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RESEARCH PAPER

# Impact of palladium nanoparticles (Pd-NPs) on the biology of neutrophils in vitro and on leukocyte attraction in vivo

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**Abstract** Recently, palladium nanoparticles (Pd-NPs) have been shown to possess pro-inflammatory activities. Herein, we investigated potential in vitro effects of Pd-NPs (primary size of 1–10 nm) on the biology of neutrophils, key player cells in inflammation. Also, the aim of this study was to evaluate the pro-inflammatory activity of Pd-NPs using the murine air pouch model, a model previously proposed to be used as a standard assay for testing in vivo pro-inflammatory effects of NPs. Although the positive controls used in vitro give the expected results in all biological functions tested, Pd-NPs do not affect the production of reactive oxygen species and that of interleukin-1β (IL-1β), IL-6, and IL-8. Pd-NPs moderately increase cellular adhesion of neutrophils onto human endothelial cells and significantly increase the capacity of neutrophils to migrate and to delay apoptosis. We conclude that Pd-NPs possess some pro-inflammatory activity in vitro but do not attract leukocytes in vivo regardless of sex.

**Keywords** Palladium · Nanoparticles · Inflammation · Neutrophils · Air pouch · Biomedicine

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## Introduction

Pd is best known for its use in the automobile industry as an active catalyst material in automobile catalytic converters. However, Pd is also used in other sectors, including electronics, jewelry, and dentistry, only to name a few (Ravindra et al. 2004). At the nanoscale, Pd-NPs possess interesting physicochemical properties, including thermal and chemical stability, electronic properties, photocatalytic activity, and optical properties (Phan et al. 2019). Pd-NPs are used in catalytic processes, electronics, wastewater treatment, and biomedicine. Of note, Pd-NPs are known to have great potential for the development of photothermal, photoacoustic, antitumor, and antimicrobial agents as well as gene and drug vectors, etc. However, there are increasing evidences that Pd-NPs are also able to induce undesired toxic effects in both in vitro and in vivo models (Leso and Iavicoli 2018; Phan et al. 2019). In vitro, Pd-NPs are known to exert cytotoxic and immunomodulatory effects. The studies focus mainly on the ability of Pd-NPs to induce apoptosis and the production of several cytokines, including pro-inflammatory cytokines (Boscolo et al. 2010; Reale et al. 2011). In vivo, some adverse effects of Pd-NPs have been reported on the renal, endocrine, and immune systems (Leso et al. 2018; Leso et al. 2019).

Inflammation is increasingly reported as one of the most undesired effect of nanoparticle (NP) exposures in both in vitro (Cho et al. 2012; Goncalves et al. 2010; Lu et al. 2009) and in vivo (Chen et al. 2013; Durocher and Girard 2016; Yuan et al. 2020) models. Although high

neutrophil counts are frequently used as a marker of inflammation after NP exposure in several in vivo models, studies aiming at understanding the direct impact of NPs on the biology of neutrophils have been less investigated. To overcome this, in the last decade, we (Babin et al. 2013, 2015; Durocher et al. 2017; Goncalves and Girard 2014; Liz et al. 2015; Noel et al. 2016; Poirier et al. 2014, 2015) and others (Abrikossova et al. 2012; Dianzani et al. 2006; Moeller et al. 2012) have investigated how some NPs could alter the biology of human neutrophils, key player cells in inflammation and the most predominant leukocyte subtype found in the human blood. From these studies, it could be concluded that NPs possess the potential to alter the most important neutrophil responses involved in all steps of the inflammatory responses, including adherence, migration, phagocytosis, degranulation, reactive oxygen species (ROS) production (this is, however, not generalized), apoptosis, protein synthesis, and cytokine production. Although several NPs have been investigated by us and others, including titanium dioxide, cerium dioxide, zinc oxide, gadolinium oxide, and gold and silver NPs (Abrikossova et al. 2012; Babin et al. 2013; Durocher et al. 2017; Goncalves et al. 2010; Goncalves and Girard 2014) palladium NPs (Pd-NPs) have never been investigated in neutrophils. However, by studying different types of NPs, we recently documented that the capacity of eosinophils (the neutrophil sister cells) to adhere onto endothelial cells was increased after treatment with NPs, including Pd-NPs (Murphy-Marion and Girard 2018). Then, we demonstrated the importance of the actin cytoskeleton during Pd-NP-induced eosinophil adhesion as well as their inability to induce ROS production and apoptosis (Chhay et al. 2018). In this study, we reported how Pd-NPs can alter the biology of neutrophils and demonstrated their inability to attract neutrophils in vivo.

## Materials and methods

### Chemicals

RPMI-1640, HEPES, penicillin/streptomycin (P/S), $H_2O_2$, *Viscum album* agglutinin-I (VAA-I), lipopolysaccharides (LPS) from *Escherichia coli*, trypan blue, and dextran were purchased from Sigma-Aldrich Ltd. (Saint-Louis, Missouri); recombinant human tumor necrosis factor-alpha (TNF-α) and interleukin-8 (IL-8) were purchased from PeproTech Inc. (Rocky Hill, NJ, USA).

### Pd-NPs

The PD-NPs (product number 106PD) were purchased from Sciventions (Toronto, ONT) with a primary size of 1–10 nm, by transmission electronic microscopy, and were obtained at an original concentration of 1.5 mg/mL, stabilized by sodium polyacrylate. These Pd-NPs used in this study have been described previously and were used at a concentration of 100 μg/mL, unless specified (Chhay et al. 2018). This concentration was selected according to our previous works showing that Pd-NPs induce human eosinophils adhesion, and in addition, this concentration was suitable not only for Pd-NPs but also with other NPs, allowing comparison within different NPs (Chhay et al. 2018; Murphy-Marion and Girard 2018). In fact, several granulocyte functions can be altered at this concentration without activating cell necrosis in both human eosinophils and neutrophils. The endotoxin level of the Pd-NP suspension was determined by the classical *Limulus amebocyte* lysate (LAL) assay using the ToxinSensorTM Chromogenic LAL Endotoxin Assay Kit (Genscript Biotech Corp., Piscataway, NJ). Measurements were performed at a concentration of 100 μg/mL. In addition, we incubated the Pd-NP suspension in Lysogeny broth agar plates for 72 h for testing sterility and observed the presence or absence of colonies. As a positive control, we used a suspension voluntary contaminated with human hear. Pictures of the plates were taken with a Motorola Moto g(7) play cell phone.

### Scanning electron microscopy

For scanning electronic microscopy (SEM), a cover glass (disc) was cleaned with acetone and coated with 3 nm gold/palladium (Leica CPD300). Using a conductive silver paint (Ted Pella), the disc was fixed on a stub. A 100 μL drop of the stock solution was put on the cover glass and let it dry. After drying, the sample was examined with a Hitachi Regulus 8220 scanning electron microscope.

### Neutrophil isolation

Neutrophils were isolated from venous blood of healthy volunteers by dextran sedimentation, followed by

centrifugation over Ficoll-Hypaque (Pharmacia Biotech, Inc., Quebec, Canada) as described previously (Babin et al. 2013). Blood donations were obtained from informed and consenting individuals according to institutionally approved procedures. In this study, blood was obtained from both men and women (age ranging from ~ 20 to 62 years old). Cell viability was monitored by trypan blue exclusion and found to be consistently ≥ 97%. Cell purity (≥ 98%) was verified by cytology from cytocentrifuged preparations colored by Hema-3 stain set (Biochemical sciences Inc., Swedesboro, NJ). Cell viability was evaluated systematically before and after each treatment. Neutrophils were then resuspended $10 \times 10^6$ cells/mL in RPMI-HEPES (25 mM), penicillin (100 U/mL)/streptomycin (100 μg/mL) for all experiments, but ROS production (see under).

### Detection of intracellular ROS

Cells ($10 \times 10^6$ cells/mL) were suspended in HBSS containing 10 μM CM-$H_2$DCFDA (Invitrogen/Molecular Probes, Camarillo, CA) for 15 min at 37 °C as published (Simard et al. 2011). Cells were then washed twice before being incubated in the presence of buffer or Pd-NPs for 5, 15, 30, and 60 min. In these experiments, $H_2O_2$ (3%, final concentration) was simply used as a positive technical control. ROS production was determined using a Spectra Max M5 plate reader (Molecular Devices, San Jose, CA) with an excitation/emission wavelength of 485/538 nm and was expressed as relative fluorescence units (RFU).

### Neutrophil cell adhesion assay

The human umbilical vein cell line EA.hy926 (ATCC® CRL-2922™) was purchased from American Type Culture Collection (Manassas, VA) and was grown in RPMI-1640 supplemented with 10% fetal calf serum and antibiotics. Cell viability was systematically evaluated before and after each treatment, and mortality never exceeded 5%. These endothelial cells were used as a substratum for neutrophil cell adhesion. To do so, neutrophils were treated with buffer (Ctrl), Pd-NPs, or TNF-α (used as a positive control) (Murphy-Marion and Girard 2018; Pelletier and Girard 2005) for 30 min and then labeled for 30 min with 5 μM calcein-AM (Molecular Probes, Inc., Eugene, OR, USA). The number of adherent neutrophils was calculated by counting the number of fluorescent cells from five randomly selected high-power fields (× 400) observed with a photomicroscope Leica DMRE equipped with an ebq 100 dc epifluorescent condenser. Images were taken with a Cooke Sensicam High performance camera coupled to the Image Proplus® (version 4·0) program.

### Cytokine production

The concentration of IL-8, IL-6, and IL-1β was determined using a commercially available ELISA kit for IL-8, IL-6, or IL-1β (Life Technologies, Carlsbad, CA). Freshly isolated human neutrophils were incubated in the presence of buffer (Ctrl), Pd-NPs, or 1 μg/mL LPS (positive control) as above at 37 °C in 5% $CO_2$ for 24 h in a 24-well plate containing RPMI-1640 supplemented with 5% heat-inactivated fetal calf serum. Supernatants were harvested after centrifugation and stored at − 70 °C before performing ELISA.

### Assessment of neutrophil apoptosis

Neutrophils ($10 \times 10^6$ cells/mL in RPMI 1640-HEPES-P/S, supplemented with 10% heat-inactivated autologous serum) were treated with buffer, Pd-NPs, or 1 μg/mL of the potent proapoptotic plant lectin *Viscum album* agglutinin-I (VAA-I) (Lavastre et al. 2002; Savoie et al. 2000) for 24 h. Cytocentrifuged samples of neutrophils were prepared, stained with the Hema-3 Stain staining kit according to the manufacturer's instructions, and processed as documented previously (Lavastre et al. 2002).

### Neutrophil migration

In vitro migration was performed in a 48-well microchamber (Neuro Probe Inc., Gaithersburg, MD, USA) using a 3–5 μm polycarbonate membrane filter as described previously (Durocher et al. 2017). The bottom wells were loaded (final volume, 25 μL) with buffer, Pd-NPs, or the positive control CXCL8/IL-8 (25 nM), and the polycarbonate membrane was placed over the wells; then the top layer of the chamber was added over the membrane. Neutrophils (50 μL from a RPMI suspension of $1 \times 10^6$ cells/mL) were added into the upper wells. The chamber was incubated at 37 °C for 60 min in a humidified incubator in the presence of 5% $CO_2$. After the incubation, the top of the chamber was removed, and the upper side of the membrane was wiped carefully with the rubber scraper provided by

the manufacturer. The polycarbonate membrane was fixed in methanol, colored with Hema 3 staining kit, mounted on a glass slide, and examined with a light microscope under × 400 magnification. The number of cells in five random fields was counted, and the results were expressed as relative neutrophil migration (number of cells from tested group/number of cells from control).

### Murine air pouch model

CD-1 male and female mice (6–8 weeks of age) were obtained from Charles River Laboratories (St-Constant, Canada). A period of acclimation of 1 week was allowed prior to initiation of experiments with the animals. On days 0 and 3, mice were anesthetized with isoflurane, and 3 mL of sterile air was injected subcutaneously, in the back, with a 26-gauge needle to form an air pouch as published previously (Durocher and Girard 2016; Durocher et al. 2017; Pelletier et al. 2004). On day 6, 1 mL of HBSS (Ctrl), 100 or 500 μg/mL of Pd-NPs, or 1 μg/mL LPS (positive control) was injected into the air pouch. Mice were killed by $CO_2$ asphyxiation 6 h after the treatment, and the pouches were washed once with 1 mL and then twice with 2 mL of HBSS containing 10 mM EDTA. Exudates were centrifuged at 100×*g* for 0 min at 4 °C, and cells were resuspended at $0.5 \times 10^6$ cells/mL, spread onto microscope slides, stained with Hema-Stain (Fisher Scientific, Ottawa, Canada) for identification of leukocyte cell subpopulations, and were counted with a hemocytometer. All experiments were performed as per protocols approved by Animal Use and Care Committees at INRS.

### Statistical analyses

Statistical analyses were performed using repeated measures ANOVA, and differences between groups were assessed using the Dunnett's multiple comparison test with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). Statistical significance was established at $p < 0.05$.

## Results

### Sterility of Pd-NP suspension and ROS production

As assessed by the LAL assay (*data not shown*), the Pd-NP suspension reveals a level of endotoxins under the detection limit of 0.01 EU/mL ($n = 3$). Since some NPs could interfere with classical LAL assay for determining endotoxin level (Dobrovolskaia and McNeil 2007), we incubated the Pd-NP suspension (100 μg/mL) in Lysogeny broth agar plates for 72 h for further confirming sterility. As illustrated in Fig. 1a, unlike the positive control, no colony was observed on the plate containing Pd-NP suspension. A SEM image of the Pd-NP suspension is illustrated in Fig. 1b and indicates that most of the Pd-NPs possess a spherical form close to a size of 10 nm. However, some aggregates (short and intermediate chain-like structures observable close to the white asterisk) are also observable agreeing with our previous results obtained by dynamic light scattering analysis (Chhay et al. 2018). Knowing the importance of ROS production by neutrophils (Dahlgren et al. 2019; Johansson et al. 1995), we next determined the impact of Pd-NPs on this biological response. Because Pd-NPs were previously found to increase or not ROS production in a variety of cells other than neutrophils, we decide to carefully determine this using 31 different blood donors (15 males + 16 females). Figure 1c shows that, unlike $H_2O_2$ that give the expected results, Pd-NPs do not increase ROS production after 5, 15, 30, and 60 min of treatment. To discriminate between a potential differential effect associated with sex, the data were separate taking this parameter into account. When comparing the results between neutrophils isolated from men and women (Fig. 1d), clearly, Pd-NPs do not induce a different response in male vs female neutrophils. In fact, the results were remarkably similar when plotted together (males and females) or separately (males or females alone). These results clearly indicate that Pd-NPs do not induce ROS production in human neutrophils. In addition, our experiments indicate that neutrophils isolated from men or women also respond in the same manner to $H_2O_2$.

### Effect of Pd-NPs treatment on neutrophil adherence onto endothelial cells

As illustrated in Fig. 2a, Pd-NPs can slightly to moderately increase the capacity of neutrophils to adhere onto human endothelial cells. Although a clear trend is observed with a ratio of 1.5 ± 0.6 (mean ± SD, $n = 11$, vs Ctrl), the results were not statistically significant. By comparison, the positive control TNF-α gives a ratio of 2.5 ± 0.7.

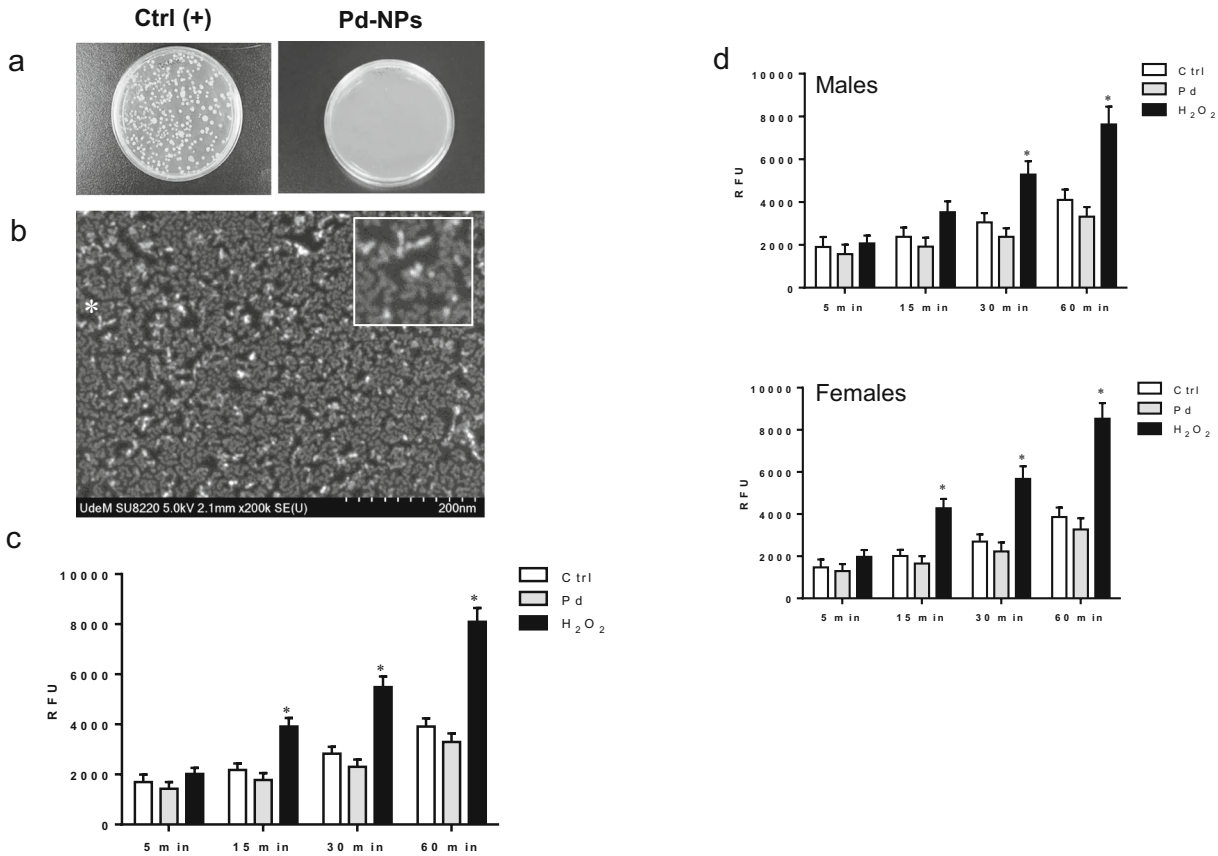


**Fig. 1** Pd-NPs do not induce ROS production in human neutrophils. **a** Aliquots of a voluntary contaminated (human hair) or Pd-NP (100 μg/mL) suspension were deposited on Lysogeny broth agar plates and incubated for 72 h. Sterility was confirmed by absence of any colony vs the positive control. Pictures of the plates were taken with a Motorola Moto g(7) play cell phone and are representative of four experiments conducted at different periods of time during the whole study. **b** SEM image obtained as described in Materials and Methods. The region close to the white asterisk is zoomed out (top right part of the figure). **c** and **d** Freshly isolated human neutrophils were treated with buffer (Ctrl), Pd-NPs, or $H_2O_2$ for the indicated periods of time and ROS production, expressed as relative fluorescence units (RFU), and were determined as described in Materials and Methods. Results are expressed as means ± SD (**c**, $n = 31$ blood donors; **d**, $n = 15$ males or 16 females). *$p \leq 0.05$ vs Ctrl

## Pd-NPs do not increase the production of IL-1β, IL-6, and IL-8 in human neutrophils

As illustrated in Fig.3, Pd-NPs do not increase the production of IL-1β ($n = 23$), IL-6 ($n = 23$), and IL-8 ($n = 30$) when compared with Ctrl. However, cells were responsive since LPS significantly increases the production of all the tested cytokines.

## Modulation of neutrophil apoptosis and migration by Pd-NPs

As illustrated in Fig. 4a, Pd-NPs treatment lead to a weak to moderate, but significant, decrease of spontaneous neutrophil cell apoptosis from 50.2 ± 15.6% (mean ± SEM, $n = 40$) to 40 ± 14.3%. As expected, VAA-I increases the apoptotic rate in almost 100% of cells. Figure 4b shows that Pd-NPs increase the ability of neutrophils to migrate by a ratio of 1.6 ± 0.5 (mean ± SEM, $n = 22$, vs Ctrl). As expected, IL-8 increases the migration by a ratio of 1.9 ± 0.7.

## Pd-NPs do not induce acute inflammation in vivo in both male and female mice

Because a delay in neutrophil apoptosis and an increase capacity to migrate in vitro are two events known to be associated with inflammation, and since it is impossible to recreate inflammation in vitro, we next determined whether or not Pd-NPs could induce inflammation

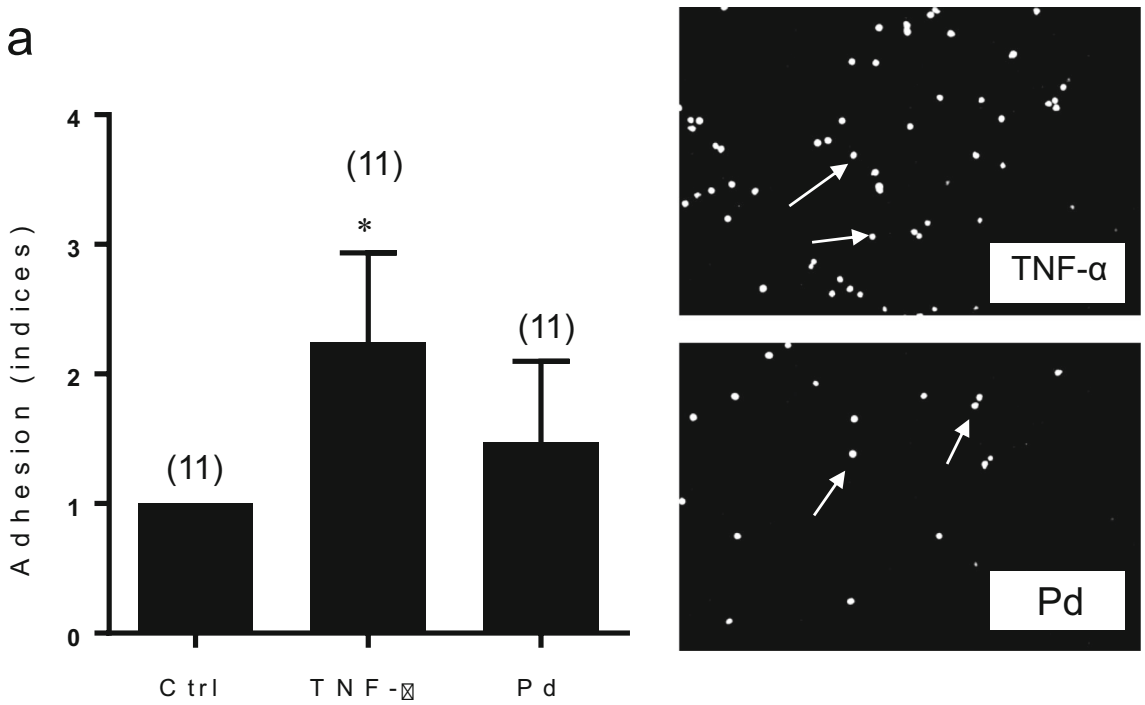


**Fig. 2** Pd-NPs induce cellular adhesion. Freshly isolated neutrophils were incubated with buffer (Ctrl), TNF-α, or Pd-NPs, and their capacity to adhere onto endothelial cells was determined as described in Materials and Methods. Inset, each dot illustrates a fluorescent neutrophil (white arrow) adhering onto endothelial cells. Results are mean ± SD, and the number of blood donors is indicated within parentheses. *$p \leq 0.05$ vs Ctrl set as 1

in vivo. Figure 5a shows that administration of 100 and 500 μg/mL Pd-NPs into murine air pouches does not induce inflammation in vivo in both male and female mice as judged by the similar number of total leukocytes recruited as compared with controls (Fig. 5a). As expected with this model (Durocher and Girard 2016; Durocher et al. 2017), LPS was found to induce a potent inflammation resulting by the infiltration of several millions of leukocytes into the exudates. Although no major difference was observed, LPS was slightly more efficient in female mice. By determining differential counts (Fig. 5b), PD-NPs, unlike LPS, do not preferentially attracted neutrophils, one of the two major leukocyte populations observed in this model, the other being mono-mac.

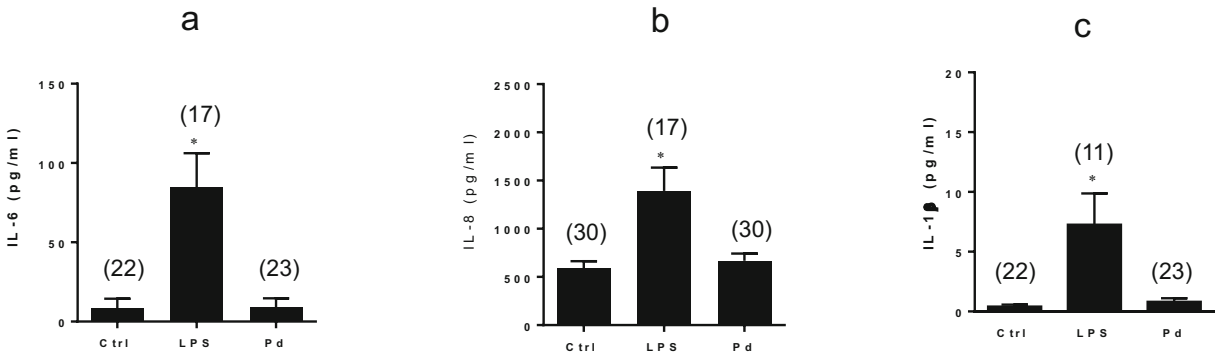


**Fig. 3** Pd-NPs do not increase IL-8, IL-6, and IL-1β production in neutrophils. Freshly isolated human neutrophils were incubated for 24 h with buffer (Ctrl), LPS, or Pd-NPs, and the concentration of secreted cytokines was determined by ELISA as described in Materials and Methods. Results are mean ± SD, and the number of blood donors is indicated within parentheses. *$p \leq 0.05$ vs Ctrl

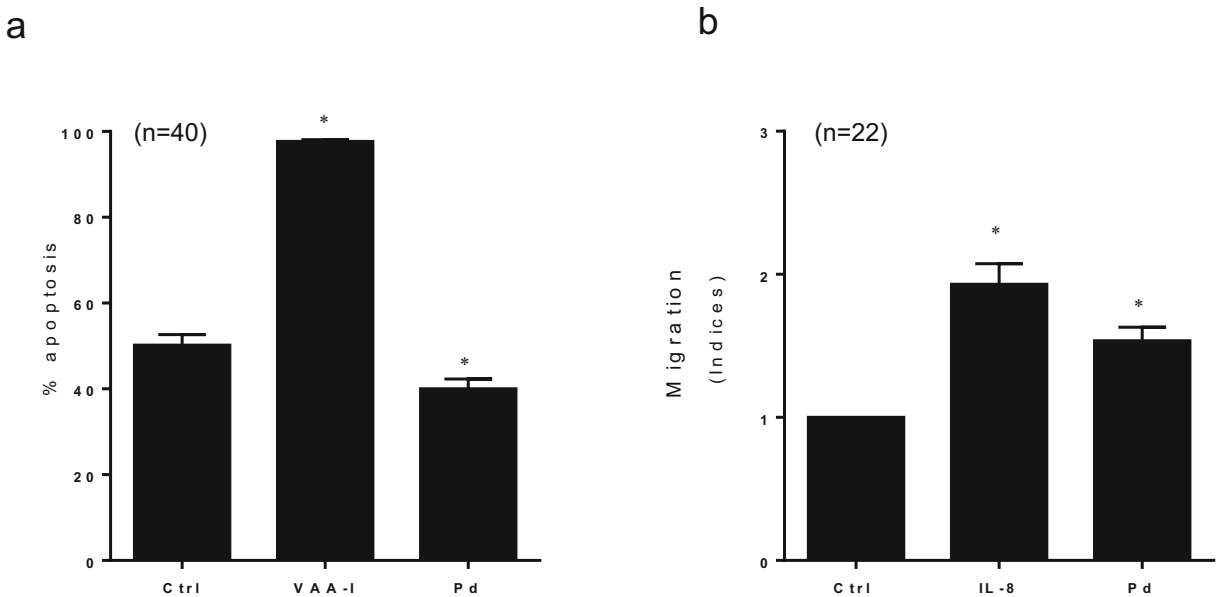


**Fig. 4** Pd-NPs can delay human neutrophil spontaneous apoptosis and increase their capacity to migrate. Neutrophils were freshly isolated and incubated with the indicated agonists, and apoptosis (**a**) or migration (**b**) was determined after 24 h or 1 h, respectively, as described in Materials and Methods. Results are expressed as means ± SD (**a**, $n = 40$ blood donors; **b**, $n = 22$ blood donors) *$p \leq 0.05$ vs Ctrl

## Discussion

A unique study reported pro-oxidative interactions of palladium metal with neutrophils; however, the metal source of Pd was not at the nanoscales (Theron et al. 2004). Therefore, the current study is the first one investigating the role of Pd-NPs on neutrophil cell biology and one of the very few investigating potential differences on neutrophil cell biology incorporating a sex-based analysis, all agents combined. Herein, the capacity of Pd-NPs to increase the adhesion of human neutrophils onto endothelial cells agrees with the previous data where Pd-NPs were found to increase the adhesion of human eosinophils onto a cell substratum (Chhay et al. 2018; Murphy-Marion and Girard 2018). However, in the present study, only a moderate (no significant) increase was observed after treatment with Pd-NPs. Of note, the same human umbilical vein cell line EA.hy926 was used in both studies, and yet, it is not clear why eosinophils (Chhay et al. 2018, Murphy-Marion and Girard 2018) adhere more efficiently onto these cells as compared to neutrophils (this report). It is highly plausible that differential expression and/or function of cell surface adhesion molecules is involved. In this respect, IL-4 was reported to induce adherence of human eosinophils and basophils but not neutrophils to endothelium, and this was associated with expression of VCAM-1 (vascular cell adhesion molecule-1) known to counteract with the integrin VLA-4 (very late antigen-4) not or very weakly expressed in human neutrophils (Schleimer et al. 1992). Interestingly, the capacity of Swiss mouse 3T3 fibroblasts to adhere onto gold, platinum, and palladium surfaces was previously reported to be differently modulated (Turner et al. 2004). Cells on palladium surfaces were more weakly attached as compared to gold and platinum. Whether or not adhesion onto these surfaces could also be a cell-specific-dependent mechanism would be important to determine in the future.

We found that Pd-NPs do not induce the production of ROS in human neutrophils. These results agree with those observed in human eosinophils (Chhay et al. 2018) and other primary human cells such as bronchial epithelial cells (Wilkinson et al. 2011) and different human immortalized cells including alveolar carcinoma cell line A549 (Wilkinson et al. 2011), eosinophilic cell line AML-14.3D10 (Chhay et al. 2018), epithelial colorectal adenocarcinoma cell line Caco-2 (Hildebrand et al. 2010), and keratinocyte cell line HaCaT (Hildebrand et al. 2010). In contrast, others reported that Pd-NPs could increase ROS production in rat embryo fibroblast cell line Rat-1 and human lung carcinoma

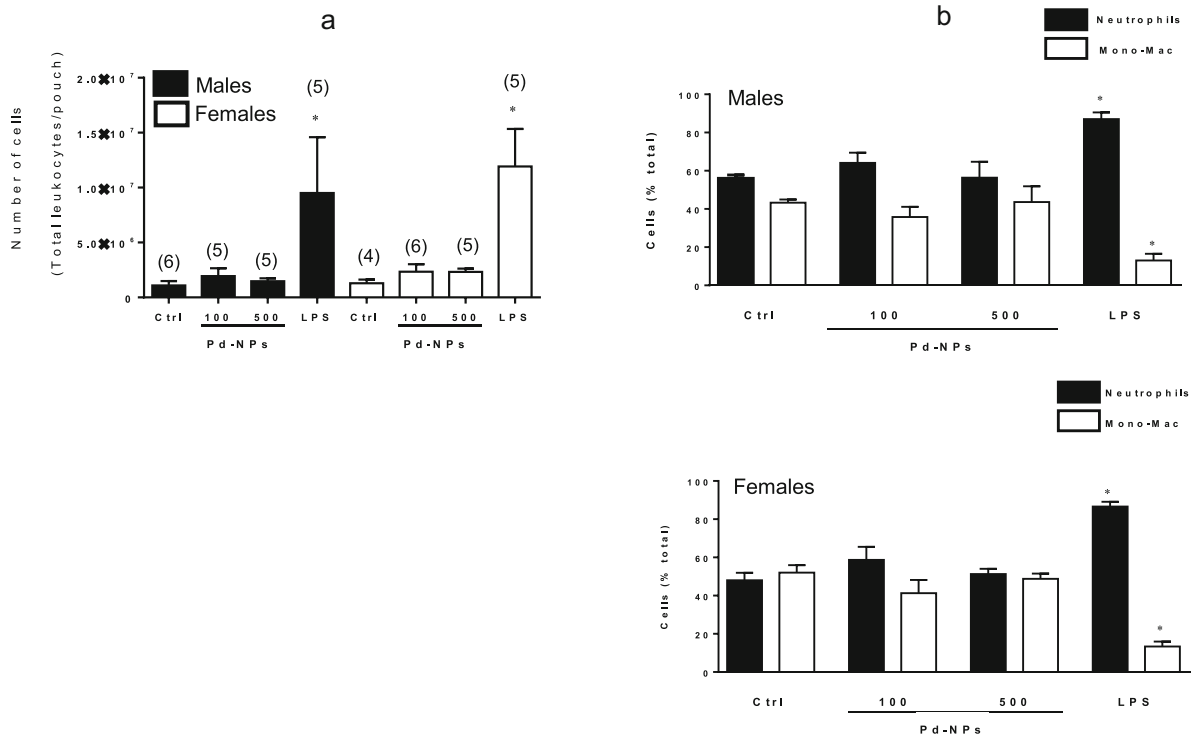


**Fig. 5** Pd-NPs do not induce leukocyte infiltration in vivo. After creation of air pouches, buffer, LPS, or 100 and 500 μg/mL Pd-NPs were directly administered into murine air pouches, and exudates were harvested after 6 h. The total leukocyte numbers (**a**) and differential counts (% of monocytes–macrophages (mono-mac) and neutrophils in males and female (**b**) were then determined as described in Materials and Methods. Results are mean ± SD, and the number of mice is indicated within parentheses. *$p \leq 0.05$ vs Ctrl

epithelial cell line A549 (Iavicoli et al. 2017), human monocyte-like cell line THP-1 (Neubauer et al. 2015), human ovarian cancer cell line A2780 (Gurunathan et al. 2015), human skin malignant melanoma cell line A375 (Alarifi et al. 2017), and human peripheral blood mononuclear cells (Petrarca et al. 2014). However, in this latter study, the ROS production was only moderately and nonsignificantly increased. The experiments were performed with only three donors, and cells were incubated with Pd-NPs in vitro for 48 h. Herein, we incubated cells with Pd-NPs for 0–60 min only since we were interested in the respiratory burst occurring via NADPH activation, the main source of ROS in neutrophils. In addition, we have performed 31 different experiments (31 different blood donors: 15 men and 16 women) to conclude inability of inducing ROS production regardless of sex. Further, our results are supported by the fact that Pd-NPs do not induce neutrophil apoptosis, a biological process frequently associated with an initial ROS production. Of note, in the above studies reporting that Pd-NPs induce ROS production in a variety of cell types, cytotoxicity and/or apoptosis was also observed (Alarifi et al. 2017; Gurunathan et al. 2015; Hildebrand et al. 2010; Iavicoli et al. 2017). Herein, Pd-NPs rather delay neutrophil apoptosis and do not induce cell necrosis.

Pd-NPs were found to increase the ability of neutrophils to migrate. Although a protocol similar to the one used in the current study has been established by others (Skoczen et al. 2011), the literature is lacking regarding the ability of NPs do induce neutrophil migration/chemotaxis in vitro. However, the present results agree with our previous study in which gold NPs were also found to increase migration of neutrophils (Durocher et al. 2017). Using the murine alveolar macrophage cell line J774.2, one study reported that carbon black NPs moderately increased macrophage migration in vitro using a system like the one used by us (Barlow et al. 2005). Of note, when carbon black NPs were incubated with serum, the response was markedly increased. In our hands, serum was not added. It is difficult to explain how Pd-NPs could attract neutrophils during the

migration assay used. Indeed, in our system, as most other ones, neutrophils normally undergo morphological cell shape changes to pass through the filter. We cannot exclude the possibility that some neutrophils could nonspecifically pass via simple gravity (observable in the negative control) and that in presence of Pd-NPs would degranulate and/or produce some potent chemo-attractants. This remains to be determined, but previously, we demonstrated that some NPs could induce degranulation in human neutrophils (Babin et al. 2013). When testing the capacity to produce and secrete cytokines in Pd-NP-induced neutrophils, we did not observe increase levels of IL-1β, IL-6, and IL-8 when compared to controls. However, as expected, LPS increased cytokine production. This further supports the results obtained with the LAL assay and the agar plate indicating absence of contaminated endotoxins in Pd-NPs; otherwise, the production of these cytokines would have been increased (particularly IL-6 and IL-8). It is important to mention here that our results do not rule out the possibility that Pd-NPs could modulate the production of cytokines other than those tested in the current study. This remains to be determined.

The murine air pouch model of acute inflammation was recently proposed to be added as a future standard assay for testing in vivo pro-inflammatory effects of several types of NPs (Girard 2014; Vandooren et al. 2013). This model is especially useful to determine the pro-inflammatory activity of an agent when administered into the air pouch, giving general information on the inflammatory response including the three important phases, namely, initiation, amplification, and resolution depending on the dose-response and kinetics studied. We used this model for several years for testing different types of agents, including more recently NPs where some were found to be pro-inflammatory and others not, as judged by the leukocyte infiltration, especially neutrophils (Durocher and Girard 2016; Durocher et al. 2017; Goncalves and Girard 2011, 2013). However, we never incorporated a sex-based analysis. The current study indicates that Pd-NPs, in the experimental conditions used, do not induce leukocyte infiltration regardless of sex, but LPS induces a more pronounced effect in female mice when compared to males. This agrees with the general immune scenario in which, in general, innate and adaptive immune responses are more potent in females than males. Females are, however, less susceptible to many types of infection but in contrast are more prone to develop autoimmune diseases (Jaillon et al. 2019; Klein and Flanagan 2016). Although Pd-NPs did not induce a neutrophilic infiltration by themselves, it is possible that, as we have documented for fullerenols (polyhydroxylated $C_{60}$ Fullerene NPs), Pd-NPs could act in concert with other agents, namely, LPS, for causing inflammation, a situation that is likely to occur in vivo during infections. In this respect, further studies need to be performed.

In conclusion, because Pd-NPs can alter the biology of human neutrophils, and knowing that differences observed between men and women immune responses and inflammation have been previously reported, we recommend to, whenever possible, systematically test in parallel in vitro and/or in vivo potential impact of a given NPs by incorporating sex-based analysis into future studies. This will certainly not only increase our knowledge on the mode of action of a given NP but will also improve our understanding of sex as an important variable that has to be considered in nanotoxicology or related fields.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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