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► To cite this version:

S. Barillet, E. Fattal, S. Mura, N. Tsapis, M. Pallardy, et al.. Immunotoxicity of poly (lactic-co-glycolic acid) nanoparticles: influence of surface properties on dendritic cell activation. *Nanotoxicology*, 2019, 13 (5), pp.606-622. <10.1080/17435390.2018.1564078>. <hal-02323703v2>

HAL Id: hal-02323703

<https://hal.science/hal-02323703v2>

Submitted on 7 Nov 2019

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HAL Authorization

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

3 Barillet S.^a, Fattal E.^b, Mura S.^b, Tsapis N.^b, Pallardy M.^a, Hillaireau H.^{b,o},
4 Kerdine-Römer S.^{a,o,*}

5 *^aUMR996 Inflammation, Chemokines and Immunopathology, INSERM, Univ. Paris-*
6 *Sud, Université Paris-Saclay, Châtenay-Malabry, France; ^b Institut Galien Paris-Sud,*
7 *Univ. Paris-Sud, CNRS, Université Paris-Saclay, Châtenay Malabry, France.*

8 ^oequal contribution

9 *corresponding author: UMR996, Faculté de Pharmacie, Univ. Paris-Sud, 5 rue J.B.
10 Clément, 92296 Châtenay-Malabry, France.

11 email: saadia.kerdine-romer@u-psud.fr

12

13 Running Title

14 Immunotoxicity of PLGA nanoparticles on dendritic cell activation

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

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

26

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

28

1 **Introduction**

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

19 In the specific context of nanotoxicology, the field of immunotoxicology is
20 gaining a growing interest, as the interactions between NPs and the immune system
21 have proven pivotal in the response to NP exposure (Dobrovolskaia & McNeil, 2007;
22 Dobrovolskaia et al., 2009; Zolnik et al., 2010; Blank et al., 2011; Di Gioacchino et al.,
23 2011; Oostingh et al., 2011). The immune response to foreign chemical and/or
24 biological molecules as well as to particles is typically initiated through a protective
25 innate inflammatory response. With the exception of some scarce studies, PLGA-based

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

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

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

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

10 **Methods**

11 *Preparation of nanoparticle suspensions*

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

18 Neutral NPs (PLGA/PVA NPs) were prepared by dissolving 100 mg of PLGA
19 (75:25 Resomer RG756) in 5 mL of a dichloromethane/acetone (1/1 v/v) mixture. This
20 organic solution was pre-emulsified with 20 mL of a 0.25 % (w/v) PVA (molecular
21 weight 30–70 kDa) aqueous solution by vortexing for one minute. The pre-emulsion
22 was kept on ice and sonicated for one minute using a VibraCell sonicator (Fisher
23 Scientific, Illkirch, France) at 40% power. As for positively charged NPs (PLGA/CS
24 NPs), the organic solution of PLGA was pre-emulsified with a 0.6 % (w/v) aqueous CS

1 solution containing 0.5 % (w/v) of Mowiol 4-88 PVA by vortexing for one minute. This
2 preemulsion was kept on ice and sonicated for 2 minutes using a VibraCell sonicator at
3 40 % power. Then, for both the neutral and positively charged NPs, the organic phase
4 was allowed to evaporate at room temperature with magnetic stirring (600 rpm). NP
5 dispersion was then completed to 20 mL. The excess of stabilizers was removed by
6 centrifugation of NPs at $37,000 \times g$ for one hour at 4 °C, and the pellet of NPs was
7 resuspended in ultrapure water.

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

16 Rhodamine B-labelled NPs were prepared as described earlier (Mura et al.,
17 2011a) by dissolving in the organic solvent a 70/30 % (w/w) mixture of PLGA
18 (Resomer RG756) and PLGA covalently bound to rhodamine B.

19 *Nanoparticle suspension characterization*

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

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

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

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

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

16 The generation of human DCs derived from monocytes (MoDCs) was performed as
17 described elsewhere (Antonios et al., 2010) from human peripheral blood supplied by
18 the French Blood Institute (EFS, Rungis, France). First, peripheral blood mononuclear
19 cells (PBMCs) were sorted from phosphate buffer saline (PBS) diluted buffy coats by
20 density centrifugation on a Ficoll gradient (650 × g, 20 min, 19 °C). Monocytes were
21 then isolated through positive magnetic selection using MiniMacs separation columns
22 and anti-CD14⁺ antibodies coated on magnetic beads (Miltenyi Biotec, Paris, France).
23 Finally, CD14⁺ cells were differentiated immature monocyte-derived DCs (MoDCs) for
24 5 days in RPMI 1640 supplemented with Glutamax-I, 10 % heat inactivated fetal calf
25 serum (FCS), 550 U/mL granulocyte-macrophage colony-stimulating factor (rh-GM-

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

3 The generation of murine DCs (BM-DCs) was performed as described
4 elsewhere (El Ali et al., 2017). In brief, DCs were derived from bone-marrow
5 hematopoietic progenitors collected from the femurs and tibiae of 8–10-week-old
6 C57BL/6 mice (Janvier SAS, Saint Berthevin, France). Cells were then differentiated to
7 immature BMDCs for 7 days in Iscove's modified Dulbecco's medium (IMDM)
8 containing 10 % FCS, 50 μ M β -mercaptoethanol and 10 % culture supernatant from
9 mouse GM-CSF-producing J558 cells (a kind gift of S. Amigorena, Institut Curie),
10 corresponding to a final concentration of about 40 ng/mL GM-CSF. Mice were handled
11 in accordance with the principles and procedures in accordance with the decree n°2013-
12 1118 of 1 February 2013 on the protection of animals, used for scientific purposes
13 Article R214.89.

14

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

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

22 *Nanoparticle internalization by DCs*

23 For microscopy analysis, after exposure to NPs, cells were harvested, washed with cold
24 PBS, fixed for 15 min in PBS supplemented with 2 % paraformaldehyde (PFA) and
25 1.5 % sucrose. Cells were then washed twice with PBS, incubated for 10 min in 50 mM

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

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

10 *Cytotoxicity evaluation*

11 Cell viability was first assessed by the MTT (methylthiazolyldiphenyl-tetrazolium
12 bromide) test. Immature cells were seeded at 10^5 cells/100 μ L/well into 96-well plates
13 and exposed to 1-400 μ g/mL PLGA NPs for 24 h (5 replicate wells per treatment
14 group). At the end of the exposure, 10 μ L of a 5 mg/ml MTT solution was added to
15 each well. After 3 h at 37 °C, medium was replaced by 100 μ L of dimethyl sulfoxide
16 (DMSO) to dissolve the resulting formazan crystals and optical density was measured at
17 550 nm with the reference filter set to 620 nm. Cell viability values were finally
18 determined as percent differences relative to untreated (negative) control values.

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

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

1 ***Phenotypic analysis***

2 To assess the phenotypic effects of NP exposure on DCs, several surface maturation
3 markers were studied. After 24 h of exposure to either 200 µg/mL PLGA NPs, distilled
4 water (negative control) or 25 ng/mL lipopolysaccharide (LPS, positive control), DCs
5 were collected, washed with PBS and incubated for 15 min in Fc Block (BD
6 Biosciences, Le Pont de Claix, France) to avoid antibody preparations binding via their
7 Fc portions to low-affinity receptors for complexed IgG. Cells were then incubated for
8 20 min either with fluorochrome-conjugated monoclonal antibodies (mAbs) or with
9 appropriate isotype control antibodies (IgGs). For MoDCs, the following mouse anti-
10 human mAbs were used: FITC-conjugated anti-CD40 (clone 5C3), APC-conjugated
11 anti-CD54 (ICAM-1, clone HA58), FITC-conjugated anti-CD80 (clone L307.4), APC-
12 conjugated anti-CD83 (clone HB15e), FITC-conjugated anti-CD86 (clone 2331(FUN-
13 1)), FITC-conjugated anti-CD274 (PD-L1, clone MIH1), PE-conjugated anti-CD197
14 (CCR7) and FITC-conjugated anti-HLA-DR (clone G46-6) (BD Biosciences). For
15 BMDCs, the following rat anti-mouse mAbs were used: FITC-conjugated anti-CD40
16 (clone 3/23), FITC-conjugated anti-CD86 (clone GL1), PE-conjugated anti-CD197
17 (CCR7, clone 4B12) (Beckman Coulter - Immunotech, Marseille, France). After mAbs
18 or IgGs incubation, DCs were washed twice with PBS supplemented with 0.5% BSA,
19 then once with PBS, and finally fixed in PBS supplemented with 1 % PFA.

20 Cells unstimulated with NP were used to determine nonspecific staining. A minimum of
21 20,000 cells were analyzed on a FACSCalibur cell analyzer with CellQuest software
22 (BD Biosciences). Living cells were identified on the basis of their physical
23 characteristics [forward scatter and side scatter. Results were expressed as percentage of
24 positive cells (*i.e.*, cells that express the marker) as well as using the relative
25 fluorescence intensity (RFI, *i.e.* mean fluorescence intensity of cells incubated with a

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

5 ***Cytokine analysis***

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

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

1 ***Intracellular signalling pathways***

2 Western blot analyses of both mitogen activated protein kinases (MAPK) and NFκ-B
3 (nuclear factor kappa-light-chain-enhancer of activated B cells) were performed as
4 previously described (Larangé et al., 2012). Immature DCs (10^6 cells/mL) were either
5 exposed to distilled water, PLGA NPs (200 µg/mL), or LPS (25 ng/mL) for 15, 30, 60
6 or 120 min. At the end of their stimulation, cells were harvested, washed with PBS and
7 pelleted by centrifugation. Cell lysates were then prepared by resuspending cell pellets
8 in lysis buffer (20 mM Tris pH 7.4, 137 mM NaCl, 2 mM EDTA pH 7.4, 2 mM sodium
9 pyrophosphate, 1 % Triton X-100, 10 % glycerol, 1 mM PMSF, 1 mM sodium
10 orthovanadate, 25 mM β-glycerophosphate, 10 µg/mL aprotinin, 10 µg/mL leupeptin and
11 100 µg/mL pepstatin). Total protein extracts were then collected after a 20 min
12 15,000 rpm centrifugation. Fifty µg of denaturated protein were then loaded onto
13 12.5 % SDS-PAGE gel and transferred on polyvinylidene fluoride (PVDF) membrane
14 (Amersham Biosciences, Les Ulis, France). Membranes were then incubated with
15 antibodies directed to the phosphorylated forms of p38MAPK (Thr180/Tyr182), JNK
16 1/2 (Thr183/Tyr185), ERK 1/2(Thr202/Tyr204), NFκ-B p65(Ser536) (all from Cell
17 Signaling Technology, Ozyme, St-Quentin-en-Yvelines, France) or to IκB-α (C-21,
18 Santa Cruz biotechnology, Santa Cruz, CA). Total p38MAPK was used as a loading
19 control with an antibody raised against total p38MAPK (Cell Signaling Technology).
20 After proper washes and incubation with secondary HRP-linked antibody,
21 immunoreactive bands were detected bychemiluminescence (ECL solution, Amersham
22 Biosciences) using a ChemiDoc XRS Imager (Bio-Rad, Marnes-La-Coquette, France).
23 Bands were quantified by densitometry using theImageLab software.

24 ***T-lymphocyte proliferation***

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

13 *Statistical analysis*

14 Significance of obtained results was calculated using nonparametric one-way analyses
15 of variance on ranks approach (Kruskall-Wallis) combined with Mann-Whitney U tests
16 for multiple comparisons (Statistica 7.0, Statsoft, Chicago, IL, USA). Data are
17 expressed as means \pm S.E.M. Effects were designated significant at $p < 0.05$ (*) or at p
18 < 0.001 (**).

19 **Results**

20 *PLGA nanoparticle properties*

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

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

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

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

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

2 NP uptake by DCs was assessed from a quantitative point of view using flow cytometry
3 by monitoring rhodamine-labelled NP fluorescence associated to DCs. In human DC
4 (MoDC), results show that PVA-coated PLGA NPs were the most efficiently taken up
5 by human DCs (Figure 2A). With these particles, fluorescence measured after 24 h of
6 exposure was about 70 times higher than cellular autofluorescence, while CS- and
7 PF68-coated PLGA NPs entry resulted in a 40-fold increase of cellular fluorescence.
8 Interestingly, fluorescence measurements carried out at early time points indicated that
9 for both PVA- and CS-coated NPs, the major part of the 24 h NP entry within cells
10 occurred within the very first hours of exposure (about 75 % after 3 h and 90 % after
11 6 h). Conversely, PF68-coated NP uptake by cells went on according to a slower
12 mechanism since only 40 % of the 24-h NP entry within cells occurred within the first
13 6 hours of exposure. In murine DC (BMDC), results show the major part of the 24h
14 NP entry within cells occurred after 3 hours of exposure. PVA-coated PLGA NPs were
15 also the most efficiently taken up by BMDC (Figure 2B). With these particles,
16 fluorescence measured after 24 h of exposure was about 50 times higher than cellular
17 autofluorescence compared to 40 % with CS-coated NPs. Regarding PF68-coated NP,
18 uptake by BMDC is very low.

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

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

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

10 *Surface protein modifications in dendritic cells after PLGA nanoparticle* 11 *exposure*

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

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

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

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

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

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

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

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

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

6 ***Investigation of signalling pathways activated as a result of PLGA nanoparticle*** 7 ***exposure***

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

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

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

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

9 **Discussion**

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

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

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

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

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

15 Noticeable phenotypic changes induced in both human and murine DCs in
16 response to PLGA NP exposure as evidenced by significant upregulations (CD86,
17 CD80, CD40, CD83), are consistent with a change allowing the transition from an
18 antigen-capturing to an antigen-presenting state of DCs (Banchereau and Steinman,
19 1998). The observed effects are attributed to PLGA NP free of endotoxin since the
20 endotoxin levels were below the USP prescribed content in water for injection (0.25
21 endotoxin units/mL, as tested by the LAL assay). Even if all tested PLGA NPs had an
22 impact on DC maturation, PVA NPs were the most potent since they exerted a higher
23 induction of all surface markers (*i.e.* CD40, CD54, CD80, CD83, CD86 and PDL-1 in
24 MoDCs; CD40, CD86 and CCR7 in BMDCs). This maturation can be correlated with
25 the higher and rapid phagocytosis of PVA-coated NPs. However, Human DCs are more

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

3 Although some literature describes the absence of DC maturation upon PLGA
4 particle exposure, both in MoDCs (Walter et al., 2001; Waeckerle-Men et al., 2004;
5 Fischer et al., 2007; Bivas-Benita et al., 2009) and BMDCs (Sun et al., 2003; Clawson
6 et al., 2010; Ma et al., 2011), our study confirms a large number of studies
7 demonstrating phenotypical changes induced in both human and murine DCs exposed to
8 PLGA films and particles (Yoshida & Babensee, 2004, 2006a; Babensee & Paranjpe,
9 2005; Diwan et al., 2003; Sun et al., 2003; Elmanchili et al., 2004; Yoshida et al., 2007).
10 Moreover, we demonstrate a relationship between PLGA particle charge and the
11 acquired DC phenotype, which remained an open question in several studies (Fischer et
12 al., 2007; Jilek et al., 2004).

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

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

5 The effect of the surfactant themselves have been raised in previous reports on
6 PLGA NPs (Guedj et al., 2015; Menon et al., 2012). Our results show that the effects of
7 PVA alone, exposed at an equivalent concentration to that associated to NPs, had no
8 own effect on DC maturation. This can be related to previous observations that PVA
9 remains strongly associated to PLGA NPs even after thorough purification (Spek et al.,
10 2015). Regarding the contribution of the PLGA core on the cell response, previous
11 reports by our group (Grabowski et al., 2015) showed that dendritic cell surrogates do
12 not show any difference in terms of mitochondrial activity and necrosis induction
13 following exposure of PVA-coated PLGA nanoparticles and surfactant-free PLGA
14 nanoparticles, which were obtained by a specific preparation technique, at concentration
15 ranges used in the present study (<1 mg/mL).

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

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

4 5 **Conclusions**

6 Our study indicates that PLGA-NPs induce clear consequences on the pivotal
7 properties of DCs *in vitro*. Exposure of immature DCs to three PLGA NP types with
8 different surface coatings and surface charge resulted in marked particle uptake by the
9 cells, but no significant cell death. Nevertheless, these NPs were not as inert as expected
10 since alterations in DC phenotype were reported, as assessed by the expression levels of
11 cell surface markers of DC maturation. In addition, the functional secretory activity of
12 DCs was largely altered upon phagocytosis of the different NPs. The study presented
13 herein lays a foundation for studying the effects of PLGA nanomaterials on the immune
14 system prior to their use as vehicles in nanomedicine. This study allowed us to indicate
15 that interspecific differences are important to take into account since human activation
16 of CD80 is higher than that of murine CD80. Human DCs are and This is the first study
17 that demonstrates MAPK activation by PLGA exposure in DCs, and the potential role
18 of surfactants in the effectively taken pathways. Further studies are needed to study the
19 role of encapsulated bioactive compounds on the effects of such PLGA nanocarriers,
20 which are relevant for drug and vaccine delivery purposes.

21 22 **Acknowledgements**

23 This study was supported by the the ANSM “Emerging risks” program and the Agence
24 Nationale de la Recherche (under reference Nanobiotox).

1 **Declaration of interest**

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

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1 **Table**

2 **Table 1. PLGA nanoparticle characterization.**

3 Data are presented as mean \pm SD. Abbreviations: CS, chitosan; PF68, poloxamer 188;

4 PLGA, poly(lactide-co-glycolide); PVA, poly(vinyl alcohol); rhod, rhodamine B; TEM,

5 transmission electron microscopy.

6

1 **Figures captions**

2 **Figure 1. Effects of PLGA NPs on cell viability.** Human MoDCs (solid lines) and
3 murine BMDCs (dashed lines) were exposed to PLGA NPs (0-500 $\mu\text{g}/\text{mL}$) for 24 h.
4 Cell death was assessed by the MTT test.

5 **Figure 2. PLGA nanoparticle uptake and internalization. A. Confocal microscopy**
6 **observations.** *Up.* Human DCs were exposed for 24 h to rhodamine coated-PLGA NPs
7 (200 $\mu\text{g}/\text{mL}$). From left to right: PLGA/PVA, PLGA/CS, PLGA/PF68. Lysosomes were
8 labeled using a green LysoTracker® probe (InVivoGen). **Flow cytometry fluorescence**
9 **analysis** *Down.* Human DCs were exposed for 3, 6 or 24 h to rhodamine-coated PLGA
10 NPs (0-400 $\mu\text{g}/\text{mL}$). From left to right: PLGA/PVA, PLGA/CS, PLGA/PF68. Red
11 fluorescence was then measured by flow cytometry. Fluorescence intensity of NP
12 exposed cells was then divided by fluorescence intensity of control cells (i.e.
13 autofluorescence). **B. Confocal microscopy observations.** *Up.* Murine DCs were
14 exposed for 24 h to rhodamine coated-PLGA NPs (200 $\mu\text{g}/\text{mL}$). From left to right:
15 PLGA/PVA, PLGA/CS, PLGA/PF68. Lysosomes were labeled using a green
16 LysoTracker® probe (InVivoGen). **Flow cytometry fluorescence analysis** *Down.*
17 Murine DCs were exposed for 3, 6 or 24 h to rhodamine-coated PLGA NPs (0-400
18 $\mu\text{g}/\text{mL}$). From left to right: PLGA/PVA, PLGA/CS, PLGA/PF68. Red fluorescence was
19 then measured by flow cytometry. Fluorescence intensity of NP exposed cells was then
20 divided by fluorescence intensity of control cells (i.e. autofluorescence).

21 **Figure 3. Phenotypic analysis of dendritic cells treated by PLGA NPs.** Human
22 MoDCs (**A**) and murine BMDCs (**B**) were exposed for 24 h to distilled water
23 (unexposed cells, white), PLGA NPs (200 $\mu\text{g}/\text{mL}$, grey) , or lipopolysaccharide (LPS,
24 25 ng/mL, positive control, black). Phenotypic markers of maturation were then studied
25 using flow cytometry analysis of fluorescent immunolabelling (statistically significant
26 differences from unexposed cells: ~~grey star~~, $p < 0.1$; ~~black star~~, *, $p < 0.05$; **, $p < 0.01$,
27 $n=5$).

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

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

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

13

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

15 MoDCs pre-loaded with purified protein derivative (*PPD*) or not were stimulated with
16 NPs. Treated MoDCs were washed and then cultured with CFSE-loaded CD4^+ T
17 autologous-T cells at 3 different ratio a 1:8, 1:4, 1:2 DC/T-cell ratio for 5 days. On day
18 5, CD4^+ T-cells proliferation was analyzed by flow cytometry and expressed as the
19 percentage of CFSE low T cells present in the sample ($n = 2$).