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Intracellular accumulation and immunological properties of fluorescent gold nanoclusters in human dendritic cells

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ABSTRACT

We have studied the effect of highly fluorescent gold nanoclusters (Au NCs) (⌀ < 3 nm) stabilized by different ligands on the intracellular accumulation and immune response of human derived-monocyte dendritic cells (DCs). Results indicate that the high uptake efficiency of Au NCs is strongly related to their small size and to the nature of the ligand, with zwitterionic ligands being more effective than PEGylated ones. Evidence from flow cytometry and microscopy demonstrate time and concentration-dependent Au NCs internalization by endocytic pathway(s) involving amorphous and laminar organelles, while maintaining their discrete size and photoluminescence properties. The uptake of zwitterionic ligand-stabilized Au NCs induced very low cytotoxicity and a strong immunosuppressive response (Th1/Treg pattern), associated with a DC maturation state. This behavior contrasts to the effect of bigger particles (~12 nm size) which induced a cytotoxic response involving Natural Killer (CD56) cells. Overall, this study stresses the critical importance of particle size and ligand type on the immunostimulation of DCs and highlights the remarkable potential of this new class of nanomaterial as a novel vaccine platform.

1. Introduction

Interest in applying engineered nanoparticles (NPs) to the field of immunotherapy [1,2], notably for vaccination against cancer [3–5] and allergy [6,7] has grown dramatically over the last decade. NPs can be designed to inhibit or suppress the immune system [1,8,9]. One of the most efficient strategies relies on targeting antigen presenting cells (APCs) such as macrophages, B cells or dendritic cells (DCs) [8]. In fact, DCs represent an ideal target because they reside in peripheral tissues and in the lymphoid system, where they act as sentinels regulating innate and adaptive immunological responses [10–12]. DCs are specialized APCs that display antigens to naïve T cells and, when activated, also express co-stimulators and cytokines that function in concert with antigens to polarize T cell responses [13].

One of the advantages of NPs is their ability to act as both adjuvant and antigen delivery system, thus simultaneously activating the immune response while lowering antigen dosage by efficient APC targeting [14–19]. NPs have significant potential in allergen immunotherapy, whose ability to act as delivery agents for epitopes leading to efficient long-term down-regulation of the reactivity toward specific allergens has been demonstrated [20,21]. Indeed, many studies have confirmed the potential of NPs for vaccination and answered fundamental questions about the nature of NP-DC interactions by passive or active targeting in terms of uptake efficiency [22,23], biodistribution [24] and immunological effects [9,25–28]. The physico-chemical properties of NPs have proved to play an essential role in the modulation of DC immune responses during maturation, T lymphocyte proliferation and cytokine secretion. NP type [8,18], size [29–32], shape [33], surface charge [34] and hydrophobicity [35] strongly affect the inflammatory responses of DCs and the level of cytotoxicity [29,36]. For instance, several in vitro and in vivo studies with polymeric and metallic NPs suggest that a size lower than 200 nm enhances DC

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uptake, improves immunoresponse induction and the delivery of antigens [27,30]. Gold NPs with sizes ranging from 5 to 200 nm have been extensively studied for immunotherapy purposes with the aim of optimizing size and shape, and functionalizing their surface with organic ligands and biomolecules. For example, it has recently been shown that the degree of hydrophobicity of gold NPs can induce an inflammatory response with high TNFα secretion [35]. Another study has shown that gold cubes and spheres are more effective than rods in promoting the secretion of tumor necrosis factor-R (TNF-R), IL-6, IL-12 [33].

Metal nanoclusters (NCs) made up of 10–100 atoms are an emerging family of nanomaterials, which exhibit properties that are distinct from NPs (size > 3 nm) such as chirality [37], magnetism [38], and photoluminescence [39,40]. These features are directly related to the quantum confinement of electrons in a size regime comparable to the Fermi wavelength of electrons (ca. ~ 0.7 nm for gold and silver). Recent progress in the development of synthetic routes over the last 5 years has led to the production of a large variety of gold (Au) and silver (Ag) NCs stabilized by thiol organic ligands [41,42], polymers [43,44], and by biological scaffolds such as proteins [45,46], peptides [47–49] or DNA [50]. Au and Ag NCs show remarkable sensitivity to their environment and have excellent potential for sensing [51,52] and bioimaging [53–55] applications. In fact, due to their excellent photophysical properties (high photostability, tunable fluorescence, long fluorescence lifetime, two-photon excitation), their small size [42], good colloidal stability in biological media [41], enhanced passive targeting [56], high clearance [57] and low toxicity [58], Au NCs are seen as a promising theranostic agent for in vitro/in vivo studies [54,56,59].

Because particle size has a direct impact on their intrinsic properties such as diffusion and ligand coverage, NCs with a size below 3 nm are expected to exhibit significantly different properties in terms of particle uptake and DC immune responses.

In this paper, we show the intracellular accumulation of Au NCs and the immunological responses of human monocyte-derived DCs in the presence of this new nanomaterial for the first time. Three different thiol ligands stabilizing the Au NCs known for their enhanced stability [60,61]: zwitterion (Zw), polyethyleneglycol (mPEG) and a mixture zwitterion/alkyl acid (ZwMUA) were chosen to investigate the influence of surface charge on cell uptake and immune responses (Fig. 1a). Cell uptake, colocalization, cytotoxicity, maturation, lymphocyte proliferation and cytokine secretion in the presence of Au NCs were fully characterized and compared to Au NPs coated with the same ligands in order to evaluate the influence of size and ligand type with the goal of developing efficient carriers for DC targeting.

Our results indicate more efficient internalization of NCs stabilized by zwitterionic ligands than by PEGylated ligands. Zwitterion-stabilized NCs were internalized in a dose (5–25 μg/mL) and time (1 h–48 h) dependent manner. DC uptake of 1–2 nm NCs was also enhanced compared to the larger but otherwise identical 12 nm NPs. Au NCs did not show any cytotoxicity even at the highest concentrations tested and were internalized by the endocytic pathway in late endocytic compartments. NC aggregates were not observed in the nucleus or outside of organelles or vesicles. Immunological analysis demonstrated that zwitterions stabilized Au NC uptake was associated with DC maturation, low level T cell proliferation, stimulation of the Th1/Treg response and the absence of Natural killer cell responses or cytotoxic effects in contrast to Au NP treatment. The ability to track Au NCs by multimodal imaging, their efficient cell uptake and immunosuppressive properties make these ultra-small carriers very promising candidates as future nanovaccines for immunotherapeutic applications such as specific allergen immunotherapy.

2. Materials and methods

All chemical products were purchased from Sigma Aldrich (Spain) except N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and sulfoNHS from Thermo Fisher (Spain). Ultra pure MilliQ water was used for the synthesis.

Synthesis of the ligands. Thioctic-zwitterion (Zw, M~412 g mol^{-1}) and thioctic polyethyleneglycol (PEG750) methyl ether (mPEG, M~926 g mol^{-1}) were synthesized following the protocols described elsewhere [62,63] (refer to the ESI for details of NMR, FTIR and mass spectroscopy analyses).

Synthesis of Au NCs. Au NCs with the different ligands: zwitterion (Zw), zwitterion/mercaptoundecanoic acid mixture (ZwMUA) and methylpolyethyleneglycol (mPEG) were prepared by the addition of gold salt ($HAuCl_4.3H_2O$, 50 mM) to a basic solution (pH 10) containing the ligand in the presence of the strong reducing agent $NaBH_4$ (50 mM) and stirred for 15 h. Zwitterion-stabilized Au NCs (*AuZw*) was synthesized with a 1:10:2 Au:Zw:$NaBH_4$ molar ratio. A mixture of zwitterion and mercaptoundecanoic ligands stabilized Au NCs (*AuZwMUA*) was synthesized with a 1:5:5:2 Au:MUA:Zw:$NaBH_4$ molar ratio mPEG-stabilized Au NCs (*AumPEG*) were prepared with a 1:10:2 Au:mPEG:$NaBH_4$ molar ratio. Afterwards, solutions were filtered twice with Amicon 3 kDa cut-off filters at 13,600 rpm for 20 min to remove excess free ligands, concentrated to 500 μg gold/mL in water and kept refrigerated until use.

Synthesis of Au NPs. Citrate-stabilized Au NPs with a diameter of approximately 12 nm were prepared by the standard Turkevitch method. Zw or mPEG ligand exchange on the Au NP surface was obtained by the simple addition of ligands in excess to a colloidal gold solution (100 μg/mL of gold in water) and then stirred for 15 h at basic pH~ 9. Zw and mPEG coated *Au NPs* were centrifuged twice at 13,600 rpm for 15 min, resuspended in water at 500 μg gold/mL and kept refrigerated before use.

Conjugation of fluorescein amine to AuZwMUA. In eppendorf tubes, 150 μL of concentrated (500 μg/mL) aqueous solutions of *AuZw* (Au NCs stabilized by a zwitterion ligand) and *AuZwMUA* (Au NCs stabilized by a mixture of zwitterion ligand and a ligand made of an aliphatic chain with a terminal carboxyl group) were diluted to 1.5 mL in a MES (2-(*N*-morpholino)ethanesulfonic acid) buffer (50 mM; pH 6.1) containing EDC (1.25 mM) and sulfoNHS (0.37 mM) with the molar ratio EDC: sulfoNHS = 1:4 and shaken for 30 min. Afterwards, 50 μL of fluorescein amine (300 μM in MES buffer (50 mM; pH 6.1)) was added to each of the two solutions and shaken in the dark overnight. Fluorescein-labeled *AuZw* and *AuZwMUA* samples were filtered with Amicon cut-off filters (3 kDa) at 13,600 rpm for 20 min and diluted with water to 1.5 mL.

Fluorescence measurements were performed after each filtration with excitation at 400 nm to observe both the emission from fluorescein at $\lambda_{em.} = 520$ nm and that from the Au NCs at λ_{em} ~ 680 nm. Fluorescence intensity was normalized to the maximum emission of Au NCs assuming the absence of cluster degradation during the centrifugation steps.

Characterization of NCs and NPs. NMR spectroscopy of Zw and mPEG ligands was performed using a Brucker Ascend™ 400 MHz NMR using deuterium oxide for Zw and deuterium chloroform for mPEG. Freeze-dried samples were characterized by Infrared spectroscopy using a Jasco FTIR-4100 from 700 cm^{-1} to 4000 cm^{-1}. PAGE electrophoresis of the NCs: *AuZw*, *AuZwMUA* and *AumPEG* on a 15% polyacrylamide gel was carried out using the Bio-Rad mini-Protean system (Hercules, CA, USA) at 100 V for 120 min. Each well was loaded with 20 μL of concentrated sample mixed with 10 μL of glycerol. Molecular weight was determined with a Precision Plus Protein Dual Xtra standard indicator™ (2–250 kDa). NC and NP hydrodynamic diameters and zeta potentials in water, PBS buffer and RPMI 1640 + 10% Fetal Calf Serum (FCS) were analyzed using a Nano ZS Zetasizer (Malvern). Absorption spectra over a 190–900 nm range were collected using a Cary 100Bio UV-visible spectrophotometer (Varian). Steady-state fluorescence measurements were obtained with diluted samples on a Perkin Elmer LS45 Fluorescence Spectrometer.

The amount of Au NCs or NPs uptake by DCs was determined by inductively coupled plasma high resolution mass spectrometry (ICP-HRMS) on an ELEMENT XS (Thermo Fisher) system after digesting cells (average of 100,000 cells per sample) with strong acid. Gold concentration per cell was determined using Thermo Element software (Thermo Fisher). Results were expressed as particles/cell, calculated assuming average NC and NP sizes of 2 nm and 12 nm, respectively, as described by Lewis et al. [64]. Briefly, the number of gold atoms per particle was estimated using the following formula:

$$N_{atom} = (R_{particle}/R_{atom})^3 \text{ with } R_{atom} = 0.137 \text{ nm } (R = \text{radius})$$

and the number of gold atoms from ICP: N_{ICP} = mass of gold measured/M_{gold} *$N_A$with $N_A = 6.02 \times 10^{23}$; $M_{gold} = 197$ g mol^{-1}

Finally particle/cell = $N_{ICP.}/(N_{atom}{}^{*}\text{cell}_{numb.})$ with $\text{cell}_{numb.} = 100{,}000$.

Confocal Microsopy. Samples were analyzed using a Leica DM6000 inverted microscope connected to a Leica SP5 laser scanning confocal system. For the calculation of Au NC fluorescence intensities single optical sections were captured from unstained DCs using excitation at 488 nm and detection of Au NC fluorescence emission between 580 and 700 nm, with an independent brightfield channel (transmitted light) to allow cell detection. For DCs stained with phalloidin or clathrin, ATTO488/Cy2 and Au NC fluorescence were detected simultaneously using 488 nm excitation with a ~495–520 nm detection window for ATTO488/Cy2 and