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Activation of human neutrophils by titanium dioxide (TiO_2) nanoparticles

D.M. Gonçalves, S. Chiasson, D. Girard *

Laboratoire de recherche en inflammation et physiologie des granulocytes, Université du Québec, INRS-Institut Armand-Frappier, Laval, QC, Canada H7V 1B7

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ABSTRACT

This paper describes the *in vitro* effects of titanium dioxide (TiO_2) nanoparticles (NPs) upon human neutrophils. Kinetic experiments revealed no cell necrosis after 24 h of treatment with TiO_2 (0–100 μg/ml). In contrast, TiO_2-induced change in cellular morphology in a concentration-dependent manner in neutrophils over time, indicating its potential to activate these cells. To further support this, we demonstrated that TiO_2 markedly and rapidly induced tyrosine phosphorylation events, including phosphorylation of two key enzymes, p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinases-1/2 (Erk-1/2). We also determined the effects of TiO_2 on two neutrophil functions requiring a longer exposure period between NPs and cells: apoptosis and cytokine production. Interestingly, at concentrations $\geqslant$20 μg/ml, TiO_2 inhibited neutrophil apoptosis in a concentration-dependent manner after 24 h of treatment. Supernatants from TiO_2-induced neutrophils were harvested after 24 h and tested for the presence of 36 different analytes (cytokines, chemokines) using an antibody array assay. TiO_2 treatment increased production of 13 (36%) analytes, including IL-8, which exhibited the greatest increase ($\sim$16 $\times$ control cell levels). The increased production of IL-8 was confirmed by ELISA. We conclude that TiO_2 exerts important neutrophil agonistic properties *in vitro*.

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1. Introduction

The use of nanoparticles (NPs) in various fields, including biotechnology, has gained increasing attention in the past few years (Moghimi et al., 2005). For example, NPs have great potential as tools for drug delivery in medicine (Laroui et al., 2009). However, the rapid development of nanotechnology has resulted in a surge of interest in nanotoxicology, since exposure of humans to NPs resulting from industrial activity is unavoidable.

Titanium dioxide (TiO_2) is used extensively as an industrial nanomaterial. TiO_2 NPs are used in a variety of products including anti-fouling paints, coatings, ceramics, sunscreens, and also as additives in pharmaceuticals and food colorants (Jin et al., 2008; Vamanu et al., 2008). Titanium (pure or in alloys) is widely used in several types of implanted medical devices, including cardiovascular stents, joint replacements and dental implants (Brien et al., 1992; Buly et al., 1992; Arys et al., 1998). Several studies evaluating potential health risks of TiO_2 NPs to humans have received increasing attention (Puccetti and Leblanc, 2000; Radnai et al., 2000; Vamanu et al., 2008; Schanen et al., 2009). It appears that, TiO_2 NPs possess pro-inflammatory properties similar to those exhibited by other nanomaterials (Blackford et al., 1997; Park et al., 2009; Schanen et al., 2009). Analysis of the pertinent scientific literature indicates that the reported pro-inflammatory effects of TiO_2 NPs were observed *in vitro* tests conducted with pulmonary cells, or from *in vivo* studies in animals following lung instillation.

Polymorphonuclear neutrophils (PMNs) are primordial players in innate immunity and provide an effective defense against bacterial and fungal infections (Whyte et al., 1997; Akgul et al., 2001). They are terminally mature non-dividing phagocytic cells which develop in the bone marrow from $CD34^+$ stem cells. It has been estimated that $\sim 5 \times 10^{10}$ cells per day, in a normal adult, are released from the bone marrow, representing one of the fastest rates of cell turnover in the human body. Therefore, PMN cell turnover must be under strict control. Interestingly, PMNs are known to spontaneously undergo apoptosis, without apparent stimulation, explaining, in part, why the number of PMNs remains relatively stable in healthy individuals. In contrast, during inflammation, the number of PMNs markedly increases but, under normal circumstances, resolution of inflammation is largely achieved by elimination of apoptotic PMNs by professional phagocytes, including macrophages.

Curiously, despite the fact that neutrophils play a key role during inflammation, and that increased PMNs have been observed in TiO_2-induced lung inflammation *in vivo,* there is a lack of literature concerning the role of TiO_2 NPs on neutrophil cell physiology, particularly human PMNs. One study conducted more than 20 years ago reported that pure rutile or anatase TiO_2 preparations elicited only a weak chemiluminescent response, indicating that these NPs

* Corresponding author. Address: INRS-Institut Armand-Frappier, 531 boul. des Prairies, Laval, QC, Canada H7V 1B7. Tel.: +1 450 687 5010x8847; fax: +1 450 686 5309.
E-mail address: denis.girard@iaf.inrs.ca (D. Girard).

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did not significantly increase reactive oxygen species production by these cells (Hedenborg, 1988).

In the present study, we investigated the potential agonistic effect of TiO_2 NPs on several human neutrophil responses/functions, some known to occur rapidly and others necessitating a prolonged exposure to NPs for measurement. We found that TiO_2-induced changes in cellular morphology and caused the rapid and vigorous tyrosine phosphorylation of several proteins, including p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinases-1/2 (Erk-1/2). TiO_2, also induced suppression of PMN apoptosis and increased the production of several cytokines/chemokines, including IL-8, the predominant chemokine produced by these cells.

2. Materials and methods

2.1. Chemicals, agonists and antibodies

The chemicals agonist and antibodies used in this were Titanium dioxide (TiO_2) nanoparticles (anatase crystals) (Vivenano, Toronto, ONT); PD98059 and SB203580 inhibitors (Sigma–Aldrich, Saint-Louis, Missouri); anti-phosphospecific p38 ($pTpY^{180/182}$) and Erk-1/2 ($pTpY^{185/187}$) (BioSource, Camarillo, CA); antibodies against the non-phosphorylated form of p38 (sc-535) (Santa Cruz, Biotechnology, Santa Cruz, CA); polyclonal Erk-1/2 (Upstate Biotechnology, Lake Placid, NY); anti-phosphotyrosine (Cell Signalling Technology, Inc., Danvers, MA); GM-CSF (PeproTech Inc., Rocky Hill, NJ, USA); and arsenic trioxide (As_2O_3 or ATO) (Sigma–Aldrich, Saint-Louis, Missouri).

2.2. 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 previously described (Pelletier et al., 2004; Binet et al., 2008). Blood donations were obtained from informed and consenting individuals according to institutionally approved procedures. Cell viability was monitored by trypan blue exclusion and the purity (>98%) was verified by cytology from cytocentrifuged preparations colored by Diff-Quick staining (Fisher Scientific, Ottawa, Canada). Cell viability was systematically evaluated before and after each treatment and was always $\geqslant$99%.

2.3. Microscopic observations of neutrophils

These were performed essentially as previously reported (Girard et al., 1997). Cells (10×10^6 cells/ml) were incubated at 37 °C in 5% CO_2 in 96-well plates for up to 24 h in the presence of buffer or an increasing concentration of TiO_2 (0, 2 10, 20, 50 or 100 μg/ml). Morphological changes in cells were observed under light microscopy (200×), and photomicrographs were taken using a Nikon Eclipse TS100 camera.

2.4. Phosphorylation events

Neutrophils (40×10^6 cells/ml in RPMI 1640) were incubated for the indicated periods of time at 37 °C with buffer or the indicated agonist in a final volume of 120 μl. Reactions were stopped by adding 125 μl 2× Laemmli's sample buffer, as described previously (Pelletier et al., 2000). Aliquots corresponding to 1×10^6 cells were loaded onto 10% SDS–PAGE and transferred from gel to a nitrocellulose membrane (Amersham Pharmacia Biotech Inc.) Non-specific sites were blocked with 1% BSA in TBS-Tween (25 mM Tris–HCl, pH 7.8, 190 mM NaCl, 0.15% Tween-20) for 1 h at room temperature. After washing, the membranes were incubated with monoclonal anti-phosphotyrosine UB 05-321 (1:4000; United Biomedical Inc. (UBI), Hauppauge, NY, USA) for 1 h at room temperature. Membranes were then washed and incubated with a HRP-conjugated goat anti-mouse IgG + IgM (1:10,000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for 1 h at room temperature in fresh blocking solution. Membranes were washed three times with TBS-Tween, and phosphorylated bands were revealed with the ECL Western blotting detection system (Amersham Pharmacia Biotech Inc.). Protein loading was verified by staining the membranes with Coomassie blue at the end of each experiment.

For MAPK activation, aliquots (corresponding to 1×10^6 cells) were prepared as above and transferred to nitrocellulose (p38) or polyvinylidene difluoride membranes (Erk-1/2). Membranes were blocked for 1 h at room temperature in Tris-buffered saline (TBS)-Tween containing 1% bovine serum albumin (BSA). After washing, an anti-phosphotyrosine antibody, the anti-phospho-specific p38 or Erk-1/2 antibody or the antibody directed against the corresponding unphosphorylated form were added at a final dilution of 1:1000 for phosphospecific antibody or 1:400 for the unphosphorylated form respectively, in TBS-Tween. The membranes were kept overnight at 4 °C and were then washed with TBS-Tween and incubated for 1 h at room temperature with a goat anti-rabbit horseradish peroxidase (HRP) secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:20,000 in TBS-Tween + 5% BSA or a goat anti-mouse HRP secondary antibody (Jackson ImmunoResearch Laboratories) at 1:20,000 in TBS-Tween + 5% nonfat dry milk followed by several washes. Protein expression was revealed with the ECL system as described above.

2.5. Assessment of neutrophil apoptosis

Freshly isolated human PMNs (10×10^6 cells/ml in RPMI 1640-HEPES-P/S, supplemented with 10% heat-inactivated autologous serum) were treated with or without increasing concentrations of TiO_2 for 24 h. Cytocentrifuged samples of PMNs were prepared using a Cyto-tek® centrifuge (Miles Scientific, Naperville, IL), stained with the Hema 3 Stain staining kit according to the manufacturer's instructions and processed as documented previously (Pelletier et al., 2004; Binet et al., 2006). Cells were examined by light microscopy at 400× final magnification, and apoptotic neutrophils were defined as cells containing one or more characteristic, darkly stained pyknotic nuclei.

2.6. Proteome profiler™ array

The human cytokine array panel A was purchased from R&D Systems Inc. (Minneapolis, MN) and all the steps for the simultaneous detection of 36 different analytes were performed within 2 weeks of harvesting cells, as per the manufacturer's recommendation. Previous experiments conducted with two membranes probed with the exudates from two different blood donors treated with LPS revealed that the results were reproducible (*data not shown*). Consequently, we pooled the supernatants ($n = 3$ experiments) harvested from HBSS-treated (control) or TiO_2-induced human neutrophils to probe the membranes. The chemiluminescent signal from the bound cytokines/chemokines present in the supernatants was detected on Kodak X OMAT-RA film. The signal intensity of each analyte (in duplicate) was normalized to the membrane's positive controls. Results are expressed as fold-increase (ratio of tested group/control). Ratios $\geqslant$1.2 were considered slightly positive and those $\geqslant$1.5 were considered strongly positive. Protein array membranes were scanned and densitometry analysis

was performed using the Multi-Analyst program (Bio-Rad, Hercules, CA).

2.7. IL-8 production

The measurement of IL-8 production was determined using a commercially available ELISA kit (Medicorp), essentially as previously described (Pelletier et al., 2004) Freshly isolated human neutrophils were incubated in the presence or absence of TiO_2 at 37 °C in 5% CO_2 for 20 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.

2.8. Statistical analysis

Statistical analyses were performed, using Student-*t*-test with SigmaStat for Windows (version 3.0). Statistical significance was established at $p < 0.05$.

3. Results

3.1. IL-4 induced morphological changes in neutrophils

The induction of morphological changes in PMNs is a rapid and simple assay used as a reflection of neutrophil activation (Girard et al., 1997). PMNs were incubated with increasing concentrations of TiO_2 (0–100 μg/ml) and morphology was observed, overtime, under light microscopy. As shown in Fig. 1, untreated neutrophils remained spherical (panel A), whereas cells incubated with an increasing concentration of TiO_2 exhibited morphological changes. Results illustrated in Fig. 1 were obtained after 1 h of incubation. The differences in cell morphology were observed as early as 30 min, but were less evident (*data not shown*). Morphology of cells treated with concentrations ⩽20 μg/ml TiO_2 was similar to that of untreated cells. However, clear effects were observed at 50 μg/ml (panel C) and 100 μg/ml (panel D).

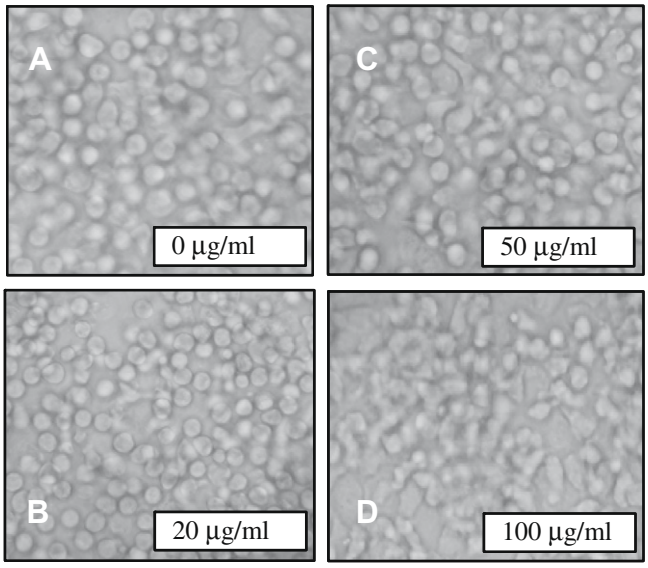

Fig. 1. TiO_2-induced morphological changes in PMNs in vitro. PMNs (10×10^6 cells/ml) were incubated in the presence or absence (A) of 20 (B), 50 (C) or 100 μg/ml TiO_2 at 37 °C in 5% CO_2 for up to 24 h in 96-well plates and morphological changes were observed by cytology, as described in Materials and Methods. Pictures were taken 60 min after treatment and the results are representative of three different experiments. Note the spherical shape of naïve untreated cells (A) that changes to an increasingly irregular shape observed in a concentration-dependent fashion in response to TiO_2.

3.2. TiO_2-induced phosphorylation events in human PMNs

The above results indicated that TiO_2 activated neutrophils. Because of this activation and the fact that phosphorylation events in response to TiO_2-induced human PMNs have never been investigated, we then treated freshly isolated cells with TiO_2 and tested its ability to induce general tyrosine phosphorylation events. As assessed by western blot, TiO_2-induced rapid tyrosine phosphorylation of different proteins as early as 15 s after TiO_2 exposure (Fig. 2). The maximal effect was observed after 60 s, followed by a decline in intensity of the tyrosine phosphorylation signal after a few minutes; however, the signal remained elevated for up to 30 min as compared to control cells (last vs. the first lane). The cytokine GM-CSF was used as a positive control (Pelletier et al., 2000).

3.3. TiO_2 activated p38 and Erk-1/2 MAPK in human PMNs

Next, we determined whether or not TiO_2 could specifically activate Erk-1/2 and/or p38 MAPKs, two key kinases involved in a variety of PMN functions (Krump et al., 1997; Mocsai et al., 2000). As illustrated in Fig. 3, TiO_2 activated both Erk-1/2 (upper panel) and p38 (lower panel) MAPKs, even at the lowest concentration of 0.002 μg/ml. Interestingly, Erk-1/2 phosphorylation was more intense after 5 min of treatment with TiO_2 when compared to p38. In contrast, p38 phosphorylation was more intense than Erk-1/2 after 15 min of treatment, suggesting that TiO_2 activates Erk-1/2 prior to p38 activation.

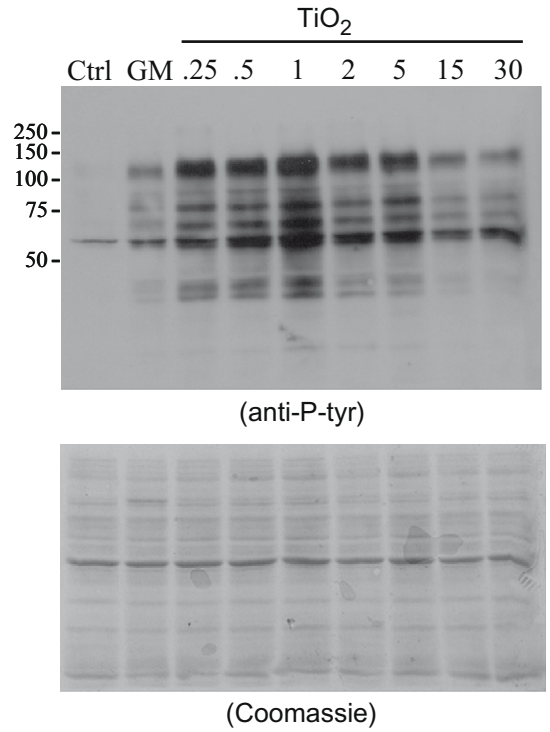

Fig. 2. TiO_2-induced phosphorylation events in human PMNs. Freshly isolated human PMNs (10×10^6 cells/ml) were incubated with buffer (Ctrl), 65 ng/ml GM-CSF (GM) for 1 min or 100 μg/ml TiO_2 0.25–30 min, and the expression of tyrosine-phosphorylated proteins (anti-P-tyr) was assessed by Western blot, as described in Section 2. Protein loading was verified by staining the membranes with Coomassie blue at the end of each experiment. Results are from one representative experiment out of four. Molecular weights are indicated on the left.

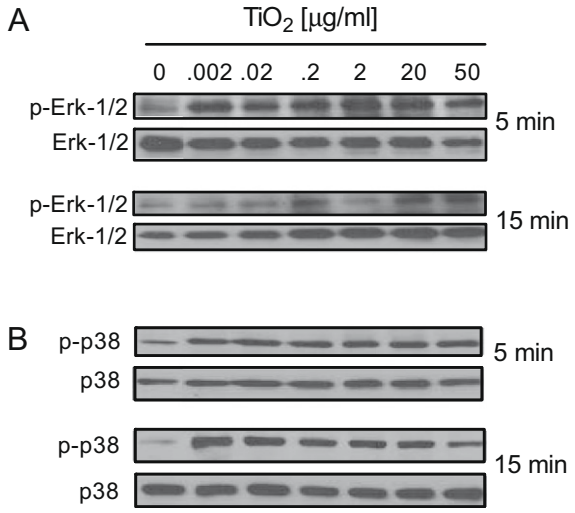

Fig. 3. TiO_2 activated both Erk-1/2 and p38 MAPKs in PMNs. Cells were treated with the indicated concentrations of TiO_2 for 5 or 15 min and phosphorylation of Erk-1/2 (A) or p38 (B) MAPK was assessed by immunoblotting, using specific antibodies as described in Section 2. Results are from one representative experiment out of three.

3.4. TiO_2 nanoparticles inhibited human PMN apoptosis

Fig. 4 illustrates that TiO_2 inhibited PMN apoptosis in a concentration-dependent manner. The neutrophil apoptotic rate was similar when cells were incubated with 0.002 (56.5 ± 10.7%, mean ± SEM, $n = 5$), 0.02 (57.9 ± 10.4%) or 2 μg/ml TiO_2 (55.5 ± 7.1%), as compared to SA (59.9 ± 9.7%). This basal apoptotic rate decreased when neutrophils were incubated with 20 μg/ml (30.5 ± 9.5%), but this decrease was not significant. In contrast, concentrations of 50 or 100 μg/ml TiO_2 significantly inhibited PMN apoptosis (20.4 ± 6.5% and 11.2 ± 5.7%, respectively).

3.5. TiO_2 induced the production of cytokines and chemokines in human PMNs: predominance of IL-8

As illustrated in Fig. 5, TiO_2 increased the production of several analytes in PMNs. Among 36 analytes, 13 (36%) displayed a ratio >1 (right of the dotted line in the figure); 12 (33%) with a ratio $\geqslant 1.2$; 7 (19%) with a ratio $\geqslant 1.5$; and 2 (5.5%) with a ratio $\geqslant 2$. These last two are the known potent chemokines GRO-α (ratio of 4.1) and IL-8, with the highest ratio of ~16.

3.6. Measurement of IL-8 production in TiO_2-induced human PMNs

As illustrated in Fig. 6, TiO_2 increased the production of IL-8 in a concentration-dependent manner. The basal level of IL-8 was 422.0 ± 104 pg/ml (mean ± SEM, $n = 3$) and this increased to 647.3 ± 217, 1129 ± 263, 1233 ± 44, and 1237 ± 133 pg/ml for cells treated with 2, 20, 50 or 100 μg/ml TiO_2, respectively.

4. Discussion

Different cellular responses and functions were investigated in order to clearly establish that TiO_2 is a human PMN agonist. We initially eliminated the possibility that TiO_2-induced cell necrosis in human PMNs. Our results agree with those of a previous study

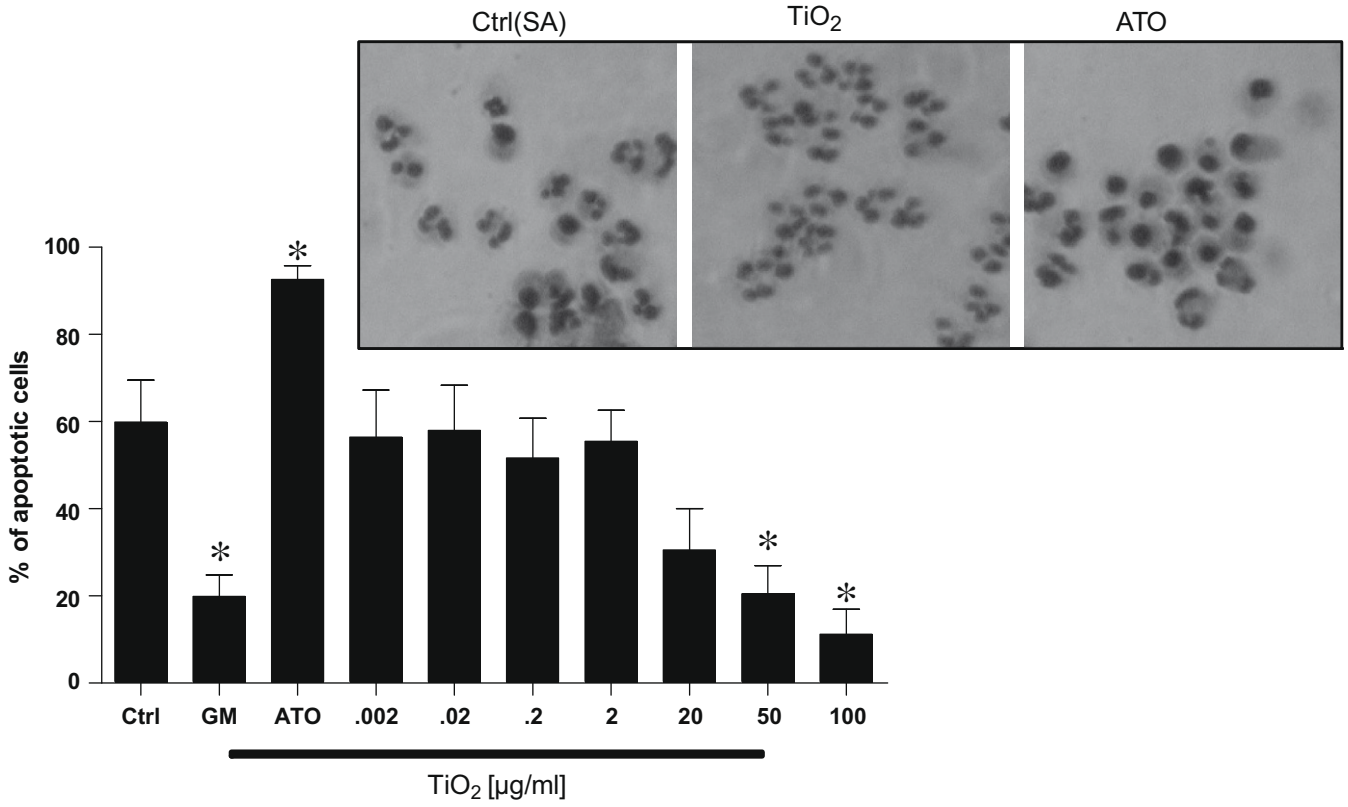

Fig. 4. High concentrations of TiO_2 inhibited human neutrophil apoptosis. PMNs (10×10^6 cells/ml) were incubated with buffer (Ctrl), 65 ng/ml GM-CSF, 5 μM arsenic trioxide (ATO) or the indicated concentrations of TiO_2 and apoptosis was assessed by cytology. Results are means ± SEM ($n \geqslant 3$). $^*P < 0.05$ vs. Ctrl. Inset, note the anti-apoptotic effect of TiO_2 at 100 μg/ml (middle panel) where almost all cells possess the characteristic poly-lobed nucleus, as opposed to the pyknotic nuclei observed after the treatment with the potent pro-apoptotic molecule ATO.

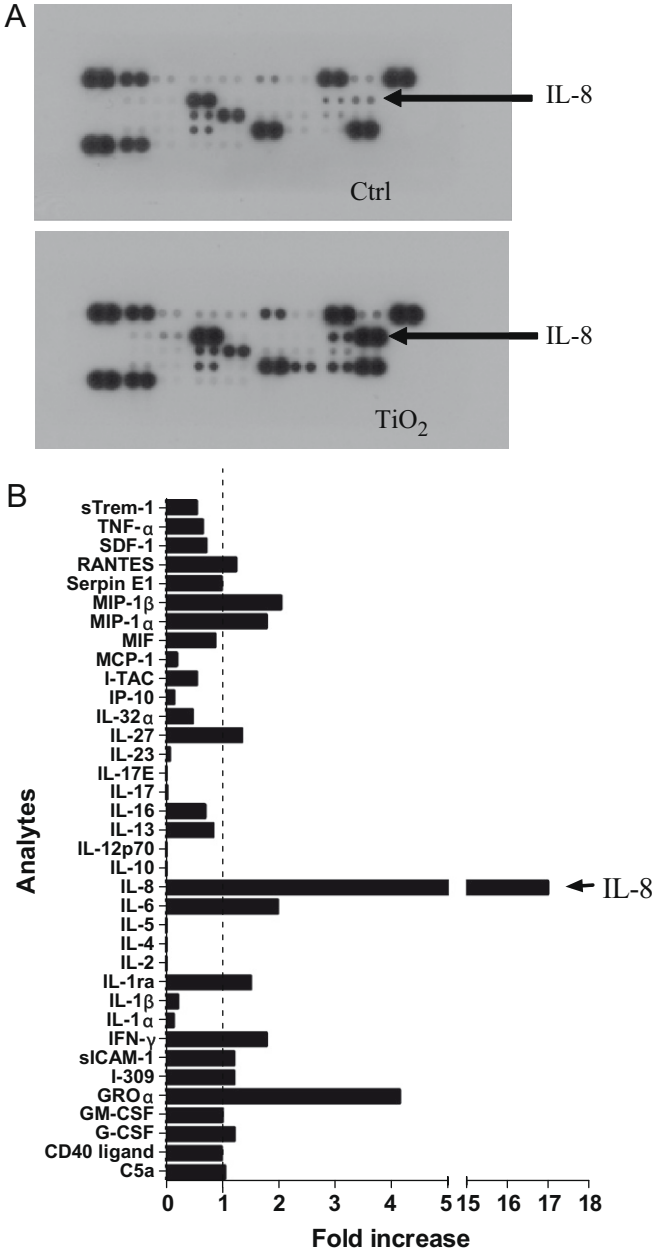

Fig. 5. Analysis of different analytes measured by an antibody array assay revealed that TiO_2 significantly increased the production of IL-8 in human PMNs. Cells (10×10^6 cells/ml) were incubated with buffer (Ctrl) or 100 μg/ml TiO_2 for 24 h and supernatants from three separate experiments were pooled. Production of 36 different analytes was detected by an antibody array assay as described in Section 2. (A) Membranes containing raw data that were analyzed to create the bar graph (B). Arrows indicate the two spots (duplicate) corresponding to IL-8.

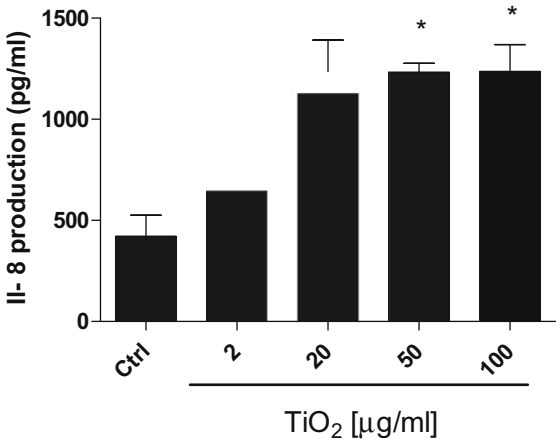

Fig. 6. Measurement of IL-8 production in TiO_2-induced human PMNs. Neutrophils (10×10^6 cells/ml) were incubated with buffer (Ctrl) or 2, 20, 50 or 100 μg/ml TiO_2 for 24 h and supernatants were harvested; the concentration of IL-8 was quantified by ELISA as described in Section 2. Results are means ± SEM ($n = 3$). $^*P < 0.05$ vs. Ctrl.

which reported that a concentration of 200 μg/ml TiO_2 did not affect cell viability of human PMNs, as assessed by lysozyme release and trypan blue exclusion (Hedenborg, 1988). Although the maximum concentration of TiO_2 used in our study is 100 μg/ml, rather than 200 μg/ml (Hedenborg, 1988), we verified PMN cell viability for a longer period of time (up to 24 h), whereas the Hedenborg study determined this for only 15 or 30 min. Therefore, these observations confirm that TiO_2 does not induce cell necrosis in human PMNs. It is important to mention that these cells appear more refractory to necrosis, as compared to other immune cells. For example, PMNs were shown to be more resistant than human peripheral blood mononuclear cells to mercury-induced cytotoxicity (Moisan et al., 2002). In fact, most compounds induced apoptosis instead of necrosis in human PMNs, probably because these cells possess unique apoptotic machinery and spontaneously undergo apoptosis.

In the present study, TiO_2 affected rapid responses in human PMNs based on changes in cell morphology and in phosphorylation events. Cell morphology changes are known to be associated with rearrangement of the cytoskeletal components (Bennett et al., 1993; Girard et al., 1997). Interestingly, the fact that TiO_2-induced rapid phosphorylation in PMNs (few seconds) indicates that TiO_2 NPs have great potential to induce a variety of different cell functions. This is particularly true for apoptosis, since both p38 and Erk-1/2 MAPKs are known to be involved in the regulation of PMN apoptosis under various experimental conditions (Klein et al., 2000; Akgul et al., 2001; Harter et al., 2002; Pelletier et al., 2002; Ward et al., 2005; Binet and Girard, 2008). The ability of TiO_2 to inhibit PMN apoptosis concurs with its non cytotoxic effects in human PMNs (Hedenborg, 1988). Of note, this is the first study to report the ability of TiO_2 to delay or suppress cell apoptosis. Interestingly, in contrast to most studies, we have used mature non-dividing primary cells. In previous studies conducted with cells other than PMNs, TiO_2-induced cell necrosis in mouse L929 fibroblasts (Osano et al., 2003; Jin et al., 2008) and apoptosis in rodent osteoblasts (Pioletti et al., 1999) and brain cells (Long et al., 2007). In humans, TiO_2-induced necrosis or apoptosis in U937 monoblastoid cells (Vamanu et al., 2008), apoptosis in a bronchial epithelial BEAS-2B cell line (Park et al., 2008), and in phytohemagglutinin-stimulated lymphocytes (Kang et al., 2009). The ability to delay apoptosis in PMNs suggests, however, that TiO_2 can also disturb normal homeostasis by prolonging the life-span of PMN, which from an inflammatory perspective, can be deleterious to the immediate environment by releasing toxic compounds. This may also explain the accumulation of PMNs observed in broncho-alveolar lavage fluids in TiO_2-induced pulmonary inflammation (Bermudez et al., 2004; Nemmar et al., 2008) or in lung tissue after intraperitoneal injection (Chen et al., 2009).

PMNs are among the first immune cells to arrive at the site of inflammation, where they initiate a rapid, nonspecific, phagocytic response and the release of soluble mediators (Smith, 1994; Serhan and Savill, 2005; Kennedy and DeLeo, 2009). Some of these mediators attract other PMNs, while others attract various leukocytes. In this study, we found that a prolonged exposure of human PMNs to TiO_2 NPs caused increased expression of important pro-inflammatory mediators, including the chemokines MIP-1α and MIP-1β

(or Macrophage Inflammatory Protein-1 alpha/beta). Although MIP-1α and MIP-1β are not chemotactic for PMNs *in vitro*, they elicit a rapid influx of neutrophils to the site of injection when administered *in vivo* (Alam et al., 1994). They are known to attract and activate macrophages, lymphocytes, NK cells, eosinophils and dendritic cells (Maurer and von Stebut, 2004). In addition, activation of PMNs by TiO_2 induced the production of IL-6, Gro-α and IL-8. Interleukin-6 (IL-6) is a pleiotropic cytokine known to be produced by many different cell types and plays a role in a wide range of responses (hematopoiesis, immune responses, acute-phase reactions, inflammation, etc.), while Gro-α and IL-8 are particularly recognized for their ability to be chemoattractants for PMNs (Pliyev, 2008). Interestingly, IL-8, one of the best PMN activators, was released in the greatest quantity in response to TiO_2 activation, suggesting that TiO_2 NPs possess a potent modulatory activity on human PMNs.

In the past, we have used the different cell responses/functions described in this study to identify cytokines (Girard et al., 1996a,b, 1997) and xenobiotics (Pelletier et al., 2000; Binet and Girard, 2008) as human neutrophil agonists. The results obtained here lead us to conclude that TiO_2 possesses important neutrophil agonistic properties *in vitro*, corresponding to its pro-inflammatory activity recently reported.

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