# *Analysis of the toxicity of gold nano particles on the immune system: effect on dendritic cell functions*

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## Abstract

**The effect of manufactured gold nanoparticles (NP) on the immune system was analysed through their ability to perturb the functions of dendritic cells (DC), a major actor of both innate and acquired immune responses. For this purpose, DCs were produced in culture from mouse bone marrow progenitors.**

**The analysis of the viability of the cells after their incubation in the presence of gold NP shows that these NP are not cytotoxics even at high concentration. Furthermore, the phenotype of the DC is unchanged after the addition of NP, indicating that there is no activation of the DC. But the analysis of the cells at the intracellular level reveals important amounts of gold NP amassing in endocytic compartments. Furthermore, the secretion of cytokines is significantly modified after such internalisation indicating a potential perturbation of the immune response.**

**Author Keywords** Nanoparticles ; gold ; immune system ; dendritic cells ; cytotoxicity ; cytokine

## Introduction

Nanotechnology is an emerging field involving production and utilisation of nanometric particles (NP) and materials; increasing exploitation of these industrially manufactured nano objects raises questions about their potential toxicity and their appropriate safe use by occupational workers and more generally, the risk assessment for all potentially exposed populations. In this context, the European Commission proposed to reinforce the knowledge on the potential toxicity of these materials. The development of new and appropriate in vitro methods to assess the toxicological profile of such materials is a prerequisite for these analyses. Our work, in the frame of the European programme CellNanoTox, aims to understand the interactions of NPs with the immune system which may provide appropriate evaluation procedures for risk assessment in this field. Beside their use for medical purpose, the penetration of NP into the organism results from the crossing of barriers (skin, lung, intestinal track for example) which are all patrolled and controlled by the immune system. Furthermore, when they are used for medical treatment, most of nano materials are also in contact, in the blood, with cells and soluble proteins involved in the defence of the organism. The analysis of the toxicity of such material is difficult, that is reflected by apparent controversial already published results: for example, it was shown that multi-walled carbon nanotubes did not induce lung inflammation or tissue damage, but caused alterations of systemic immune function in a mice model (Mitchell 2007 ) whereas, Chou et al. (Chou 2008 ) showed that single-walled carbon nanotubes induced alveolar macrophage activation with chronic inflammatory responses and severe pulmonary granuloma formation. The toxicity of these materials may vary according numerous parameters (Warheit 2008 ) like nature, size, roughness, form, aggregation state, coating, etc, making difficult to provide general results.

The impact of nanomaterials on the immune system was already shown using various models like carbon nanotubes (Shvedova 2008 ) or poly (D, L-lactic acid-co-glycolic acid) NP (Elamanchili 2004 ) for example. The particularity of some of these materials was also used in order to stimulate the immune system through an adjuvant effect as for polybutyl cyanoacrylate nanoparticles (Schneider 2008 ) or to reduce allergic responses as in the case of fullerene (Ryan 2007 ).

The immune response involves numerous humoral and cellular partners among which, dendritic cells (DCs) play a major role as the only professional antigen presenting cells able to initiate the specific immune response. Thus the persistence of their integrity is particularly important for the regulation of the immune system. DCs are able to detect, phagocyte, process and present to T lymphocytes antigens which are issued from proteins. In addition, by their capacity to secrete various cytokines, they are also able to activate cells acting on the innate immune response such as natural killer (NK) cells. Furthermore, the reactivity of DCs is greatly influenced by their local conditions in the body (stress, inflammation, etc.). For all these reasons, we have proposed to use DCs as a model system to study the impact of nano materials on the immune system to report on their potential deleterious effects. Here, we report the results of the exposition of DCs to gold NPs.

## Materials and Methods

### Gold nanoparticles

Gold NPs were synthesized by Daniele Bonacchi from Colorobia (Italy) in aqueous medium containing sodium citrate and poly-(N-vinyl)-2-pyrrolidone. Pure gold particles have a mean size of 10 nm as determined by scanning transmission electron microscopy, a zeta potential of -13,0 at pH 7,4 and are stable in the medium used for cell culture as shown by analysis by electron microscopy (data not shown). The particles were provided at the gold concentration of 13mM. Throughout the experiments, NPs were used at a final gold concentration of 0.5mM.

**Dendritic cells**

DCs were generated from bone marrow extracted from C57BL/6 mice (Charles River, l'Arbresle, France) as previously described ( Faure 2004 ). Briefly, bone marrow cells were isolated by flushing from the femurs. Erythrocytes and GR1 positives cells were removed by magnetic cell sorting and the remaining negatively sorted cells were resuspended at $5\times10^5$ cells/ml in complete Iscove's modified Dubelcco's medium (IMDM) supplemented with factors GM-CSF, FLT-3L and IL-6 and cultured at 37°C in the presence of 5% CO2. The transformation of the progenitors into fully active DC occurs during a 10 days culture.

**Incubation with nanoparticles**

After washing, DCs ($10^6$ cells/ml) were incubated in culture medium in 24 wells plates for 24 hours; then NPs were added and cells further incubate at 37°C as indicated in the text. The impact on DCs was tested for various parameters, such as apoptosis, activation or cytokine secretion.

Apoptosis and necrosis were analysed by flow cytometry after 4, 24 or 48 hours incubation together with NPs, cell were stained in the presence of phycoerythrin labelled Annexin V and 7-Amino-Actinomycin D (7AAD). Cellular activation was analysed by flow cytometry after cell staining using fluorescent antibodies against IAb (Major Histocompatibility Class II (MHC-II) molecules) and CD11b, both markers for DCs. The analysis were performed on a FACSAria (BD Bioscience) and the results recalculated using FCS Express V3 (De Novo Software). Each experiment was performed at least three times with cells issued from different cultures.

**Cytokines Immunoassays**

Cytokines were measured in the supernatant of cell cultures by immuno assays using OptEIA for mouse cytokines (Pharmingen) according to the procedures recommended by the manufacturer. Each experiment was performed at least three times with cells issued from different cultures and each measurement is performed in duplicate. The supernatants are harvested after 24 hours incubation at 37°C.

**Statistical analysis**

The results are presented as a mean ±standard error of the mean (SEM). Statistical analysis was performed using SPSS software, version 15.0 (SPSS Inc Chicago, Il USA). The non parametric Wicoxon and Mann-Whitney U–test were used to compare quantitative variables. Differences were considered as statistically significant when p-value was less than 0.05.

## Results

In order to determine if gold NPs are cytotoxic, DCs were incubated with NPs for 4, 24 and 48 hours and then labelled using Annexin V and 7-AAD for detection of apoptosis and necrosis respectively. As shown in Fig. 1 , the labelling of the DCs incubated with NPs is similar to what is obtained for the untreated DCs: indicating that there is no induction of apoptosis in the presence of gold NPs after 24 hours incubation. The same results were obtained whatever the incubation time (4, 24 and 48 hours). A positive control for cell death (Fig 1B ) was realised by incubating DC with di-ethylen glycol (2%) for 24 hours which leads to a high dyes incorporation (90% of apoptotic and necrotic cells).

While the gold NPs did not induce DCs death, they strongly accumulated in the cells as shown by microscopy (Fig 2 ), this is likely due to the high capacity of phagocytosis of these cells.

Such internalisation may induce the activation of the cells; to assess this eventuality, DCs were labelled, after incubation, with anti-IAb (MHC-II) and anti-CD11c antibodies: The level of expression MHC-II molecules is correlated to the activation of the DCs which are themselves characterized by their high expression of CD11c molecules. As shown in Fig. 3 , the percentages of activated DCs were similar whether the cells were incubated or not in the presence of NPs. These percentages must be compared to the value obtained (38%) when bacterial Lipo-Poly-Saccharides (LPS) was added to the cell culture as positive control for DC activation. These results indicate that in the presence of these gold NPs, the cells are not activated after 24 hours incubation; same results were obtained for shorter (4 hours) or prolonged incubation (48 hours), data not shown.

The apparent harmlessness of the gold NPs on DCs was further assessed by analysis of functional parameters: Their effect on the capacity of DCs after activation to secrete cytokines (one of the most important functions of these cells) was measured. The cells were incubated in the presence of NPs together with LPS which induces the cell activation and the resulting cytokine secretion. The

supernatants were harvested after 24 hours incubation: it was shown previously that this corresponds to the period where the major part of the cytokines secretion occurs after DCs activation (Bueno 2001 ). Two cytokines were measured: IL-12p70 and IL-6, these two were chosen because of their importance in the functions of the DCs, they are responsible for the induction of an immune response through the stimulation of T lymphocytes and of the induction of inflammation reaction respectively. As shown in Fig. 4 , secretion of IL-12 p70 was significantly inhibited ($p<0.05$) in the presence of NPs, whereas the amount of IL-6 was not affected by the addition of NPs. In the absence of LPS, there is no secretion of cytokines even after incubation in the presence of NPs.

## Conclusion

The results presented here indicate that the gold NPs used for the experiments, are not cytotoxic: they do not induce the death of the cells nor their activation, but they accumulate inside the cells which is in agreement with the strong phagocytic capacity of DCs (Savina 2007 ). Indeed, such accumulation of NPs inside the DCs, especially in compartments dedicated to antigen processing, may be a handicap for antigen presentation. DCs activation induces morphological transformations with development of long dendrites and increases the amount of MHC-II molecules at the cell surface, molecules which present the antigens to T lymphocytes. These modifications of DCs facilitate the contact of DCs with the other cells involved in the immune responses. Furthermore, there is induction of the secretion of many factors among which interleukins (IL-1, IL-6, IL-12, IL-10, TNF, etc…..). We have chosen to measure two very important cytokines: IL-6 and IL-12p70. IL-6 is implicated in the inflammation; we show here that the internalisation of NPs did not influence its secretion. IL-12p70 corresponds to the active complex formed by the association of IL12p35 and IL12p40 which is directly involved in T lymphocyte activation and thus in the regulation of the antigen specific immune response. The fact that there is a strong inhibition of this secretion in presence of NPs indicates that, in the case of simultaneous presence of NPs and antigens, the specific immune response may be altered.

We have shown that the gold NPs are not cytotoxic for DCs but we demonstrate also that these NPs reduce the secretion of IL12p70 when the cells are simultaneously activated by addition of LPS, such, these results point out that it is not possible to account for the impact of NPs on the organism by the sole measurement of cytotoxic effects. Such analysis is too restrictive and does not reflect the potential deleterious effects of NPs in the organism, especially in the case of long term or chronic exposures. Therefore, further analysis on the functional aspects must be precisely defined to perform assays, which are relevant to determine accurately the biological modifications resulting of the presence of new nano materials.


### Ackowledgements:

We acknowledge members of CellNanoTox project (FP6-NMP-CT-2006-032731) of the European Community, especially to Dr Daniele Bonacchi for synthesis and production of the NPs.


### References:


- Bueno C , Almeida J , Alguero MC . 2001 ; Flow cytometric analysis of cytokine production by normal human peripheral blood dendritic cells and monocytes: comparative analysis of different stimuli, secretion-blocking agents and incubation periods . Cytometry . 46 : 33 - 40
- Chou CC , Hsiao HY , Hong QS . 2008 ; Single-walled carbon nanotubes can induce pulmonary injury in mouse model . Nano Lett . 8 : 437 - 445
- Elamanchili P , Diwan M , Cao M , Samuel J . 2004 ; Characterization of poly(D,L-lactic-co-glycolic acid) based nanoparticulate system for enhanced delivery of antigens to dendritic cells . Vaccine . 22 : 2406 - 2412
- Faure M , Villiers CL , Marche PN . 2004 ; Normal differentiation and functions of mouse dendritic cells derived from RAG-deficient bone marrow progenitors . Cell Immunol . 228 : 8 - 14
- Mitchell LA , Gao J , Wal RV , Gigliotti A , Burchiel SW , McDonald JD . 2007 ; Pulmonary and systemic immune response to inhaled multiwalled carbon nanotubes . Toxicol Sci . 100 : 203 - 214
- Ryan JJ , Bateman HR , Stover A . 2007 ; Fullerene nanomaterials inhibit the allergic response . J Immunol . 179 : 665 - 672
- Savina A , Amigorena S . 2007 ; Phagocytosis and antigen presentation in dendritic cells . Immunol Rev . 219 : 143 - 156
- Schneider T , Becker A , Ringe K , Reinhold A , Firsching R , Sabel BA . 2008 ; Brain tumor therapy by combined vaccination and antisense oligonucleotide delivery with nanoparticles . J Neuroimmunol . 195 : 21 - 27
- Shvedova AA , Fabisiak JP , Kisin ER . 2008 ; Sequential exposure to carbon nanotubes and bacteria enhances pulmonary inflammation and infectivity . Am J Respir Cell Mol Biol . 38 : 579 - 590
- Warheit DB , Sayes CM , Reed KL , Swain KA . 2008 ; Health effects related to nanoparticle exposures: Environmental, health and safety considerations for assessing hazards and risks . Pharmacol Ther . 120 : 35 - 42

**Fig. 1**

Analysis of the cytotocity of gold NPs

Dendritic cells were incubated at 37°C in presence of gold NPs (0.5mM), then, after washing, the cells were stained for the determination of apoptosis and/or necrosis using Annexin V and 7-AAD respectively. A) Control DCs incubated without NPs. B) Positive control with DCs incubated for 24 hours in presence of DEG (2%, v/v). C) DCs incubated with NPs. The circle indicates the area used to determine the dead cells. The results which are presented correspond to 24 hours incubation with NPs, similar results were obtained after 4 and 48 hours incubation. This figure is representative of three independents experiments

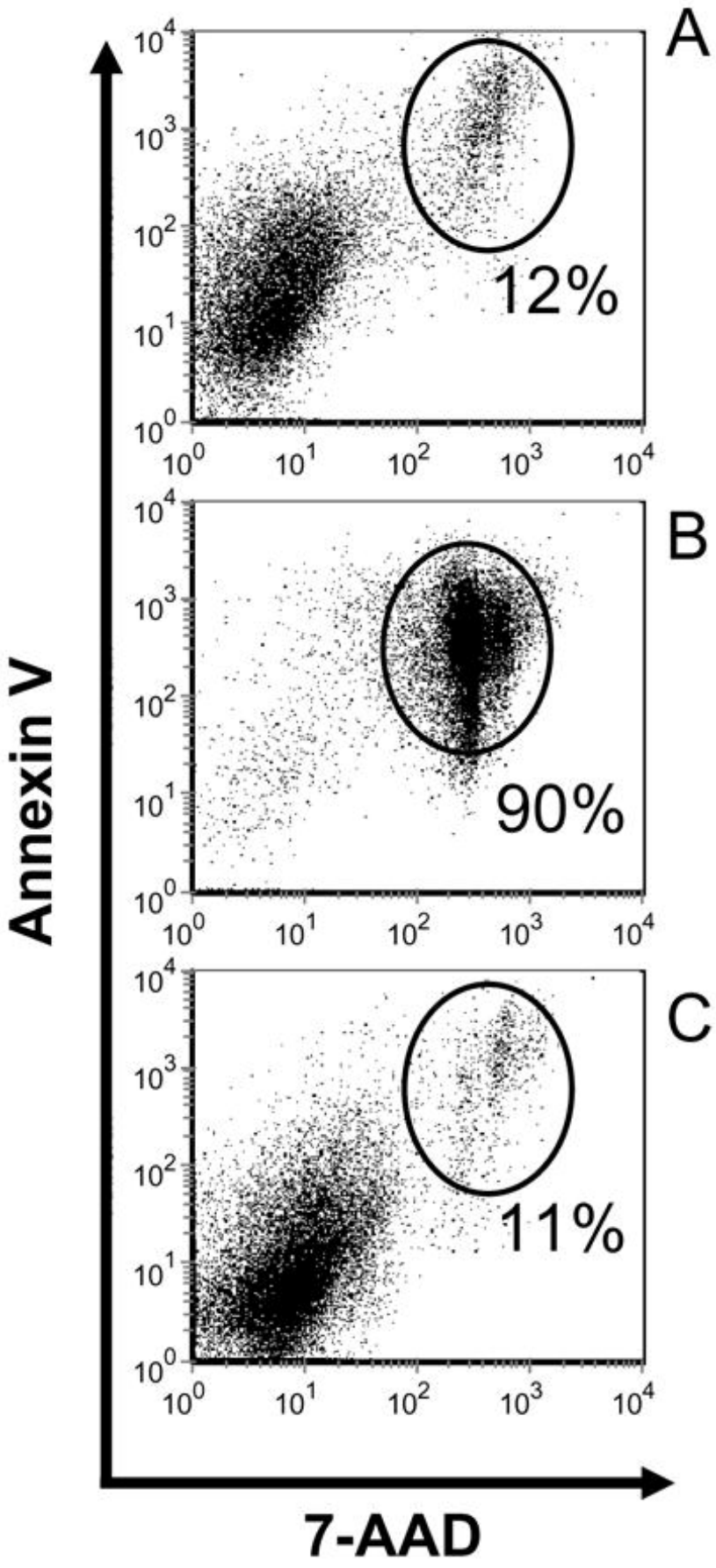

**Fig. 2**

Visualisation of internalised gold NPs by DCs

DCs were incubated at 37°C in presence of gold NPs (0.5mM) for 24 hours and then, after washing, they were observed under microscope, the arrows indicate the visible accumulation of NPs in the cells. The bar correspond to 40µm (A) or 5µm (B).

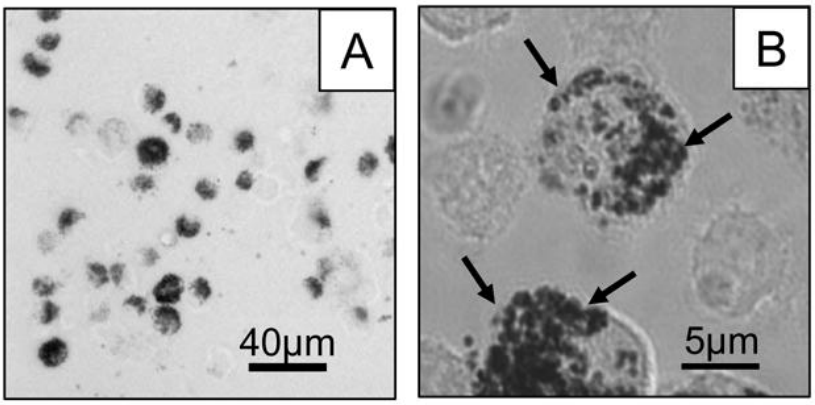


**Fig. 3**

Analysis of the activation of DCs in presence of gold NPs

DCs were incubated at 37°C in presence of gold NPs (0.5mM), then, after washing, they were stained in order to determine their activation using fluorescently labelled antibodies anti-IAb (MHC-II) and anti-CD11c markers. A) Control DCs incubated without NPs. B) Positive control with DCs incubated for 24 hours in presence of LPS (2µg/ml). C) DCs incubated with NPs. The circle indicates the area used to determine the percentage of activated cells. The results which are presented correspond to 24 hours incubation with NPs, similar results were obtained after shorter (4 hours) or longer (48 hours) incubation. This figure is representative of three independents experiments.

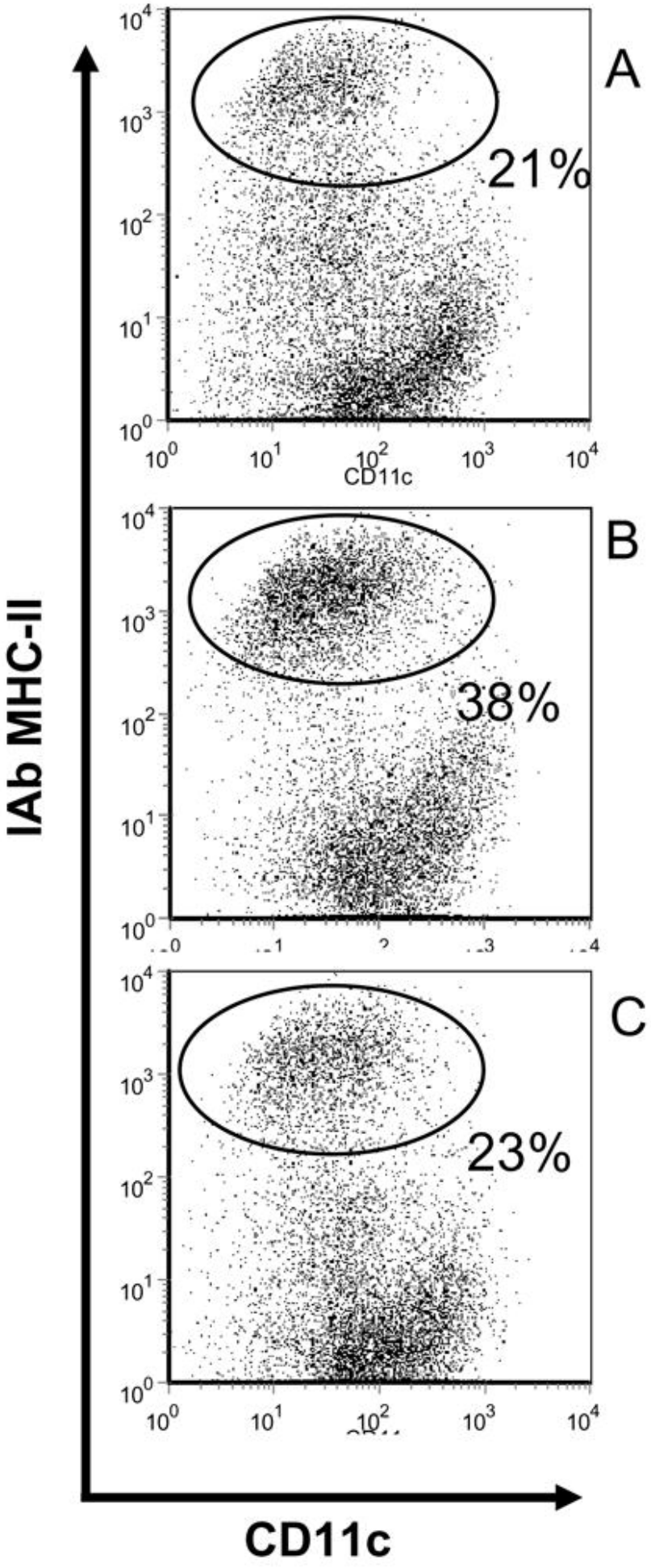
A
21%
B
38%
C
23%
IAb MHC-II
CD11c
CD11c

**Fig. 4**

Quantification of the cytokines secreted by DCs in presence of NPs

DCs were incubated at 37°C in the presence of LPS (2μg/ml) and/or gold NPs (0.5mM) for 24 hours, then the supernatants were collected and used to determine the concentration of IL-6 and IL-12p70 as indicated in the material and methods section. A control was performed without additive: LPS and NPs. The results correspond to the mean value of three independents experiments. ** denotes a significant difference between the indicated experimental groups ($p<0.05$).