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RESEARCH ARTICLE

Impact of emerging pollutants on pulmonary inflammation in asthmatic rats: ethanol vapors and agglomerated TiO₂ nanoparticles

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Abstract

Context: Titanium dioxide nanoparticles (nano-TiO₂) and ethanol vapors are air contaminants with increasing importance. The presence of a pathological pulmonary condition, such as asthma, may increase lung susceptibility to such contaminants.

Objective: This study aimed to investigate if exposure to inhaled ethanol vapors or nano-TiO₂ can modulate the rat pulmonary inflammatory response resulting from an allergic asthmatic reaction.

Materials and methods: Brown Norway rats were sensitized (sc) and challenged (15 min inhalation, 14 days later) with chicken egg ovalbumin (OVA). Leukocytes were counted in bronchoalveolar lavages (BAL) performed at 6, 24, 36, 48 and 72 h following the challenge and either after ethanol exposures (3000 ppm, 6 h/day, daily) or at 48 h (peak inflammation) for nano-TiO₂ exposures (9.35 mg/m³ aerosol for 6 and 42 h after the OVA challenge). For the nano-TiO₂ exposures, plasma and BAL cytokines were measured and lung histological analyzes were performed.

Results: Exposure to ethanol did not significantly affect BAL leukocytes after OVA challenge. Exposure to nano-TiO₂ significantly decreased BAL leukocytes compared to OVA-challenged controls. Plasma and BAL IL-4, IL-6, and INF- γ levels were also decreased in the nano-TiO₂ group.

Discussion: While ethanol vapors do not modify the pulmonary inflammation in rats during an asthmatic response, a surprising protective effect for agglomerated nano-TiO₂ was observed. A putative mechanistic basis involving a decrease in the Th2 response caused by OVA is proposed.

Conclusion: Allergic pulmonary inflammation is not up-regulated by inhalation of the pollutants ethanol and nano-TiO₂. On the contrary, nano-TiO₂ decreases lung inflammation in asthmatic rats.

Keywords: Ethanol, nanoparticules, TiO₂, asthma, lung inflammation, eosinophils, ovalbumin

Introduction

Asthma affects approximately 300 million individuals worldwide (WHO, 2008) and is characterized by bronchial obstruction, airway hyperresponsiveness and a sustained inflammatory response; children and adults

suffering from asthma are more likely to be affected by several inhaled pollutants including volatile organic compounds (Rumchev et al., 2004; Arif & Shah, 2007). Amongst these, alcohol and titanium dioxide (TiO₂) are relatively new agents for which there have been few

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studies assessing their potential toxicity when inhaled by susceptible individuals.

Very little is known on the effects of ethanol through inhalation exposure. Although this entry route has been neglected in studies on ethanol toxicity, it is of growing concern. Indeed, the reemergence of ethanol blended gasoline in several countries (Health Canada, 2010), the increasing use of hydro alcoholic solutions and gels in the health care sector, and other occupational exposures stemming from industrial uses such as in paints, varnishes, detergents, and distilleries/wineries have raised concerns over potential toxicity when ethanol vapors are inhaled. In France, ethanol represents 11% of all solvents, and 50% of all alcohols used in the industrial sector (Triolet, 2005). Such uses imply that human beings find themselves exposed to various concentrations of ethanol vapors in the ambient air.

The impact of ethanol inhalation on the health of patients suffering from asthma has yet to be studied. A study on six healthy subjects showed that acute inhalation of ethanol vapors (2000 ppm, 30 min) caused a significant decrease in flow rates on partial expiratory flow-volume (PEFV) curves for up to 90 min without significantly affecting FEV1 (volume that has been exhaled at the end of the first second of forced expiration) values (Zuskin et al., 1981). Ingestion of ethanol (30 g over 5–10 min) did not change FEV1 in a group of 30 volunteers, but caused a fall in FEV1 associated with a rise in blood acetaldehyde and histamine concentrations in 15 of 32 asthmatic subjects (Takao et al., 1998). Inhalation exposure to acetaldehyde caused bronchoconstriction indirectly via histamine release in asthmatic subjects, and nonspecific bronchial hyperresponsiveness appeared to be a necessary precondition for the expression of acetaldehyde-induced bronchoconstriction (Myou et al., 1993). In addition, in patients with alcohol-induced asthma, the evaluation of bronchial responsiveness showed that ascending doses of inhaled ethanol did not significantly affect FEV1, but that a higher dose of metacholine was necessary to decrease FEV1 by 20% following ethanol inhalation (Myou et al., 1996). The authors concluded that in alcohol-induced asthma, ethanol can reduce non specific bronchial responsiveness. To this day, there have been no scientific articles studying ethanol as a potential modulator of the allergic airway response *in vivo*. Considering that about 90% of asthmatics suffer from allergic asthma, this question needs to be addressed.

Nanotechnologies are growing at a tremendous pace and today represent a multi billion dollar industry (Lux research, 2006). Due to their wide range of uses in various fields, although relatively new, nanoparticles (≤ 100 nm) are an important source of concern in occupational and environmental toxicology. TiO_2 nanoparticles (nano- TiO_2), which are chemically stable and relatively inexpensive, are used in industries for the manufacturing of paints, cosmetics, sunscreens, packaging, and many more (Chen & Mao, 2007). When inhaled by workers, nano- TiO_2 enters the lungs generally in a particulate

form. Thus, aerosolized nano- TiO_2 in experimental studies represents the most realistic route of exposure (Pauluhn & Mohr, 2000; Pauluhn, 2005; Mühlfeld et al., 2008; Jacobsen et al., 2009). Although the initial size of nano- TiO_2 is in the nanometer range (<100 nm), the actual size of the particles entering the lungs can be higher because of its tendency to agglomerate, especially at high concentrations (Hinds, 1999; Murr et al., 2004; Grassian et al., 2007; Ma-Hock et al., 2007, 2009). Characterization of nano- TiO_2 administered to experimental animals, despite its complexity, is therefore necessary in order to make adequate conclusions regarding nanoparticle exposure characteristics and resulting biological responses (Oberdörster et al., 2005; Warheit et al., 2007a, 2007b, 2007c, 2008, 2010; Pauluhn, 2009).

TiO_2 is classified as possibly carcinogenic to humans (group 2B) and is believed to induce inflammatory responses when inhaled as nanoparticles (Ma-Hock et al., 2009; Bermudez et al., 2002). While Rossi et al. (2010) observed that healthy mice elicited pulmonary neutrophilia accompanied by significantly increased chemokine CXCL5 expression when exposed to nano- TiO_2 by inhalation and that the allergic pulmonary inflammation was dramatically suppressed in asthmatic mice exposed to nano- TiO_2 , Hussain et al. (2011) recently reported that intrapulmonary doses of TiO_2 or gold nanoparticles can aggravate pulmonary inflammation and airway hyper-reactivity in a mouse model of diisocyanate-induced asthma. Given the importance of asthma worldwide and the increased use of engineered nanomaterials, notably nano- TiO_2 , it is relevant to confirm their potential for toxicity in such susceptible populations.

This study aimed to determine if exposure to ethanol vapors or nano- TiO_2 can modulate the pulmonary inflammatory response associated with allergic asthma, through changes in the development, maximum response intensity or recuperation time. Brown Norway (BN) rats were sensitized subcutaneously with the allergen ovalbumin (OVA) and then challenged by inhalation with the same allergen 14 days later to induce lung inflammation. Bronchoalveolar lavages (BAL) were performed at various times following the challenge and pulmonary toxicity was assessed by counting leucocytes in BAL fluid, measuring cytokines levels in the blood, and analyzing histological samples of the lungs. The effects of repeated exposures to ethanol vapors or a single exposure to nano- TiO_2 were assessed.

Materials and methods

Choice of animal model

In a previous study (Scarino et al., 2009), we assessed the pulmonary toxicity of ethanol vapors in Sprague-Dawley (SD) rats carrying a type 2 acetaldehyde dehydrogenase (ALDH2) deficiency. BN rats, 6–8 weeks of age, were used in the present study instead of SD rats because of their higher pulmonary sensitivity to atmospheric pollutants (Tesfaigzi et al., 2005). Furthermore, BN rats

are commonly used in animal studies on allergic asthma induced by OVA sensitization and challenge (Elwood et al., 1991; Renzi et al., 1993). Given that the only available data on ethanol inhalation toxicity was obtained using SD rats, we could not make the assumption that the BN strain would have the same metabolic, toxicological, and gender profiles. Therefore, the first step of this study was to gather information on the pulmonary and toxicokinetics of inhaled ethanol in both male and female BN rats in order to insure the absence of toxicity before moving on to asthmatic BN rats. A comparison of the toxicological profiles between both strains, i.e. BN rats from this study and SD rats from our previous study (Scarino et al., 2009), is presented in the "Results" section.

Induction of allergic asthma in rats

Male BN rats (6–8 week-old) were used in this study. These animals are known to have an enhanced Th2 response, making them ideal for asthma studies. Chicken egg OVA, a common allergen for this type of study, was used to induce allergic asthma in two phases, i.e. sensitization and challenge. Animals were sensitized by s.c. injection of 1 mL solution containing 0.9% saline (w/v), 1 mg ovalbumin, and 3.5 mg aluminum hydroxide (adjuvant). Fourteen days later, animals were challenged for 15 min by inhalation with a freshly prepared 0.9% saline solution containing a specific OVA concentration (see below) (Renzi et al., 1992). A DevilBiss 5650D compressor was used to vaporize OVA solution from the nebulizer to the cage (2 rats/exposure).

To verify, if ethanol vapors or nano-TiO₂ could modulate, upwardly or downwardly, the inflammatory asthma response caused by allergen inhalation, it was important to verify that OVA challenge alone did not cause maximum pulmonary inflammation. To that end, various OVA concentrations (1.5–5%) were tested in order to find one that would cause around 30–40% of maximal pulmonary inflammation (defined as the response for the 5 % level).

The pulmonary response is determined by measuring increases in leukocytes, mainly eosinophils, in BAL and their mediators. The temporal profile following OVA challenge was assessed to determine the development, maximal intensity, and recuperation phases of the inflammatory response.

Ethanol exposure protocol

Animals ($n = 8$ per group) were kept on a 12-hour light/dark cycle in a humidity- and temperature-controlled room prior to experimentation. During inhalation exposures, animals were individually caged in the exposure chamber and were not provided with food or water. Animals were exposed to 3000 ppm ethanol (95%, Les Alcools de Commerce Inc., Montreal, Quebec), 6 h/day, every day starting immediately following OVA sensitization until the animals were killed.

Ethanol exposures were conducted in a dynamic exposure chamber of 500 L (Unifab, Kalamazoo, MI) for which the air-flow rate was set at 280 L/min. A high-pressure

liquid chromatography (HPLC 2510) pump (Varian Canada, Montreal, Quebec) was used to deliver ethanol into the inhalation chamber. A Hewlett-Packard HP-5890 Series II gas chromatograph equipped with an Air Cadet pump (Cole Parmer model 7530-40) was used to monitor the ethanol concentration in the exposure chamber at 10-min intervals (Scarino et al., 2009).

Generation and measurements of nano-TiO₂ aerosols

Anatase nano-TiO₂ with a primary particle size of 5 nm were purchased from Nanostructured & Amorphous Materials Inc. (Houston, TX, USA). Nano-TiO₂ aerosols were generated over 6.83 h in the same type of chamber used for ethanol exposures (500 L Unifab, Kalamazoo, MI, USA), but equipped with six conical tubes placed on one wall of the inhalation chamber for rodent nose-only exposures. A small fan was placed inside the chamber at the opposite side of the wall with the conical tubes. The aerosols were generated by a dry NP powder dispersion technique, using a fluidized bed 3400A device (Fluidized Bed, TSI Inc., Shoreview, MN, USA). The low and high dose concentrations were achieved by adjusting the various feed rates of the generator. Each aerosol was generated with compressed air. No aerosol neutralizer was used.

Air samples were collected throughout the experiment on cassettes (Sure Seal, SKC Inc. Eighty Four, PA, USA) using 37-mm polyvinyl chloride (PVC) filters at a flow rate of 4 L/min for gravimetric analysis. The mass concentrations were followed and adjusted in real time using a Model 8520 Dust Trak Aerosol Monitor (TSI Inc., Shoreview, MN, USA) previously calibrated with TiO₂ by comparison with the gravimetric method. Numbers and particle size distributions were monitored in real time with an electrical low pressure impactor (ELPI) (Dekati Ltd., Tampere, Finland) which was operated at a flow rate of 10 L/minute in the filter stage configuration. Cumulative size distributions were acquired for the entire aerosol generation period. The ELPI was also used to determine the median aerodynamic diameter based on the number (NMAD). Air samples were all collected in the area of the inhalation chamber corresponding to the breathing zone of the animals.

Nano-TiO₂ exposure protocol

Animals ($n = 6$ per group) were exposed nose-only to nano-TiO₂ aerosols for 6 h (single exposure to 9.4 or 15.7 mg/m³), 42 h following the OVA challenge. Upon reception, animals were kept at the animal quarters for 1 week. Prior to TiO₂ exposure, animals were acclimatized to the inhalation system, i.e. individually placed in cylindrical tubes with a small opening for the nose at the conical end, insuring a nose-only exposure, for 6 days where they were exposed to compressed air: 15 min/day for the first 3 days and 3 h/day for the following 3 days. The animals were killed immediately after the 6 h exposure, i.e. 48 h following the OVA challenge. Thus, there was no recuperation time between nanoparticle exposure and sacrifice.

Determination of blood ethanol concentration

Ethanol being quickly metabolized in the organism using multiple pathways (Parkinson, 2001), blood samples were collected immediately (i.e. 2 min) following exposure to allow the assessment of peak blood ethanol concentration (BEC). A detailed method was previously described (Scarino et al., 2009). Briefly, blood samples (100 µL) were taken at 2, 20, and 40 min post-exposure on days 1 and 22, and kept in sealed vials at -80°C until analyzed, within 72 h. Concentrations were measured using a Varian Compak CP-3800 gas chromatograph with a Combi-Pal automatic injector from CTC Analytics (head-space sampling).

Bronchoalveolar lavages

For ethanol exposures, BAL were performed in different groups of rats at 6, 24, 36, 48, and 72 h following OVA challenge, always immediately after the last ethanol exposure. For nano-TiO₂ exposures, BAL were performed 48 h following OVA challenge, immediately after nanoparticles exposure. The BAL preparation method was previously described (Scarino et al., 2009). Briefly, animals were anesthetized with isoflurane and exsanguinated from the descending abdominal artery. Saline (0.9%) was injected (5 × 5 mL) into the lungs through the trachea, using a 16G needle, and then removed. Following centrifugation of pooled BAL, supernatant was removed and the cells were resuspended in 500 µL of saline (100 µL aliquot was fixed 1:1 with formalin for cell count). The lungs and the trachea were fixed *in situ* with buffered formalin and then removed and placed in containers with formalin at room temperature for 24 h, before being transferred into phosphate buffer solution and kept at 4°C.

Cell count

Fixed white blood cells (WBC) were mixed 1:1 with methylene blue and then counted using a hemacytometer. To determine the proportion of WBC represented by macrophages, eosinophils, neutrophils, or lymphocytes, 100 µL of the previously resuspended cells in saline were centrifuged at 600 rpm for 2 min using a cytospin, followed by fixation and staining with Hema 3 solutions (Fisher Diagnostics no cat. 122-911A, 122-911B, and 122-911C). Slides were analyzed using a photonic microscope.

Cytokine analysis in plasma and BAL

The plasma concentrations of seven cytokines relevant to asthma inflammation were measured using the Milliplex Rat Cytokine Kit (Millipore, Ottawa, Canada): MCP-1, IL-1β, IL-4, IL-6, IL-10, INF-γ, and TNF-α. Assays were performed using Luminex xMAP Technology (Bio-Plex 200 System, Biorad, Ottawa, Canada). The relative levels of 29 cytokines were measured in BAL fluid using Rat Cytokine Array Panel A (R & D Systems). BAL samples for each group (6 samples/group) were respectively pooled together to perform the assay.

Lung histology

Lungs were first sliced into sections 2–3-mm thick. Following dehydration of the sections in a series of

ethanol solutions ranging from 65 to 100%, ethanol was removed using Citrisolv (Fisher Scientific, Montreal, Quebec, Canada) as solvent and sections were then embedded with paraffin. Using a microtome, sections were sliced into 5 µm cuts and colored using H & E staining protocol. Pictures were taken using a Cooke SensiCam High Performance camera mounted on a Leica DMRE microscope.

Statistical analysis

Results are presented in the form of the mean ± SEM and statistical significance was determined using ANOVA and Tuckey's test with $p < 0.05$.

Results

Strain differences in BEC

Following the first day of ethanol exposure (day 1), significant ($p < 0.05$) gender differences in BEC for BN rats were observed at 2 min (mean ± SEM of $357.2 \pm 17.7 \mu\text{m}$ for males vs. $308.9 \pm 14.6 \mu\text{m}$ for females) and 20 min ($167.2 \pm 12.5 \mu\text{m}$ for males vs. $134.3 \pm 27.4 \mu\text{m}$ for females) post-exposure (half life was 13.2 min in males and 13.6 min in females) (Figure 1). On day 22, BEC in males were significantly higher than levels in females at 2 min ($266.7 \pm 27.6 \mu\text{m}$ vs. $228.3 \pm 23.5 \mu\text{m}$, respectively) and lower at 40 min ($24.9 \pm 4.0 \mu\text{m}$ vs. $39.3 \pm 13.0 \mu\text{m}$, respectively) post-exposure (half life was 11.1 min in males and 15.0 min in females). The relative change in BEC from day 1 to day 22 for each gender was similar, i.e. a 25.3% decrease for males versus a 26.1% decrease in females. Further, when comparing BN and SD rat strains within gender, the only statistically significant change on day one occurred between males at 20 min post exposure. On day 22, although females BN vs. SD had significantly different BEC at all times, males showed no significant changes at any timepoint analyzed (Figure 1).

Development of the allergic asthma model

Exposure to 1.5, 3.0, or 5.0% (w/v) OVA for the challenge dose showed that the optimal allergen concentration to use to reach the moderate response target, i.e. 30–40% of maximal inflammatory response, was reached at 1.5% (yielding 39% of the maximal inflammatory response) (Figure 2). As dose-response experiments were conducted prior to time-response experiments, the precise peak inflammatory response was unknown. Therefore, BAL were performed 36 h following the OVA challenge based on an estimation of peak inflammation resulting from past studies (Renzi et al., 1993).

Results for the time-response experiments, using 1.5% OVA for the challenge, showed that peak inflammatory response takes place 48 h post-challenge. These results indicate that the development phase of pulmonary inflammation following induction of the asthmatic response occurs from the time of the challenge until 48 h post-challenge and that recovery occurs after this time point (Figure 3, OVA group).

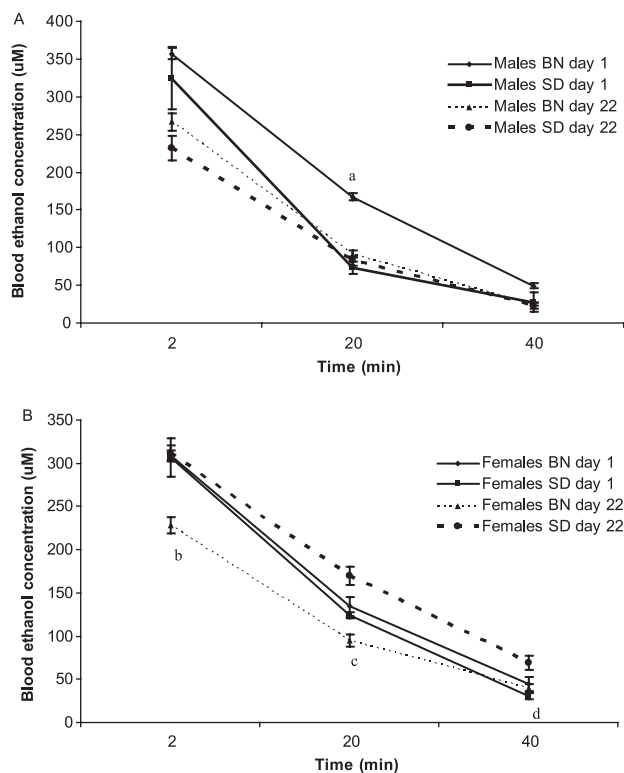


Figure 1. Blood ethanol concentration 2, 20, and 40 min following ethanol exposure on day 1 and day 22 (mean \pm SD) for males (A) and females (B). Data for Sprague Dawley rats were extracted from Scarino et al. (2009). a, $p < 0.05$ for males BN versus males SD at 20 min post-exposure; b, $p < 0.05$ for females BN versus females SD at 2-min post exposure; c, $p < 0.05$ for females BN versus females SD at 20 min post-exposure; d, $p < 0.05$ for females BN versus females SD at 40-min post exposure.

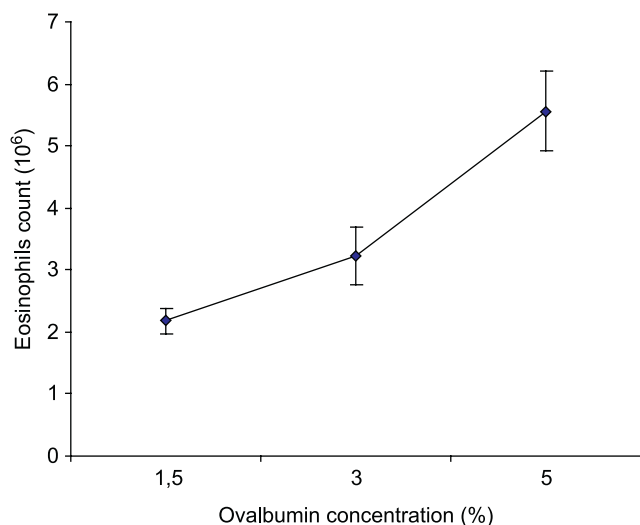


Figure 2. Dose-response effects of ovalbumin challenge on eosinophils collected in bronchoalveolar lavages (BAL) 36 h following challenge for concentrations ranging between 1.5 and 5% (mean \pm SEM).

Evaluation of pulmonary toxicity following ethanol exposure

When comparing leucocytes present in the BAL fluid of BN rats, results showed statistically significant ($p < 0.05$)

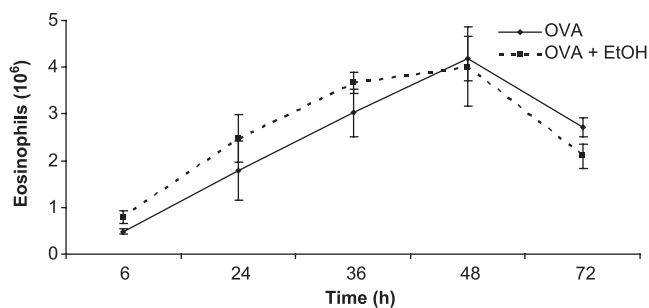


Figure 3. Time-response for the effect of a repeated exposure to ethanol vapors on lung inflammation for rats challenged with 1.5 % ovalbumin (mean \pm SEM). * $p < 0.05$ at 6 h following challenge for OVA versus OVA + TiO₂. OVA, ovalbumin.

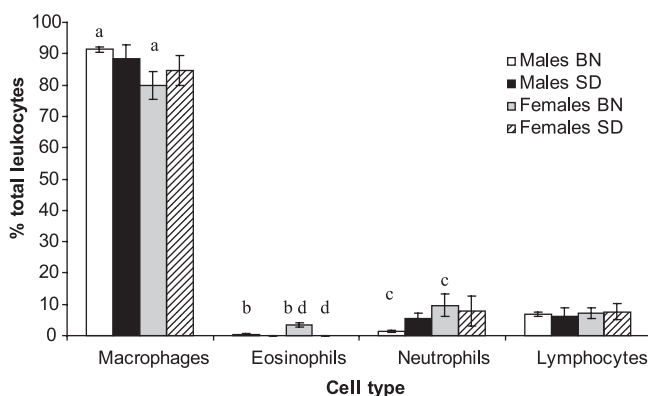


Figure 4. Comparison of the proportion of each type of leukocyte found in the BAL fluid between rat strains (BN vs. SD) in males (A) and females (B) (mean \pm SEM). a, $p < 0.05$ for macrophages in males BN versus females BN; b, $p < 0.05$ for eosinophils in males BN versus females BN; c, $p < 0.05$ for neutrophils in males BN versus females BN; d, $p < 0.05$ for eosinophils in females BN versus females SD.

gender differences in the percentage of macrophages (mean \pm SEM of 91.4 ± 0.9 vs. 79.8 ± 4.5), eosinophils (0.4 ± 0.2 vs. 3.4 ± 0.8), and neutrophils (1.4 ± 0.3 vs. 9.7 ± 3.5) for males versus females, respectively (Figure 4). Within a gender, no significant strain differences were seen in males for any of the four types of leukocytes counted but a significant difference was seen in females only for eosinophils (3.4 ± 0.8 for BN vs. 0.0 ± 0.0 for SD), indicating a similar pulmonary inflammatory pattern between both strains (Figure 4).

In OVA-sensitized and challenged animals, exposure to ethanol every day yielded no significant differences in eosinophil counts at most points when compared to rats exposed to OVA alone. The only exception was found at 6 h following OVA challenge (mean \pm SEM of $0.481 \times 10^6 \pm 0.059$ for OVA alone vs. $0.792 \times 10^6 \pm 0.128$ for OVA + ethanol, $p = 0.045$) (Figure 3). Results for other leukocytes are presented in Table 1 and the only significant difference ($p < 0.05$) is found in neutrophils 72 h following OVA challenge. As previously reported, inhalation of ethanol alone did not cause pulmonary inflammation as demonstrated by BAL leukocytes counts and histology results (Scarino et al., 2009).

Table 1. White blood cells in BAL fluid at different times following ovalbumin exposure. BAL were performed immediately following the final ethanol exposure (Results are shown as mean \pm SEM).

Cell type	Group	Time following ovalbumin challenge (h)				
		6	24	36	48	72
Total leukocytes (10^6)	OVA	2.11 \pm 0.26	4.41 \pm 1.56	6.29 \pm 1.06	9.39 \pm 1.06	7.52 \pm 0.59
	OVA + EtOH	2.91 \pm 0.47	5.88 \pm 1.22	7.24 \pm 0.44	10.24 \pm 2.14	7.05 \pm 0.84
Macrophages (10^6)	OVA	1.24 \pm 0.15	1.78 \pm 0.35	2.40 \pm 0.40	3.54 \pm 0.40	3.85 \pm 0.30
	OVA + EtOH	1.56 \pm 0.25	2.34 \pm 0.49	2.51 \pm 0.15	4.04 \pm 0.84	4.11 \pm 0.49
Neutrophils (10^6)	OVA	0.32 \pm 0.04	0.71 \pm 0.14	0.73 \pm 0.12	1.26 \pm 0.14	0.74 \pm 0.06*
	OVA + EtOH	0.46 \pm 0.07	0.93 \pm 0.19	0.97 \pm 0.06	1.77 \pm 0.37	0.53 \pm 0.06*
Lymphocytes (10^6)	OVA	0.07 \pm 0.01	0.13 \pm 0.03	0.13 \pm 0.02	0.40 \pm 0.05	0.21 \pm 0.02
	OVA + EtOH	0.10 \pm 0.02	0.14 \pm 0.03	0.09 \pm 0.01	0.42 \pm 0.09	0.21 \pm 0.31

OVA, ovalbumin; EtOH, ethanol.

*Significant difference ($p < 0.05$) between OVA and OVA + EtOH groups.

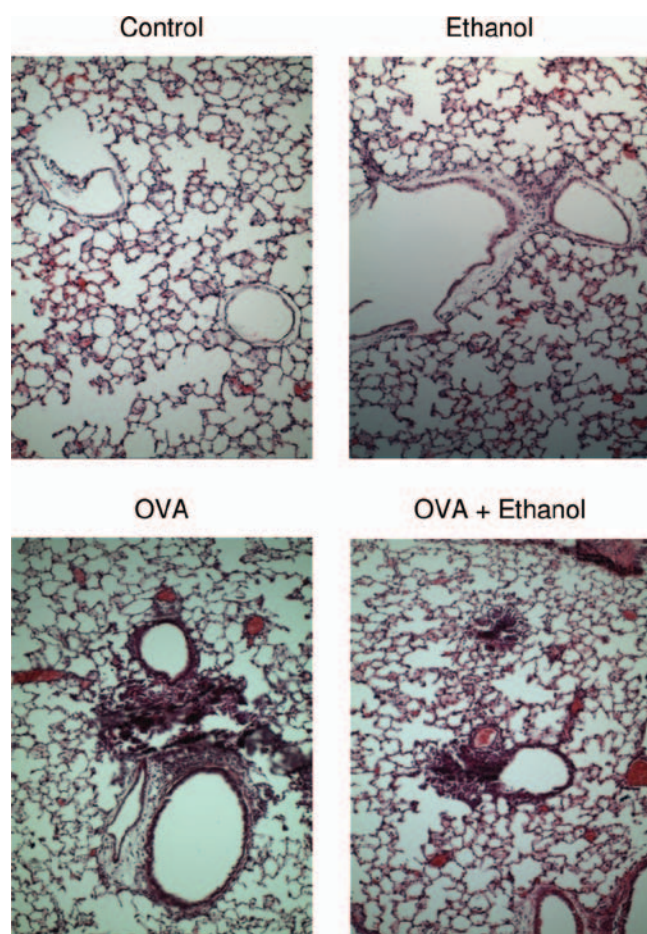


Figure 5. Photomicrograph (5 μ m slices, H & E staining) of rat lungs for control (top left), ethanol alone (top right), ovalbumin alone (bottom left) and ovalbumin + ethanol (bottom right) exposed rats (100 \times).

Furthermore, histological analysis of lung tissue showed that OVA exposure alone induced pulmonary inflammation, as shown by the leukocytes surrounding bronchioles and to a lesser extent, alveoli, when compared to healthy animals. Lung tissue of animals exposed to both OVA and ethanol did not differ from those exposed to OVA alone with regards to the changes assessed (Figure 5).

Table 2. Nano-TiO₂ aerosols characterization.

Parameters	Low dose of nano-TiO ₂	High dose of nano-TiO ₂
Primary particle size (nm)	5	5
Mass concentration (Cassette) (mg/m ³)	9.4	15.7
Min and max concentrations (Dust trak) (mg/m ³)	6.4 and 13.5	14.9 and 17.4
Number concentration (ELPI) (/cm ³)	76 169	617 709
Number median aerodynamic diameter (NMAD) (D ₅₀) (ELPI) (nm) ^a	168	171
D ₂₅ and D ₇₅ ELPI (nm) ^a	75 and 1000	70 and 321
Percentage of nano-agglomerates (<100 nm) in aerosol (%)	37	31

^aAerodynamic diameters for which 25% (D₂₅), 50% (D₅₀ or NMAD) or 75% (D₇₅) of the particles in the aerosol are smaller than this value.

Characterization of nano-TiO₂ exposures

Characterization of the nano-TiO₂ aerosols generated for the nose-only animal exposures is found in Table 2. NMADs measured with the ELPI were of 168 and 171 nm and appreciatively 30 % of the particles present in the aerosols were of nanometric dimension. Cumulative size distributions of the nano-aerosols generated are shown in Figure 6.

Evaluation of pulmonary toxicity following exposure to nano-TiO₂

In asthmatic rats, exposure to a low dose of nano-TiO₂ (9.4 mg/m³) 42 h after allergen challenge significantly ($p < 0.05$) decreased total leukocytes (7.12E⁶ \pm 0.93 for OVA vs. 4.18E⁶ \pm 0.52 for OVA + TiO₂) and eosinophils (3.2E⁶ \pm 0.42 for OVA vs. 1.41E⁶ \pm 0.17 for OVA + TiO₂, Figure 7). In addition, changes in the levels of plasma cytokine levels were also found (Figure 8). Levels of MCP-1, IL-4, IL-6, and INF- γ in asthmatic rats exposed to nano-TiO₂ (OVA + nano-TiO₂) decreased by 21, 54, 53, and 92%, respectively, when compared to asthmatic rats exposed to compressed air (OVA) ($p > 0.05$) (levels of IL-1 β , IL-10, and TNF- α were below detection limit for all animals). Similar results were found in BAL, the

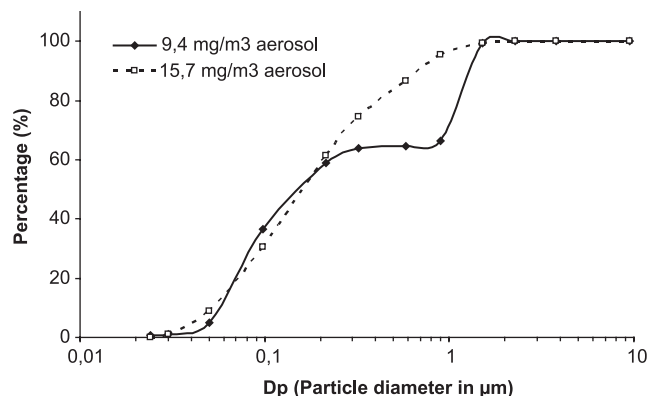


Figure 6. Cumulative size distribution of nano-TiO₂ aerosols (measured by ELPI).

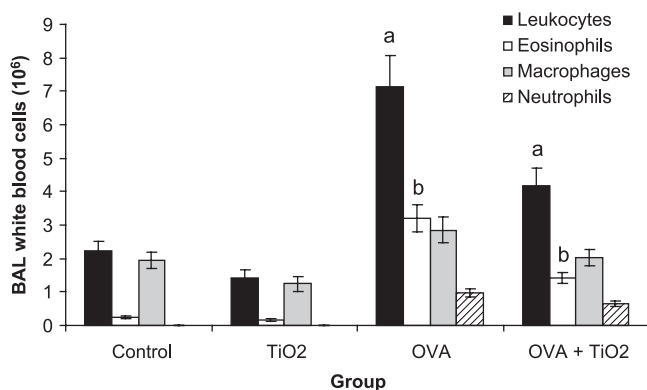


Figure 7. Effect of exposure to a low dose of nano-TiO₂ (9.4 mg/m³) on pulmonary inflammation of exposed rats (mean \pm SEM). a, $p < 0.05$ for total leukocytes in OVA versus OVA + TiO₂; b, $p < 0.05$ for eosinophils in OVA versus OVA + TiO₂. OVA, ovalbumin.

levels of relevant cytokines decreased in OVA + nano-TiO₂ group when compared to OVA group. Levels of IL-4, IL-6, and INF- γ decreased by 36, 17, and 10%, respectively (data not shown).

To determine the effect of nano-TiO₂ in normal (not asthmatic) BN rats, exposure to a high dose (15.7 mg/m³) of nano-TiO₂ was tested. Nano-TiO₂ did not induce inflammation, yielding no significant differences in total leukocytes when compared to animals exposed to filtered air (mean \pm SEM of $1.412E6 \pm 0.267$ for TiO₂ group vs. $2.235E6 \pm 0.297$ for control group), eosinophils ($0.154E6 \pm 0.029$ for TiO₂ group vs. $0.247E6 \pm 0.033$ for control group), macrophages ($1.242E6 \pm 0.235$ for TiO₂ group vs. $1.949E6 \pm 0.259$ for control group), and neutrophils ($0.010E6 \pm 0.002$ for TiO₂ group vs. $0.006E6 \pm 0.001$ for control group).

Interestingly, histological analyses of lung tissue lesions support BAL cellular and molecular findings (Figure 9A). On one hand, animals exposed to OVA alone had increased lung inflammation when compared to healthy animals, as expected. On the other hand, animals exposed to OVA and nano-TiO₂ showed decreased leukocyte infiltration when compared to those exposed to OVA alone. Figure 9B shows macrophages with phagocytosed nano-TiO₂ agglomerates in

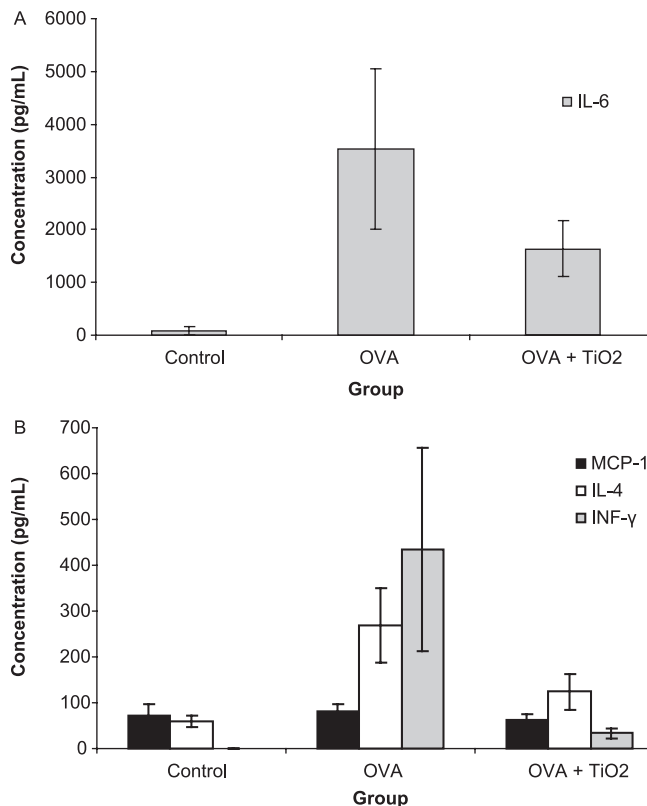


Figure 8. Effect of nano-TiO₂ aerosols on the plasma levels of various plasma cytokines involved in the inflammatory reaction: IL-6 (A) and MCP-1, IL-4, INF- γ (B) (mean \pm SEM).

exposed animals when compared to those exposed to filtered air.

Discussion

In the present study, we assessed whether nano-TiO₂ or ethanol affected pulmonary inflammation in asthmatic BN rats. Our objective was not to compare both pollutants between them, but rather to assess their level of toxicity individually. We used leukocyte count as our primary endpoint and histological analyses of the lungs as our secondary endpoint to determine the level of inflammation. The results of these two endpoints provided us with an overview of pulmonary inflammation and allowed us to determine if more endpoints were necessary in order to support cellular results. In order to be cost efficient, further analyses (e.g. cytokine analyses) would only be pursued in the presence of significant changes among groups for the primary and secondary endpoint, as others have done when studying multiple pollutants (Cho et al., 2010). Moreover, given that we were not comparing ethanol toxicity with that of nano-TiO₂, we were able to customize study designs for each pollutant that would provide answers to our objectives while ensuring an optimal ethical use of animals.

We first assessed whether a single high dose of nano-TiO₂ caused pulmonary inflammation in healthy, unsensitized and unchallenged BN rats. A high dose (15.7 mg/m³) did not induce pulmonary inflammation

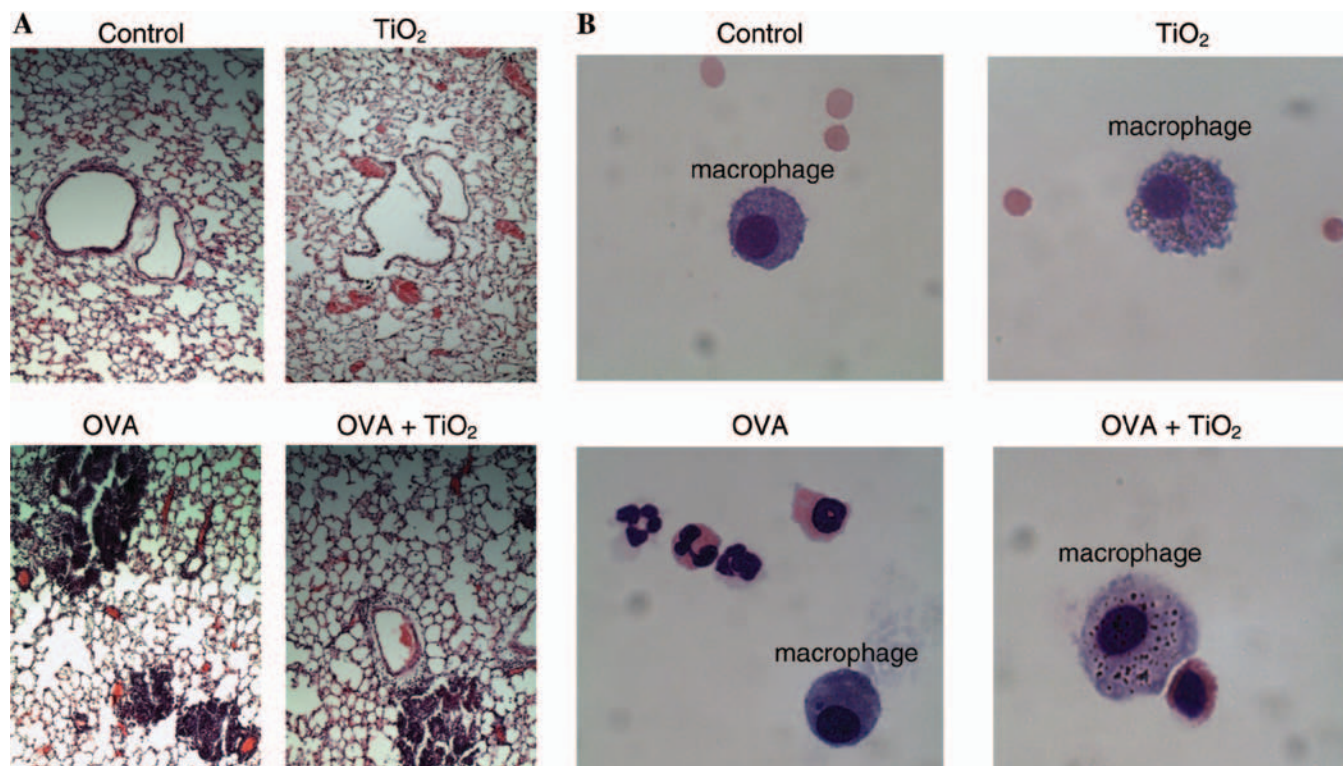


Figure 9. (A) Photomicrograph (5 μ m slice, H & E staining) of rat lungs for control (top left), nano-TiO₂ alone (top right), ovalbumin alone (bottom left), and ovalbumin + nano-TiO₂ (bottom right) exposed rats (100 \times). (B) Photomicrograph showing nano-TiO₂ phagocytosis by a macrophage in an exposed rat (1000 \times).

as measured in BAL immediately following 6 h exposure. This result is in line with other studies that have shown that exposure to particulate matter, such as wood smoke and diesel exhaust particles, at concentrations of 1, 10, and 20 mg/m³, did not cause an acute lung inflammatory response in BN rats (Tesfaigzi et al., 2002; Dong et al., 2005). However, other studies have shown that exposure to nano-TiO₂ at concentrations higher than 10 mg/m³ can significantly induce pulmonary inflammation in Wistar and Fisher rats (Oberdörster et al., 1994; Baggs et al., 1997; Bermudez et al., 2002; Ma-Hock et al., 2009; Van Ravenzwaay et al., 2009). The reason for this discrepancy remains unclear but may be related to either a difference in the size of the particulates of TiO₂ administered (and thus their effects on the immune response) or in a difference in the immune response between the different strains of rats, the BN rat known to have less of an acute phase response and more of a humoral response to different substances (Siegel et al., 2004).

We also assessed whether a single low dose of nano-TiO₂ (9.4 mg/m³) would affect a lung inflammatory response that is ongoing in the BN rat. Nano-TiO₂ was administered to BN rats 42 h after allergen challenge and lung BAL was retrieved. The level of nano-TiO₂ used in asthmatic rats was 31 times higher than the current NIOSH time weighted average (TWA) acceptable level of 0.3 mg/m³. We found that nano-TiO₂ reduced allergic inflammation, as measured by total inflammatory cells and decreased systemic and BAL cytokines. This reduction was observed with anatase nano-TiO₂, which

is considered more toxic than rutile nano-TiO₂ (Jiang et al., 2008; Warheit et al., 2007b). These results were unexpected, since it had previously been shown in a mouse model of diisocyanate-induced asthma that oropharyngeal exposure to anatase nano-TiO₂ increased the lung inflammatory response (Hussain et al., 2011). Also, rats are reported to be more sensitive than mice to the pulmonary toxic effects induced by nano-TiO₂ (Bermudez et al., 2002). We have considered the possibility, as suggested by Persson & Uller (2010), that inflammatory cells might persist in the lung interstitium during lung disease/injury instead of migrating towards the airway lumen, therefore decreasing the number of leukocytes found in BAL fluid. However this hypothesis was rejected following lung histology analyses, which showed that the level of inflammation in the lungs was proportional to BAL cellular results. Others have also reported results that are similar to ours. While healthy mice exposed to nano-TiO₂ had an increase in pulmonary neutrophilia accompanied by significantly increased chemokine CXCL5 expression, reduced inflammation after nano-TiO₂ exposure was observed after allergen challenge by Rossi et al. (2010). They found that in OVA-sensitized mice, inhalation of aerosolized needle-like silica-coated rutile nano-TiO₂ (10 mg/m³, 2 h/day, three times per week for 4 weeks, geometric mean size of particles in aerosol of 199 nm as measured by an ELPI) significantly reduced the OVA-induced increase in the number of BAL eosinophils and lymphocytes (without changing the level of neutrophils), lowered the level of macrophages, decreased Th2

cytokines, airway hyperresponsiveness and the number of mucus-producing goblet cells, two important aspects of an asthmatic reaction. It is interesting to note that Rossi et al. (2010) obtained similar results when exposing mice to fine TiO₂ particles (<5 µm) instead of nano (<100 nm) particles. Thus, both rutile nano-TiO₂ in mice and anatase nano-TiO₂ in rats have been shown to cause a reduction of allergic lung inflammation in this model.

Although it is unclear why studies show contradictory effects regarding either enhancement (Hussain et al., 2011; De Haar et al., 2006) or suppression (Rossi et al., 2010 and the present study) of allergic pulmonary inflammation in rodents following nano-TiO₂ exposure, one possibility may also reside in the way the particles reach the lungs. Grassian et al. (2007) reported that the route of exposure, instillation versus inhalation, can have an impact on the agglomeration state of nano-TiO₂ and thus their potential physiologic effects. Hussain et al. (2011) exposed rodents oropharyngeally to nano-TiO₂ in suspension, de Haar et al. (2006) exposed mice intranasally to nano-TiO₂, whereas inhalation of aerosolized nano-TiO₂ powder was used in the study by Rossi et al. (2010) and in the present study. Thus, different exposure routes between the two groups of studies may have lead to the formation of different TiO₂ nanoparticles agglomerates, which can lead to various effective concentrations in the different lung compartments. Therefore, the effective nanoparticle biological dose may have been different and could be partly responsible for the discrepancy in the results. Finally, Hussain et al. (2011) used TDI as the allergen and studies on the mechanisms of asthma caused by TDI, which can induce a Th1 or Th2 response, are controversial (Ban et al., 2006) and may not fully mimic the Th2 response in humans.

Nano-TiO₂ inhalation caused a decrease in both Th2 cytokines and the Th1 cytokine (INF-γ) retrieved in either plasma or BAL. Whether the decrease in cytokines caused a decrease in inflammatory cells, or a decrease in inflammatory cells led to a decrease in the release of cytokines is a question that remains unanswered. Amongst the possible mechanisms by which this occurred one may speculate that nano-TiO₂ induced a decrease in release of proinflammatory mediators from macrophages, thus down-regulating the immune response, such as a reduction in IL-1 and/or a release of the soluble receptor of IL-1. Another possibility is that nano-TiO₂ directly affected the cells that are known to release IL-4 and INF-γ (lymphocytes, mastocytes) and caused a down-regulation of their cytokine production/release. In this regard, the effects of nano-TiO₂ on the immune response seem similar to what has been demonstrated in OVA challenge with agents such as black seed oil (Shahzad et al., 2009) and thymoquinone (El Gazzar et al., 2006).

As inflammatory kinetics are cell-dependent, i.e. the various leukocytes peak at different times during the course of an allergic inflammatory reaction, we can hypothesize that the effect of nano-TiO₂ exposure on specific inflammatory cells may differ according to timing of exposure.

For instance, neutrophils peak 4–8 h following allergen challenge (Lukacs et al., 1998), whereas eosinophils peak 48 h following the challenge. The timing of exposure may therefore account for differences in effects induced by nano-TiO₂ in rodents by either promoting inflammation when TiO₂ exposure occurs prior to challenge (de Haar et al., 2006; Larsen et al., 2010; Hussain et al., 2011) or suppressing it when exposure is subsequent to allergen challenge, as demonstrated by our results.

Macrophages are the main cells responsible for particle clearance through phagocytosis in the alveolar region. We observed that macrophages from BAL fluid have engulfed nano-TiO₂ agglomerates (Figure 9B). Phagocytosis activity of alveolar macrophages is optimal for micrometer particles, but as reported in several studies, seems to be less efficient for particles in the nanoscale range (Oberdörster, 1988, 2001; Oberdörster et al., 1997; Takenaka et al., 2001, 2006; Geiser et al., 2008). Accordingly, the optimal particle size range for phagocytosis by alveolar macrophages would be between 0.5 and 3 µm and particles having a size below 0.26 µm would not mainly undertake this clearance pathway (Oberdörster, 1988; Morrow, 1988; Yang et al., 2008; Mansour et al., 2009). Hence, given that nano-TiO₂ tends to agglomerate when administered by aerosol, especially at high concentrations, the size of nanoparticle agglomerates in the physiological environment may have a crucial impact on macrophage phagocytosis, as it represents the real size that could elucidate a pulmonary immune cell response.

We then assessed the effects of ethanol on the response to allergen in the BN rat. The level for ethanol vapors was three times higher than the current TWA NIOSH or American Conference of Industrial Hygienists (ACGIH) level, and Occupational Safety and Health Administration (OSHA) permissible exposure limit (PEL), of 1000 ppm. We found that repeated exposure to ethanol vapors by inhalation did not exacerbate the allergic inflammatory response caused by the OVA challenge. Results showed that, aside from 6 h following OVA challenge, there were no statistically significant differences between exposed groups (OVA + ethanol) and control groups (OVA only) for all phases, i.e. the development, peak, and recuperation of the lung inflammatory response. Furthermore, no significant differences were observed when either 0.1 or 0.5% OVA was used instead of 1.5%, with BAL performed 24 h following challenge, which allowed us to evaluate the effect of ethanol inhalation on a smaller inflammatory response (data not shown). Although this study does not focus on alcohol-induced asthma, our results are in accordance with what has been suggested in some studies on alcohol-induced asthma, i.e. that ethanol is not the substance causing the pulmonary response. Studies have shown that acetaldehyde, a direct metabolite of ethanol, causes pulmonary responses following inhalation (Fujimura et al., 1999), by increasing pressure at the airway opening (Myou et al., 1993); blood acetaldehyde was increased in patients suffering from alcohol-induced asthma with changes in FEV1

higher than 20%, but not in those with a response lower than 20%, while blood alcohol levels remained unchanged (Watanabe, 1991). Acetaldehyde has been detected in the expired air of human volunteers during and after a 6 h acute exposure to 25–1000 ppm ethanol (Tardif et al., 2004). Further, Takao et al. (1998) showed that while ingestion of ethanol (30 g over 5–10 min) did not change FEV1 in a group of 30 volunteers, it caused a fall in FEV1 associated with a rise in blood acetaldehyde and histamine concentrations in asthmatic subjects. The response was positive in 19% of 16 patients with normal homozygote ALDH2 genotype, 71% of 14 patients with type mutant heterozygote, and 100% of two patients with type mutant homozygote ALDH2 genotype. It was therefore suggested that alcohol-induced asthma is probably caused by increased blood acetaldehyde concentration resulting from abnormalities of ALDH2, an enzyme responsible for metabolizing acetaldehyde. In a previous study, we have shown, however, that repeated exposure (13 weeks) to ethanol vapors (3000 ppm) did not induce pulmonary toxicity in normal Sprague-Dawley (SD) rats nor in a subgroup carrying a deficiency in ALDH2 (Scarino et al., 2009).

In conclusion, data show that acute exposure to the emerging pollutant nano-TiO₂ agglomerates decreases allergic lung inflammation in rats. This response occurs in association with a decrease in both Th2 and Th1 cytokines. Characterization of the nano-TiO₂ aerosols and timing of the nanoparticle exposure in relation to the induction of the asthmatic lung inflammation may play a crucial role in this protective effect. Inhalation exposure to another emerging pollutant, ethanol vapors, does not exacerbate allergic lung inflammation in a rat model. Results obtained with the OVA-induced Brown Norway rat model are relevant to humans as (1) it is a validated asthma model for the human disease, (2) exposure was done using the inhalation route, and (3) levels were representative of human exposure. Considering the increasing number of individuals exposed to contaminated urban air with various pollutants, data reported herein contributes to public health risk assessment.

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Declaration of interest

There are no conflicts of interest for all authors included in this manuscript.

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