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## Focussing on Neutrophils for Evaluating In vitro and In vivo Inflammatory Activities of Nanoparticles

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Additional information is available at the end of the chapter

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### Abstract

A tremendous interest to use nanomaterials for medical diagnosis and therapeutic purposes has increased in the past few years. Although a lot of investigations focus on the use of nanoparticles (NPs) for drug delivery in cancer therapies, there are several studies investigating the potential use of NPs as carriers to detect allergies or to alleviate inflammatory symptoms. However, although this represents a very interesting interest and a potential avenue to use nanodrug systems, there are some potential toxic risks. For example, cytotoxicity, oxidative stress, genotoxicity, and inflammation have been reported both in in vitro and in vivo models for testing NPs. In addition to medicine, a variety of other sectors, including electronics, cosmetics, aerospace, textile industries, and even in food, used NPs. Consequently, the probability of human exposure to NPs has risen, leading to the possibility that NPs may reach the blood circulation and interact with immune blood cells. Therefore, it is crucial to evaluate the risk that NPs represent to human health. In different studies using in vivo models of inflammation, especially those investigating airway NP exposure, an increased number of leukocytes, mainly neutrophils, in the lungs and bronchoalveolar lavages have been reported. In fact, neutrophil counts are used as biomarkers of inflammation. Despite this and knowing that neutrophils are key player cells in inflammation, it is intriguing that few nanotoxicology studies have focused on how NPs can directly alter the biology of these cells. However, an increasing amount of studies, including some from my laboratory, demonstrate that NPs can activate human neutrophils by different manners in vitro and can attract them or not in vivo. The focus of this review will be to cover this new area of research.

**Keywords:** Inflammation, neutrophils, nanotoxicology

## 1. Introduction

The use of nanoparticles (NPs) has increased in the past few years in various fields, including textile, sport, defense, aerospace, electronics, biology, medicine, etc. There is also a growing interest to use NPs in different applications, including diagnostic technology, bioimaging, and drug/gene delivery. Therefore, voluntary or nonvoluntary human exposure to NPs and nanomaterials is unavoidable and will certainly expand in the near future. This has led to a growing interest in nanotoxicology, the study of toxicity of nanomaterials. Especially, a number of studies reported the effects of NPs on pulmonary inflammation by investigating in vitro activation of pulmonary cells with NPs and in vivo in a variety of models in which neutrophils appear to be the predominant leukocyte cell type in the lungs and in bronchoalveolar lavages following inhalation or intratracheal instillation of NPs. It is reasonable that the first studies focussed on pulmonary effects of NPs since inhalation is one of the major routes of human exposure to NPs. However, even if several studies reported an increased number of neutrophils, the literature dealing with the direct effects of given NP with neutrophils is poorly documented and has been neglected until the last few years. In addition, since NPs are used in a variety of sectors and are already included in several consumable products, NPs could reach the blood stream and interact with immune cells. This review will summarize the current literature dealing on the direct interaction of NPs with human neutrophils as well as recent data indicating that the murine air pouch model of inflammation is suitable for evaluating the ability of NPs to attract neutrophils in vivo.

## 2. Neutrophils: A brief overview

Polymorphonuclear neutrophil cells (PMNs) are important cells of the immune system involved in host defense. In particular, they are primordial players of innate immunity and provide a very effective defense against bacterial and fungal infections. Other than erythrocytes and platelets, PMNs are the most abundant cell type in circulation, representing more than 65 % of total leukocytes. They are terminally mature nondividing cells which develop in the bone marrow from CD34<sup>+</sup> stem cells, resulting from a series of cell divisions and stages as myeloblasts, promyelocytes, myelocytes, metamyelocytes, band neutrophils, and finally, mature neutrophils. The mechanism is still not well understood; however, this occurs under the influence of regulatory cytokines [1-3]. It takes about 14 days to obtain fully mature neutrophils from the CD34<sup>+</sup> precursor cells. Of note, more than 50 % of the bone marrow is dedicated to the generation of PMNs. A huge number of neutrophils are released from the bone marrow. Indeed, this has been estimated at  $\sim 5 \times 10^{10}$  cells on a daily basis in a normal adult. This represents one of the fastest cell turnovers in the human body [2, 3]. Therefore, cell turnover must be under strict control in order to prevent diseases. The number of PMNs remains relatively constant in healthy individuals, and this is due to the limited life span (half-life of  $\sim 12$  h in circulation) of these cells. In addition, PMNs are known to undergo constitutive or spontaneous apoptosis, an important step for regulating cell number. Apoptosis renders PMNs unresponsive to extracellular stimuli and leads to expression of "eat-me" signals, some

molecules involved in the elimination of apoptotic PMNs by professional phagocyte, a process called efferocytosis, largely responsible for the resolution of inflammation [4-7]. Because of this, identification of proapoptotic or antiapoptotic agents is therefore of major importance. When the rate of PMN apoptosis is accelerated, this results in an increase in bacterial susceptibility. In contrast, when apoptosis is delayed or suppressed, this can aggravate inflammation and lead to autoimmune disorders [4, 7].

Although different biochemical hallmarks of apoptosis, including cell shrinkage, chromatin condensation and internucleosomal DNA degradation, appearance of pyknotic nuclei, caspase activation, flip-flop of phosphatidylserines to the extracellular (outer) surface of the cell, etc., are observable in neutrophils [4, 6, 8], these cells are different from several other cell types. In this respect, the common caspase substrates such as poly(ADP-ribose) polymerase, the catalytic subunit of DNA-dependent protein kinase, the small ribonucleoprotein U1-70 kDa, and the nuclear/mitotic apparatus proteins are not detected in PMNs [9, 10]. In addition, human neutrophils do not express caspase-2 as well as the antiapoptotic Bcl-2 (B-cell lymphoma 2) proteins [11]. In contrast, a predominant expression of the antiapoptotic protein myeloid cell leukemia-1 (Mcl-1) is observed in these cells. Also, it is important to mention that PMNs possess a very low number of mitochondria that may have a role restricted to apoptosis rather than for energy generation [12]. During apoptosis, mature neutrophils can release proteases from azurophilic granules, including cathepsin D, which may contribute to caspase-3 activation through processing of caspase-8 [13]. Finally, unusual roles for nuclear proteins have been reported in PMNs [14]. For example, unlike other cells, proliferating cell nuclear antigen (PCNA) is expressed in the cytoplasm of mature neutrophils where it could bind to procaspases, affecting their apoptotic rates.

Interestingly, both intrinsic and extrinsic pathways of cell apoptosis appear to be activated during spontaneous human neutrophil apoptosis as evidenced, for example, by caspase-9 (intrinsic) and caspase-8 (extrinsic) activation [15-17]. More recently, increasing evidences indicate that the endoplasmic reticulum (ER) stress-induced cell apoptotic pathway is also operational in human PMNs [18, 19]. These cells were found to express inositol-requiring protein-1 (IRE1), activating transcription factor-6 (ATF6), and protein kinase RNA (PKR)-like ER kinase (PERK), the three major sensors of protein folding status in the ER [20, 21].

During acute inflammation, PMNs are the first type of leukocytes to migrate to an inflammatory site, where they will produce several proinflammatory mediators including chemokines that first attract other PMNs and then other cell types like monocytes-macrophages and lymphocytes, corresponding to chronic inflammation. PMNs are phagocytes well recognized for their ability to eliminate invading pathogens via two important mechanisms: *i*) the respiratory burst, which is an oxygen-dependent process leading to the generation of reactive oxygen species (ROS), and *ii*) degranulation, an oxygen-independent mechanism by which PMNs release potent toxic degradative products stored in granules. In addition to reactive oxygen metabolites and granule enzymes, PMNs are known to be an important source of products implicated in tissue damage and inflammation such as leukotriene B<sub>4</sub>, platelet-activating factor, and various cytokines (IL-1 $\alpha$ , IL-8, IL-12, TNF- $\alpha$ , TGF- $\beta$ , GRO- $\alpha$ ), to name a few [1]. The importance of PMNs in inflammation is further supported by the observation that

various PMN priming and activating agents such as IL-1 $\beta$ , IL-8, IL-15, GM-CSF, TGF- $\beta$ , C5a, C9, etc., are present, for example, in the synovial fluids of rheumatic patients [22-24]. PMNs are also known to adhere onto cell substratum (e.g., endothelial cells) [25] or onto extracellular matrix proteins, including fibronectin [26]. Finally, PMNs can move toward a chemotactic gradient (chemotaxis) and exert phagocytosis, two important functions involved in killing and eliminating pathogens. About ten years ago, an important new discovery was made concerning the biology of neutrophils; upon activation, these cells were found to release neutrophil extracellular traps (NETs) composed of decondensed chromatin DNA in association with histones, granular proteins, and a few cytoplasmic proteins [27]. NETosis, a sort of PMNs suicide-generating NETs, was identified as a novel antimicrobial mechanism able to kill extracellular bacteria, fungi, and parasites.

It is important to mention that the same arsenal and biological responses of PMNs discussed above that are involved in host defense can also be deleterious for an organism when deregulation occurs. This phenomenon is known as the neutrophil paradox. Because of this, and knowing the role of PMNs during inflammation (PMNs are seen as conductors of inflammation), it is very important to carefully understand the mode of action of PMN agonists as well as to identify new ones, including the new actors, NPs. Because how NPs interact with PMNs is an area of research that is still in infancy and that a lot of work needs to be done, it is important to determine how a given NP will alter or not several PMN functions in order to obtain a general picture rather than investigating only one or two functions. Also, even if an agent is not a direct PMN agonist by itself, it can be indirectly proinflammatory by attracting these cells *in vivo*, as is the case with the cytokine IL-21 [28]. This aspect needs also to be studied with NPs. For this reason, the following sections will describe how several PMN functions can be studied *in vitro*. Of note, several techniques and methods can be used to determine a given PMN function; but for clarity and simplicity, I will describe those that are used by several laboratories, especially the assays that are routinely used in mine. In addition to this *in vitro* aspect, I will describe how we performed the murine air pouch model, a model recently proposed by a consortium of 18 researchers from six different countries, as a future standard assay for testing NPs *in vivo* [29].

### **3. Evaluating neutrophil functions *In vitro***

#### **3.1. Neutrophil source**

The first step for investigating neutrophil cell biology is to have access to a PMN source. Although some researchers, including ourselves, used immature human cell lines such as HL-60 and PLB-9895, these cells are not primary neutrophils and may respond differently [30]. Others used rodent PMNs for investigating the role of different agents on neutrophil biology. However, it is important to remember that unlike humans where more than 65–70 % of circulating leukocytes are neutrophils, this proportion do not exceed ~25 % in rodents [31]. In addition, although methods for human neutrophil isolation are now standardized, similar procedures for isolating PMNs from nonhuman species are not as well developed. Because



PMNs are very reactive cells, the method of isolation is extremely important to avoid cell activation during the isolation process. In respect with this, different techniques have been proposed [32] for isolating highly purified PMNs from large animals (bovine, equine, ovine), small animals (rodents, rabbit), and nonhuman primates (macaques). In my laboratory, we are using human blood as a source of neutrophils and we freshly isolate them in order to perform several studies, including the role of chemicals of environmental concern [30, 33-35], plant lectins and extracts [36, 37], cytokines [28, 38-46], myeloid-related proteins [47-49], different other compounds [19, 50-55], and, more recently, NPs [56-59]. Blood is obtained from healthy consenting individuals according to institutionally approved procedures, but it is also possible to isolate PMNs from blood of patients suffering from a given disease for comparison with age- and sex-matched healthy individuals.

### **3.2. Cell viability, necrosis, and purity**

It is important to evaluate cell viability immediately after PMN cell isolation. We propose to determine this by the trypan blue exclusion assay since, during this method, it is also possible to simultaneously evaluate if cells are activated (irregular cell shape) or not (round or spherical shape) following the isolation procedure. In addition, it is mandatory to evaluate cell viability over time, especially after 24 h of incubation, since the rate of apoptotic PMNs is normally in the range of 30–50 % without addition of any exogenous agents. Moreover, apoptotic PMNs are known to exclude trypan blue and are thus considered “viable” and not necrotic. In parallel, we strongly suggest to evaluate neutrophil purity that could be verified by cytology from cytocentrifuged preparations colored with Hema-3 stain set, a procedure allowing a differential count. We never performed experiments with samples having  $\geq 3$  % eosinophils, the most contaminant cell type observed in our preparations. Other methods can also be used to evaluate cell viability, such as the determination of the release of lactate dehydrogenase (LDH) measured by a colorimetric assay and the dimethylthiazolyl-diphenyltetrazolium (MTT) reduction assay. Several commercially available kits exist; however, it is mandatory to first verify if a given NP could interfere with the assay to avoid false assessment of toxicity [60-62].

### **3.3. Cell shape changes**

Naive non-activated neutrophils possess a round or spherical shape [41]. To study the effect of a given agent on neutrophil activation, cells have simply to be incubated in the presence of the molecule of interest, and morphological examination is monitored over time by optical microscopy [41, 58].

### **3.4. Actin polymerization**

Actin is a cytoskeletal protein involved in several (if not all) PMN functions. It is possible to study its polymerization as a marker of neutrophil activation [63] where actin monomer (G-actin) will be reorganized to form, for example, filaments or F-actin. To do so, PMNs are incubated for short periods of time (normally from 0 to 30 min) at 37 °C with buffer (control) or with a molecule of interest in a final volume of 100  $\mu$ L. Synthetic peptide N-formyl-

methionyl-leucyl-phenylalanine (fMet-Leu-Phe) is used as a positive control for the assay [36]. After incubation, digitonin and paraformaldehyde are used for permeabilization and cell fixation, respectively, and then PMNs are washed twice by centrifugation and incubated with phalloidin-FITC (binds to filamentous of actin) for 20 min at 4 °C (light protected) prior to FACS analysis.

### 3.5. Expression of tyrosine phosphorylated proteins

Another manner to determine rapid PMN activation by a given agent is to determine its potential induction of phosphorylation events, especially tyrosine phosphorylation [44, 48]. This consists of incubating PMNs with a given agent for several short periods of time (typically, 30–60 s and 5, 15, and 30 min), and then the reaction is stopped by adding Laemmli's sample buffer, as described previously [30, 48]. Aliquots corresponding to a desired number of cells (we are using normally  $1 \times 10^6$  cells) are then loaded onto 10 % SDS-PAGE and transferred from gel to a nitrocellulose or polyvinylidene difluoride membrane and nonspecific sites are treated with a blocking solution [48]; the membrane is then washed and incubated with monoclonal anti-phosphotyrosine antibody. The membrane is washed and incubated with a horseradish peroxidase-conjugated anti-mouse IgG + IgM antibodies for about 1 h, and, after washing, phosphorylated bands are revealed with ECL Western blotting detection system. In our hands, protein loading is verified by probing the membrane (after stripping) with an anti- $\beta$ -actin or anti-GAPDH antibody and/or by staining the membranes with Coomassie blue at the end of each experiment.

### 3.6. Neutrophil adherence assay

Adhesion of PMNs is an important biological response involved during inflammation. This response could be studied by several ways. In my laboratory, we determine the capacity of PMNs to adhere onto a cell substratum using the well-characterized human epithelial A549 cells [64]. Briefly, after obtaining the desired confluence of A549 cells grown onto coverslips, PMNs (pretreated or not with a given agent for a desired period of time) are stained for 30 min with calcein AM and incubated with A549 cells. The number of adherent PMNs are then calculated by counting the number of fluorescent cells from five randomly selected high-power fields, as previously published [43, 64].

### 3.7. Chemotaxis

Chemotaxis of human PMNs could be easily studied using a Boyden chamber assay. The bottom wells are loaded with buffer or agonists to be tested (typically in a final volume of 25  $\mu$ l), the membrane is then placed over the wells, and the top layer of the chamber is added over the membrane. Cells (in 50  $\mu$ l) are added to the top chamber wells, and the chamber is incubated at 37 °C for a given period of time (normally 0–60 min) in a humidified incubator in the presence of 5 % CO<sub>2</sub>. After the incubation, the top of the chamber is removed and the upper side of the membrane is wiped carefully with the rubber scraper furnished by the manufacturer. Then, the membrane is fixed in methanol, colored with Hema-3 stain kit, mounted on a glass slide, and examined under oil immersion at 400 x. The details of the

procedure have been previously published [65, 66]. In this assay, the potent neutrophil chemokine CXCL8 (IL-8) is used as a positive control [67].

### 3.8. ROS generation

#### 3.8.1. Superoxide production

Several assays can be used to evaluate ROS generation by human PMNs. Since the major source of ROS in PMNs occurred after NADPH oxidase activation leading to superoxide production ( $O_2^-$ ), we routinely used the colorimetric assay based on reduction of cytochrome c, as previously published [48, 51]. Briefly, PMNs ( $1 \times 10^6$  cells/ml) are suspended in buffer supplemented with 1.6 mM  $CaCl_2$  with or without 10  $\mu$ g/ml superoxide dismutase (SOD) with 130  $\mu$ M ferrocyanide for 5–90 min at 37 °C in the presence of various concentrations of the agonists to be tested or phorbol 12-myristate 13-acetate (PMA) at  $10^{-7}$  M, used as a positive control. The reduction of cytochrome c is then monitored at 550 nm, and the concentration of  $O_2^-$  anions produced is calculated by the difference between corresponding wells with or without SOD using an extinction coefficient of 21.1.

#### 3.8.2. Detection of intracellular ROS

Flow cytometry is frequently used to measure ROS production. Using different probes, we can evaluate the production of ROS mainly originating from the mitochondria or endoplasmic reticulum, depending on the probe used. We routinely used the CM-H2DCFDA probe and measured the fluorescence with a FACScan. ROS production is expressed as mean fluorescence intensity (MFI) [48].

### 3.9. Degranulation

Degranulation is one of the most important functions exerted by PMNs for the defense of an organism. Following activation, PMNs will rapidly release potent degradative enzymes and several receptors involved in the recognition and ingestion of pathogens. These products are localized in different kinds of granules: azurophil, specific/gelatinase, and secretory granules [68, 69]. Because granules will fuse with the cytoplasmic membranes and release some products on the cell surface and then in the external milieu, it is possible to study in the laboratory the cell surface expression of markers of the different types of granules [70]. Therefore, we routinely determine the cell surface expression of CD35, CD63a, and CD66b by flow cytometry, since these molecules are specific markers of azurophil, specific/gelatinase, and secretory granules, respectively [47, 53, 56]. Excellent antibodies to these markers are commercially available.

Although the expression of the above markers could be increased (or not) at the PMN cell surface after stimulation, it is also important to determine if a protein known to be expressed in a given type of granule is released into the external milieu. To do so, we harvested the extracellular milieu after several periods of time following stimulation and performed Western blot experiments using antibodies specific for myeloperoxidase (azurophil), matrix metalloproteinase-9 or MMP-9 (specific/gelatinase), or albumin (secretory). The details of the protocol have been previously described [47, 53].



Even if a given granule product is released by activated PMNs into the external milieu, it is interesting to know also if an enzymatic activity could be preserved in the fluids. To test this, we performed zymography assay. After stimulation of human PMNs, cells are centrifuged at 13,000 rpm for 10 min at 4 °C and the pellets are discarded. The supernatants (10–50 µl, corresponding to 50,000 cells) are then mixed with a nonreducing buffer (40 % glycerol, Tris-HCl 1 M, pH 6.8, SDS 8 %) and separated on 10 % acrylamide gels containing 0.2 % gelatin. Gels are washed twice for 30 min with 2.5 % Triton X-100 and incubated overnight in digestion buffer (Tris-HCl 50 mM, pH 7.4, NaCl 150 mM, CaCl<sub>2</sub> 5 mM). The gels are stained with Coomassie blue 0.1 % and then destained. Densitometric analysis is performed to quantify the intensity of the white zones corresponding to gelatinase activity digesting gelatin incorporated into the gel [47, 53, 71].

### 3.10. Phagocytosis

Our preferred technique for evaluating PMN phagocytosis consists of the ingestion of opsonized sheep red blood cells (SRBCs). In this assay, PMNs are treated with a given agent or the corresponding buffer for a given period of time and cells are incubated with SRBCs pretreated with a final 1/200 dilution of commercially available rabbit IgG anti-SRBCs for 30–45 min at 37 °C in a 1:5 ratio. The samples are centrifuged 200 × g at 4 °C for 10 min. Supernatants are discarded and, to eliminate noningested SRBCs, the pellets are treated with 300 µl of H<sub>2</sub>O for 20 s followed immediately by the addition of 4.5 ml ice-cold PBS [45, 48, 54, 72]. After washing, the final pellets are suspended to a final concentration of 10 × 10<sup>6</sup> cells/ml. Duplicate cytocentrifuged preparations are then stained with Hema-3 stain kit, and the phagocytosis rate is determined by counting the number of PMNs ingesting at least one opsonized SRBC.

### 3.11. Apoptosis

Apoptosis can be evaluated by several different methods. We routinely determine the apoptotic rate of human PMNs by two assays in parallel, by cytology and flow cytometry [37, 38, 40, 44, 51, 73]. For both assays, freshly isolated human PMNs are incubated (10 × 10<sup>6</sup> cells/ml in RPMI-1640 supplemented with 10 % autologous serum) at 37 °C in 5 % CO<sub>2</sub> in 96-well plates for 24 h with a given agonist and its corresponding buffer/vehicle. Cells are then harvested to perform the assays as follows.

#### 3.11.1. Cytology

For cytology, cells are cytocentrifuged on microscope slides, stained with the Hema-3 staining kit and examined by light microscopy at 400× final magnification. Apoptotic neutrophils are defined as cells containing one or more characteristic darkly stained pyknotic nuclei. Results are expressed as the percentage of PMNs in apoptosis.

#### 3.11.2. Flow cytometry

For the flow cytometric procedures, PMNs are stained with FITC annexin-v or an FITC antihuman CD16 antibody. During apoptosis, the flip-flop of phosphatidylserines occurred, leading to their expression at the cell surface. Since annexin-v possesses a very high affinity to bind to phosphatidylserines, apoptotic cells will be positive to FITC annexin-v. In this assay,

propidium iodide can also be used to measure cell necrosis in parallel. In contrast, cell surface expression of CD16 (that is very high in normal PMNs) is lost during PMN apoptosis resulting from CD16 shedding.

### 3.12. Cytokine production

PMNs are known to produce several cytokines [74]. There are several different approaches that can be used to measure the production of cytokine. We prefer to study production of cytokine at the protein level. We used an antibody array assay for screening purpose and then quantified a given cytokine by ELISA. For both assays, supernatants of activated PMNs are harvested and used for the detection of cytokines. When fluids are frozen, we normally use them within three weeks after the experiments to eliminate possible cytokine/chemokine degradation occurring over time.

#### 3.12.1. Antibody array: Proteome profiler<sup>tm</sup> array

We used a commercially available human cytokine array panel for the screening purpose. All the steps for the detection of different analytes are performed following the manufacturer recommendation and as previously described [50, 75]. To detect the different analytes (cytokines/chemokines), we used pooled supernatants harvested from neutrophils ( $10 \times 10^6$  cells/ml in RPMI1640-HEPES p/s supplemented with 10 % autologous serum) treated for different periods of time with buffer (negative control) and LPS (positive control) or with the tested agents to probe the membranes. The chemiluminescent signal from the bound analytes present in the supernatants is then detected on Kodak X-OMAT-RA film. The signal intensity of each analyte (in duplicate) is normalized to the membrane's positive controls. Protein array membranes are scanned and densitometric analysis is performed using the AlphaEaseFC (FluorChem HD2) software.

#### 3.12.2. ELISA

The measurement of a given cytokine/chemokine (such as IL-6/IL-8) is determined with commercially available specific enzyme-linked immunosorbent assay (ELISA) kits. Neutrophils are incubated as above in a 96-well plate, and the supernatants are harvested, centrifuged, and stored at  $-80^\circ\text{C}$  for no more than three weeks before performing ELISA. Unlike the antibody array assay described above, each supernatant (normally at least from five different blood donors) is used to quantify the amount of the tested analyte.

### 3.13. De novo protein synthesis

Cells ( $10 \times 10^6$  cells/ml in RPMI-1640 medium supplemented with 10 % autologous serum) are metabolically labeled with 4.625 MBq of the Redivue Pro-Mix L- $^{35}\text{S}$  in vitro cell labeling mix in the presence or absence of a given agonist or with 1–10  $\mu\text{g/ml}$  cyclohexymide (CHX), an inhibitor of protein synthesis, or a mixture of both the agonist and CHX for 24 h [38, 41, 51, 76]. Cells are then harvested, and cell lysate is prepared for SDS-PAGE as previously described. After electrophoresis, gels are stained with Coomassie blue (to verify

equivalent loading), dried, and exposed with Kodak X-OMAT-RA film at  $-80\text{ }^{\circ}\text{C}$  for 1–3 days. Absence of new polypeptides is observed in the lanes where the cells were treated with CHX.

#### 4. Evaluating PMN infiltration In vivo

Several animal models of inflammation have been developed over the years. Some are suitable for understanding the mechanisms involved in the development of inflammatory diseases. The collagen-induced arthritis model is a good example [77-79]. Other models focus on pulmonary inflammation, including mouse models of allergic asthma. Typically, in this latter model, animals are sensitized to a foreign antigen by intraperitoneal injection in the presence or absence of an adjuvant. After the sensitization period, mice are challenged with the antigen directly in the lungs or the nose, and airway inflammation is then elicited. Although PMNs are known to exert some pathological effects during arthritis and in some cases during asthma, the observed cells in these models are not necessarily PMNs, but rather mainly some lymphocytes and eosinophils in collagen-induced arthritis and asthma models, respectively. However, in several other inflammatory models where a given agent is administered by inhalation, intratracheally or directly into lungs, PMNs will be easily observed in the bronchoalveolar lavages or lungs. However, these models are time consuming, are not the cheapest, and, in addition, necessitate a certain degree of technical skills. The rodent air pouch model of acute inflammation is probably the best, simple, not time-consuming, and cheapest model for monitoring leukocyte influx, including PMNs. This model has been used for investigating the inflammatory activity of a large number of compounds, including cytokines [28, 43, 80], plant extracts and lectins [81-83], different drugs [84-86], etc.

##### 4.1. Murine air pouch

Several kinds of mice can be used to perform this model. For screening purpose, we recommended to use outbred CD-1 mice since they are less expensive than other inbred mice. Normally, we use female mice (6–8 weeks of age). A period of acclimation of about one week is allowed to animals prior to initiation of the experiments. On days zero and three, mice are anesthetized with isoflurane, and 3 cc of sterile air was injected subcutaneously, in the back, with a 26-gauge needle to form an air pouch as published previously. On day six, 1 mL of buffer control (HBSS or PBS) or an increasing concentration of a given compound is injected directly into the air pouch. Mice are then killed by  $\text{CO}_2$  asphyxiation 3, 9, 12, or 24 h after the treatment, and the pouches are washed once with 1 ml and then twice with 2 ml of buffer containing 10 mM EDTA. Exudates are centrifuged at  $100 \times g$  for 10 min at room temperature, and supernatants are collected and stored at  $-80\text{ }^{\circ}\text{C}$  for further analysis. Cells are resuspended at  $0.5 \times 10^6$  cells/ml, spread onto microscope slides, and stained with Hema-3 stain kit for identification/quantification of leukocyte cell

subpopulations. The details of the model have been previously discussed [28, 35, 50, 87, 88]. Interestingly, several kinetics could be done with this model as well as several different experiments using the exudates such as the determination and quantification of different soluble factors including cytokines and chemokines. Moreover, the exudates could be used to perform zymography experiments to determine, for example, gelatinase activity. After centrifugation of the collected exudates, cells can also be incubated in vitro for studying the different PMN functions, especially in an experimental condition such as LPS-induced murine air pouches, where more than 85 % of cells are PMNs. It is also possible to purify a given type of leukocyte before performing in vitro assays. In brief, this model allows a panoply of different experiments. For example, we have used this model to demonstrate that an intraperitoneal administration of curcumin, prior to LPS-induced air pouch, was able to inhibit the proinflammatory effect induced by LPS [50].

## 5. Nanotoxicology and PMNs

While NPs have great potential for human needs, there are increasing concerns that the same features that make them so attractive and interesting also represent potential risks to human health [89]. Consequently, a new branch of toxicology, nanotoxicology, has recently emerged. Nanotoxicology could be defined simply as a discipline evaluating the role and safety of NPs on health. Exposure to NPs has increased dramatically in the past few years due to anthropogenic sources given that NPs can be formed via a wide variety of processes/methods. These sources are numerous and include internal combustion engines, power plants, and many other sources of thermodegradation [90]. Furthermore, intense research and development by the industry and academia multiply the number of individuals potentially exposed to NPs. Nanotoxicology is, therefore, a very complex discipline, and the diversity and complexity of NPs makes chemical characterization not only more important but also more difficult [91].

One of the most adverse effects of NPs reported in the literature is certainly inflammation. A variety of NPs were found to possess proinflammatory activities, principally based on their ability to increase the production of different proinflammatory cytokines [92-95] and on the observations that NPs can exacerbate airway inflammation in vivo [96-99]. However, as previously mentioned, inflammation is a normal biological response of the body to various assaults, including microorganisms, injuries, dusts, drugs, and other chemicals. Under normal circumstances, inflammation will subside and resolve itself in a healthy individual through a series of tightly regulated responses. However, when deregulation occurs, inflammation can lead to inflammatory disorders and diseases including asthma and several pulmonary lung diseases, dermatitis, arthritis, inflammatory bowel diseases, etc. [1, 100]. Curiously, despite the importance of PMNs in inflammation and since several studies reported an increased number of PMNs in NP-induced pulmonary inflammation, there are few studies investigating the direct effects of NPs and

their mode of action in PMNs. The following sections will cover different studies that have been done regarding the effects of some NPs on PMN cell physiology.

## 6. Interaction between nanoparticles and human PMNs

### 6.1. Interaction between nanoparticles and human PMNs: Not so novel finally

Probably the first study reporting a direct effect of NPs with human neutrophils was done more than 25 years ago where Hedenborg published in 1988 that titanium dioxide ( $\text{TiO}_2$ ) dust induced the production of ROS by human neutrophils as measured by a chemiluminescence assay [101]. Different dust particles (ranging in size from 345 to 1,000 nm) were tested, and none of them were cytotoxic, as assessed by lysozyme release or trypan blue exclusion [101]. It was concluded that  $\text{TiO}_2$  stimulated the chemiluminescence activity of PMNs in a concentration-dependent manner and that particle size and surface structure of the dust were important for determining the intensity of the response. Although the nanomaterial used for this study was not-typical NPs based on the definition that the three dimensions need to be smaller than 100 nm, the size was still in the nanometer range. In fact, the definition of an NP is complex, and according to the 2011 Commission of the European Union, the definition of an NP is: *“a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1-100 nm.”* Therefore, according to such a definition, a “nano” object needs only one of its dimensions, <100 nm, to be classified as an NP, even if its other dimensions are not in that range. Strictly based on the nanometer terminology, one can define any objects in the nm range as a nanoparticle or at least as a “nano” object as was the case with this study investigating the effect of  $\text{TiO}_2$  dust.

### 6.2. Studies from different laboratories

The next section will cover studies investigating the direct interaction between NPs and human neutrophils. Before describing works performed by others, I would like to mention that if some studies have been forgotten, this is completely unintentional on my part. Also, it is important to specify that few studies reported the effects of NPs on nonhuman neutrophils, including fish [102, 103] and rat [104] PMNs that are not part of this present review. Nevertheless, in brief, the results indicate that  $\text{TiO}_2$  NPs stimulated oxidative burst and NET release in fathead minnow PMNs [102], whereas fullerenes were found to inhibit oxidative burst and suppressed the release of NETs and degranulation of primary granules [103]. In rats, poly(lactide acid) or PLA nanoparticles were reported to be more efficiently phagocytosed than PLA/poly(ethylene glycol) or PEG blends [104].

In one study, human PMNs incubated with increasing concentrations of polymethylmethacrylate (PMMA) NPs (50–60 nm) in vitro were found to release lactate dehydrogenase, lysosome, and beta-glucuronidase in a dose-dependent fashion [105]. In contrast, PMMA NPs diminished migration of PMNs in a dose-dependent manner, as assessed by measuring the



distance attained by the leading front of cells in Boyden chambers. Interestingly, polystyrene beads (50 nm in diameter) were employed as a physical control throughout the study, and they were also found to affect the same PMN functions as compared with cells incubated with the buffer alone, but the intensity of the response was inferior to that of PMMA NPs.

In 1996, using solid lipid NPs (SLN) produced by high-pressure homogenization of melted lipids (glycerolbehenate, cetylpalmitate), Müller and colleagues modified the surface of these NPs with hydrophilic poloxamine 908 and poloxamer 407 block copolymers and found slightly different results regarding the phagocytic uptake and cell viability of human PMNs [106]. Interestingly, cell viability was  $\geq 80\%$  for all studied NPs with a diameter ranging from 123 to 246 nm, as assessed by the colorimetric MTT assay. Modification of the solid lipid NPs with poloxamine 908 and poloxamer 407 reduced phagocytic uptake to 8–15 % of hydrophobic polystyrene particles. The same team also reported, in another study, the in vitro cytotoxicity of SLN as a function of lipid matrix and stabilizing surfactant not only in mature PMNs but also in human promyelocyte HL-60 cells. These latter cells, which can be differentiated in laboratory toward neutrophil-like cells with dimethyl sulfoxide, were used for comparison with fully mature PMNs isolated from the blood of healthy volunteers. The aim was to use this cell line to replace the daily PMN isolation that is costly and time consuming. They reported that the nature of the lipid had no effect on PMN and HL-60 cell viability. However, some distinct differences were found for the surfactants. For example, binding of poloxamer 184 to the SLN surface reduced the cytotoxicity of the surfactant by a factor of  $\sim 65$ . They concluded that HL-60 cells represented a potentially good model for replacement of primary PMNs. In addition to HL-60, PLB-985 cells can also be driven by chemical treatment into “neutrophil-like cells but, as we previously documented, both cell lines can respond differently than PMNs [30, 107]. Therefore, I believe that results obtained from “real” human mature PMNs are more easily interpretable for the evaluation of human risk.

The cytotoxicity of injectable cyclodextrin nanoparticles/nanocapsules (specifically,  $\beta$ -CDC6) in mouse L929 fibroblasts and human PMNs has been determined in one study [108]. The cytotoxicity was evaluated in the presence or absence of PF68, the most commonly used surfactant in NP formulations and designed to be potentially utilized as an injectable nanosized drug carrier. Depending on the formulation, the particle size distribution was between  $\sim 110$  and 350 nm. Using MTT assay, it was concluded that  $\beta$ -CDC6 NPs do not exert a significant cytotoxicity against both types of cells. Of note, although the experimental conditions were appropriate for fibroblasts (three days before performing the assay), this is not necessarily the same for human PMNs known to spontaneously undergo apoptosis when incubated in vitro; about 50 % of PMNs are already in apoptosis after only 24 h [4, 37]. In 2006, the effects of cholesteryl butyrate (chol-but) solid lipid NPs and PMNs were investigated [109]. In vitro incubation of PMNs with  $10^{-8}$ - $10^{-4}$  M cholesteryl butyrate solid lipid nanoparticles (chol-but SLNs, mean diameter of 130–160 nm) for 10–240 min did not lead to cytotoxic effects as determined by the trypan blue exclusion assay [109]. Chol-but SLNs were found to inhibit adhesion of PMNs onto fetal calf serum-coated plastic wells as well as onto human umbilical vein endothelial cells. Also, in this study, the ability of FMLP-induced  $O_2^-$  production and FLMP-induced MPO release by PMNs was inhibited by chol-but SLN. More recently, the

capacity of human immune cells to internalize rod-shaped and spherical gold NPs (AuNPs), with diameters of 15–50 nm and a variety of surface chemistries, has been determined. Interestingly, in contrast to monocytes–macrophages that were found to ingest AuNPs [110], PMNs rather “trap” them in NETs [111]. The cell-gold networks, already observed after 15 min of treatment of immune cells with the AuNPs, were predominantly observed in PMNs and, to a lesser extent, in monocytes and macrophages. This indicates that NETs act as a physical barrier for NPs. In addition, in this study, the authors demonstrated that the particle shape is not very important for particle trapping, whereas the positive charges significantly enhance this phenomenon [111]. Influence of AuNPs on activation of human PMNs was also investigated in another study where AuNPs with a size of 60 nm were found to induce generation of free radicals as assessed by a chemiluminescence assay [112]. The authors proposed that the influence of AuNPs on the membrane surface potential of PMNs was most likely the mechanism involved.

Interaction between silver nanoparticles-polyvinyl-alcohol (AgNPs-PVA) and human PMNs was recently investigated. In this study, PMNs were incubated in the presence of 10  $\mu$ M of AgNPs-PVA, and the increased ROS production was determined by flow cytometry using the DCFH-DA probe [113]. Curiously, in this study, the authors determined necrosis and apoptosis by flow cytometry after staining with PI and annexin-v in human hepatocellular carcinoma (HepG2) and in peripheral blood mononuclear cells (PBMCs), but not in PMNs. Both cell necrosis and apoptosis were significantly increased after treatment with the NPs. Further, they investigated cellular uptake in HepG2 and PBMCs based on increased SSC fluorescence intensity recorded by flow cytometry, but, again, not in human PMNs.

Several PMN functions, including viability, chemotaxis, phagocytosis, oxidative burst, and cytokine production (IL-1 $\beta$ , IL-6, and IL-8), were investigated in response to an immunosuppressive agent sirolimus (SRL) alone, SRL-loaded poly(d,l-lactide) nanoparticles (SRL-PLA-NPs), and plain PLA-NPs [114]. While phagocytic activity was markedly reduced, but recovered within 3 h, the other tested PMN functions were not affected.

In their study, Haase et al. (2014) compared the effects of AgNPs and ionic silver (Ag<sup>+</sup>) on cells of the innate immune system, in particular on PMNs and macrophages [115]. They generated five kinds of AgNPs (diameters ranging from 2 to 35 nm) and did not observe any impact on phagocytosis, oxidative burst, as well as activation of the TNF- $\alpha$  promoter. In contrast, AgNPs and Ag<sup>+</sup> were found to induce NET release and to inhibit the formation of nitric monoxide. Also, both AgNPs and Ag<sup>+</sup> were found to increase intracellular ROS levels as well as the second messenger Zn<sup>2+</sup>. Therefore, based on these data, the effect of AgNPs on human PMNs is not specific to the particles since they are also observed with Ag<sup>+</sup>.

### 6.3. Our involvement in studying interaction with NPs and human PMNs

Our laboratory has been interested in investigating the role of NPs on the biology of human PMN because one of the most reported adverse effects of NPs after administration in animals or when incubated in vitro in different type of cells is inflammation, our main expertise. Of note, we voluntarily use unloaded, naked, or plain engineered NPs in our present studies based on the fact that several kinds of NPs are commercially available and since we believe that it is

important to first establish how human PMNs will react with a given NP before trying to use this latter as potential carrier for drug delivery, for example. In addition, since these NPs are relatively easy to obtain and that several of them are probably already used by workers in different kinds of industries, it is warranted to understand their mode of action as they are handled and/or used by individuals. The first study was published in 2010, indicating that our involvement in this area of research is recent [57]. We investigated how human PMNs respond to TiO<sub>2</sub> NPs since these NPs were (and are still) the most studied NPs reported in the literature. We used a commercially available preparation of TiO<sub>2</sub> NPs (anatase crystals) of 1–10 nm in size, as determined by transmission electronic microscopy (mentioned in the technical data sheet and also confirmed by us). We first incubated freshly isolated human PMNs with increasing concentrations of TiO<sub>2</sub> NPs (0–800 µg/ml) over time and determined their potential cytotoxicity. As assessed by trypan blue exclusion assay, the NPs did not decrease cell viability and only a small portion ≤ 3 % of cells were in necrosis after 24 h of incubation at the highest concentration tested. We next determined if TiO<sub>2</sub> NPs could induce morphological cell shape changes in PMNs, an indicator of cell activation. After 24 h, the optimal concentration inducing cell shape changes was 100 µg/ml, a concentration used by others with human lymphocytes [116]. Others reported the use of TiO<sub>2</sub> NPs up to 4,000 µg/ml for in vitro studies, but in U937 human monoblastoid cell line [117]. Interestingly, we demonstrated that TiO<sub>2</sub> NPs were able to induce rapid tyrosine phosphorylation events in PMNs as quickly as 15 s, with a maximal effect at 1 min of treatment. More specifically, we identified Erk-1/2 and p38 MAP kinases, the two major enzymes involved in different PMNs functions, as targets of TiO<sub>2</sub> NPs [57]. Concordant with our data indicating TiO<sub>2</sub> NPs were not cytotoxic, we reported and demonstrated that they significantly inhibited PMN apoptosis. Using an antibody array assay allowing the simultaneous detection of different cytokines/chemokines, TiO<sub>2</sub> NPs were found to increase the production of 13 analytes, including IL-8 and Gro-α, two potent neutrophil activators. They exhibited the greatest increase (~16 times and ~4 times more vs control cells, respectively). Because antibody assay is a semiquantitative assay, we next confirmed that TiO<sub>2</sub> NPs increased IL-8 production by quantitative ELISA. Taken together, these results clearly indicate that TiO<sub>2</sub> NPs are neutrophil activators.

In another study, we focused our attention on the human PMN degranulation process not only in response to TiO<sub>2</sub> NPs but also after treatment with two other metal oxide NPs, zinc oxide (ZnO) and cerium dioxide (CeO<sub>2</sub>) [56]. This was probably the first study investigating the effect of NPs on degranulation. Because TiO<sub>2</sub> was previously found to activate PMNs, we first determine whether or not ZnO and CeO<sub>2</sub> NPs could be neutrophil modulators. We found that all NPs (having the size of 1–10 nm) were able to activate PMNs, based on induction of actin polymerization. As assessed by flow cytometry, the three types of NPs slightly downregulated cell surface expression of the granule marker CD35 but increased CD66b and CD63 expression. In addition, the protein expression of myeloperoxidase, MMP-9 and albumin stored in azurophil, specific/gelatinase, and secretory granules, respectively, was significantly increased in the supernatants of NP-induced PMNs vs supernatant from untreated cells. Also, both TiO<sub>2</sub> and CeO<sub>2</sub> were found to markedly increase the enzymatic activity of MMP-9 released into the supernatants, as determined by gelatin zymography. ZnO NPs were found to only exert a modest effect in these experiments. Therefore, the three NPs can differentially affect all steps

involved during neutrophil degranulation, namely, cell surface expression of granule markers, liberation of proteins in the supernatants, and enzymatic activity [56].

We next examined the role of AgNPs with a starting size of 20 nm (AgNP<sub>20</sub>) on human PMN apoptosis. Treatment of PMNs with AgNP<sub>20</sub> results in increased cell size, and TEM experiments revealed that AgNP<sub>20</sub> can rapidly interact with the cell membrane, penetrate inside neutrophils, localize in vacuole-like structures, and be randomly distributed in the cytosol after 24 h [59]. Treatment with 100 µg/ml AgNP<sub>20</sub> for 24 h (but not 10 µg/ml) increased the PMN apoptotic rate as determined by cytology and by flow cytometry after staining with FITC annexin-v. Also, AgNP<sub>20</sub> was found to inhibit de novo protein synthesis as demonstrated by gel electrophoresis of metabolically [<sup>35</sup>S]-labeled cells as strong as the potent protein inhibitor cycloheximide. Therefore, AgNP<sub>20</sub> was identified as potent PMN proapoptotic agents. Preliminary experiments indicate that AgNP<sub>70</sub> (70 nm), in contrast to AgNP<sub>20</sub>, delays human PMN apoptosis (*our unpublished data*) in agreement with the fact that an NP with a different size (here 20 vs 70 nm) can act completely different.

Based on these results, we were interested in investigating more in depth how ZnO NPs can alter human PMN biology. We demonstrated that ZnO increased the cell size, induced cell shape changes, activated phosphorylation events, and enhanced cell spreading onto glass, but did not induce the generation ROS [58]. In contrast to AgNP<sub>20</sub>, treatment of PMNs with ZnO markedly and significantly inhibited apoptosis and increased de novo protein synthesis. Utilization of cycloheximide reversed not only the ability to increase de novo protein synthesis but also the antiapoptotic effect of ZnO NPs. It was concluded that ZnO NPs are activators of several human PMN functions and that they inhibit apoptosis by a de novo protein synthesis-dependent and ROS-independent mechanism. In the future, it will be of interest to identify the nature of proteins that are neo-synthesized in response to ZnO NPs.

## 7. In vivo infiltration of PMNS induced by NPs using the murine air pouch model

The effect of different sets of nanoparticles were tested by Vandooren et al. [29] using the murine air pouch model. The tested NPs were CANs maghemite, type I PEI-CAN-maghemite, type II PEI-CAN-maghemite, PDMAEMA-SCPNs, PMAAc-SCPNs, PLGA-COOH, PLGA-b-PEG-COOH, Magh@PNPs, LNP LII, and CAN CIII. All details regarding their characterization (size, zeta potential, PDI, etc.) are described in their study. When compared with the negative control (phosphate-buffered saline or PBS), the number of attracted leukocytes were increased with all NPs, but PMAAc-SCPNs and CAN CIII. The highest count they observed was  $\sim 2.8 \times 10^6$  cells/pouch, after treatment with type II PEI-CAN-maghemite NPs vs  $\sim 0.8 \times 10^6$  cells/pouch. Interestingly, for almost all tested NPs, the majority of leukocytes recruited into air pouch were PMNs (sometime up to  $\sim 90$  %). From this study, it was proposed to use the air pouch leukocytosis model as a future standard assay for an in vivo test for nanoparticles. Although I am in favor, it is important to mention that the leukocyte infiltration was only determined after 24 h of treatment, a time point that is probably not the optimal one. As



previously proposed [118], it is better to perform kinetic experiments with the murine air pouch model, especially between 3 and 12 h, as we previously documented for determining the effect of different kinds of agents on inflammation [28, 35, 43, 50, 87, 88, 119]. This is particularly true for NPs, since their mode of action is still not fully understood. Moreover, time points after 24 h should also be investigated because of lack of knowledge with proinflammatory activity of NPs in such a model.

The potential proinflammatory activity of iron oxide-containing magnetic nanoparticles (MNPs) was investigated using the murine air pouch model. Administration of 1,000-MNPs and 2,000-MNPs (1,000 and 2,000 referring to 1,000  $\mu\text{g}$  or 2,000  $\mu\text{g}$  of iron in terms of CAN maghemite particles) in C57BL/6 mice induced a prominent influx of leukocytes, mainly PMNs as determined by differential count and by flow cytometry using anti-CD11b + anti-/Gr-1 antibodies. The neutrophilia was similar to the effect obtained with the positive control chlorite-oxidized oxyamylose [120]. Since several authors of this study were also authors in the original work describing the use of the murine air pouch as a standard assay for in vivo testing of NPs, they did not evaluate the effects of the NPs before the 24 h time point.

Although we have been using the murine air pouch model in our laboratory for  $\sim 13$  years for testing potential effects of different agents on inflammation, it is only recently in 2011 that we used it to demonstrate for the first time that a given NP, namely, titanium dioxide ( $\text{TiO}_2$ ), was proinflammatory. Indeed, administration of a single dose of  $\text{TiO}_2$  NPs into the air pouch attracted leukocytes after 3–9 h, where more than 80 % of cells were PMNs [75]. In addition, in this study, we reported that  $\text{TiO}_2$  NPs induced the production of several chemokines locally present in the air pouch exudates. This model is a simple model that allows investigation of an acute inflammatory response, the first step leading to chronic inflammation when deregulation occurs, such as the many studies reporting an increased number of PMNs in the lung/BALS [98, 99, 121–125]. On the other hand, the fact that an NP possesses some proinflammatory properties could be of help for the design of drug delivery by NPs for clinical purposes. In this regard, one can imagine attracting leukocytes such as PMNs into an inflammatory site by a NP delivering (or coated with) a neutrophil proapoptotic molecule. Although this is speculative at the moment, this remains an interesting avenue of research that needs to be explored in the future.

Kinetic and dose-dependent experiments performed with the murine air pouch model of acute inflammation revealed that, unlike  $\text{TiO}_2$  used as a positive control in this model,  $\text{C}_{60}(\text{OH})_n$  (fullerenols) NPs were not proinflammatory in CD-1 mice [126]. To further confirm this negative result and since, yet, no genetic susceptibility has been reported regarding the biological activity of NPs, we performed other sets of experiments using C57BL/6 and BALB/c mice. Again, no significant leukocyte attraction was observed into air pouch. However, after 3 h of treatment,  $\text{C}_{60}(\text{OH})_n$  NPs were found to amplify the effect of lipopolysaccharides (LPS) causing a rapid leukocyte influx in which the major cells observed were PMNs. Using an antibody array assay to detect different analytes present in the exudate led us to conclude that the amplification effect is explained, at least partially, by an increased local production of several cytokines/chemokines in the exudates, including the proinflammatory cytokines IL-1 $\beta$  and IL-6. In fact, the profile of analytes was different in response to LPS alone,  $\text{C}_{60}(\text{OH})_n$  alone, and the mixture of both.



Using an ELISA to quantify the amount of IL-6, we demonstrated that C<sub>60</sub>(OH)<sub>n</sub> increases the LPS-induced local production of this cytokine. Therefore, although C<sub>60</sub>(OH)<sub>n</sub> NPs alone do not exert proinflammatory activity under certain conditions, they can act in concert with other agents to cause inflammation, a situation that is likely to occur *in vivo*. These results further reinforce that the murine air pouch model is to be performed at different periods of time (kinetic experiments) as discussed above.

## 8. Conclusion

Most of the *in vitro* studies discussed above evaluated the cytotoxicity and/or only one or few PMN response(s). Yet, no study other than our own has determined the effects of NPs on the PMN apoptotic rate, a very important process for regulating the number of PMNs. However, more recent reports have investigated the interaction between NPs and PMNs by studying different functions/responses, an approach that we encourage. The fact that TiO<sub>2</sub> NPs induced the production of several analytes, including two potent chemokines (IL-8 and Gro- $\alpha$ ) is a good example demonstrating that NPs can indeed target PMNs that, in turn, could attract/activate other cells [57]. It is highly probable that, in the forthcoming years, several aspects regarding the effects of different NPs on the biology of human PMNs will be studied. Although there is a growing interest in developing *in vitro* assays in nanotoxicology [127], it is also strongly encouraged to use primary human cells as a source of *in vitro* cells for testing NPs, since cancerous cell lines of different origins will complicate data interpretation for the evaluation of human risk. In addition, determining how a given NP alters inflammation *in vivo* in other models than those exclusively targeting the lungs, such as the murine air pouch model, will help us to better evaluate the potential toxic mechanisms of NPs, especially how they alter the inflammatory process.

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