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In vitro age dependent response of macrophages to micro and nano titanium dioxide particles

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Abstract: As a result of corrosion, microparticles (MP) and/or nanoparticles (NP) can be released from the metallic implants surface into the bioenvironment. The biological response to these particles depends not only on the physico-chemical properties of the particles but also on host factors, such as age. Macrophages have attracted wide concern in biomedicine. The aim of this investigation was to study the age related biological response of macrophages to TiO_2-MP and NP *in vitro*. Alveolar macrophages (AM) obtained from young and senescent rats were cultured and exposed to TiO_2-MP and NP. Cell metabolism, superoxide anion (O_2^-) and nitric oxide (NO) generation, and cytokine release (IL-6, TNFα, IL-10) were measured. Cell metabolism was not affected by particle exposure. O_2^- and NO generation increased in a dose dependent manner. A marked increase on IL-6 release was found in the young-AM subpopulation exposed to TiO_2-MP. Conversely, both particle sizes induced a dose dependent release of TNFα in senescent-AM. Only the highest concentration of TiO_2-particles caused a significant increase in IL-10 release in AM-cultures. These observations lend strong support to the suggestion that cellular response of macrophages to TiO_2-particles is age dependent. The biological effect of the particles would seem to be more deleterious in the senescent age-group. © 2014 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A:000–000, 2014.

Key Words: macrophages, TiO_2, aging, nano and microparticles, inflammatory mediators

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INTRODUCTION

A commonly used definition of biomaterial is "a nonviable material used in a medical device, intended to interact with biological systems."[1] Implanted medical devices are essential to modern medical practice, and they improve quality of life. Titanium (Ti) is one of the most utilized biomaterials for the manufacture of biomedical implants, especially dental implants. It is a highly reactive metal, and on exposure to fluids or air, rapidly develops a layer titanium dioxide (TiO_2) at the interface, between the biological milieu and the metal structure. This layer passivates the metal determining its degree of biocompatibility and biological response.[2,3] However, due to the corrosion of the metal, ions/particles can be released into the bioenvironment as a result of the electrochemical attack.[4–8] The size of the particles that result from this process can be in the micrometer (>100 μm in diameter) and/or nanometer range (<100 μm in diameter).[9,10] Although the biological effect of micrometer-sized particles (MP) from prosthetic devices is well documented, little is known about the biological consequences of nanoparticles (NP). In keeping with other findings reported in the literature[11,12] a previous *in vivo* study conducted at our laboratory showed that the particles released from implants can deposit locally in the periimplant tissue,[13–16] or disseminate systemically transported by plasma or circulating monocytes[17] to body organs, mainly those with macrophagic activity such as the liver, spleen and lungs.[18–22]

The toxicity of some metallic particles and their oxides to macrophages has been extensively investigated in environmental toxicology studies.[23–25] Nonetheless, the mechanisms underlying the response of macrophages to metallic

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particles originating from a titanium biomedical device is not well understood. In the main, the lungs have a high rate of blood perfusion, which makes them more likely to be exposed to xenobiotics[26]; therefore, they are the most susceptible organ to oxidative damage.[27] Metallic particles can induce oxidative stress by disturbing the balance between oxidant and antioxidant species.[28] It has become indisputable that reactive oxygen species (ROS), ions that form as a natural byproduct of oxygen metabolism, play an important role in cell signaling of many physiological and pathophysiological processes. Superoxide anion, one of the main ROS, can oxidize and hence damage DNA, lipids, and proteins. We have previously shown that TiO_2 induced a significant rise in superoxide anion and a decrease in antioxidant enzyme activities in lung homogenates.[20] In addition, biological response to wear particles not only depends on the intrinsic physico-chemical properties of the particles (chemical structure, shape, concentration, and size, among others)[29] but also on host factors, such as gender, genetic profile, and age. Aging is a multifactorial process and is widely thought to be partly caused by the accumulation of random molecular damage due to ROS. Even though it has been demonstrated that macrophages from different species and different organs can respond differently to an array of stimuli (radiation, drugs, toxins, metal ions),[30,31] few and controversial studies have addressed the influence of age on the function of macrophages. Studies performed in senescent individuals show an increase in mononuclear phagocyte system function,[32–34] while others, report a decrease in immune system activity.[35–39] It is noteworthy that today, metallic biomedical devices are not only employed to treat adult patients but are increasingly being used in the young. Based on the above, the aim of the present investigation was to study the age related biological response of alveolar macrophages to titanium dioxide microparticles (MP) and nanoparticles (NP).

MATERIALS AND METHODS

Drugs

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), nitroblue tetrazolium (NBT), dimethyl sulfoxide (DMSO), polyvinylpyrrolidone (PVP), tween 20, phosphate buffered saline (PBS), RPMI-1640 medium, penicillin/streptomycin, and paraformaldehyde were purchased from Sigma–Aldrich, MI. Griess reagent was purchased from Fluka (Taufkirchen, Germany), and fetal calf serum was purchased from Bioser (Argentina). BD OptEIA™ Rat TNF, IL-6 and IL-10 ELISA Kits were provided by BD Pharmingen (San Diego, USA); 3,3′,5,5′-tetramethylbenzidine (TMB) was purchased from DakoCytomation (USA).

TiO_2 (anatase) micrometer-sized particles (MP) were purchased from Sigma–Aldrich, St. Louis, MI and TiO_2 (anatase) nanometer-sized particles (NP) from Nanostructured and Amorphous Materials, Los Alamos, NM.

Animals

Female young (3-month old) and senescent Wistar rats (18-month old) were housed in a controlled environment at the School of Science and Technology, University of San Martín. The animals received a normal protein diet and water *ad libitum*. All the experiments were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals.

Alveolar macrophage isolation, cell culture, and exposure to TiO_2 particles

Fully differentiated rat alveolar macrophages (AMs) were obtained by bronchoalveolar lavage as described Tasat and De Rey.[40] Briefly, the thoracic cavity was partly dissected and the trachea was cannulated with an 18-gauge needle. The excised lung was then gently massaged and lavaged 12 times with 1 mL of cold sterile phosphate–buffer saline (PBS, Ca++Mg free, pH 7.2–7.4). The bronchoalveolar lavage fluid (BAL) was immediately centrifuged at 800*g* for 10 min at 4°C and the total cell number was determined with a Neubauer chamber. Based on morphological criteria, control animals showed >95% of alveolar macrophages (AMs).

AMs from young and senescent animals were cultured in RPMI 1640 with penicillin (100 UI mL^{-1}), streptomycin (10 μg mL^{-1}), and 10% fetal calf serum. After 20-min incubation, cells were carefully washed to remove any nonadherent cells and were incubated overnight at 37°C in a 5% CO_2 atmosphere.

MP and NP TiO_2 particles were added to the cell cultures for 24 h in the following concentrations: 2.5, 10, 50, 100 μg mL^{-1}. Previously, all particles were heated at 220°C for 16 h in order to eliminate potential endotoxins. To ensure a uniform distribution, the particles were sonicated before use for 15 min.

Cell metabolism

Macrophage metabolism was determined by the MTT assay.[41,42] Briefly, AMs from young and senescent animals were seeded at a density of 1.25×10^5 cells/well on a 24 multiwell plate. Medium from control and TiO_2 exposed particle cultures was removed and 0.5-mL fresh complete growth medium supplemented with 50-μL MTT (4 mg mL^{-1} in PBS) was added for 3 h. Immediately after incubation, 10% SDS was added to stop the MTT reaction and to solubilize the formazan precipitate. The optical density (OD) of the final solution was measured at 570 nm in a spectrophotometer (Shimadzu UV-1201V).

Superoxide anion generation

Superoxide anion (O_2^-), a main reactive oxygen species (ROS) generated during the respiratory burst, was evaluated in control and TiO_2 particle-exposed AM cultures using the NBT test.[43] During the respiratory burst, O_2^- is formed by the reduction of O_2 by NADPH oxidase localized on the surface of the plasma membrane. The intracellular release of this active oxygen species is evidenced by the amount of blue formazan precipitate in the cells after NBT reduction. This assay was performed by adding NBT (1 mL, 0.1% in PBS) to cultured AMs. All plates were incubated with NBT for 60 min at 37°C. After incubation time, paraformaldehyde (0.4%) was added to stop the NBT reaction and fix the cells.

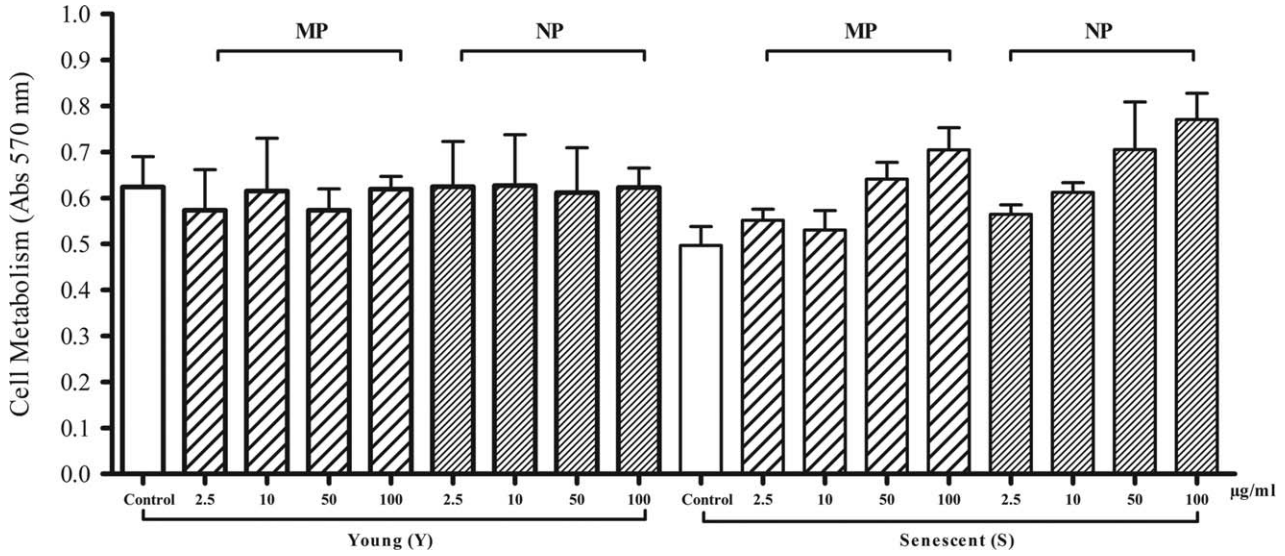

FIGURE 1. Cell metabolism of cultured alveolar macrophages (AMs) after exposure to micro (MP) or nano (NP) TiO_2 particles (2.5–100 μg mL^{-1}) for 24 h. MTT was evaluated spectrophotometrically at 570 nm. The histograms represent the mean ± SD of each experiment ($n \geq 8$). The bars represent control AMs (▭) and AMs exposed to different concentrations of micro (▨) or nano (▩) TiO_2 particles.

The presence of a blue formazan precipitate was indicative of a reactive cell, whereas cells without the precipitate were considered nonreactive. Cells were scored by light microscopy as described Molinari et al.[44] At least 200 cells per dish and three dishes for each experimental condition were scored under a Nikon Alphaphot-2 V52 microscope. Results are expressed as percentage of reactive cells.

Nitrite oxide production

To clarify and compare the effects of TiO_2 MP and NP on cell oxidative metabolism, nitric oxide (NO), a free radical produced transiently in mammalian cells for intercellular signaling, was evaluated in young and senescent AM cultures. When secreted in large quantities, as a result of macrophage activation, NO can induce cytotoxicity. Nitrite production was assessed by Griess reaction.[45] The culture supernatant was evaluated according to the manufacturer's instructions. A nitrite Standard curve was made by adding $NaNO_2$ and nitrite-free water. Absorbance was measured at 595 nm on a microplate reader (Bio-Rad, Benchmark).

Proinflammatory and anitinflammatory cytokines

To determine the effect of TiO_2 MP and NP on proinflammatory and antiinflammatory cytokine production, tumor necrosis factor alpha (TNFα) and interleukin IL-6 and IL-10 levels were assayed in young and senescent AM cultures, 24-h post-exposure. After exposure, the supernatants were collected, centrifuged to remove residual debris, and frozen at −80°C until use.

All cytokines were detected in cell-free culture supernatants using a commercially available specific enzyme linked immunosorbent assay (ELISA) kit, following the manufacturer's instructions. ELISA plates (Corning, Newark, CA) were coated with 1:125 cytokine-specific capture antibody diluted in coating buffer (0.1*M* Na_2CO_3, pH 9.5) at 4°C overnight. Wells were blocked with PBS containing 10% FCS for 1 h at RT. Cytokine standards and AM supernatants were added to wells and incubated for 2 h. Following three washes, biotinylated cytokine-specific detection antibody 1:250 was added for 1 h. After washing, streptavidin-peroxidase/TMB detection system was employed for 30 min. Absorbance was measured on a microplate reader (Bio-Rad, bench mark) at 655 nm. All samples were run in triplicates.

Statistical analysis

Results for control and exposed cultures were compared employing one-way ANOVA followed by Newman–Keuls test or Bonferroni accordingly. Statistical significance was set at $p < 0.05$.

RESULTS

Cell metabolism after TiO_2 particle exposure

As clearly shown in Figure 1, none of the tested concentrations (2.5–100 μg mL^{-1}) of either TiO_2 MP or NP caused differences in cultured AMs cell metabolism compared to non-exposed control cultures.

Superoxide anion generation after TiO_2 particle exposure

Superoxide anion generation in control and Ti exposed young AMs is shown in Figure 2(A,B), respectively. Most cells (60%) from control cultures were nonreactive, colorless cells [Fig. 2(A)]. Conversely, a large proportion of cells from AM cultures exposed to TiO_2 particles exhibited an intense (dark blue, violet) reaction as a result of formazan precipitation [Fig. 2(B)].

A dose dependent response was observed after exposure to either TiO_2 MP or NP irrespective of the maturation state of the AMs from young (3-month old) and senescent (18-month old) animals. Figure 2(C) shows that the lowest studied

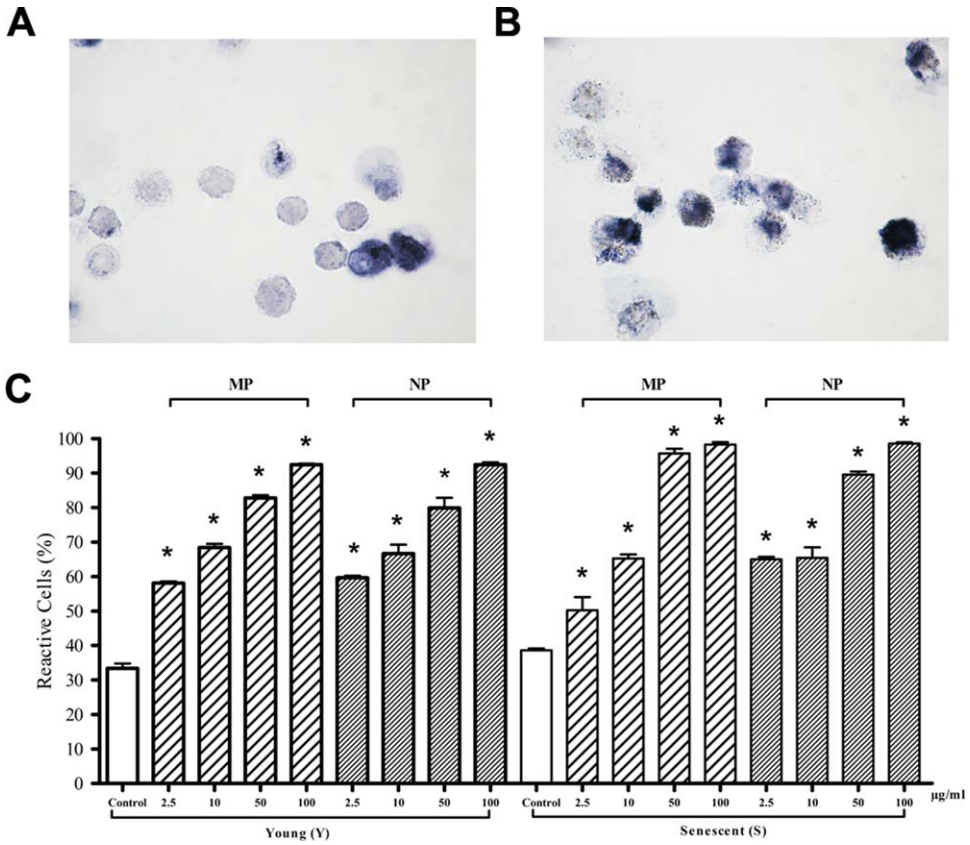

FIGURE 2. (A and B) Microphotographs of NBT reaction. Control (A) and TiO_2 particle-exposed (B) cultured young AMs. The majority of the cells in control cultures were non reactive whereas cells exposed to TiO_2 particles elicited a large proportion of reactive cells. Orig. Mag. ×1000. (C) Quantification of the NBT reaction. Young (Y) and senescent (S) AMs exposed to micro (MP) or nano (NP) TiO_2 particles (2.5–100 µg mL^{-1}) for 24 h. The histograms represent the mean ± SD of each experiment ($n \geq 4$). The bars represent control AMs (□) and AMs exposed to different concentrations of micro (▨) or nano (▩) TiO_2 particles. *$p < 0.05$ with respect to controls for each group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

concentration (2.5 µg mL^{-1}) significantly increased the percentage of reactive positive cells in both young and senescent AM cultures as compared to untreated controls. O_2^- generation increased in a dose dependent manner in both the studied age-groups, irrespective of particle size. It is noteworthy that higher particle concentrations (50 and 100 µg mL^{-1}) induced greater O_2^- generation in senescent AMs than in young AMs [Fig. 2(C)].

Nitric oxide production after TiO_2 particle exposure

Figure 3 shows a dose-dependent increase in NO production in both young and senescent particle-exposed AM cultures irrespective of particle size. Unlike O_2^- generation, NO levels did not increase in young and senescent AM cultures exposed to the lower particle concentration (2.5 µg mL^{-1}). It must be pointed out that, in both young and senescent AM cultures, the 10 µg mL^{-1} concentration of NP caused a significant increase in NO, whereas only the ≥50 µg mL^{-1} concentration of MP had the same significant effect.

Proinflammatory and antiinflammatory cytokine production after TiO_2 particle exposure

Exposure to TiO_2 did not affect TNF-α levels of young AMs except for the highest concentration of TiO_2 NP (100 µg mL^{-1}), which caused a significant increase. Exposure to MP caused a dose dependent increase in TNF-α release in senescent AMs but only the highest concentrations of NP caused a marked increase in TNF-α release in this cell population [Fig. 4(A)].

Only the highest concentration of TiO_2 MP (100 µg mL^{-1}) caused an increase in IL-6 secretion in young AMs, whereas none of the concentrations of NP caused changes in IL-6 secretion in this cell population. None of the studied concentrations (2.5–100 µg mL^{-1}) of either particle size of TiO_2 induced changes in IL-6 secretion in senescent AM cultures [Fig. 4(B)].

As regards the release of anti-inflammatory cytokine IL-10, a concentration-dependent release was observed in young TiO_2-exposed AM [Fig. 4(C)]. Whereas the two lowest TiO_2 particle concentrations employed in this study did not alter IL-10 release, the latter increased significantly when cultures were exposed to 50 and 100 µg mL^{-1} of TiO_2 MP and NP. As to the effect of TiO_2-exposure on cytokine IL-10 production by senescent cultures, only the highest concentration (100 µg mL^{-1}) of MP and NP induced a significant increase.

DISCUSSION

In this study we sought to demonstrate that biological response of TiO_2-exposed alveolar macrophages depends

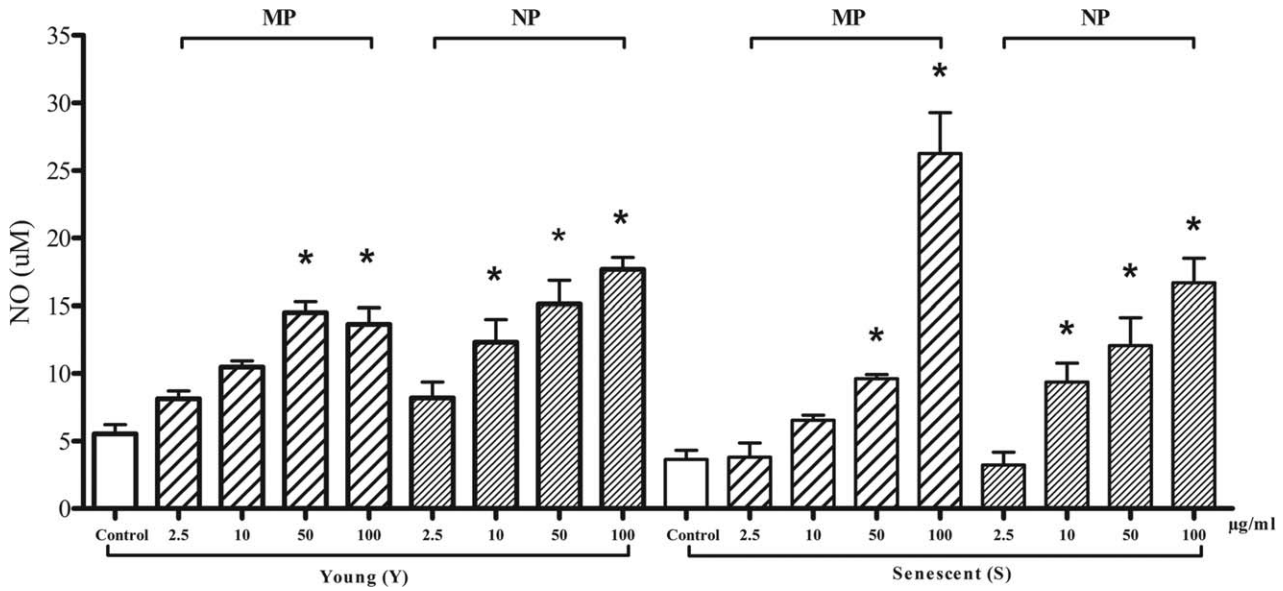

FIGURE 3. Production of nitric oxide. Nitric oxide (NO) was quantified in supernatants from cultured AMs by Griess assay. AMs were exposed to micro (MP) or nanoparticles (NP) of TiO_2 (2.5–100 µg mL^{-1}) for 24 h. NO production was evaluated spectrophotometrically at 540 nm. The histograms represent the mean ± SD of each experiment ($n \geq 4$). The bars represent control AMs (□) and AMs exposed to different concentrations of micro (▨) or nano (▩) TiO_2 particles. *$p < 0.05$ with respect to controls for each group.

not only on the physico-chemical properties of the particles but also on the instrinsic characteristics of the individual.

In agreement with Kang et al.,[46] the results obtained herein indicate that *in vitro* exposure of alveolar macrophages to TiO_2 microparticle (MP) or nanoparticle (NP) concentrations in the range of 2.5–100 µg mL^{-1} for 24 h did not significantly compromise cell viability. However, based on the capacity of mitochondrial dehydrogenase enzymes present in living cells, MTT is currently used to measure cell viability and proliferation.[47] This method can be influenced by a number of conditions, including particle size, specifically, nanoparticles. In fact, Wang et al.[48] recently showed that measuring cell viability or proliferation by the reduction of tetrazolium salts may yield inaccurate results when conditions in cultured cells increase superoxide formation. In our study, the increase in reactive positive cells indicating increased generation of superoxide anion (O_2^-), proved to be dose dependent, irrespective of the age of the animal. The finding reported here that TiO_2 MP and NP induced an increase in the formation of O_2^- is in line with previous reports,[49,50] and lends support to the suggestion that the MTT assay underestimates cytotoxicity by overestimating cell viability.

Yet controversies regarding the use of this assay on cell cultures exposed to titanium particles still remain. Several studies have shown a positive MTT reaction in different cell lines.[51,52] Conversely, a study by Shukla et al.[53] on genotoxicity induced by TiO_2 NP in human epidermal cells, showed a decrease in cell viability after 48-h exposure. Although inconsistencies in the published data are generally attributed to the size and concentration of the particles as well as to the cellular system employed, a new variable is introduced in the study by Shukla et al., that is, exposure time.

Published results regarding macrophage ingestion capacity in young and adult AMs are contradictory and depend not only on the type of particle ingested,[54,55] but also on the species, type (alveolar, peritoneal, etc.) and exact age of the tissue.[54,56]

As was previously reported by our group,[39] and in agreement with other researchers,[57] rat alveolar macrophages elicited age-dependent functional changes showing differences in the biological response when AMs obtained from young rats were compared to old adults animals. In those studies, regarding phagocytosis, a reduced particle uptake was observed in AMs from young rats. In this study, irrespective of the maturation state of the AMs, TiO_2 MP and NP were able to provoke an increase in almost all parameters assayed.

It is well known that metallic particles are able to cause an increase in reactive oxygen species (ROS), which could lead to oxidative stress. As pointed out by Knaapen et al.[58] generation of ROS could be due to: (1) active redox cycling on the surface of the particles, particularly of metal-based particles, (2) particle functional oxidative groups and, (3) particle-cell interactions, mainly in the lungs, where neutrophils and macrophages (inflammatory phagocytes) are powerful generators of ROS.

Because the dynamics of the aging process comprises a broad range of cellular processes describing functional decline and/or adaptive regulatory mechanisms, we herein studied the involvement of signaling oxidative molecules (O_2^- and NO) and cytokines (TNFα, IL-6 and IL-10) in AMs obtained from animals of different age groups.

Although it has been demonstrated that due to their greater surface/volume ratio compared to MP, NP have an increased ability to induce inflammation and cytotoxicity as a result of increased oxidative activity,[10,59,60] the results of the

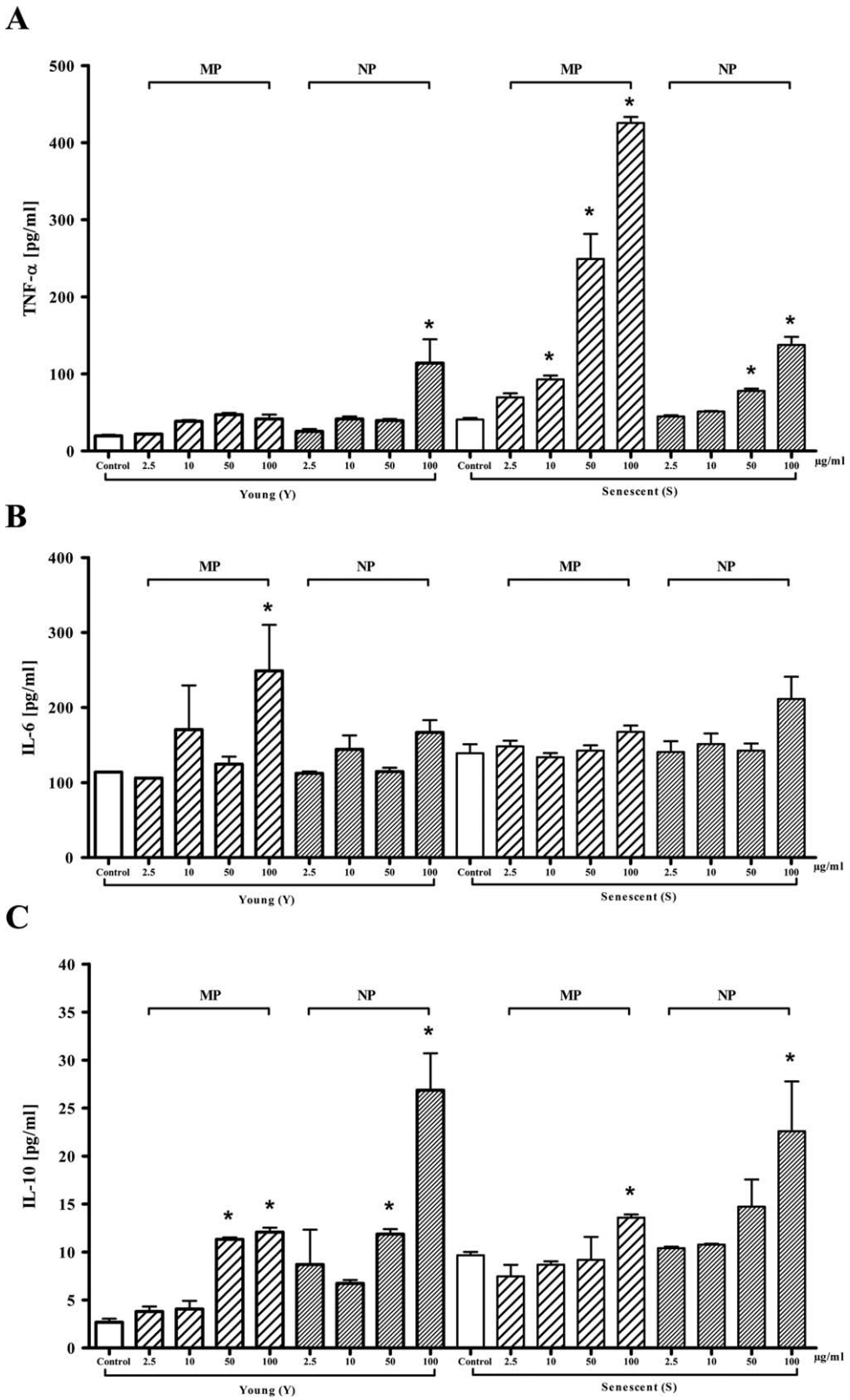

FIGURE 4. (A–C). Proinflammatory and anti-inflammatory cytokine secretion. Levels of cytokines TNF-α (A), IL-6 (B),s and IL-10 (C) were quantified in supernatants from cultured AMs by ELISA. AMs were exposed to micro (MP) or nanoparticles (NP) of TiO_2 (2.5–100 μg mL^{-1}) for 24 h. The histograms represent the mean ± SD of each experiment ($n = 3$). The bars represent control AMs (□) and AMs exposed to different concentrations of micro (▨) or nano (▩) TiO_2 particles. *$p < 0.05$ with respect to controls for each group.

present study showed that exposure to TiO_2 particles irrespective of particle size, increased both superoxide anion (O_2^-) and nitric oxide (NO) generation to the same extent. This discrepancy may be explained by the fact that the MP used here were very small (150 nm), almost at the lower limit of the micrometer range (100 nm). Studies reporting substantial differences in biological response to MP used particle sizes in the order of 300 nm.

It is well known that overproduction of ROS activates a series of cytokine cascades which generally include upregulation of tumor necrosis factor alfa (TNFα) and proinflammatory interleukins (IL).[61] In this regard, two main proinflammatory cytokines, TNFα and IL6, were evaluated. Interestingly, AMs from young rats were not able to produce detectable TNFα or IL-6 with the exception of those exposed to 100 μg mL^{-1}, the highest TiO_2 concentration used.

Conversely, when cultured senescent AMs were exposed to TiO_2 MP or NP a marked and significant ($p < 0.05$) dose dependent increase in TNFα levels was seen. The latter results are in agreement with Goldman et al.[39] who showed that cultured macrophages from adult animals (9- to 12-months old) were able to secrete significant amounts of TNFα after PMA stimulation.

The antiinflammatory cytokine IL-10 plays an essential role in suppressing the production of proinflammatory cytokines, such as TNF-α and IL-1b, and the antigen-presenting capacity of monocytes/macrophages.[62] Again, clear differences between the cellular response of young and senescent cultured AM were found. The low basal TNFα level maintained in young AMs exposed to TiO_2 particles, throughout the range of doses, could probably be due to the increase in IL-10 secretion, indicating a cell adaptive response. In senescent AMs, only the highest concentration of TiO_2 MP and NP induced a significant rise in IL-10 secretion, though the increase was not sufficient to prevent the release of TNFα.

Although particle size undoubtedly affects biological response, the difference in size of the particles used in the present study was very small, so that particle size did not play a significant role in the cellular response.

All these observations lend strong support to the suggestion that cellular response of macrophages to TiO_2 particles is age dependent, with the main mediator molecules involved in the biological response being ROS and NOS.

Based on the results described above, it is important to consider that the influence of particles resulting from a corrosion process may have a different impact on young and older adult patients. The biological effect of the particles would seem to be more deleterious in the latter age-group, which is more likely to need a metallic biomedical device. It must be highlighted that in the clinical practice, most implants are placed in older adults who often present mild insufficiencies (cardiac, renal, or respiratory). These insufficiencies must be taken into account, in view of the potential adverse clinical effects of MP and NP, especially when there is a risk of particle dissemination.

Further research evaluating known cell targets for ROS, such as membrane lipoperoxidation, DNA strand break, and protein carbonylation might provide further evidence of our findings.

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