bhol and Schechter, 2005 and Tian et al., 2007).

Exposure of mice or rats by instillation to large quantities of C_{60} -NPs is usually associated with pro-inflammatory responses (Morimoto et al., 2010) and Park et al., 2010). However, the pre-treatment of mice with low concentrations of fullerenol reduced inflammatory response associated with an exposure to quartz particles (Roursgaard et al., 2008). C_{60} -NPs reduced delayed-type hypersensitivity induced by methylated bovine serum albumin in mice (Yamashita et al., 2009).

In the immune system, T lymphocyte plays a central role. T cell activation through its antigen receptor lead to interleukin-2 synthesis (Gaffen and Liu, 2004). IL-2 is mostly produced by activating CD4⁺ T cells and is a key mediator of growth, proliferation, and differentiation of effector CD4⁺ T cells (Gaffen, 2001 and Liao et al., 2011). Moreover, IL-2 have also a wide spectrum of effects on the immune system and are involved in the regulation of self-tolerance through their essential roles in the development and homeostasis maintenance of regulatory T cells (Treg) (Malek and Bayer, 2004 and Walsh et al., 2006). Major studies in nanotoxicologie of immune system address the effect of nanomaterial on T cell proliferation (Chen et al., 2013 and Tulinska et al., 2013). Capacity of T cells to respond at IL-2 by their own IL-2 receptor (IL-2R) is rarely determined. IL-2R is consisted of three chains, the CD122, the vc and the CD25. CD25 increases the receptor affinity for IL-2 and its expression is induced following antigenic stimulation. The objective of our research was to determine whether Ag or C₆₀-NPs could interfere with IL-2-dependent CD4⁺ T cell proliferation. This will allow us to evaluate NPs' effects on proliferating, instead of guiescent, CD4⁺ T cells, which are the most studied. An *in vitro* approach was chosen in order to reject signals from other cytokines and to establish NPs' mechanism of action on signaling pathways activated by IL-2R.

2. Experimental methods

2.1. Nanoparticles – Ag-NPs and C_{60} -NPs

Manufactured silver (Ag) (cat. no. 576832, vendor size <100 nm) and fullerene (C_{60}) (572500, vendor size <100 nm) nanoparticles were purchased from Sigma Aldrich (Oakville, On, Canada). Ag-NPs arrived as nanopowder and were coated with polyvinylpyrrolidone (PVP) to increase their stability and avoid agglomeration. C_{60} -NPs were purified by column chromatography in toluene, then heat-treated under vacuum to remove solvent residue in order to obtain a highly crystalline sublimed material with low solvent and metal content. Ag-NPs had a

purity of ≥99.5% and C₆₀-NPs of ≥99.85% as determined by trace metals analysis and HPLC respectively. Stock solutions of nanoparticles (2 mg/ml) were prepared in sterile deionized water. Nanoparticles were dispersed by sonication on ice for 5 min, followed by addition of BSA and PBS 10× to attain a concentration of 15 mg/ml and 1× respectively. It was shown that albumin stabilizes nanoparticles dispersion (<u>Bihari et al., 2008</u>). The hydrodynamic diameter and the zeta-potential of the dispersed particles were measured by dynamic light scattering (DLS) with a Malvern Zetasizer Nano ZS (Malvern, Worcestershire, UK). Hydrodynamic diameter and zeta-potential were determined in complete RPMI 1640 medium (Sigma–Aldrich) [RPMI 1640 supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine (Sigma–Aldrich), 100 U/mL penicillin, 0.1 mg/mL streptomycin (Sigma–Aldrich) and 10 mM HEPES (Sigma–Aldrich) pH 7.2].

2.2. Cell culture

The IL-2-dependent T lymphoblastoid WE17/10 cell line was maintained in completed RPMI 1640 and 50 U/ml of recombinant human IL-2 (PeproTech, Rocky Hill, NJ, USA). Cells were maintained in 5% CO₂ humidified incubator at 37 °C. Day before the experiment, cells were washed and incubated in the presence of fresh medium and IL-2. Before each experiment, cells were washed with medium and resuspended in complete medium containing 30 U/ml of IL-2 at 500,000 cells/ml. For nanoparticles exposure, cells (500,000 cells/ml plus 30 U IL-2) were immediately treated with freshly prepared nanoparticles from the stock solution to obtain a range of concentrations from 5 to 100 μ g/ml. Controls were treated with the vehicle alone. Incubation were performed in flat bottom 96 or 24 wells plate at 50,000 cells or 500,000 cells by well respectively.

2.3. Cell viability assay

Cell viability was determined at different time following nanoparticles treatment in 96 wells plate by propridium iodide staining. Propidium iodide (PI; Sigma Aldrich) prepared in PBS was added to cells to yield a concentration of 10 µg/ml. Fluorescence was then immediately read by flow cytometry with a FACScan® (BD Biosciences, Mississauga, On, Canada). Dead cells show nuclear staining from the PI, which stains both late-stage apoptotic and necrotic cells displaying permeable membranes. Viability percentages consisted of the proportion of cells expressing a low fluorescence level in relation to total cell number. NPs were excluded from the analysis based on absence of fluorescence signal and light forward scatter (FSC) and side scatter (SSC) characteristics. Analysis was performed on 10,000 collected events using the WinMdi 2.9 software.

2.4. Proliferation measurement using carboxyfluorescein succinimidyl ester labeling

Proliferation was monitored with Carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) as described before (<u>D'Elia et al., 2009</u> and <u>D'Elia et al., 2010</u>). Briefly, cells were diluted at 10⁷ cells/ml in a solution of HBSS supplemented with 0.1% BSA and incubated for 10 min at 37 °C with CFSE at a final working concentration of 5 μ M. CFSE was subsequently neutralized by adding ice-cold complete RPMI to cells, followed by 5 min of incubation at room temperature and washed once with ice-cold complete RPMI. CFSE-labeled WE17/10 cells (5,000,000 cells/ml) were cultured for 72 h in 24-well plates in presence of nanoparticles. Cells were analyzed as previously described on a FACScan® (BD Biosciences) flow cytometer by gating only live cells. Results are presented as the inverse ratio of the average Gmean read following an exposure to NPs to the Gmean of control cells.

2.5. Nanoperticles cellular uptake determination by flow cytometry

The kinetics of cellular binding and uptake of Ag and C₆₀-NPs into WE17/10 cells were examined by flow cytometry as describe by <u>Suzuki et al. (2007)</u>. The side scatter distribution ratio was chosen as a measure of cellular uptake. Cells' analysis was carried out after 72 h incubation in the presence of NPs. Cells were sampled and directly analyzed by flow cytometry with FACScan® (BD Biosciences). Analysis was performed as described above. Cellular uptake of NPs was calculated by dividing the SSC values of particle-treated cells by the SSC values in control cells.

2.6. Intracellular reactive oxygen species measurement

Reactive oxygen species (ROS) generation were determined as we have previously describe with 2',7'-dichlorofluorescin-diacetate (DCFH-DA; Invitrogen) (<u>D'Elia et al., 2009</u>). Briefly, cells were treated with different concentrations of nanoparticles for 24 h or 48 h. After washing, cells were incubated with 5 μ M DCFH-DA prepared in PBS containing 1 g/L of glucose for 45 min in the dark (incubator, 37 °C). At the end of incubation, cells were washed and the fluorescence intensity was measured by flow cytometry with FACScan® (BD Biosciences, Mississauga, On, Canada). To ensure cells' capability of ROS production, positive control samples were activated with 1.5 μ M PMA during the 45 min of loading with DCFH-DA. NPs were excluded from the analysis based on absence of fluorescence signal and forward-scattered light (FSC) and SSC characteristics. Histogram analyses were performed using WinMdi software by gating only live cells.

2.7. Phenotypic analysis

Phenotypic analysis of WE17/10 cells was performed following treatment with 25 µg/ml NPs for 24 h, 48 h and 72 h. Cells (100,000 cells) were washed twice with PBS and non-specific binding sites were blocked with 10 µg/ml human IgG (Sigma–Aldrich) for 30 min on ice. Cells were stained with 0.2 µg of mouse antihuman: CD25 Alexa Fluor 488 conjugated antibody (eBioscience, San Diego, CA, USA) for 30 min. Cells were then washed twice with ice-cold PBS and analyzed for fluorescence by flow cytometry with FACScan® (BD Biosciences). Analysis was performed as described above.

2.8. Western immunoblotting

Following exposure to nanoparticles, WE17/10 cell extracts were prepared in lysis buffer [50 mM Tris–HCI (pH 7.4), 150 mM NaCl, and 1% Triton X-100] supplemented with 1 mM EDTA, 1X Halt protease inhibitor cocktail (78430; Pierce Biotechnology, Rockford, IL, USA) and 1X Halt phosphatase inhibitor cocktail (78428; Pierce Biotechnology). The soluble protein concentration was determined using the BCA protein assay (Pierce Biotechnology). Protein samples (30 µg) were separated by SDS–PAGE and transferred onto a PVDF or nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% non-fat dry milk or 3% BSA diluted in Tris-buffered saline-Tween (TBS-T; 50 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Tween) for 1 h at room temperature and hybridized overnight at 4 °C with anti-phosphoStat5 (9359; Cell Signaling Technology, Beverly, MA, USA), anti-phosphoERK1/2 (44680G; Invitrogen), or anti-phosphoJNK (4671; Cell Signaling Technology) prepared in blocking buffer. Specific antibody-antigen complexes were identified using a horseradish peroxidase (HRP) linked sheep anti-rabbit IgG (NA934; GE Healthcare, Little Chalfont, United Kingdom) and SuperSignal West Femto

Chemiluminescent Substrate (Pierce Biotechnology). Membranes were stripped and reprobed with anti-Stat5 (9358; Cell Signaling Technology), anti-ERK1/2 (44654G; Invitrogen) or anti-JNK (9258; Cell Signaling Technology) to evaluate protein loading in each sample.

2.9. Statistical analysis

Results are presented as mean \pm standard error of the mean (SEM) from at least 3 independent experiments performed on different days. Each experiment was performed in triplicate. ANOVA pairwise comparisons against the respective controls were performed using two-sided Dunnett's test as a post hoc analysis with the InStat statistics program (GraphPad Software, San Diego, CA). Differences were considered significant when P < 0.05.

- 3. Results
- 3.1. NPs characterization

Summary of Ag and C₆₀-NPs characteristics can be found in <u>Table 1</u>. Since it is known that NPs will agglomerate to some extent when dispersed in the media (<u>Murdock et al., 2008</u>), dynamic light scattering (DLS) was performed to determine size distribution and agglomerates' sizes in media with serum at a concentration of 25 μ g/ml of NPs. Results have shown that C₆₀-NPs' agglomerates had an average diameter of 478 ± 93 nm, while Ag-NPs' diameter was 185 ± 44 nm, showing that C₆₀-NPs form much larger agglomerates than Ag-NPs. The zeta potential was also measured and results showed no significant difference between Ag and C₆₀-NPs, with values of -10.1 ± 0.9 mV and -11.4 ± 0.7 mV respectively.

Table 1.

Characterization of Ag and C_{60} NPs.

Nanoparticles	Description	Average size	Purity	Surface area	Hydrodynamic diameter [⊴]	Zeta (ζ) potential	Formula weight
Ag	Silver nanopowder ^a	≼100 nm	≥99.5% ^b	5.0 m ² /g	185 ± 44 nm <i>(91.6%)</i> [≘]	−10.1 ± 0.9 mV	107.87 g/mol
					. ,		
					32 ± 6 nm		
					(8.4%) ^e		
C ₆₀	Fullerene crystals (sublimed)	≼100 nm	≥99.85% [£]	n/a	478 ± 93 nm	−11.4 ± 0.7 mV	720.64 g/mol
					(89.8%) 🖻		
					18 ± 8 nm		
					(10.2%) 🖻		

Contains polyvinylpyrrolidone (PVP) as dispersant. b Trace metals analysis. c HPLC. d Using dynamic light scattering. e % of total naparticles preparation.

3.2. Effect on cell viability

The viability of IL-2 proliferating T cells was measured by flow cytometry using propidium iodide in order to determine at which concentration the NPs would have a toxic effect leading to cell death. The assay was carried out following a 72 h exposure. It was shown that the viability was not affected by an exposure to C_{60} -NPs at up to 100 µg/ml (data not shown). For Ag-NPs, significant decrease in cell viability was observed following an exposure to a concentration of 100 µg/ml (data not shown).

3.3. Nanoparticles uptake potential

It is well demonstrated that nanomaterials, by endocytic mechanisms and/or by passive processes, are capable of entering live cells. To evaluate the uptake potential of these NPs, we measured the flow cytometry light scatter (SSC) modifications associated with exposure to NPs. Several authors have shown the relevance of this method when assessing NPs' uptake (Suzuki et al., 2007, Zucker et al., 2010 and Greulich et al., 2011). Our results indicated a different uptake between Ag and C₆₀-NPs (Fig. 1). There is a significant increase of cellular uptake of Ag-NPs following an exposure to 25–100 µg/ml. C₆₀-NPs uptake is limited, and significant only after an exposure to 75–100 µg/ml. Considering the loss of viability, the following tests made with Ag-NPs were done using concentrations from 5 to 50 µg/ml in order to avoid any loss of cell viability (see Fig. 2).

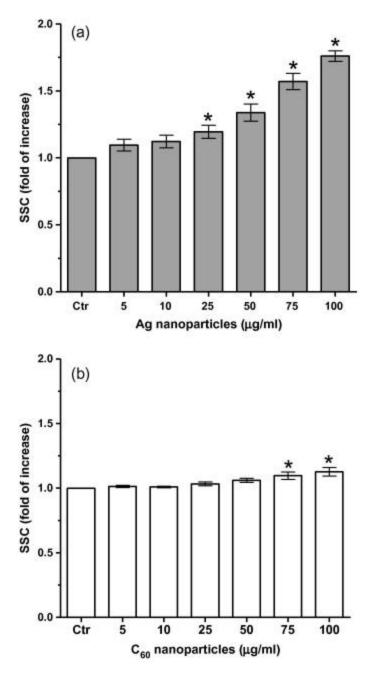
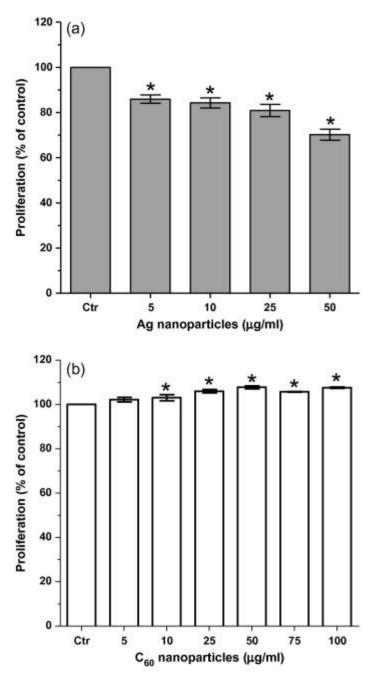


Fig. 1.

Cellular binding and uptake of Ag-NPs and C₆₀-NPs by proliferating T cells. Concentration effects on cellular binding and uptake into WE17/10 cells of (a) Ag-NPs and (b) C₆₀-NPs. The cells were treated with 5, 10, 25, 50, 75 and 100 µg/ml of particles for 72 h. Scatter distribution (SSC) ratio was chosen as a measure of cellular binding/uptake. Data are expressed as means ± SEM from three or four independent experiments performed at different days, *(p < 0.05), **(p < 0.01), ***(p < 0.0001).





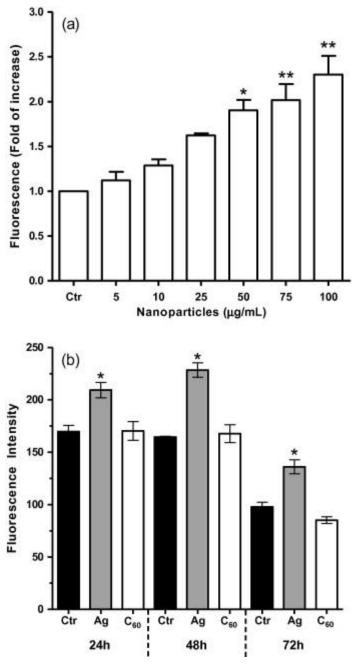
Effect of Ag-NPs and C₆₀-NPs on IL-2 dependent T cells proliferation. WE17/10 cell proliferation was determined in presence of (a) Ag-NPs and (b) C₆₀-NPs. Cells were initially labeled with CFSE as described in Section <u>2</u>. Cells were then treated with 5, 10, 25 and 50 µg/ml for Ag-NPs, and an addition of 75 and 100 µg/ml for C₆₀-NPs for 72 h. IL-2-dependent proliferation was determined by measuring CFDA fluorescence. Data are expressed as means ± SEM of three or four independent experiments, performed at different days $\cdot(p < 0.05)$, $\cdots(p < 0.0001)$.

3.4. Nanoparticles' influence on cell proliferation in response to IL-2

We have next measured the effects of Ag and C₆₀-NPs on cell proliferation in response to IL-2. Results have shown that Ag-NPs significantly decrease the proliferative response to IL-2 (Fig. 2). On the contrary, C₆₀-NPs slightly, but significantly, increase proliferative response to IL-2 at all doses tested. Significant effects on proliferation are observed from 5 μ g/ml for Ag-NPs and 10 μ g/ml for C₆₀-NPs. Ag-NPs had a much stronger effect than C₆₀-NPs and a dose-dependent relation is observed unlike for C₆₀-NPs. Considering that there was no significant effect on cell viability at above 75 μ g/ml for Ag-NPs, these observations do not result from viability loss.

3.5. Assessment of ROS generation

Several nanoparticles induce an increase in oxidative stress in cells (<u>Oberdorster</u> <u>et al., 2005</u>). To evaluate if an exposure to Ag and C₆₀-NPs affects the level of oxidative stress in IL-2 proliferating WE17/10 T cells, ROS levels have been measured after 24 h exposure to NPs with DCFH-DA probes. In the protocol, Ag-NPs caused a quenching effect on DCFH-DA assays and could not be considered for measuring ROS (<u>Supplement data S1</u>). C₆₀-NPs do not interfere with DCFH-DA, and ROS determination after 24 h showed a significant dose-ependent increase from 50 µg/ml to the higher dose used (<u>Fig. 3</u>).





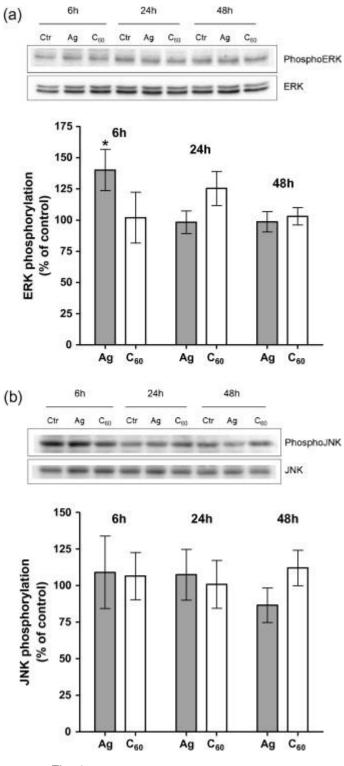
Effect of NPs on ROS generation and high affinity IL-2R expressions (a) ROS production following exposure to C₆₀-NPs. WE17/10 cells were treated with 5, 10, 25, 50, 75 and 100 µg/ml of NPs for 24 h. ROS production was determined by measuring H₂DCFDA probe fluorescence as described in Section <u>2</u>. Data are expressed as means ± SEM of two independent experiments performed at different days, (p < 0.05), (p < 0.01). (b) IL-2R α expression in WE17/10 cells in presence of Ag and C₆₀-NPs. WE17/10 cells were incubated with 25 µg/ml of Ag and C₆₀-NPs for 24 h, 48 h or 72 h. IL-2R α expression was analyzed by flow cytometry using Alexa Fluor 488-conjugated anti-human IL-2R α as described in Section <u>2</u>. Data are expressed as means ± SEM of three independent experiments performed at different days, (p < 0.05), (p < 0.01), (p < 0.0001).

3.6. Determination of IL-2R expression after exposure to nanoparticles

In order to determine whether NPs can act directly to the IL-2 receptor expression to influence cell proliferation, levels of CD25 expression were measured at 24 h, 48 h and 72 h following exposure to 25 μ g/ml of NPs. Results have shown that the presence of Ag-NPs lead to a higher surface expression of CD25 (Fig. 3b). This effect was seen following 24 h of incubation and stayed relatively consistent throughout the incubation, although the level of CD25 expression decreased in all conditions at 72 h. C₆₀-NPs did not show any effects on CD25 expression at any time point. The percentage of CD25⁺ cells have also been measured, and the results showed no significant effect on this aspect for both types of NPs (data not shown).

3.7. Interference signaling processes

IL-2 binding causes signal transduction in cells mediated by $\beta\gamma$ chain complex. In order to evaluate NPs' effects on signaling pathways, we have determined their influence on the MAPK pathway and the JAK-STAT pathway. In IL-2 proliferating T cells, ERK1/2 analysis following exposure to Ag-NPs have shown that after 6 h, there was a significant increase in ERK1/2 activation followed by a return to the normal level after 24 h (Fig. 4a). C₆₀-NPs caused no significant changes in ERK1/2 activation in same condition. JNK pathway was not affected by both NPs up until 48 h of exposure (Fig. 4b).





ERK and JNK activation in response to IL-2 in presence of Ag and C_{60} -NPs. (a) ERK and (b) JNK activation in IL-2 proliferative response of WE17/10 cells in presence of Ag and C_{60} -NPs. WE17/10 cells exposed to IL-2 were challenged with 25 µg/ml Ag and C_{60} -NPs

for 6 h, 24 h and 48 h. Western immunoblotting was used to detect (a) ERK and (b) JNK levels of phosphorylation. Values for protein levels were obtained by densitometry analysis using Image Lab software and normalized against non-phosphorylated protein levels. Results shown are of one experiment that is representative of at least four other experiments. Densitometry analysis corresponding to phospho-ERK or JNK divided by total ERK or JNK was reported in% of control. Data are the mean \pm SEM of four independent experiments, performed at different days, \cdot (*p* < 0.05).

Next, we have determined the effects of NPs on Stat5 activation, a protein directly involved in the transcription of specific genes in response to IL-2. Stat5 analysis has shown that both Ag and C₆₀-NPs have no effects on its level of activation in the first 24 h in IL-2 proliferating T cells (Fig. 5a). Surprisingly, a significant decrease was noted at 48 h exposure to C₆₀-NPs. This effect was even stronger following a 72 h incubation (data not shown). We hypothesized that this resulted from proliferation increase caused by the presence of C₆₀-NPs, which enhance cells' consumption of IL-2, leading to cells' deprivation of the cytokine from 48 h incubation time and up. To confirm this hypothesis, IL-2 has been added to cells after 48 h incubation time and Stat5 phosphorylation was determined 24 h later. Results have shown that this permitted the cells to recover the same level of activation as the control cells (Fig. 5b). Therefore, the C₆₀-NPs' measured effect following long-term incubation was in fact an indirect effect resulting from an increase in proliferation.

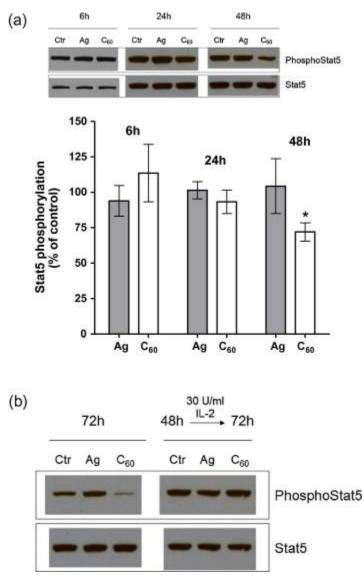


Fig. 5.

Stat5 activation in response of T cells to IL-2 in presence of Ag and C₆₀-NPs. Stat5 activation in IL-2 response of WE17/10 cells in presence of Ag and C₆₀-NPs was determined. (a) IL-2 proliferating WE17/10 cells were challenged with 25 µg/ml Ag and C₆₀-NPs for 6 h, 24 h and 48 h. Western immunoblotting was used to detect Stat5 levels of phosphorylation. Values for protein levels were obtained by densitometry analysis using Image Lab software and normalized against non-phosphorylated protein levels. Results shown are of one experiment that is representative of at least four other experiments. Densitometry analysis corresponding to phospho-Stat5 divided by total Stat5 was reported in% of control. Data are the mean ± SEM of four independent experiments, performed at different days, (p < 0.01). (b) IL-2 proliferating WE17/10 T cells were challenged with 25 µg/ml Ag or C₆₀-NPs for 48 h and 30 U/ml of IL-2 was

added and cell incubated for an additional 24 h. Western immunoblotting was used to detect phosphor-Stat5 and total Stat5. Data are the mean \pm SEM of three independent experiments, performed at different days.

4. Discussion

Silver NPs and C_{60} -NPs are among the most promising types of nanomaterials today. Their chemical compositions are very different, as C₆₀-NPs are relatively inert and hydrophobic, while Aq-NPs are reactive and possess antimicrobial properties. Characterization of both NPs using DLS has shown that agglomerates' sizes in media with serum were much larger for C₆₀-NPs than for Ag-NPs. This could be accounted by the hydrophobic properties of C_{60} -NPs and by Ag-NPs' stock nanopowder form, which included PVP as dispersant. Presence of PVP enabled more effective dispersion and diminished tendency towards aggregation by steric hindrances (Foldbjerg et al., 2009). To evaluate uptake potential of both NPs, SSC analysis by flow cytometry has been carried out after a 72 h exposure. This method is based on the principle that SSC analysis reflects the inner density of particles (Suzuki et al., 2007). Increase of SSC is mainly caused by the NPs both adsorbed on the cell surface and enclosed within endosomes (Xia et al., 2008). Results showed an uptake of Ag-NPs from 25 μ g/ml to 100 μ g/ml. On the contrary, the uptake of C₆₀-NPs was only observed at 75–100 µg/ml. Since NPs can enter T cells by pinocytosis or by passive diffusion, smaller Ag-NPs are able to penetrate cell membranes more efficiently than C₆₀-NPs. Greulich et al. have shown that Ag-NPs agglomerates cannot penetrate T cells (Greulich et al., 2011). Surprisingly, the uptake of Ag-NPs was not associated with an increase in cell death. Indeed, it was shown in several cell lines that Aq-NPs significantly decreased cell viability (Guo et al., 2013, Wei et al., 2010 and Asharani et al., 2009). Prasad et al. had established that the smaller (10 nm) Ag-NPs were more toxic (Prasad et al., 2013). Since we used a formulation with different sizes below 100 nm, it could explain the decrease in cytotoxicity compared to a uniform suspension with a size range between 1 and 30 nm (Guo et al., 2013). The C₆₀-NPs did not affect viability at any concentration, possibly resulting from their low reactivity and aggregate size, which could reduce cellular uptake.

In this study, we used WE17/10 T cells, an IL-2-dependent cell line, to determine the impact of Ag-NPs or C_{60} -NPs upon IL-2 stimulation. Activation by IL-2/IL-2R leading to proliferation in the presence of Ag-NPs or C_{60} -NPs was perturbed. Cell

proliferation was negatively inversely related and dose-dependent to Ag-NPs. Fullerene C₆₀-NPs slightly, but significantly increased proliferation in response to IL-2. A decrease in proliferation could be caused by the release of silver ions from Ag-NPs after oxidation in the media and could be associated with an increase of ROS (Kim and Ryu, 2013). In our hand, Ag-NPs quenched the DCFH-DA probes. We confirmed quenching effect by the absence of ROS formation after addition of PMA on cells incubated with Ag-NPs (Supplement S1). C_{00} -NPs caused a dose-dependent increase of ROS associated with low, but significant increase of proliferation. Exposure to fullerene caused an increase of the intracellular ROS level in keratinocytes and lung cell lines (Horie et al., 2010). However, C₆₀-NPs can prevent oxidative stress in thymocytes and PBMCs (Monti et al., 2000 and Hrebinyk et al., 2012). Interestingly, fullerene can also improve the immune response (Liu et al., 2009). In our model, the increase of ROS was associated with the increase in proliferation. This latter association could be explained by the capacity of IL-2 to rescue apoptosis as was demonstrated with dexamethasone (Rebollo et al., 1995 and Mor and Cohen, 1996). Modulation of proliferative responses can be explained by down-regulation of high-affinity IL-2 receptors or by a perturbation of signaling pathways. CD25 expression was measured following exposure to both NPs to determine if they caused a modulation of high affinity IL-2 receptor. Its expression is regulated by signaling pathways triggered by the binding of IL-2 to its receptor (Malek, 2008). The presence of C_{60} -NPs had no effect on CD25 expression, while Ag-NPs significantly increased the level of expression up until 72 h of culture. These results indicate an increase of high affinity receptors, which are complexes formed by $\alpha\beta\gamma$ chains. However, we have observed no modulation of β chains (data not shown), which are critical for IL-2-dependent signal transduction (Hatakeyama et al., 1989). The number of high affinity IL-2 binding sites appears limited by the total number of IL-2R β subunits on the cell surface and an excess of α -chains at the cell surface represents a low affinity IL-2R without signaling capacity (Hemler et al., 1984). Thus, an excess of IL-2Rα can compete with IL- $2R \alpha\beta\gamma$ or IL-2R $\beta\gamma$ for IL-2 available in cell culture, slightly reducing proliferation. Ag-NPs' effects were also associated with ERK1/2 activation after 6 h of exposure. This increase might result from ROS production due to the presence of Ag-NPs. It is well accepted that ROS activate the MAPK pathway (Torres and Forman, 2003). Eom et al. have measured no effects on either ERK or JNK activation following exposure of Jurkat T cells to dispersed tetrahydrofuran AgNPs (Eom and Choi, 2010). Ag-NPs' formulation and doses used could explain the differences in ERK results in the present study. Stat5 transcription factor is known to be involved in the regulation of CD25 expression (Malek, 2008) and in cell cycle progression in response to IL-2 (Moriggl et al., 1999). Analysis of Stat5 phosphorylation has shown no perturbation by Ag-NPs. However, a decrease of Stat5 by C₆₀-NPs was noted at 48 h although proliferation was increased and CD25 expression not affected. Therefore, we have speculated that the decrease in Stat5 activation was related to a decrease in IL-2 availability caused by the increase in proliferation of WE17/10 cells. Addition of fresh IL-2 restores activation of Stat5, confirming our hypothesis. C_{60} -NPs have been known to have immunostimulatory properties by increasing Th1 cytokine production (Liu et al., 2009).

Overall, our study has shown that Ag-NPs perturb the IL-2 proliferation by decreasing cell proliferation due to an increase of low-affinity receptors for this cytokine. C_{60} -NPs increase the turnover of IL-2-dependent proliferating cells at concentrations that do not significantly increase ROS formation.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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