Inhalation exposure of nano-scaled titanium dioxide (TiO2) particles alters the inflammatory responses in asthmatic mice

Article *in* Inhalation Toxicology · March 2013

DOI: 10.3109/08958378.2013.770939 · Source: PubMed

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Genetic variation influences immune responses in sensitive rats following exposure to TiO_2 nanoparticles

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ARTICLE INFO

Article history:
Received 28 August 2014
Received in revised form 7 October 2014
Accepted 8 October 2014
Available online 14 October 2014

Keywords:
Titanium dioxide
Inflammation
Asthma
Ovalbumin
Aerosol inhalation
Immune response

ABSTRACT

This study examines the immunological responses in rats following inhalation to titanium dioxide nanoparticles (TiO_2 NPs), in naïve rats and in rats with induced allergic airway disease. The responses of two different inbred rat strains were compared: the Dark Aguoti (DA), susceptible to chronic inflammatory disorders, and the Brown Norwegian (BN), susceptible to atopic allergic inflammation. Naïve rats were exposed to an aerosol of TiO_2 NPs once daily for 10 days. Another subset of rats was sensitized to the allergen ovalbumin (OVA) in order to induce airway inflammation. These sensitized rats were exposed to TiO_2 NPs before and during the allergen challenge.

Naïve rats exposed to TiO_2 NPs developed an increase of neutrophils and lymphocytes in both rat strains. Airway hyperreactivity and production of inflammatory mediators typical of a T helper 1 type immune response were significantly increased, only in DA rats.

Sensitization of the rats induced a prominent OVA-specific-IgE and IgG response in the BN rat while DA rats only showed an increased IgG response. Sensitized rats of both strains developed airway eosinophilia following allergen challenge, which declined upon exposure to TiO_2 NPs. The level of neutrophils and lymphocytes increased upon exposure to TiO_2 NPs in the airways of DA rats but remained unchanged in the airways of BN rats.

In conclusion, the responses to TiO_2 NPs were strain-dependent, indicating that genetics play a role in both immune and airway reactivity. DA rats were found to be higher responder compared to BN rats, both when it comes to responses in naïve and sensitized rats. The impact of genetically determined factors influencing the inflammatory reactions pinpoints the complexity of assessing health risks associated with nanoparticle exposures.

1. Introduction

The increased productions of engineered nanomaterials (ENMs) augment the risk for harmful exposure in occupational settings. In these environments inhalation is the main route of exposure and may lead to adverse effects on the respiratory system, such as airway inflammatory reactions and decline in lung function. Up to date there is an absence of regulations of occupational exposure limits for most ENMs. This is due to limited understanding of biological uptake and toxicity as well as lack of standardized protocols for ENMs (Kuempel et al., 2012). Another concern is the increasing number of individuals with respiratory disorders, for example asthma (AAAAI, 2014; GINA, 2012; Holgate, 2012). When such sensitive individuals are exposed to particulate matter in occupational environments it may result in aggravated asthmatic disease (Peden, 2005). With a larger part of the population being highly sensitive and with an increased risk of being exposed to ENMs there is a recognized need to understand the toxicity of such materials (Savolainen et al., 2013).

Previous studies have indicated a correlation between genetically determined susceptibility and exposure to particulate matter

Abbreviations: AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; BN, Brown Norwegian; C_{RS}, respiratory compliance; DA, Dark Aguoti; ENMs, engineered nanomaterials; *G*, tissue resistance; *H*, tissue elastance; MCh, methacholine; MMAD, mass median aerodynamic diameter; MPPD, multiple path particle dosimetry models; NPs, nanoparticles; OVA, ovalbumin; PBS, phosphate-buffered saline; R_{RS}, respiratory resistance; TiO_2, titanium dioxide.

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http://dx.doi.org/10.1016/j.tox.2014.10.004
0300-483X/

in the development of allergic asthma (Heidenfelder et al., 2009; Li et al., 2008; Peden, 2005). In animal models it is shown that different inbred mouse strains respond differently to particulate air pollution, providing experimental evidence of a genetic contribution to the health effects (Ichinose et al., 1997; Miyabara et al., 1998; Ohtsuka et al., 2000a,b). It is also suggested that genetic variation in humans also affect the response to particulate matter (Granum and Løvik, 2002).

Several studies have been published on the effects of ambient particulate exposure on allergic airway disease (Arantes-Costa et al., 2008; Kim et al., 2011) and, more recently, on the impact of ENMs on rats with allergic diseases (Hussain et al., 2011; Koike et al., 2008; Nygaard et al., 2009; Rossi et al., 2010a,b). The results from these studies vary, probably as a consequence of using different protocols, differing in choice of strains and sensitization schedule. Furthermore the physicochemical properties of the ENMs used in the studies varied.

Titanium dioxide (TiO_2) nanomaterials are manufactured worldwide in large quantities due to their versatile functions in varying products. Traditionally, TiO_2 particles have been considered to exhibit low solubility and relatively low toxicity (Donaldson et al., 2008; Kreyling et al., 2006; Shi et al., 2013; Warheit et al., 2007). Despite the relative low toxicity of TiO_2 nanoparticles (NPs), however, we have previously demonstrated that TiO_2 NPs (P25, crystalline structure of 80% anatase and 20% rutile) induce oxidative stress and pro-inflammatory response in human bronchial epithelial cells (Ahlinder et al., 2013; Andersson et al., 2011; Ekstrand-Hammarstrom et al., 2012). In an *in vivo* study performed on rats, we showed that one exposure of TiO_2 NPs to the lung resulted in an inflammatory response in the airways, with an influx of neutrophils and macrophages. This innate immune response was followed by an adaptive immune activation that lasted for at least three months (Gustafsson et al., 2011). In a subsequent study on mice we demonstrated that inhalation of TiO_2 NPs may aggravate allergic airway disease, depending on the dose and the timing of the exposure. The mice were sensitized to the allergen ovalbumin (OVA) and when exposed to TiO_2 NPs, before and during an allergen challenge, they lost body weight, their general health condition declined, and there was an increase of neutrophils in the airways (Jonasson et al., 2013). The data in that study imply that the inhalation of TiO_2 NPs by individuals with allergic airway disease may increase the risk to develop symptoms similar to that of severe asthma.

In order to improve the understanding of how genetics influence the effect of TiO_2 NP exposure on allergic airway inflammatory disease, we compared the responses of two inbred rat strains exhibiting different susceptibility to inflammatory disorders. For this purpose we examined immune activation and airway hyperresponsiveness (AHR) after repeated exposure to TiO_2 NPs in healthy Dark Agouti (DA) and Brown Norway (BN) rats, as well as in sensitized rats during a period of allergen challenge. DA rats were previously reported to be highly susceptible to chronic inflammatory disorders, such as arthritis (Carlson et al., 2000) and encephalomyelitis (Jagodic et al., 2005). The Brown Norwegian (BN) rat is naturally atopic with high levels of allergen-specific immunoglobulin E (IgE) antibodies and is susceptible to inflammatory responses characterized by eosinophilic infiltration of tissues (Granum and Løvik, 2002; Hylkema et al., 2002; Martin and Tamaoka, 2006).

The specific aims of this study were to (i) determine if the DA and BN rats respond differently to inhalation of TiO_2 NPs in terms of AHR, inflammatory response, immune activation and decline in general health condition, and to (ii) determine if the inhalation to TiO_2 NPs aggravated the response in terms of AHR, inflammatory response, immune activation and decline in general health condition in rats with induced allergic airway inflammation.

2. Materials and methods

2.1. Animals

Healthy inbred male rats, 85 DA/OlaHsd (8–10 weeks old) and 85 BN/SsNOlaHsd (6–8 weeks old), were purchased from Harlan Laboratories (Netherlands). Upon arrival the rats were acclimatized, for at least one week, at a facility approved for housing animals. The rats were housed in groups of four in plastic cages with a 12 h light cycle, a temperature of 22 °C and 50–60% humidity. The rats had access to food (R 36, Lantmännen Lantbruk, Sweden) and water *ad libitum*. The study was approved by the Animal Research Ethical Committee, Umeå, Sweden, in compliance with Swedish law.

2.2. Particle characterization, particle aerosol exposure and particle deposition

Nano-sized TiO_2 P25 particles (crystalline structure: 80% anatase, 20% rutile) (Degussa AG, Frankfurt, Germany) were used in this study. The primary particle size is 21 nm according to the manufacturer. Particle solutions were prepared in phosphate-buffered saline (PBS, Sigma–Aldrich). Aggregation and agglomeration behavior of these particles, both in their dry state and in PBS solution, as well as particle charge (ζ-potential) have been

Table 1
Physicochemical properties of TiO_2 (P25).

Property	TiO_2 (P25)
Crystalline structure[a]	80% anatase and 20% rutile
Primary size[b]	21 nm
Median agglomeration size within PBS[c]	4.2 μm
Mass median aerodynamic diameter measured at the batelle port[d]	2.12 μm
Volumetric mean diameter of TiO_2 within the droplet[e]	1–2.5 μm (85% of the fraction) 100–200 nm (15% of the fraction)
ζ- potential in PBS (pH 7.4)[f]	−24 mV
Endotoxin[g]	Non detectable
Mass deposition in lung per animal[h]	168 ± 12 μg DA rat (n = 5) 159 ± 48 μg BN rat (n = 5)

[a] According to the manufacturer.
[b] Determined from Scherrer analysis of the diffraction peak in XRD, results from Andersson et al., (2011).
[c] Static light scattering experiments, results from Andersson et al., (2011).
[d] Malvern Mastersizer X.
[e] Photon cross correlation spectroscopy.
[f] Malvern Zetasizer Nano ZS, results from Andersson et al. (2011).
[g] Limulus amebocyte lysate (Chromogenic Endotoxin Quantification Kit).
[h] ICP-SFMS.

previously reported by our group (Andersson et al., 2011); the data are detailed in Table 1.

Rats were placed in individual nose-only containers (EMMS, UK), which were coupled to a Battelle tower connected to a 6-jet Collison nebulizer (Waltham, MA). During all aerosol exposures the flow rate was 7.8 ± 0.2 l/min. The concentration of TiO_2 in the PBS suspension was 10 mg/ml. The characteristics of the inhaled aerosol, the droplet size distribution and the mass median aerodynamic diameter (MMAD), were measured at the exit port of the Batelle tower by Malvern Mastersizer X (Malvern instruments, Malvern, UK). The agglomerated particle hydrodynamic size distribution within the droplet was measured by collecting the aerosol (flow 1 l/min) in an impinger system, as previously described (Jonasson et al., 2013), and subsequently measuring it with photon cross correlation spectroscopy (Nanophox, Sympatec, Clausthal-Zellerfeld, Germany).

The total deposition of TiO_2 NPs in the lungs of the rats was measured after one single exposure in a group of rats; the group consisted of five rats from each strain and was dedicated to determination of TiO_2 NPs deposition only. To determine the TiO_2 content, the lung was digested in HNO_3 and HF and diluted in MilliQ-H_2O before being analyzed with ICP-SFMS for quantification of titanium. These measurements were carried out by ALS Scandinavia AB (Luleå, Sweden).

To estimate the deposition fraction in different regions of the respiratory system following inhalation to TiO_2 NPs, a multiple path particle dosimetry model was used http://www.ara.com/products/mppd.htm (MPPD v 2.11, 2013). The deposition fractions of the head, trachea–bronchial, and pulmonary regions were calculated with default settings for the rat model and additional selected settings; a size range of 0.01 μm–10.0 μm, a leaning-forward body orientation with "constant exposure", and "deposition only". In order to calculate deposited mass (% of total aerosol) in different regions of the respiratory system, the deposition fractions derived with MPPD (corresponding to the geometric midpoint of each size bin returned by Malvern Mastersizer) were multiplied with the aerosol size distribution (intensities of every bin is given as %wt. of total aerosol) and finally the result was summed over the particle size.

A Limulus amebocyte lysate Chromogenic Endotoxin Quantification Kit (Thermo Scientific, Nordic Biolabs AB, Stockholm, Sweden) was used, according to the manufacturer's instructions, to ensure that the TiO_2 NPs were not contaminated with endotoxin.

2.3. Experimental design

This work was divided in two parts: in the first part we sacrificed rats and analyzed the samples one day after the last TiO_2 NPs exposure. In the second part, we included additional rats, which were sacrificed and analyzed 70 days after the last TiO_2 NP exposure. The experimental protocol is outlined in Fig. 1A and B. The different rat strains were analyzed with identical procedures but not on the same day. In the first part we performed respiratory physiology and examined general health status, antibodies detected in serum, cells differentiation in BALF, measured cytokine, and chemokine levels in BALF. In the second part only general health status, antibodies detected in serum, and cell differentiation in BALF was examined. In both studies naïve rats, from both strains, were exposed to either vehicle control (PBS group) or to TiO_2 NPs in a PBS solution (TiO_2 group) for 2 h/day for 10 subsequent days (Fig. 1A). Additional groups were sensitized with OVA on two occasions during the first two weeks and were exposed to three OVA challenges (OVA group) during the last week of the schedule. One group from each strain was exposed to TiO_2 NPs for 2 h/day for 10 subsequent days, both before and during the OVA challenges (TiO_2/OVA group) (Fig. 1B). In more detail, the rats were sensitized with two intraperitoneal injections (i.p) of 100 μg ovalbumin (OVA, Sigma–Aldrich) emulsified in sterile saline and $Al(OH)_3$ (Alu-Gel-S suspension 1.3%, research grade sterile, cat no: 12261, SERVA electrophoresis, Thermo Fisher Scientific Inc.) (1:3) on days −23 and −10. The sensitized rats were then challenged with aerosolized 1% OVA in H_2O, nose-only for 30 min on days 6, 8 and 10. The TiO_2 NP exposures were performed daily before and during the OVA challenges (days 1–10).

2.4. Antibodies in serum

Rats were sacrificed by a lethal dose of sodium pentobarbiturate i.p. (Apoteket AB, Stockholm, Sweden), followed by exsanguination from descending aorta, during which blood samples were collected. The blood was centrifuged and the serum samples were stored at −80 °C until the analyses. Total IgE, OVA specific IgE, and

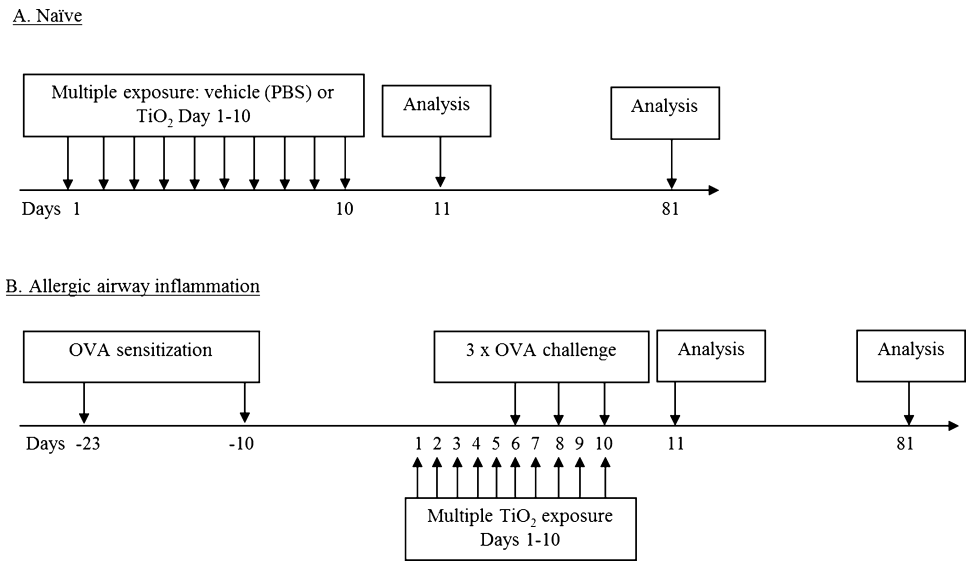

Fig. 1. Schematic illustration of the TiO_2 NPs exposures. Procedures for the TiO_2 NPs exposures and analysis are shown for naïve rats (A) and rats sensitized and challenged with ovalbumin (OVA) (B). Sampling of cells in bronchoalveolar lavage fluid (BALF), inflammatory and immune mediators from both BALF and serum, and measurement of respiratory physiology were performed on day 11. On the late time-point, day 81 only cells were differentiated and analyzed.

OVA specific IgG was analyzed using enzyme-linked immunosorbent assay (ELISA), performed, with some modification, according to a previously described protocol (Svensson et al., 2003). Nunc-Immuno plates (96-wells) with Max Sorb surface (Tamro MedLab AB, Mölndal, Sweden) were coated with 50 μl anti-IgE (BD Pharmingen, clone B41-1) and diluted to 10 μg/ml for total IgE analysis or 14 μg/ml for OVA-specific IgE analysis. In a pilot experiment for IgE validation, a serial dilution of serum was analyzed in order to ensure that the measurement was done within the linear range of the O.D. Results are shown for the serum dilution total IgE 1:4 and OVA-specific IgE 1:4 The plates were then incubated with 50 μl of serum and bound IgE-antibodies were detected after incubation with either 50 μl biotin-labeled anti-IgE antibody (4 μg/ml, BD Pharmingen, clone B41-3) or 50 μl biotinylated OVA (16 μg/ml). For OVA-specific IgG analysis the wells were coated with 3 μg of OVA to which the serum was then added. In a pilot experiment for IgG validation, a serial dilution of serum was analyzed in order to ensure that the measurement was done within the linear range of the O.D. Results are shown for the serum dilution 1:60 000. Bound OVA-specific IgG-antibodies were detected after incubation with 50 μL biotin-labeled anti-IgG polyclonal antibody (0.5 $\mu g/mL^{-1}$ KPL, Gaithersburg, MD).

Bound antibodies were quantified with a ready-to-use streptavidin–peroxidase system (Roche Diagnostics, Basel, Switzerland) together with tetramethylbenzidine (TMB) substrate according to the manufacturer's instructions. The soluble product was analyzed at 450 nm and 620 nm in a Thermo Labsystems iEMS ELISA reader (Vantaa, Finland). The background level of the ELISA as determined in wells incubated with PBS (IgE) or BSA (IgG), to which all substrates and serum were added, was subtracted from all data.

2.5. Isolation of BALF cells

The lungs were lavaged with ice cold Ca^{2+}/Mg^{2+}-free Hanks balanced salts solution (HBSS, Sigma–Aldrich, Germany) by cannulating the trachea. Two aliquots were collected, the first 2 ml were collected separately and the following 23 ml (4 × 5 ml + 3 ml) were pooled in another tube. After centrifugation (10 min, 4 °C, 1500 rpm), the supernatant from the first 2 ml was used for detection of cytokines and chemokines. The cell pellets from both aliquots were pooled for total cell count using trypan blue exclusion in a Bürker chamber. Differential cell counts of lymphocytes, eosinophils, macrophages, and neutrophils were determined, in duplicate, with cytospin preparations of 30,000 cells stained with May-Grünwald–Giemsa solutions (Shandon© cytospin 3 cyto-centrifuge, Runcorn, UK). Three hundred cells were counted from each slide in a blinded manner.

2.6. Flow cytometry analysis of BALF cells

Lymphocytes in BALF were analyzed with flow cytometry, using a BD FACSort™ (Becton, Dickinson and Company, San Jose, CA, USA). For staining, 250,000 cells were distributed into tubes and washed in PBS containing 0.1% fetal bovine serum (FBS, Sigma–Aldrich, Germany). Isotype-matched antibodies were used as negative controls. The lymphocytes were stained using the monoclonal antibodies (mAbs), all from BD Bioscience Pharmingen (San Diego, USA) CD3-FITC, CD3-PE, αβ-TCR-PerCP, γδ-TCR-FITC, CD45RA-PE, NKR-P1A-PE, CD8a-PerCP, CD4-PE-Cy5, and CD25-PE as follows: non-specific binding was blocked by incubating the cells with 4 μl rat Fc Block™ (BD Biosciences Pharmingen) and 15 μl FBS for 5 min on ice. Then, mAbs were added and the cells were incubated for 30 min in darkness. Red blood cells were lysed using FACS™ Lysing solution and cells were fixed with BD CellFIX™ (BD Biosciences, San Jose, CA) before analysis on BD FACSort™. B cells were defined as $CD45RA^+$ and $CD3^-$, T_H cells as $CD4^+$and $CD3^+$, T_C cells as $CD8^+$ and $CD3^+$, NK cells as $NKR\text{-}P1A^+$ and $CD3^-$ and NKT cells as $NKR\text{-}P1A^+$ and $CD3^+$. Results are presented as total numbers of the different lymphocytes.

2.7. Inflammatory cytokines and chemokines in BALF and serum

Inflammatory mediators in the BALF and the serum were analyzed measured with a multiplex kit (Bio-Plex™ Pro Rat Cytokine 23-plex panel) for the presence of interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12(p70), IL-13, IL-17, IL-18, EPO, CSF1, CSF2, CSF3, GRO, IFN-γ, CCL3, CCL20, CCL5, TNF-α and VEGF according to the manufacturer's instructions and analyzed with a Bio-Plex™ system (Luminex Bio-Plex™ 200 System, Bio-Rad Hercules, CA).

2.8. Measurement of respiratory physiology

Twenty four hours after the last exposure, respiratory resistance and compliance were measured following exposure to methacholine (MCh, acetyl-β-methylcholine chloride, Sigma–Aldrich). The rats were weighed and anesthetized with pentobarbital sodium (50 mg/kg, i.p., purchased from local suppliers). The rats were tracheostomized with a 15-gauge cannula and mechanically ventilated with a small animal ventilator (flexiVent™, SCIREQ) at a frequency of 1.5 Hz (90 breath/min) and a tidal volume (V_T) of 10 ml/kg body weight. To exclude the rats' intrinsic respiration and respiratory function, and to make a strict respiratory mechanics measurement possible, the rats were paralyzed with pancuronium (0.1 mg/kg, i.p., from local suppliers). A positive end-expiratory pressure of 3 cm H_2O was applied. A warming pad prevented cooling of the animal. In order to establish stable baseline respiratory mechanics and to ensure a similar volume history before the experiments, four sigh maneuvers at $3 \times V_T$ were performed at the beginning of the experiment. The rats were then allowed a 2 min resting period before the experiment began. Dynamic respiratory mechanics were measured by applying sinusoidal standardized breathing, and the resulting data were analyzed using the single compartment model and multiple linear regression, to give respiratory resistance (R_{RS}) and compliance (C_{RS}). More thorough evaluations of the respiratory mechanics of the peripheral components were made using forced oscillation technique (FOT). During the forced oscillatory maneuver, the ventilator piston delivers 13 superimposed sinusoidal frequencies, ranging from 1.0 to 20.5 Hz, in a 2 s period (Quick-Prime 2, impedance (Zrs). The primary parameters obtained are tissue resistance (G) and tissue elastance (H). All parameters were measured continuously and the maximum response to a given concentration of 5–45 mg/ml MCh was determined. MCh was diluted in saline and a volume of 20 μl was given over 10 s with an aerosol with a volume diameter of 4–6 μm (Aeroneb™, SCIREQ). The MCh dose was aerosolized without any interference to the ventilation pattern.

2.9. Statistical analysis

The results are presented as the mean ± standard error of means (SEM) or mean ± standard deviation (SD). Statistical significance was assessed by parametric methods using one-way analysis of variance to determine differences between selected groups, followed by Dunnett's post hoc test. When only two groups were compared, a Student's unpaired t-test was used. A statistical result with $p < 0.05$ was considered significant. The statistical analyses were carried out and graphs were prepared with

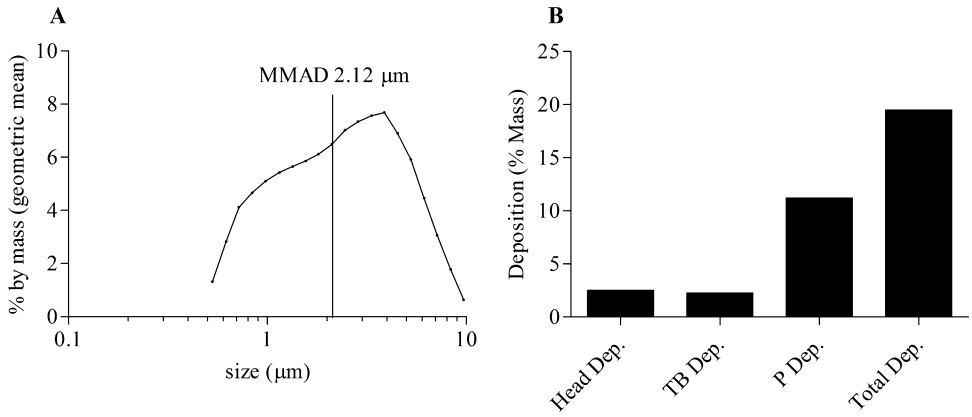

Fig. 2. Aerosol size distribution and lung particle deposition. (A) Size distribution of the aerosol at the exit port of the Battelle tower, showing the geometric mean and the mass median aerodynamic diameter (MMAD). (B) Regional deposition of the particles in the head (Head Dep), trachea bronchiolar (TB Dep) region and the pulmonary (P Dep) region as well as the total deposition (Total Dep). The dose deposition was calculated with the multiple path particle dosimetry model (MPPD) and multiplied with each fraction from the geometric mean size range in A.

GraphPad Prism program (version 6.0 GraphPad software Inc., San Diego, CA).

3. Results

3.1. Particle characterization, exposure and deposition

The droplet size at the exit port of the Battelle tower displayed a bimodal distribution, ranging from 0.5 μm (the cutoff for the instrument) to 10 μm with an MMAD of 2.12 μm (Table 1 and Fig. 2A). The volumetric diameter of the TiO_2 NPs within the droplet was distributed into two fractions: the largest fraction, consisting of about 85%, ranged from 1 to 2.5 μm (1.52 ± 0.16 μm (mean ± SD)). The remaining 15% of the NPs ranged from 100 to 200 nm, with an average size close to 100 nm ($n = 5$).

The deposition of NPs in the lung after one single 2 h NPs exposure was 168 ± 12 μg in the DA rat lung and 159 ± 48 μg in the BN rat lung. Calculations using the MPPD model, (aerosol droplet size ranged from 0.01 to 10.0 μm) show that, based on the three examined regions, most of the NPs were deposited in the pulmonary alveolar region. The second largest fraction was deposited in the head (nasal cavity), and a yet smaller fraction in the trachea bronchiolar region (Fig. 2B).

There was no contamination of endotoxin in the TiO_2 NPs samples as indicated by the Limulus endotoxin assay.

3.2. TiO_2 NP exposure in rats with allergic airway inflammation (sensitive rats)

3.2.1. General health effects

There were no visible signs of impaired general health conditions or reduced body weight in rats with allergic airway inflammation after exposure to TiO_2 NPs in either of the strains when examined on day 11 and day 81.

3.2.2. IgG and IgE responses to OVA

In both strains, similar levels of allergen-specific IgG were detected in serum on day 11 (Fig. 3). In the BN rat, OVA also induced an increase of OVA-specific-IgE antibodies, which was not observed in DA rats. The total IgE levels in serum did not change after sensitization with OVA, but naïve BN rats had higher baseline levels of IgE than naïve DA rats. TiO_2 exposure of OVA sensitized rats did not result in any significant change of the total IgE, the OVA specific-IgE, or the OVA specific-IgG levels in any of the strains.

3.2.3. Inflammatory responses

In both rat strains, the OVA-treated rats had increased levels of eosinophils, neutrophils, lymphocytes, and macrophages in the BALF when compared to naïve rats on day 11 (Fig. 4). In both strains, the TiO_2/OVA-groups showed a decrease in the number of eosinophils compared to the OVA groups. In DA rats it was

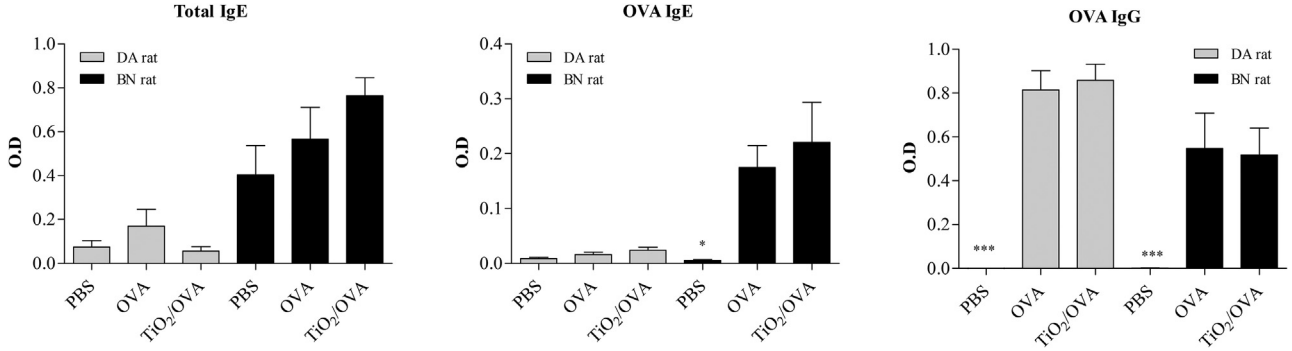

Fig. 3. Analysis of antibodies in serum from sensitive rats on day 11. Total IgE, OVA specific IgE and OVA specific IgG were analyzed in serum. Optical density (OD) values from the immunoassays are shown. From both rat strains, rats exposed to PBS (PBS-groups), and groups that received TiO_2 nanoparticles before and during the OVA challenge (TiO_2/OVA-group) were compared to the sensitized group with OVA challenge only (OVA-group). Differences within each strain were calculated. Data are expressed as means ± SEM ($^{*}p < 0.05$ and $^{***}p < 0.001$, all groups: $n = 8$).

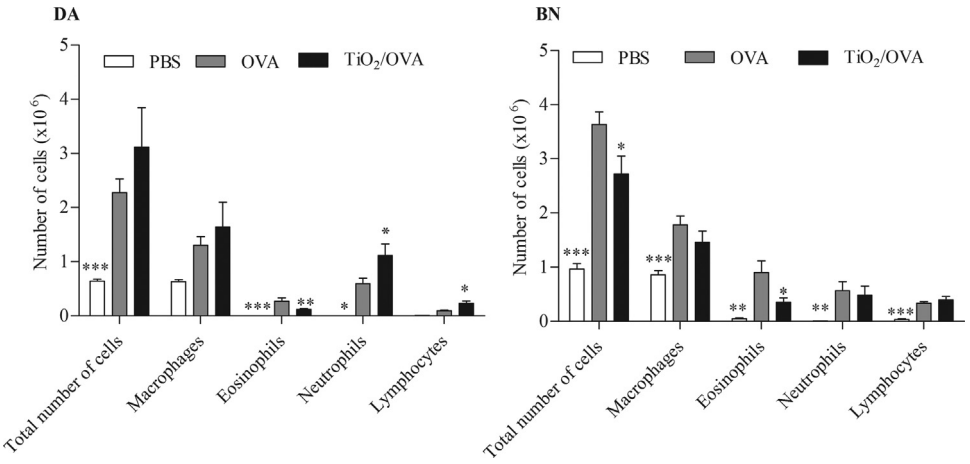

Fig. 4. Analysis of cells in BALF from sensitive rats on day 11. Analysis of cells in the BALF following exposure to TiO_2 NPs before and during the development of allergic airway inflammation. Total number of cells and differential cell count in BALF from both DA rats and BN rats. Control groups with naïve individuals exposed to PBS (PBS-group) and sensitized groups exposed to TiO_2 nanoparticles before and during the OVA challenge (TiO_2/OVA-group) were compared to the sensitized group with OVA challenge only (OVA-group). Differences within each strain were calculated. Data are expressed as means ± SEM ($^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, all groups: $n = 8$).

observed that the numbers of neutrophils and lymphocytes had increased following the TiO_2 NP exposure, which was not observed in BN rats.

For both strains the lymphocytes in the TiO_2/OVA-groups were predominately T_H cells ($CD3^+$, $CD4^+$) carrying the αβ-TCR receptor (Fig. 5). The TiO_2/OVA-groups did not affect the numbers of T cells, B cells, or NK cells. The levels of IL-17, CCL20, and CXCL-1 were increased in the BALF of both the OVA- and TiO_2/OVA-groups (data not shown).

On day 81, 70 days after the last OVA exposure, the number of eosinophils remained elevated in BN rats (Fig. 6). In the BN rats the number of neutrophils was increased in the TiO_2/OVA-group compared to the OVA-group ($p < 0.01$). A similar difference was observed in DA rats, although without statistical significance.

3.2.4. Effects on respiratory physiology

At the higher concentrations of MCh (15–45 mg/ml), all groups in both strains were hyperreactive to a similar degree. Differences in AHR between groups and strains were only possible to detect at the lowest concentration of MCh (5 mg/ml) (Fig. 7). The OVA sensitization and the aerosol challenge with OVA resulted in AHR in DA rats, as indicated by an increase of respiratory resistance (R_{RS}), tissue resistance (G) and tissue elastance (H) on day 11 when compared to the control group receiving PBS only. In BN rats similar differences were observed but without statistical significance (Fig. 7). The increase of R_{RS} in DA rats was accompanied by a decrease in respiratory compliance (C_{RS}). The TiO_2 exposures to the TiO_2/OVA-group further decreased C_{RS} in DA rats, compared to the OVA-group, while no change was observed for the other respiratory parameters. BN rats showed no changes in respiratory parameters following TiO_2 exposure.

3.3. TiO_2 NP exposure in naïve rats

3.3.1. General health effects

There were no visible signs of impaired general health condition or reduced body weight in either of the strains, neither on day 11 nor on day 81.

3.3.2. Inflammatory responses

Both rat strains showed an increase of neutrophils and lymphocytes in BALF, one day after the last exposure to TiO_2 NPs (day 11), when compared to the control group exposed to PBS only (Fig. 8A). DA rats showed an increase of macrophages in the BALF which was not observed in BN rats. There were no significant increase of eosinophils to BALF following TiO_2 NPs exposure

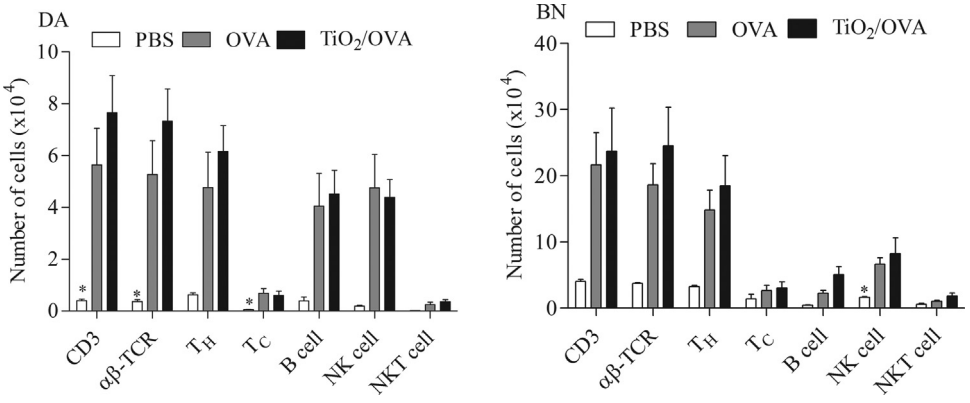

Fig. 5. Analysis of lymphocytes in BALF from sensitive rats on day 11. Analysis of sub-populations of lymphocytes in the BALF following exposure to TiO_2 NPs before and during the development of allergic airway inflammation. Data on cell numbers were derived from flow cytometry analysis and the results are shown from both DA rats and BN rats. Control groups with naïve individuals exposed to PBS (PBS-group) and sensitized groups exposed to TiO_2 NPs before and during the OVA challenge (TiO_2/OVA-group) were compared to the sensitized group with OVA challenge only (OVA-group). Differences within each strain were calculated. Data are expressed as means ± SEM ($^*p < 0.05$, all groups: $n = 8$).

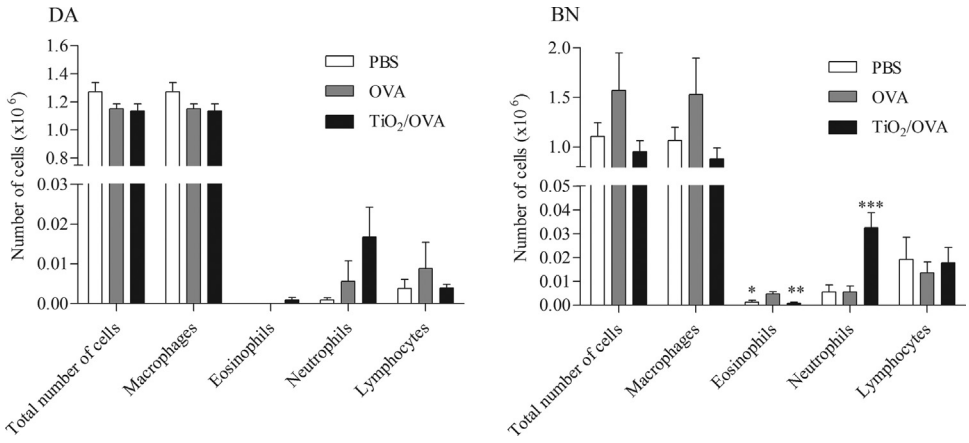

Fig. 6. Analysis of cells in BALF from sensitive rats on day 81, the late-phase response. Total number of cells and differential cell count in BALF from both DA rats and BN rats. Control groups with naïve individuals exposed to PBS (PBS-group) and sensitized groups exposed to TiO_2 nanoparticles before and during the OVA challenge (TiO_2/OVA-group) were compared to the sensitized group with OVA challenge only (OVA-group). Differences within each strain were calculated. Data are expressed as means ± SEM ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, all groups: $n = 6$).

(day 11) in either of the strains. In both strains, the expanded lymphocytes were predominately T helper cells (T_H) carrying the αβ T cell receptor (data not shown).

The day after the last TiO_2 NP exposure, both strains showed increased concentrations of IL-1α, IL-1β, CXCL1, CSF-1, CCL3, and TNF-α, in the BALF compared to the control group (Table 2). DA rats displayed additional increase of IL-6, IL-7, IL-12(p70), IL-13, IL-18, CSF3, CSF2, IFN-γ, CCL20, and VEGF.

On day 81, 70 days after the last TiO_2 exposure, the number of neutrophils was still elevated in the BALF of both strains (Fig. 8B). Flow cytometry analyses showed elevated numbers of lymphocytes in the TiO_2 groups from both strains compared to the PBS groups (DA, $p < 0.05$ and BN $p < 0.05$). The late-phase lymphocyte response was dominated by T_H cells carrying the αβ-TCR receptor. An increase of T cells, carrying the γδ-TCR receptor, and NK cells was also observed (data not shown). BN rats had increased levels of T_C cells and NKT cells which were not observed in DA rats.

3.3.3. Effects on respiratory physiology

As described previously for sensitized rats, the lowest dose of MCh (5 mg/ml) is presented since the sensitivity between groups was only possible to measure at this concentration. Inhalation of TiO_2 NPs in naïve DA rats resulted in increased R_{RS},G, and decreased C_{RS} when compared to rats exposed to PBS only (Fig. 9). In BN rats, TiO_2 NPs exposure did not change the respiratory parameters.

4. Discussion

Our study demonstrates that concomitant exposure to TiO_2 NPs and respiratory allergens in pre-sensitized individuals may potentiate neutrophil inflammation and lymphocyte responses. By comparing the response in two different inbreed rat strains we showed that the aggravated inflammation was influenced by genetic factors. The animals with inflamed airways and AHR represented sensitive populations, specifically individuals with

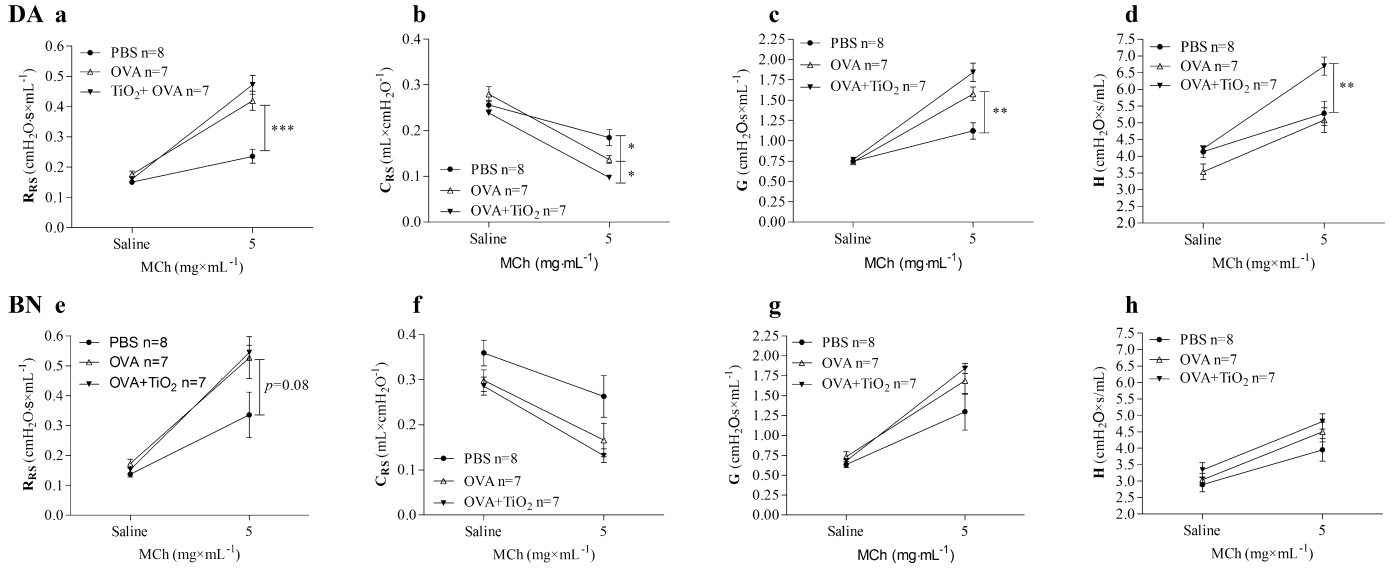

Fig. 7. Measurement of respiratory physiology in sensitive rats on day 11. Measurement of respiratory physiology following exposure to TiO_2 NPs before and during the development of allergic airway inflammation. Control groups with naïve individuals exposed to PBS (PBS-group) and sensitized groups exposed to TiO_2 NPs before and during the OVA challenge (TiO_2/OVA-group) were compared to the sensitized group with OVA challenge only (OVA-group). Measurements of MCh-induced respiratory resistance R_{RS} (a and e) and respiratory compliance C_{RS} (b and f) were performed with the single compartment model. Measurement of tissue resistance G (c and g) and tissue elastance H (d and h) were performed with the FOT (Zrs measurements, Prime-2). Differences within each strain were calculated. Data are expressed as means ± SEM ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, all groups: $n = 8$).

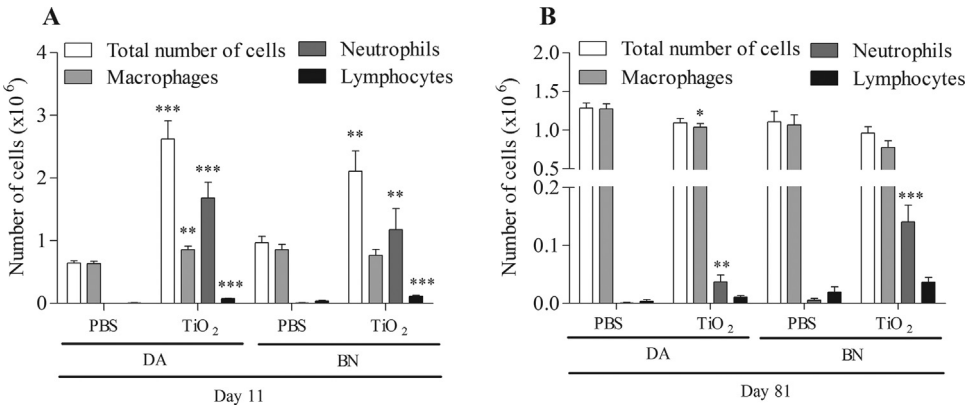

Fig. 8. Analysis of leukocytes in BALF from naïve rats on day 11 or 81. Total number of cells and differential cell count in BALF from both DA and BN rats on day 11 (A) and on day 81 (B). Control groups with naïve individuals exposed to PBS (PBS-group) were compared to the TiO_2 NPs group within each strain. Data are expressed as means ± SEM (**$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$, all groups: $n = 6–8$).

allergic asthma. The study was motivated by epidemiological reports pointing to severity of symptoms following particulate exposure in humans with pre-existing respiratory disease (Di Giampaolo et al., 2011; Gilliland, 2009; Holgate et al., 2003; Koike et al., 2008; Nordenhäll et al., 2001) and by the notion that mechanisms for the influence of particulate matter on asthma and the development of asthma are still unclear (Heidenfelder et al., 2009). In this context, particles in the nano-size range are of certain interest since it is suggested that nanoparticles have a higher deposition in the alveolar spaces of individuals with chronic inflammatory diseases, such as asthma and chronic obstructive pulmonary disease, compared to naïve individuals (Byrne and Baugh, 2008; Oberdörster et al., 2005).

The typical eosinophilic airway response occurring in sensitized rats following inhalation to respiratory allergens was found to be suppressed in both strains by TiO_2 NP exposure. The suppression of eosinophilic induction has previously been reported to occur in allergic BALB/c mice following TiO_2 exposure (Jonasson et al., 2013). The differential responses of eosinophils versus neutrophils and lymphocytes, observed in the DA rat, indicate that the cell reduction is not due to a general cytotoxic activity of the NPs. Another possibility is that TiO_2 NP exposure affects cytokine mediators that are specifically influencing eosinophilic recruitment to the lung. In our study we analyzed a panel of cytokines one day after the last OVA challenge, but we were not able to detect a decrease in T_H2 associated cytokines involved in the eosinophilic inflammatory responses. However, from our data on TiO_2 NPs exposure in naïve rats it is clear that NPs induce a T_H1 type of immune response. Such response might supress the T_H2 type immune activation, thereby reducing eosinophilic recruitment to the airways. The study by Jonasson et al. demonstrated that neutrophil inflammation was aggravated following TiO_2 exposure in allergic BALB/c mice. In our study a similar increase of airway neutrophils was identified in DA rats but not in BN rats. Another similarity between DA rats and BALB/c mice is the exacerbated decline in respiratory compliance. These

Table 2
Analysis of cytokines and chemokines in BALF from naïve rats on day 11. Concentrations of cytokines and chemokines (pg/ml) was analysed in the BALF one day after the last TiO_2 NPs exposure (day 11).

	DA		BN	
	PBS	TiO_2	PBS	TiO_2
IL-1α	32.2 ± 13	85.6 ± 16***	.3	42.6 ± 19*
IL-1β	33.7 ± 24	310.7 ± 81***	63.8 ± 48	146.2 ± 90*
IL-2	43.6 ± 16	54.9 ± 16	25.5 ± 12	26.4 ± 9
IL-4	25.1 ± 7	30.1 ± 9	16.9 ± 8	16.6 ± 8
IL-5	11.8 ± 7	13.7 ± 5	7.1 ± 4	8.4 ± 4
IL-6	14.0 ± 8	35.2 ± 8***	16.6 ± 17	13.7 ± 12
IL-7	38.9 ± 17	111.2 ± 17***	50.3 ± 27	59.0 ± 27
IL-10	32.1 ± 14	41.2 ± 15	20.6 ± 10	23.7 ± 5
IL-12(p70)	32.0 ± 18	69.4 ± 9***	19.6 ± 20	25.6 ± 14
IL-13	29.1 ± 15	70.6 ± 27**	12.8 ± 13	15.3 ± 13
IL-17	46.4 ± 20	50.7 ± 18	77.3 ± 54	54.6 ± 44
IL-18	45.1 ± 23	66.9 ± 10*	34.6 ± 12	47.6 ± 20
EPO	79.7 ± 42	59.6 ± 17	40.2 ± 13	58.3 ± 25
CSF3	17.0 ± 7	34.0 ± 6***	9.5 ± 5	12.6 ± 9
CSF2	19.4 ± 11	48.4 ± 13***	16.7 ± 14	18.5 ± 10
CXCL1	14267.5 ± 5405	24590.4 ± 400***	17267.9 ± 4722	23293.1 ± 2236**
IFN-γ	20.8 ± 10	32.6 ± 10*	16.7 ± 11	13.2 ± 10
CSF1	338.4 ± 91	1216.1 ± 373***	351.6±	921.6 ± 686*
CCL3	43.3 ± 35	488.8 ± 64***	38.6 ± 49	1243.3 ± 1104**
CCL20	7722.8 ± 3864	13149.5 ± 5302*	12297.4 ± 5375	9884.6 ± 5808
CCL5	24.8 ± 14	36.4 ± 10	10.2 ± 7	17.6 ± 10
TNF-α	38.0 ± 24	188.4 ± 39***	65.8 ± 34	132.9 ± 79*
VEGF	22429.6 ± 1343	23882.4 ± 682*	15288.2 ± 3970	15598.7 ± 2784

Statistical significance was calculated between groups exposed to TiO_2 and control groups (PBS) within each strain. Data are expressed as means ± SEM (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, all groups: $n = 8$).

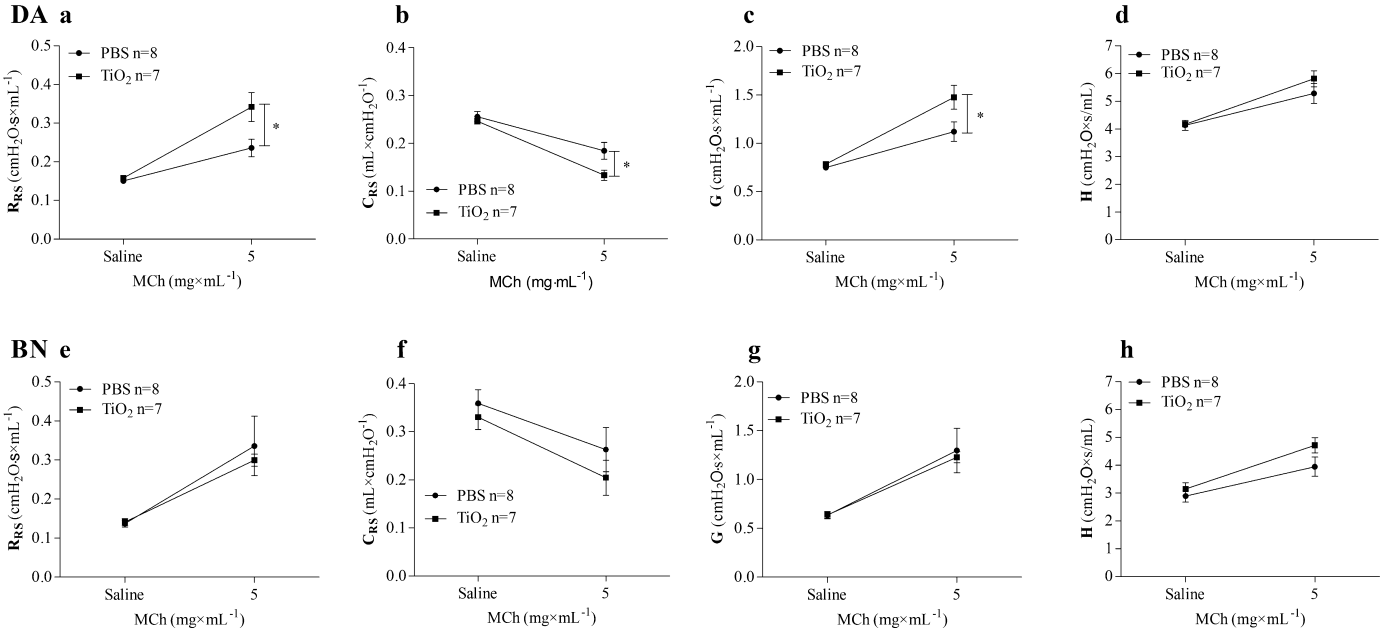

Fig. 9. Analysis of respiratory physiology in naïve rats on day 11. Analysis of respiratory physiology in naïve DA and BN rats exposed to repeated doses of TiO_2 NPs. Measurement of MCh-induced respiratory resistance R_{RS} (a and e) and respiratory compliance C_{RS} (b and f), were performed with the single compartment model. Measurement of tissue resistance G (c and g), and tissue elastance H (d and h) were performed with the FOT (Zrs measurements, Prime-2). Statistical significance was calculated between groups exposed for TiO_2 NPs and control groups (PBS) within each strain. Data are expressed as means ± SEM (*$p < 0.05$, all groups: $n = 8$).

comparisons of rats and mice indicate that genetic determinants strongly influence the nature of innate immune activation following particle exposure, and that the discrepancy of respiratory responses can be more pronounced within one species than between species. Inhalation exposure to TiO_2 NPs in pre-sensitized rats, before and during aerosol challenges to OVA allergen, did not cause any decline in general health condition in either of the two strains, as indicated by maintained increase in body weight and the absence of visible symptoms of adverse effects. This observation differs from the study by Jonasson et al., in which exposure to TiO_2 NPs before and during the OVA challenge leads to aggravated health effects in mice (Jonasson et al., 2013). This discrepancy illustrates that species differences may influence the outcome of the general symptoms of disease. Furthermore this study used a different TiO_2 NPs exposure protocol than Jonasson et al., in terms of dosing and timing of particle and allergen challenge, which also may have influenced the outcome.

By comparing two inbred rat strains, with different genetic susceptibility to inflammatory disorders, we were able to determine the genetic influence on the immune and respiratory responses in airways. We observed that sensitization of BN rats induced allergen-specific IgE and IgG responses, and that this strain developed a predominance of eosinophils in the airways after challenge with OVA. The sensitized DA rat developed an allergen-specific IgG response but no IgE antibodies, and following the OVA challenge a predominance of neutrophils was observed in the airways. Since B cells are the cells responsible for production of immunoglobulins, we hypothesized that the high levels of OVA-specific IgE antibodies in BN rats is due to relatively high numbers of B cells. When compared to the number of B cells in DA rats it was evident that this is not the case in either the BALF or in the lymph nodes (mediastinal). As an alternative hypothesis, DA rats may harbor a genetically determined bias towards a T_H1-type immune response that would counteract immunoglobulin switch to IgE, which would deviate from the suggested T_H2 bias immune response in BN rats. Such deviation of T_H-responses was not detected in the rats following the OVA-challenge. However, the data from naïve rats support a general strain divergence in T_H1/T_H2 regulation by the increased expression of the T_H1-marker IFN-γ in the BALF of DA rats following TiO_2 exposure; this was not observed in BN rats. Furthermore, our study highlights the genetic divergence regarding MCh-induced AHR, where DA rats were more reactive, both in central and peripheral airways, compared to BN rats. In addition to this genetically determined susceptibility, DA rats has previously been identified as being prone to developing chronic inflammatory diseases (Bäckdahl et al., 2014; Carlson et al., 2000), involving macrophage activation and neutrophil recruitment to injured tissues (Gustafsson et al., 2011, 2014; Zhang et al., 2011), while BN rats are susceptible to developing a prominent humoral immune response with IgE production and eosinophil inflammation (Granum and Løvik, 2002; Martin and Tamaoka, 2006). These results indicate that these strains are useful for further investigations of genetic influence of asthma pathology due to their different responses in terms of immune activation, AHR, and inflammatory profile.

We exposed rats to TiO_2 NPs through inhalation of aerosol droplets with particle characteristics implying that the largest fraction of TiO_2 remained in the alveolar region followed by the nasal cavity in the head and the trachea bronchiolar regions. The fraction of the deposited particles in the conducting airways is normally cleared by mucociliary escalator up to the mouth and then swallowed (Geiser and Kreyling, 2010; Jud et al., 2013; Kreyling et al., 2006; Schmid et al., 2009). The particles move by diffusion and therefore efficiently deposit in the entire respiratory tract, and the lower they deposit, the longer they will be retained in the respiratory system (Geiser and Kreyling, 2010). We have previously reported that TiO_2 NPs can be retained in the alveolar macrophages and lower respiratory epithelium up to 90 days after lung exposure (Gustafsson et al., 2011).

An inflammatory response developed in the airways of the naïve rats following repeated aerosol exposure to TiO_2 NPs. A prominent airway neutrophilia along with a lymphocyte response was detected one day after the last exposure. Among the lymphocytes recovered from airways, T_H cells expressing the αβ

T cell receptor dominated. Other lymphocyte subsets, such as T_C cells, B cells, and NK cells, were detected but found at considerably lower numbers than the T_H cells. The neutrophils were sustained in the airways for at least 70 days, indicating that the particles continued to trigger an inflammatory response for an extended period of time. These results are in agreement with previous studies performed in mice (Park et al., 2009; Roursgaard et al., 2011) and rats (Gustafsson et al., 2011; Kobayashi et al., 2009; Sager and Castranova, 2009). The influx of inflammatory cells to the airways was accompanied by increased expression of pro-inflammatory cytokines (TNF-α, IL-1α, IL-1β) and chemokines (CSF1, CCL3, CXCL1) in the BALF of both strains. DA rats had a more pronounced expression of the pro-inflammatory cytokines IL-1α, IL-1β, and TNF-α. Increase of IL-6, IL-7, IL-12, IL-13, IL-18, CSF2, CSF3, CCL20, IFN-γ and VEGF was only detected in DA rats. These results indicate a strain difference in the magnitude of pro-inflammatory responses to particulate exposure. The increase of TNF-α, IL-1β, IL-6, IL-12, IFN-γ and CCL3, indicate that the DA rat has a stronger macrophage population with M1 (classical) phenotype in BALF (Martinez and Gordon, 2014). Some of those mediators were also found to be present in the BN rat, although in lower levels, indicating a weaker M1 response. The M1 phenotype is known to favor a T_H1 response, whereas a M2 (alternative) phenotype favors a T_H2 cell response (Sica and Mantovani, 2012). Our data on M1 predominance in the DA rats and the previously reported arthritis susceptibility of this strain (Carlson et al., 2000; Griffiths et al., 1981) is consistent with a linkage of the M1 subpopulation to arthritis (Sica and Mantovani, 2012). The activation towards a M1 phenotype is reported to be induced by IFN-γ and toll receptor (TLR4) (Sica and Mantovani, 2012). Recently, Chen et al. showed that TiO_2 NPs exposure *in vitro*, resulted in promotion of inflammatory response through the TLR4 receptor (Chen et al., 2013), supporting a mechanism by which TiO_2 NPs induce a M1-type of macrophage response through TLR4 engagement. The presence of TNF-α and CSF2 stimulates T_H1 cell activation (Hubbell et al., 2009) and the increase of IFN-γ indicates a T cell shift towards a T_H1 immune deviation, which was further favored by the induction of IL-12 and IL-18. These two cytokines are mainly produced by macrophages and dendritic cells: the main cellular stimulators of cell mediated immunity (Commins et al., 2010; Feldman et al., 2001). After secretion, these cytokines play important roles in the T_H1 response by promoting expression of IFNγ and cytotoxic activity by T cells, as well as by suppressing T_H2-dependent IgE production. Our finding of increased levels of CCL20 may also support involvement of cell mediated immune responses since CCL20 is a strong chemo-attractant for recruitment of immature dendritic cells to the airway epithelium and underlying mucosa (Holgate, 2012). In a recently published study it was reported that innate immune activation can lead to excessive IL-7 expression in the lungs, which in turn potentiate recruitment of T_H1-biased IFN-γ- producing T cells (Jin et al., 2013; Jin and Yu, 2013). Our data on increased IL-7 expression in the BALF of DA rats exposed to TiO_2 NPs provide additional support for a strong bias towards T_H1 immune responses in this rat strain. Such a shift towards a T_H1 immune response is also reported to occur in DA rats exposed to triggers for inflammatory joint disease (Mussener et al., 1997). It is likely that the predisposition to induce T_H1 immune responses is a generic feature of DA rats when responding to immunoactivating challenges in the environment. The mechanisms by which TiO_2 NPs trigger the adaptive immune response is not defined, as these particles themselves have not been shown to function as an immunogen or as a carrier for immunogenic haptens (Di Gioacchino et al., 2011). It is, though, well described that particles interacting with components of the respiratory lining fluid and blood will be coated with a film of endogenous biomolecules forming a corona, and that the nature of this coating is highly dependent on the physicochemical properties of the particle (Fadeel, 2012; Lundqvist et al., 2008; Monopoli et al., 2012; Nel et al., 2009). Formation of such corona may induce conformational changes of adsorbed proteins leading to loss of function, but may also result in the presentation of novel epitopes to the immune system. Hypothetically, such interplay between particles and components of the body fluids may lead to immune reactivity to self-epitopes resulting in autoimmune reactions.

5. Conclusion

In this study, we examined whether rats with airway inflammation and AHR develop aggravated symptoms of disease when exposed to TiO_2 NPs. The study was motivated by epidemiological reports pointing to the severity of symptoms following particulate exposure in humans with pre-existing respiratory disease, and by the lack of understanding of the influence of nano-sized particulate matter on allergic asthma. We demonstrated that concomitant exposure to TiO_2 NPs and respiratory allergens in pre-sensitized individuals may potentiate neutrophil inflammation and lymphocyte responses, whereas eosinophilic responses in airways were found to be suppressed. This response was, however, strain-dependent, indicating that genetic determinants influencing the susceptibility of an individual to develop a certain type of an inflammatory response play a major role. We identified DA rats, harboring an inherent susceptibility to chronic inflammatory disorders, to be a high responder, while BN rats, susceptible to atopic allergic eosinophil inflammation, were found to be relatively resistant to TiO_2 NPs inhalation exposure. The impact of genetically determined factors influencing the inflammatory reactions pinpoints the complexity of assessing health risks associated with nanoparticle exposures.

Conflict of interest

There is no conflict of interests of any of the co-authors.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgements

The authors would like to thank Lina Ågren for performing the ELISA analyses and contributing with data analysis. To Christine Akfur, Elisabeth Wigenstam, and Barbro Ekstrand-Hammarström, for contributing on the days of analysis. To Bo Koch for technical assistance in the measurements of rat lung physiology. To Christian Lejon, Ola Nerbrink, and Kristoffer Svensson for their work on characterizing the aerosol. To Sofia Eriksson for proof reading. This study was financially supported by the Swedish Research Councils Formas, FAS and Umeå Center for Environmental Research, and by the Swedish Ministry of Defence.

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