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Running Title

Immunotoxicity of PLGA nanoparticles on dendritic cell activation

# Immunotoxicity of poly(lactic-co-glycolic acid) nanoparticles: influence of surface properties on dendritic cell activation

Modified nanoparticles (NPs) can interact with the immune system by causing its activation to fight tumors or for vaccination. During this activation, dendritic cells (DCs) are effective in generating robust immune response. However, the effect of nanomaterials on dendritic cell (DC) maturation, and the associated adjuvant effect, should be assessed as a novel biocompatibility criteria for biomaterials since immune consequences may constitute potential complications in nanomedicine. Among emerging biomaterials, poly(lactic-co-glycolic acid) NPs (PLGA NPs) are widely explored for various applications in which the degree of desired adjuvant effect may vary. As contradictory results are reported regarding their effects on DCs, we aimed at clarifying this point with particular emphasis on the relative impact of particle surface properties. To that end, NP uptake and effects on the viability, phenotype and secretory activity of DC primary cultures. Intracellular signaling pathways were ~~additionally~~ explored were evaluated. Immature human and murine DCs, were exposed to cationic, neutral, or anionic PLGA NPs. Particle uptake was ~~first~~ assessed by both confocal microscopy and flow cytometry. Cell viability was then evaluated prior to the study of maturation by examination of both surface marker expression and cytokine release. Our results demonstrate that PLGA NPs are rapidly engulfed by DCs and do not exert cytotoxic effects. However, upon exposure to PLGA NPs, DCs showed phenotypes and cytokine secretion profiles consistent with maturation which resulted, at least in part, from the transient intracellular activation of mitogen-activated protein kinases (MAPKs). Interestingly, NP-specific stimulation patterns were observed since NP surface properties had sensible influence on the various parameters measured.

Keywords: Nanoparticles, poly(lactic-co-glycolic acid), immunotoxicity, dendritic cell

## Introduction

Nanotechnology applications in medicine, termed as nanomedicine, have opened doors to a plethora of possible answers to several impediments of conventional medicine. Among the broad panel of available materials in nanomedicine, poly(lactic-co-glycolic) acid (PLGA) has been extensively investigated, as this copolymer is generally viewed as biocompatible and biodegradable (Panyam and Labhasetwar, 2003 ; Bala et al., 2004; Mundargi et al., 2008; Lu et al., 2009; Xu et al., 2009). It has been approved by the US FDA for drug delivery and other applications in clinics, and widely investigated for applications in cardiovascular diseases, cancer, vaccine and tissue engineering (Lu et al., 2009). In this context, NPs of PLGA are explored as carriers for the controlled delivery of small molecules such as anticancer cytotoxic compounds, and macromolecular therapeutics such as proteins, peptides, vaccines, genes, antigens and growth factors (Mundargi et al., 2008). Because of this increasing interest for these PLGA NPs, it is obvious that a careful assessment of their potential toxicity is crucial. Despite such a need for risk assessment studies to be carried out, the current state of knowledge about biological effects and toxicity of PLGA NPs is well characterized for some aspects (Bisht & Rupenthal, 2016) but remains limited for most of exposure routes.

In the specific context of nanotoxicology, the field of immunotoxicology is gaining a growing interest, as the interactions between NPs and the immune system have proven pivotal in the response to NP exposure (Dobrovolskaia & McNeil, 2007; Dobrovolskaia et al., 2009; Zolnik et al., 2010; Blank et al., 2011; Di Gioacchino et al., 2011; Oostingh et al., 2011). The immune response to foreign chemical and/or biological molecules as well as to particles is typically initiated through a protective innate inflammatory response. With the exception of some scarce studies, PLGA-based

NPs would not seem to induce such an innate response, as shown by our group and others (Dailey et al., 2006; Mura et al., 2011a, 2011b, Aragao-Santiago et al., 2015). In particular, PLGA-based NPs induced moderate but significant suppression of of T-lymphocytes proliferation (Aragao-Santiago et al., 2015). However, little is known about the effects of PLGA NPs on the activation of antigen-specific cells (APCs) such as dendritic cells (DCs). DCs play a major role in innate immunity by driving the specific reaction by the adaptive immune system. A myriad of cytokines (TNF-α, GMCSF, IL-1, …) may be produced by our innate immune system in response to a xenobiotic allowing inflammation and contributing to immunotoxic effects (Petrarca *et al.*, 2015).

Given the importance of DCs, NP impact on these cells raises growing concerns. Among the deleterious effects of NPs on DCs currently expected, immunosuppression has been suggested (Ilinskaya & Dobrovolskaia, 2014). More insidiously, NPs could act as immune adjuvants, inducing exacerbation or modification of the type of immune response to a given antigen, hence resulting in hypersensitivity or allergic reactions. Possible impacts on the immune system stress the need for a careful immunotoxicological evaluation, particularly for regulatory purposes (Dobrovolskaia *et al.* 2009, 2016).

To elucidate PLGA NP-dependent outcomes on DCs, we herein present an *in vitro* study of the effect of 100-250 nm PLGA NPs on both human monocyte-derived DCs (MoDCs) and murine bone-marrow derived DCs (BMDCs). Since the performance of nanotechnology-based drug delivery systems is strongly influenced mostly by surface properties (charge and hydrophilicity), surfactants may have a strong impact on resulting biological effects of particles, especially in terms of uptake and intracellular fate (Bhardwaj et al., 2005; Yue et al., 2011; Sadat et al., 2016; Chen et al., 2018). We

therefore conducted our study on PLGA NPs formulated with three different stabilizers: polyvinyl alcohol (PVA), chitosan (CS) or poloxamer 188 (commercially named Pluronic F68 [PF68]), resulting in neutral, positively or negatively charged particles, respectively. NP uptake by DCs was first examined. Viability, phenotypical changes and cytokine release of DCs were then assessed. To further understand underlying mechanisms involved in observed responses, intracellular signaling pathways were also investigated. We believe that this approach assessing the immune response to PLGA NPs may contribute to a better understanding of their biological fate and to a robust assessment for their use as pharmaceutical ingredients.

## Methods

### *Preparation of nanoparticle suspensions*

PLGA NPs were prepared by solvent evaporation-based methods (Mura et al., 2011a). Positively and negatively charged as well as neutral NPs were tailored by coating their surface with chitosan (CS, Protasan® UP CL113, 75%–90% deacetylation, molecular weight 50–150 kD), poloxamer 188 (commercially named Pluronic F68 [PF68]), or polyvinyl alcohol (PVA, 87%–89% hydrolyzed, molecular weight 30–70 kDa), respectively.

Neutral NPs (PLGA/PVA NPs) were prepared by dissolving 100 mg of PLGA (75:25 Resomer RG756) in 5 mL of a dichloromethane/acetone (1/1 v/v) mixture. This organic solution was pre-emulsified with 20 mL of a 0.25 % (w/v) PVA (molecular weight 30–70 kDa) aqueous solution by vortexing for one minute. The pre-emulsion was kept on ice and sonicated for one minute using a VibraCell sonicator (Fisher Scientific, Illkirch, France) at 40% power. As for positively charged NPs (PLGA/CS NPs), the organic solution of PLGA was pre-emulsified with a 0.6 % (w/v) aqueous CS

solution containing 0.5 % (w/v) of Mowiol 4-88 PVA by vortexing for one minute. This preemulsion was kept on ice and sonicated for 2 minutes using a VibraCell sonicator at 40 % power. Then, for both the neutral and positively charged NPs, the organic phase was allowed to evaporate at room temperature with magnetic stirring (600 rpm). NP dispersion was then completed to 20 mL. The excess of stabilizers was removed by centrifugation of NPs at 37,000 × *g* for one hour at 4 °C, and the pellet of NPs was resuspended in ultrapure water.

Negatively charged NPs (PLGA/PF68 NPs) were prepared using PF68 as stabilizer. PLGA was dissolved in 10 mL ethyl acetate and added dropwise into 20 mL of a 1 % (w/v) PF68 aqueous solution under vigorous magnetic stirring. The pre-emulsion was vortexed for one minute and then sonicated for another minute using a VibraCell sonicator at 40 % power. Water (20 mL) was added to the emulsion, in order to promote diffusion of the organic solvent into the external phase, leading to formation of the NPs. The organic phase was allowed to evaporate at room temperature with magnetic stirring (600 rpm).

Rhodamine B-labelled NPs were prepared as described earlier (Mura et al., 2011a) by dissolving in the organic solvent a 70/30 % (w/w) mixture of PLGA (Resomer RG756) and PLGA covalently bound to rhodamine B.

***Nanoparticle suspension characterization***

NPs were characterized in terms of size, zeta potential and surface chemical composition, confirming modifications provided by hydrophilic stabilizers. NP size (hydrodynamic diameter) and polydispersity were determined after dilution in MilliQ® water at 25°C by dynamic light scattering using a Nano ZS (Malvern Instrument, UK) set at a 173° scattering angle. The zeta potential was determined using the same instrument after dilution in 1 mM NaCl. All measurements were done in triplicate.

The amount of stabilizers associated to NPs after their purification was assessed through the formation of colored complexes of PVA and CS, or by 1H NMR in the case of PF68, as previously described (Mura et al., 2011a).

The morphology of NPs in aqueous suspension was studied by transmission electron microscopy (TEM) using a JEOL JEM-1400 microscope operating at 80 kV (filament current of 55 µA). 5 µL of NP aqueous suspensions was deposited onto copper grids covered with formvar film (400 mesh) for one minute. PLGA samples were then stained using 2% phosphotungstic acid (PLGA/PVA, PLGA/PF68) or 2% uranyl acetate (PLGA/CS) for 30 seconds. The excess liquid was blotted off using filter paper, and the grids were dried before observation. Images were acquired using a postcolumn high-resolution (11 megapixels) high-speed camera (SC1000 Orius; Gatan) and processed with Digital Micrograph (Gatan) and ImageJ.

Endotoxin level in NP suspensions was <0.05 EU/ml as tested by limulus amebocyte lysat (LAL) assay (GenScript, Piscataway, NJ, USA).

***Generation of primary cultures of human and murine dendritic cells***

The generation of human DCs derived from monocytes (MoDCs) was performed as described elsewhere (Antonios et al., 2010) from human peripheral blood supplied by the French Blood Institute (EFS, Rungis, France). First, peripheral blood mononuclear cells (PBMCs) were sorted from phosphate buffer saline (PBS) diluted buffy coats by density centrifugation on a Ficoll gradient (650 × *g*, 20 min, 19 °C). Monocytes were then isolated through positive magnetic selection using MiniMacs separation columns and anti-$CD14^+$ antibodies coated on magnetic beads (Miltenyi Biotec, Paris, France). Finally, $CD14^+$ cells were differentiated immature monocyte-derived DCs (MoDCs) for 5 days in RPMI 1640 supplemented with Glutamax-I, 10 % heat inactivated fetal calf serum (FCS), 550 U/mL granulocyte-macrophage colony-stimulating factor (rh-GM-

CSF), 550 U/mL interleukin-4 (rh-IL4), 1 % sodium pyruvate, streptomycin and penicillin.

The generation of murine DCs (BM-DCs) was performed as described elsewhere (El Ali et al., 2017). In brief, DCs were derived from bone-marrow hematopoietic progenitors collected from the femurs and tibiae of 8–10-week-old C57BL/6 mice (Janvier SAS, Saint Berthevin, France). Cells were then differentiated to immature BMDCs for 7 days in Iscove's modified Dulbecco's medium (IMDM) containing 10 % FCS, 50 µM β-mercaptoethanol and 10 % culture supernatant from mouse GM-CSF-producing J558 cells (a kind gift of S. Amigorena, Institut Curie), coresponding to a final concentration of about 40 ng/mL GM-CSF. Mice were handled in accordance with the principles and procedures in accordance with the decree n°2013-1118 of 1 February 2013 on the protection of animals, used for scientific purposes Article R214.89.

***In vitro exposure of immature dendritic cells to PLGA nanoparticles***

After proper differentiation, immature DCs were collected, washed, and resuspended in fresh complete culture medium (RPMI or IMDM for human and murine cells respectively, supplemented with 10 % FCS, 1 % sodium pyruvate and antibiotics) at a final density of $10^6$ cells/ml. Cells were then exposed to either PLGA NPs, their vehicle (distilled water, negative control), or any other chemical or biological molecule that would constitute an adequate positive control for a given analysis.

***Nanoparticle internalization by DCs***

For microscopy analysis, after exposure to NPs, cells were harvested, washed with cold PBS, fixed for 15 min in PBS supplemented with 2 % paraformaldehyde (PFA) and 1.5 % sucrose. Cells were then washed twice with PBS, incubated for 10 min in 50 mM

ammonium chloride, and washed twice with PBS once again. Cells were then mounted onto microscope slides using an appropriate mounting medium (DakoCytomation mounting medium supplemented with 4′,6-diamidino-2-phenylindole (DAPI) for nucleus labeling) and examined under a confocal microscope equipped with epifluorescence.

For cytometry analysis, after exposure to rhodamine-labelled NPs, cells were harvested, washed with cold PBS, and analyzed on a FACSCalibur® cell analyzer using the CellQuest® software (BD Biosciences). Results were expressed as percentage of positive cells.

***Cytotoxicity evaluation***

Cell viability was first assessed by the MTT (methylthiazolyldiphenyl-tetrazolium bromide) test. Immature cells were seeded at $10^5$ cells/100 µL/well into 96-well plates and exposed to 1-400 µg/mL PLGA NPs for 24 h (5 replicate wells per treatment group). At the end of the exposure, 10 µL of a 5 mg/ml MTT solution was added to each well. After 3 h at 37 °C, medium was replaced by 100 µL of dimethyl sulfoxide (DMSO) to dissolve the resulting formazan crystals and optical density was measured at 550 nm with the reference filter set to 620 nm. Cell viability values were finally determined as percent differences relative to untreated (negative) control values.

The trypan blue dye exclusion method was additionally used to assess cytotoxicity. Viable and dead cells were counted with a hemocytometer, distincted thanks to the use of trypan blue, a vital stain that only enters dead cells.

Obtained cytotoxicity curves were used to identify NP exposure conditions that do not induce more than 30 % cell death so as to investigate NP effects on DC maturation.

***Phenotypic analysis***

To assess the phenotypic effects of NP exposure on DCs, several surface maturation markers were studied. After 24 h of exposure to either 200 µg/mL PLGA NPs, distilled water (negative control) or 25 ng/mL lipopolysaccharide (LPS, positive control), DCs were collected, washed with PBS and incubated for 15 min in Fc Block (BD Biosciences, Le Pont de Claix, France) to avoid antibody preparations binding via their Fc portions to low-affinity receptors for complexed IgG. Cells were then incubated for 20 min either with fluorochrome-conjugated monoclonal antibodies (mAbs) or with appropriate isotype control antibodies (IgGs). For MoDCs, the following mouse anti-human mAbs were used: FITC-conjugated anti-CD40 (clone 5C3), APC-conjuguated anti-CD54 (ICAM-1,clone HA58), FITC-conjuguated anti-CD80 (clone L307.4), APC-conjugated anti-CD83 (clone HB15e), FITC-conjugated anti-CD86 (clone 2331(FUN-1)), FITC-conjugated anti-CD274 (PD-L1, clone MIH1), PE-conjugated anti-CD197 (CCR7) and FITC-conjugated anti-HLA-DR (clone G46-6) (BD Biosciences). For BMDCs, the following rat anti-mouse mAbs were used: FITC-conjugated anti-CD40 (clone 3/23), FITC-conjugated anti-CD86 (clone GL1), PE-conjugated anti-CD197 (CCR7, clone 4B12) (Beckman Coulter - Immunotech, Marseille, France). After mAbs or IgGs incubation, DCs were washed twice with PBS supplemented with 0.5% BSA, then once with PBS, and finally fixed in PBS supplemented with 1 % PFA.

Cells unstimuled with NP were used to determine nonspecific staining. A minimum of 20,000 cells were analyzed on a FACSCalibur cell analyzer with CellQuest software (BD Biosciences). Living cells were identified on the basis of their physical characteristics [forward scatter and side scatter. Results were expressed as percentage of positive cells (*i.e.*, cells that express the marker) as well as using the relative fluorescence intensity (RFI, *i.e.* mean fluorescence intensity of cells incubated with a

given mAb divided by mean fluorescence intensity of cells incubated with its corresponding isotype) and the "fold increase RFI" (*i.e.* NP- or LPS-exposed cells RFI divided by control cells RFI). This latter parameter (fold increase RFI) therefore reflects the mean expression level of a given maturation marker within the total cell population.

***Cytokine analysis***

The BD Biosciences cytometric bead array (CBA) technique was performed to measure cytokine concentrations within DC supernatants at the end of NP exposure. This technique employs microbeads coated with a capture antibody specific for a given soluble protein. For human MoDC supernatants, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8, IL-10, interferon-γ-induced protein (IP)-10 (also known as CXCL10), monocyte chemotactic protein (MCP)-1 (also known as CCL2) and RANTES (regulated upon activation, normal T-cell expressed and secreted, also known as CCL5) were tested. For murine BMDC supernatants, TNF-α, IL-6, IL-10, MCP-1 and CCL5 were tested.

Briefly, cell culture supernatants were either diluted or not and serial dilutions of each cytokine standard (from 10 to 2500 pg/mL) were prepared. Test samples and standard dilutions (50 µL/test) were then incubated with capture beads for 1 h at room temperature, protected from light. Then, PE detection reagents were added and an additional incubation step was performed (2 h, room temperature, protected from light). After proper washes, a three-color cytometric analysis was carried out using a FACScalibur flow cytometer (BD Biosciences). Results were generated using the FCAP Array™ Software.

***Intracellular signalling pathways***

Western blot analyses of both mitogen activated protein kinases (MAPK) and NFκ-B (nuclear factor kappa-light-chain-enhancer of activated B cells) were performed as previously described (Larangé et al., 2012). Immature DCs ($10^6$ cells/mL) were either exposed to distilled water, PLGA NPs (200 µg/mL), or LPS (25 ng/mL) for 15, 30, 60 or 120 min. At the end of their stimulation, cells were harvested, washed with PBS and pelleted by centrifugation. Cell lysates were then prepared by resuspending cell pellets in lysis buffer (20 mM Tris pH 7.4, 137 mM NaCl, 2 mM EDTA pH 7.4, 2 mM sodium pyrophosphate, 1 % Triton X-100, 10 % glycerol, 1 mM PMSF, 1 mM sodium orthovanadate, 25 mM β-glycerophophate, 10 µg/mL aprotinin, 10 µg/mL leupeptin and 100 µg/mL pepstatin). Total protein extracts were then collected after a 20 min 15,000 rpm centrifugation. Fifty µg of denaturated protein were then loaded onto 12.5 % SDS-PAGE gel and transferred on polyvinylidene fluoride (PVDF) membrane (Amersham Biosciences, Les Ulis, France). Membranes were then incubated with antibodies directed to the phosphorylated forms of p38MAPK (Thr180/Tyr182), JNK 1/2 (Thr183/Tyr185), ERK 1/2(Thr202/Tyr204), NFκ-B p65(Ser536) (all from Cell Signaling Technology, Ozyme, St-Quentin-en-Yvelines, France) or to IκB-α (C-21, Santa Cruz biotechnology, Santa Cruz, CA). Total p38MAPK was used as a loading control with an antibody raised against total p38MAPK (Cell Signaling Technology). After proper washes and incubation with secondary HRP-linked antibody, immunoreactive bands were detected bychemiluminescence (ECL solution, Amersham Biosciences) using a ChemiDoc XRS Imager (Bio-Rad, Marnes-La-Coquette, France). Bands were quantified by densitometry using theImageLab software.

***T-lymphocyte proliferation***

The capacity of MoDCs to activate T cells has been evaluated by the use of T CD4+ T-cells lymphocytes labelled with 0.5 µM CFSE following the manufacturer's instructions. T cells are isolated from PBMC by positive selection with the MACS CD4 isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). These T cells are confirmed to have purity greater than 95%, based on CD4 (555349, BD Biosciences) expression evaluated by flow cytometry. MoDCs are pre-loaded with purified protein derivative (*PPD*) or not and then stimulated with NPs. Treated MoDCs were washed and then cultured with autologous T cells at 3 different ratio a 1:8, 1:4, 1:2 DC/T-cell ratio for 5 days in RPMI 1640 Glutamax supplemented with 10% AB human serum, in round bottomed 96-well plates. On day 5, CD4+T-cells proliferation is analyzed by flow cytometry and expressed as the percentage of CFSE low T cells present in the sample.

***Statistical analysis***

Significance of obtained results was calculated using nonparametric one-way analyses of variance on ranks approach (Kruskall-Wallis) combined with Mann-Whitney U tests for multiple comparisons (Statistica 7.0, Statsoft, Chicago, IL, USA). Data are expressed as means ± S.E.M. Effects were designated significant at $p < 0.05$ (*) or at $p < 0.001$ (**).

## Results

***PLGA nanoparticle properties***

As shown in Table 1, the selection of different stabilizers allowed the modification of NP surface charge. PLGA/PF68 NPs had a mean diameter of 100 nm, while both PLGA/PVA and PLGA/CS NPs were around 200 nm. All formulations had a narrow size distribution, with a polydispersity index of 0.1–0.2. Transmission electron

microscopic observations showed spherical NPs with a smooth surface. Zeta potential measurements confirmed that the stabilizers influence NP surface charge. CS-coated NPs exhibited a positive zeta potential (+32 ± 3 mV), whereas PVA-coated NPs were almost neutral (−5 ± 1 mV), and PF68-coated NPs exhibited a negative zeta potential (−24 ± 1 mV). The presence of stabilizers on the NP surface was quantified by spectrophotometric methods and $^{1}$H NMR spectroscopy. Between 10 mg and 30 mg of stabilizer (per 100 mg of PLGA) was found to be associated with the NPs (Table 1). The use of Rhod-PLGA to render fluorescent NPs did not influence their size distribution nor their surface charge. The only exception was Rhod-PLGA/CS NPs, the size and zeta potential of which slightly decreased (Table 1) due to the presence of rhodamine moieties on the NP surface. An important point is to monitor the possible aggregation of NPs in serum-containing media (Hinderliter et al., 2010), especially since the PLGA NPs used in this study are expected to be transported by sedimentation (DeLoid et al., 2017). The dispersion of the three NP types in fetal bovine serum-containing cell culture medium resulted in only minor modifications of the mean hydrodynamic diameters for up to 72 h incubation, and the same trends in zeta potential values (Table S1), despite the complexity of the cell culture medium.

***Cytotoxic effects of PLGA nanoparticles on dendritic cells***

Our toxicological evaluation of PLGA NPs first consisted in the evaluation of the cytotoxic impact of NP exposure on DCs. Results obtained by both the MTT test (Figure 1) and the trypan blue dye exclusion assay (data not shown) showed that human and murine DCs underwent about 5 to 10 % cell death after 24 h of exposure to concentrations as high as 0.5 mg/ml NPs. Given such relatively high NP concentrations needed to induce only moderate cell death within the DC populations, these first results clearly indicate that PLGA NPs exert very low cytotoxic effects on DCs.

***PLGA nanoparticle uptake and internalization into dendritic cells***

NP uptake by DCs was assessed from a quantitative point of view using flow cytometry by monitoring rhodamine-labelled NP fluorescence associated to DCs. In human DC (MoDC), results show that PVA-coated PLGA NPs were the most efficiently taken up by human DCs (Figure 2A). With these particles, fluorescence measured after 24 h of exposure was about 70 times higher than cellular autofluorescence, while CS- and PF68-coated PLGA NPs entry resulted in a 40-fold increase of cellular fluorescence. Interestingly, fluorescence measurements carried out at early time points indicated that for both PVA- and CS-coated NPs, the major part of the 24 h NP entry within cells occurred within the very first hours of exposure (about 75 % after 3 h and 90 % after 6 h). Conversely, PF68-coated NP uptake by cells went on according to a slower mechanism since only 40 % of the 24-h NP entry within cells occurred within the first 6 hours of exposure. In murine DC (BMDC), Rresults show the major part of the 24h NP entry within cells occurred after 3 hours of exposure. PVA-coated PLGA NPs were also the most efficiently taken up by BMDC (Figure 2B). With these particles, fluorescence measured after 24 h of exposure was about 50 times higher than cellular autofluorescence compared to 40 % with CS-coated NPs. Regarding PF68-coated NP, uptake by BMDC is very low.

To better understand the PLGA NP interaction with DCs, we then used rhodamine-labelled NPs to assess NP intracellular localization within DCs. Confocal microscopic observations showed that, after 24 h of exposure, very large quantities of PLGA NPs were encountered within the cytoplasm of both human DCs and murine (Figures 2A & B).

The herein demonstrated capacity of DCs to internalize large quantities of PLGA NPs is in good accordance with their constitutive ability to constantly sample

their surrounding environment since they act as sentinels of the immune system. Interestingly, surfactants exert a clear effect on NP internalization, which might be due to differences of the electric charge present at the surface of particles. According to our present results, PVA-coated NPs, which are uncharged particles, are the most rapidly and efficiently internalized. Regarding CS- and PF68-coated NPs, which are charged particles, similar quantities of NPs entered the cells after a 24-h exposure period. However, negatively charged particles (i.e. PF68-coated ones) seemed to be internalized according to a slower kinetic pattern than both uncharged and positively charged particles.

***Surface protein modifications in dendritic cells after PLGA nanoparticle exposure***

To assess the phenotypic effects of NP exposure on DCs, we first studied the modification of membrane markers of maturation. Obtained results clearly indicate that a 24-h exposure to 200 µg/mL PLGA NPs induced moderate modifications of herein tested maturation markers. Regarding human DCs (Figure 3A), we noticed subtle (fold increases generally <2) but significant enhancement of the surface expression of the CD83 maturation marker, the CD54 adhesion molecule (also known as Inter-Cellular Adhesion Molecule 1, ICAM-1), as well as of costimulatory molecules such as the CD40 receptor that interact with the CD40L of T cells and several members of the B7 family ligands (CD80/B7-1, CD86/B7-2 and PD-L1/CD274/B7-H1) that bind to the CD28 family receptors of T cells. Similar results were obtained in murine DCs at the surface of which expressions of CD40, CD86 and CCR7 (a chemokine receptor) molecules were significantly increased (Figure 3B).

Both human and murine DCs can therefore be considered as in an activated state, able to be engaged in immunological synapses with T cells. However, it is

noticeable that ~~PVA-coated~~ NPs generally exerted ~~the most marked~~ slight effects effects on murine DC phenotype compared to human DC. ~~, while CS- and PF68-coated NPs induced only slight effects.~~

The effect of a soluble surfactant exposed to cells at an equivalent concentration to that associated to NPs was evaluated in the case of PVA vs. PVA-coated NPs (Figure S1A). For all human DC markers, no significant increase~~d~~ was noticed for free PVA.

***Cytokine secretion profile of dendritic cells exposed to PLGA nanoparticles***

As a second approach of the phenotypic impact of PLGA NP exposure on DCs, we evaluated the secretory activity of DCs (Figure 4). Eight cytokines and chemokines were tested in the culture medium supernatants of human MoDCs collected after 24 h of exposure to NPs. Results showed that the pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α were induced by human DCs. A similar induction was observed for the CXCL10 and CCL5 chemokines (Figure 4A). Conversely, in murine BMDCs, no effect on secretory activity of cells was observed as a result of NP exposure. Interleukine-6 and TNF-α cytokines even tended to be encountered in cell media at lower concentrations when cells were exposed to PLGA NPs. However, such tendency was not statistically significant (Figure 4B).

Our results therefore indicate that human MoDCs but not murine BMDCs enhanced their cytokine secretory activity as a result of NP exposure. In human CDs, the three categories of NPs induce the same cytokines (IL-6, IL-8 and CXCL10) in identical proportions. In murine CDs, the base level of cytokine production is higher, therefore no effect is reported on cytokine production by the NPs.

~~As for membrane maturation markers, surfactants had an impact on cytokine secretions since PF68-coated NPs generally exerted the slightest effects~~. As for maturation markers, the effects of PVA alone were tested (Figure S1B). With the

exception of IL-8 secretion, no significant differences were observed compared to control conditions, suggesting that surfactant effect was only due to its direct association to NPs. In the case of IL-8, a slight but significant increase in its secretion was noticed as compared to controls. However, effects of PVA alone were lower than PVA-coated NP effects.

***Investigation of signalling pathways activated as a result of PLGA nanoparticle exposure***

Considering the results obtained regarding both surface maturation marker expressions and cytokine secretions, we then tried to elucidate the underlying signalling pathways involved in DC maturation. We first carried out western blot experiments to follow the time evolution of the intracellular contents of the phosphorylated forms of mitogen activated protein kinases (p38MAPK, JNK 1/2, ERK 1/2) and of the p65 subunit of NFκ-B, as well as the time evolution of IκB-α content, its cytoplasmic inhibitor (Figure 5). We showed that exposure to PVA- and PF68-coated PLGA NPs induce the significant activation of both JNK and ERK 1/2 pathways as indicated by the increase of the phosphorylated forms of these proteins in human DCs. Regarding CS-coated PLGA NP exposure, JNK pathway was not activated but both p38 and ERK 1/2 kinases were phosphorylated. NFκ-B pathway study did not show any significant activation.

***T lymphocyte proliferation is not modulated in response to PLGA nanoparticle***

MoDCs pre-loaded purified protein derivative (*PPD*) were treated with NPs, then washed and cultured with purified autologous ~~CD4+ T lymphocytes~~ CD4+ T-cells cells at 3 different ratio a 1:8, 1:4, 1:2 DC/T-cell ratio for 5 days. On day 5, ~~CD4 cells~~ CD4+ T ~~lymphocytes~~ cells proliferation was analyzed by flow cytometry and expressed as the percentage of CFSE low T cells. On day 5, <20% of total cells were CFSElow at ratio

1/4 in absence of NP. MoDC loaded with PPD and treated with LPS allow us to appreciate the maximal of proliferation of the cells, 50 % at ratio 1/4. MoDCs preloaded or not with PPD have a quit similar proliferation in regard to the ratio. NP did not affect the proliferation of lymphocytes in response to MoDC preloaded with PPD whatever the ratio tested (Figure 6). These results suggest that NP-stimulated MoDCs do not augment the proliferation of $CD4^+$ T-cells ~~lymphocytes~~. Collectively, these findings reveal that PLGA NPs do not induce the capacity of mature moDC to activate autologous CD4+ T cells proliferation.

**Discussion**

The use of NPs in medicine requires the understanding of their possible adjuvant effect in the immune response. In the particular case of PLGA materials, some questions regarding immune consequences of their use as NP carriers still remain unclear. It is currently accepted that charging professional APC with antigens loaded onto PLGA vehicles leads to a presentation of MHC class I–antigen complexes over an extended period (Audran et al., 2003). More generally, PLGA NP carriers are considered as efficient carriers of antigens in comparison with the use of soluble forms of antigens (Diwan et al., 2003). However, to this day, professional APC activation has not been fully investigated in response to PLGA NP. The current study was therefore conducted to examine PLGA NP effects on DCs. A particular emphasis was placed on the impact of polyelectrolyte coatings on the particle uptake by cells, as well on the phenotype and secretory profile of DCs. To this purpose, PLGA NPs were coated either with CS, PVA, or PF68 stabilizers, resulting in significant differences in their zeta potential (the resulting particles being either cationic, neutral or anionic, respectively).

Regarding NP uptake, our results showed that, irrespective of their surface charge, all herein tested NP formulations were efficiently taken up and internalized by

DCs, as evidenced by cytometry analysis and confocal microscopy observations, respectively. Uptake of PLGA nano- and micro-particles has previously been demonstrated *in vitro* by human peripheral blood-monocytes-derived DCs (Walter et al., 2001; Lustiak et al., 2002; Waeckerle-Men et al., 2004; Yoshida & Babensee, 2006b; Fischer et al., 2007; Ma et al., 2011), human cord-blood CD34+ stem cells-derived DCs (Diwan et al., 2003), mouse bone marrow-derived DCs (Elamanchili et al., 2004; Ma et al., 2011), and *in vivo* upon intradermal immunization (Newman et al., 2002). Some of these authors additionally reported that PLGA NPs were not detectable inside the DCs when cells were pretreated with cytochalasin, an inhibitor of actin polymerization, or when incubation with the fluorospheres was carried out at 4°C, therefore demonstrating that NP internalization is achieved by a phagocytosis phenomenon (Lustiak et al., 2002; Yoshida & Babensee, 2006b). This conclusion was supported by electron microscopy studies, where membrane ruffling around the phagocytosed particle was visualized (Diwan et al., 2003). According to Hamdy et al. (2011), the uptake of PLGA particles is affected by surface charge. Cationic particles would indeed be particularly effective for uptake by DCs. The ionic attraction between the positively charged particles and the negatively charged cell surface initiates efficient binding and facilitate particle internalization, even though the ossible formation of a protein corona around the NPs in serum-containing media should be taken into account (Tenzer et al., 2011). In our study, cationic (CS-coated) NPs remained positively-charged in cell culture media, and were indeed rapidly engulfed by DCs since about 80 % of the particles that were encountered into the cells after 24 h of exposure actually entered the cells within the first 3 h of incubation. Anionic (PF68-coated) NPs were taken up by DCs according to a slower kinetics. Despite this difference, after 24 h of incubation, both anionic and cationic particles were internalized to a similar extent on a quantitative basis. Neutral (PVA-

coated) NPs appeared as the most efficiently internalized particles since DC fluorescence was about twice as high compared to CS- or PF68-coated NP exposed cells after 24 h. Such a difference shows that electrostatic interactions cannot account alone for internalization by cells, and could be related to the fact that neutral particles are less susceptive to attract surrounding molecules such as proteins that are present in the culture medium.

As for their cytotoxic potential, PLGA NPs do not significantly affect the survival of DCs whatever their charge, even when the cells were loaded with very large amounts of NPs, which is in total agreement with others studies (Walter et al., 2001; Waeckerle-Men et al., 2004; Fischer et al., 2007; Semete et al., 2010). Previous studies on PLGA NPs displaying positive, negative or neutral surface charge revealed that positively-charged NPs appeared to be more cytotoxic and genotoxic to different cell lines with ROS induction linked to high endocytosis and chromosomal aberrations, contrary to other NP types that appear to be relatively inert (Platel et al., 2016).

Noticeable phenotypic changes induced in both human and murine DCs in response to PLGA NP exposure as evidenced by significant upregulations (CD86, CD80, CD40, CD83), are consistent with a change allowing the transition from an antigen-capturing to an antigen-presenting state of DCs (Banchereau and Steinman, 1998). The observed effects are attributed to PLGA NP free of endotoxin since the endotoxin levels were below the USP prescribed content in water for injection (0.25 endotoxin units/mL, as tested by the LAL assay). Even if all tested PLGA NPs had an impact on DC maturation, PVA NPs were the most potent since they exerted a higher induction of all surface markers (*i.e.* CD40, CD54, CD80, CD83, CD86 and PDL-1 in MoDCs; CD40, CD86 and CCR7 in BMDCs). This maturation can be correlated with the higher and rapid phagocytosis of PVA-coated NPs. However, Human DCs are more

sensitive than murine DCs in response to nanoparticles since many surface markers are induced, as are cytokines.

Although some literature describes the absence of DC maturation upon PLGA particle exposure, both in MoDCs (Walter et al., 2001; Waeckerle-Men et al., 2004; Fischer et al., 2007; Bivas-Benita et al., 2009) and BMDCs (Sun et al., 2003; Clawson et al., 2010; Ma et al., 2011), our study confirms a large number of studies demonstrating phenotypical changes induced in both human and murine DCs exposed to PLGA films and particles (Yoshida & Babensee, 2004, 2006a; Babensee & Paranjpe, 2005; Diwan et al., 2003; Sun et al., 2003; Elmanchili et al., 2004; Yoshida et al., 2007). Moreover, we demonstrate a relationship between PLGA particle charge and the acquired DC phenotype, which remained an open question in several studies (Fischer et al., 2007; Jilek et al., 2004).

Beside the expression of maturation markers, the secretion of cytokines is indeed crucial for T-cell activation and efficient antigen presentation. We therefore investigated the influence NPs on cytokine secretion of DCs. Our results clearly demonstrated that, upon exposure to PLGA NPs, MoDCs secrete significant amounts of pro-inflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α) and chemokines (CXCL10, CCL5), consistent with the maturation of human DCs upon exposure to PLGA NPs, and with other studies demonstrating the impact of PLGA particles on cytokine secretion by DCs. The induction of IL-6, IL-8 and TNF-α by PLGA-exposed MoDCs may control a TH1 response as suggested elsewhere (Yoshida & Babensee, 2006a,b). This result is in agreement with another study in which the delivery of a model antigen with PLGA particles resulted in a TH1 response as measured by production of IgG2 isotype antibody against the antigen (Newman et al., 1998).

However, PLGA NP modules CD40 and CD86 in both DC models (BMDC or MoDC) equally. The differences mainly observed in cytokine production by MoDCs might be explained by the origin of DC. Human MoDCs are inflammatory monocyte-derived DC close to conventional DC while murine DCs are differentiated from CD34.

The effect of the surfactant themselves have been raised in previous reports on PLGA NPs (Guedj et al., 2015; Menon et al., 2012). Our results show that the effects of PVA alone, exposed at an equivalent concentration to that associated to NPs, had no own effect on DC maturation. This can be related to previous observations that PVA remains strongly associated to PLGA NPs even after thorough purification (Spek et al., 2015). Regarding the contribution of the PLGA core on the cell response, previous reports by our group (Grabowski et al., 2015) showed that dendritic cell surrogates do not show any difference in terms of mitochondrial activity and necrosis induction following exposure of PVA-coated PLGA nanoparticles and surfactant-free PLGA nanoparticles, which were obtained by a specific preparation technique, at concentration ranges used in the present study (<1 mg/mL).

Phenotypic changes and concomitant cytokine secretion are maturation hallmarks that result from intracellular signaling cascades. Different signaling pathways such as MAPKs and NF-κB play important roles in DC maturation (Neves et al., 2011). Regarding the maturation process, it has been proved that different chemicals could stimulated different signal transduction pathways and subsequently induced different phenotypic and functional changes in DC, such as MAPKs and NF-κB signalling pathways (Antonios et al., 2010). In our hands, MAPK pathways were activated in response to all PLGA NPs, but at different levels depending on the type of PLGA NPs. Our results show a nice correlation between MAPKs and human DC maturation induced by PLGA NPs.

In addition, we show that PLGA NP do not affect the activation of T lymphocytes in response to an antigen such as PPD. This confirms these NP do not modify the adaptive immune response of DC even if the phenotype is slightly activated.

## Conclusions

Our study indicates that PLGA-NPs induce clear consequences on the pivotal properties of DCs *in vitro.* Exposure of immature DCs to three PLGA NP types with different surface coatings and surface charge resulted in marked particle uptake by the cells, but no significant cell death. Nevertheless, these NPs were not as inert as expected since alterations in DC phenotype were reported, as assessed by the expression levels of cell surface markers of DC maturation. In addition, the functional secretory activity of DCs was largely altered upon phagocytosis of the different NPs. The study presented herein lays a foundation for studying the effects of PLGA nanomaterials on the immune system prior to their use as vehicles in nanomedicine. This study allowed us to indicate that interspecific differences are important to take into account since human activation of CDs is higher than that of murine CDs. Human DCs are  and This is the first study that demonstrates MAPK activation by PLGA exposure in DCs, and the potential role of surfactants in the effectively taken pathways. Further studies are needed to study the role of encapsulated bioactive compounds on the effects of such PLGA nanocarriers, which are relevant for drug and vaccine delivery purposes.

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## Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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## Table

**Table 1. PLGA nanoparticle characterization.**

Data are presented as mean ± SD. Abbreviations: CS, chitosan; PF68, poloxamer 188; PLGA, poly(lactide-co-glycolide); PVA, poly(vinyl alcohol); rhod, rhodamine B; TEM, transmission electron microscopy.

**Figures captions**

**Figure 1. Effects of PLGA NPs on cell viability.** Human MoDCs (solid lines) and murine BMDCs (dashed lines) were exposed to PLGA NPs (0-500 µg/mL) for 24 h. Cell death was assessed by the MTT test.

**Figure 2. PLGA nanoparticle uptake and internalization. A. Confocal microscopy observations.** *Up.* Human DCs were exposed for 24 h to rhodamine coated-PLGA NPs (200 µg/mL). From left to right: PLGA/PVA, PLGA/CS, PLGA/PF68. Lysosomes were labeled using a green LysoTracker® probe (InVitroGen). **Flow cytometry fluorescence analysis** *Down.* Human DCs were exposed for 3, 6 or 24 h to rhodamine-coated PLGA NPs (0-400 µg/mL). From left to right: PLGA/PVA, PLGA/CS, PLGA/PF68. Red fluorescence was then measured by flow cytometry. Fluorescence intensity of NP exposed cells was then divided by fluorescence intensity of control cells (i.e. autofluorescence). **B. Confocal microscopy observations.** *Up.* Murine DCs were exposed for 24 h to rhodamine coated-PLGA NPs (200 µg/mL). From left to right: PLGA/PVA, PLGA/CS, PLGA/PF68. Lysosomes were labeled using a green LysoTracker® probe (InVitroGen). **Flow cytometry fluorescence analysis** *Down.* Murine DCs were exposed for 3, 6 or 24 h to rhodamine-coated PLGA NPs (0-400 µg/mL). From left to right: PLGA/PVA, PLGA/CS, PLGA/PF68. Red fluorescence was then measured by flow cytometry. Fluorescence intensity of NP exposed cells was then divided by fluorescence intensity of control cells (i.e. autofluorescence).

**Figure 3. Phenotypic analysis of dendritic cells treated by PLGA NPs.** Human MoDCs (**A**) and murine BMDCs (**B**) were exposed for 24 h to distilled water (unexposed cells, white), PLGA NPs (200 µg/mL, grey) , or lipopolysaccharide (LPS, 25 ng/mL, positive control, black). Phenotypic markers of maturation were then studied using flow cytometry analysis of fluorescent immunolabelling (statistically significant differences from unexposed cells: ~~grey star, $p<0.1$~~; ~~black star~~, *, $p<0.05$; **; $p<0.01$, n=5).

**Figure 4. Cytokine production by DCs.** MoDCs (**A**) and murine BMDCs (**B**) were exposed for 24 h to distilled water (unexposed cells, white), PLGA NPs (200 µg/mL, grey), or lipopolysaccharide (LPS, 25 ng/mL, positive control, black). Cytokine concentrations were then measured in exposure medium using the cytometric bead array

technique (statistically significant differences from unexposed cells: grey star, $p<0.1$; black star, $p<0.05$; two stars, $p<0.001$; $n = 5$).

**Figure 5. PLGA NPs modulate MAPK phosphorylation in MoDCs.** Human MoDCs were exposed for 15 min, 30 min, 1 h or 2 h to distilled water (unexposed cells, white ), PLGA NPs (grey ,200 µg/mL), or lipopolyssacharide (LPS, 25 ng/mL, positive control, black). Cells were lysed and the level of phosphorylated p38MAPK, JNK and ERK was evaluated by western-blotting. Membrane was then probed with anti-p38MAPK Ab for loading control. **A.** Results of a representative experiment **B.** Quantitative time evolution of phosphorylated MAPKs. Folds represent the ratio of normalized intensity of specific band divided by the normalized intensity of unexposed cells (statistically significant differences from unexposed cells: ~~grey star, p<0.1; black star, p<0.05;~~ *, $p<0.05$; **; $p< 0.01$ $n=5$).

**Figure 6. T lymphocyte proliferation is not modulated in response to PLGA NPs.**

MoDCs pre-loaded with purified protein derivative (*PPD*) or not were stimulated with NPs. Treated MoDCs were washed and then cultured with CFSE-loaded $CD4^+$ T autologous ~~T~~ cells at 3 different ratio a 1:8, 1:4, 1:2 DC/T-cell ratio for 5 days. On day 5, CD4+ T-cells proliferation was analyzed by flow cytometry and expressed as the percentage of CFSE low T cells present in the sample ($n =2$).