

RESEARCH ARTICLE

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

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Abstract

Context: Titanium dioxide (TiO₂) nanoparticles (NPs) are regarded as relatively non-toxic in concentrations occurring in occupational environments. Nevertheless, it is conceivable that adverse health effects may develop in sensitive populations such as individuals with respiratory diseases.

Objective: We investigated whether single or repeated exposure to TiO₂ could aggravate inflammatory responses in naïve mice and mice with ovalbumin (OVA)-induced airway inflammation.

Methods: Exposure to aerosolized TiO₂ was performed during OVA sensitization, before, or during the OVA challenge period. The effects on respiratory physiology, inflammatory cells in bronchoalveolar lavage (BAL) and inflammatory mediators in BAL and serum were assessed 24 h after the last OVA challenge or TiO₂ exposure.

Results: A single exposure of TiO₂ had a marked effect on responses in peripheral airways and increasing infiltration of neutrophils in airways of naïve animals. Marked aggravation of airway responses was also observed in animals with allergic disease provided that the single dose TiO₂ was given before allergen challenge. Repeated exposures to TiO₂ during sensitization diminished the OVA-induced airway eosinophilia and airway hyperresponsiveness but concomitant exposure to TiO₂ during the OVA challenge period resulted in neutrophilic airway inflammation and a decline in general health condition as indicated by the loss of body weight.

Conclusion: We conclude that inhalation of TiO₂ may aggravate respiratory diseases and that the adverse health effects are highly dependent on dose and timing of exposure. Our data imply that inhalation of NPs may increase the risk for individuals with allergic airway disease to develop symptoms of severe asthma.

Keywords

Asthma, inflammation, nanomaterial, respiratory mechanics, titanium dioxide

History

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Introduction

Despite the increased use of nanomaterials for various applications over the past years, the potential harmful effects on various pulmonary diseases when dispersed into the environment are not well characterized. Nanosized titanium dioxide (TiO₂) is commonly used in the industry due to its photocatalytic properties and is produced in increasing amounts for energy and environmental applications, as well as use as pigment, in cosmetic manufacturing and medical applications. In contrast to other materials, e.g. carbon nanotubes, carbon black and silica that may cause toxicity by the induction of inflammation and/or fibrosis in the lung (Card et al., 2008; Inoue et al., 2006, 2009), TiO₂ nanoparticles (NPs) have relatively low toxicity and have even been used as a negative reference material for toxicity

testing of nanosized compounds (Madl & Pinkerton, 2009). However, it is reported that TiO₂ NPs can cause negative health effects (Granum et al., 2001; Larsen et al., 2010; Trouiller et al., 2009) through oxidative damage and pulmonary inflammation which in worst case may lead to lung emphysema (Chen et al., 2006, 2011; Johnston et al., 2009; Park et al., 2009). It is previously shown that single or repeated exposure of TiO₂ NPs may provoke airway inflammation in healthy animals (Bermudez et al., 2002; Johnston et al., 2009; Lindberg et al., 2012; Park et al., 2009; Warheit et al., 2007), but also that NP exposure can aggravate inflammatory responses in experimental models of ovalbumin (OVA)-induced allergic disease (Han et al., 2011; Johnston et al., 2009), toluene-2,4-diisocyanate (TDI)-induced asthma (Hussain et al., 2011), pulmonary mucus hypersecretion (Chen et al., 2011) and sepsis (Shin et al., 2010).

Growing evidence indicates that the adverse health effects of TiO₂ NPs are highly dependent on the crystal structure and size of the particles (de Haar et al., 2006; Kobayashi et al., 2009; Warheit & Frame, 2006; Warheit et al., 1997, 2007) and risk assessment of TiO₂ involves a number of other

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considerations such as dose, duration of exposure and route of administration (Johnston et al., 2009).

In a recent rat study (Gustafsson et al., 2011), we demonstrated that lung exposure of a single high-dose of TiO₂ NPs induced an innate immune activation in the airways and a long-lasting lymphocyte response implicating risks for adverse and persistent immune stimulation in individuals susceptible for immune-mediated airway diseases. Based on these results, we wanted to investigate the potential detrimental effects of TiO₂ NPs in individuals with an ongoing inflammatory disease in the airways. With the aim to investigate how a sensitive population would respond to an NP challenge, we evaluated the impact of TiO₂ exposures on allergic airway inflammation provoked by repeated inhalation of aerosolized OVA in pre-sensitized mice. We studied the interactions between NPs and allergen exposure using three different TiO₂ NP exposure settings: (1) TiO₂ exposure during the sensitization, (2) TiO₂ exposure before OVA challenge in sensitized mice and (3) TiO₂ exposure during OVA-challenge period in sensitized mice. TiO₂ exposure was also carried out in healthy animals without OVA-induced airway inflammation. A small animal ventilator and a forced oscillation technique (FOT) (FlexiVent™, SCIREQ) was used to evaluate respiratory mechanics and airway hyperresponsiveness (AHR), together with the analysis of multiple parameters of immune activation and inflammation both locally in the lung and systemically. The murine asthma model presented in this study resembles in many ways human asthma with increased airway reactivity, eosinophilia and a T_H2-mediated inflammatory response. By this approach, we have been able to address whether timing and repeated exposures of TiO₂ NPs influence multiple end-points associated with allergic airway disease.

Methods

Animals

Female BALB/c mice ($n=6-10$, 8 weeks old, Harlan laboratories, the Netherlands) were used in this study. Animals were housed in plastic cages with absorbent bedding material and were maintained on a 12 h daylight cycle. Food and water were provided *ad libitum*. Their care and the experimental protocols were approved by the regional ethics committee on animal experiments in Umeå, Sweden.

Aerosol exposure, particle characterization and lung deposition of TiO₂

Nano-sized TiO₂ (80% anatase, 20% rutile) particles (aeroxide P25, 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, St. Louis, MO). Animals were placed in individual nose-only containers (EMMS, Hampshire, UK) and coupled to an exposure system consisting of a Battelle nose-only exposure tower fitted with a six-jet Collison nebulizer (Waltham, MA) operated during all aerosol exposures at a flow rate of 7.8 ± 0.2 l/min. The Battelle exposure tower provided an equal and simultaneous exposure to the TiO₂ aerosol.

The droplet size measurement of the aerosol exiting the nebulizer was determined using a Malvern Mastersizer X (Malvern Instruments, Malvern, UK) and was expressed as volumetric mean diameter (μm) measured 0.5 cm from the laser beam and 3 cm from the lens (water in air presentation model).

In order to determine the particle aggregation and agglomeration in the solution, the aerosol was collected at an exit port of the Battelle chamber using a 1 l/min air flow glass impinger filled with 20 ml PBS solution. The particle hydrodynamic size distribution in the collected solution was measured with a photon cross correlation spectroscopy ((PCCS), Nanophox, Sympatec, Clausthal-Zellerfeld, Germany).

The deposited amount of TiO₂ in the lung measured after one single exposure and after eight repeated exposures was determined using a method performed by ALS Scandinavia AB (Luleå, Sweden). In short, the exposed lungs were digested in HNO₃ and HF and samples were then diluted in MilliQ-H₂O before analyzing with ICP-SFMS for the detection of titanium.

Experimental protocols

OVA-induced airway inflammation

The 35-day-long airway inflammation protocol (Figure 1) started with the intraperitoneal (i.p.) injection of 10 μg OVA (Sigma-Aldrich, St. Louis, MO) emulsified in Al(OH)₃ (Alu-Gel-S suspension 1.3%, research grade sterile, cat no: 12261, SERVA electrophoresis, Thermo Fisher Scientific Inc., Waltham, MA) on days 0 and 14. The mice were then challenged with aerosolized 1% OVA (OVA diluted in H₂O) nose-only for 30 min on days 29, 32, and 34. The experiment ended on day 35. Control mice did not receive any OVA i.p. (sensitization) but were challenged with aerosolized OVA using the same protocol as for the OVA-treated animals described above.

Single or repeated TiO₂ exposure

Single exposure. Mice without airway inflammation (unsensitized + OVA challenged) were subjected simultaneously to TiO₂ and compared to unexposed mice (Figure 1a, Table 1). Another group of mice with airway inflammation (OVA sensitized + OVA challenged) was subjected to a single exposure of aerosolized TiO₂ on day 28 or 33 during the 35-day OVA protocol and compared to the unexposed OVA animals (Figure 1b, Table 1).

Repeated exposures. Mice (naïve) were exposed to repeated exposure to TiO₂ (TiO₂ repeated) and compared to unexposed mice (naïve) (Figure 1c, Table 1). Mice with airway inflammation (OVA sensitized + OVA challenged) were subjected to aerosolized TiO₂ during sensitization (Group 1; exposed on days 4–8 and 11–13), before OVA-challenge (Group 2; exposed on days 19–23 and 26–28) and during the period of OVA-challenge (Group 3; exposed on days 25–29, 32–34) and compared to a group of unexposed animals with OVA-induced airway inflammation (OVA group) (Figure 1d, Table 1). A group of unsensitized mice was not exposed to TiO₂ (OVA control group, Table 1).

Figure 1. Schematic presentation of the mouse model of asthma, 35-day long protocol, with and without exposure to TiO₂. Assessment of respiratory mechanics, bronchoalveolar lavage (BAL) and serum were performed 24 h after last challenge or exposure (day of analysis). Single exposure of TiO₂ was carried out on day 28 or 33 in mice (a) without airway inflammation and (b) with airway inflammation. (c) Eight repeated exposures to TiO₂ were performed in naïve mice during 10 days and (d) repeated exposure of TiO₂ was also carried out in animals with airway inflammation using three different protocols: Group 1(Gr. 1); exposed on days 4–8 and 11–13, Group 2 (Gr. 2); exposed on days 19–23 and 26–28 and Group 3 (Gr. 3); exposed on days 25–29, 32–34.

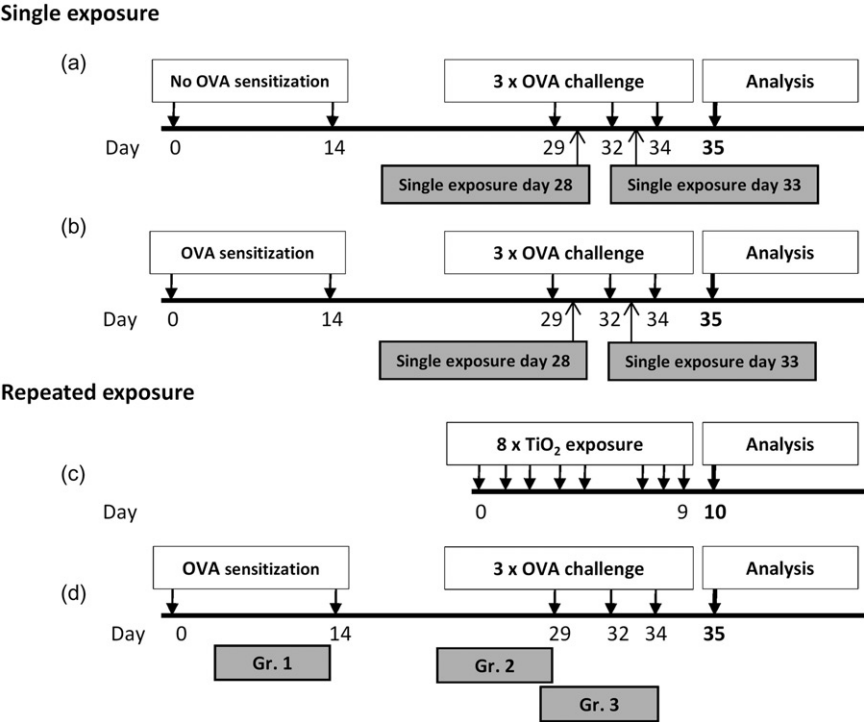


Table 1. Groups and protocol.

Group name	OVA sensitization	OVA challenge	TiO ₂ exposure
Single exposure			
Control	No	Yes	No
TiO ₂ day 28	No	Yes	day 28
TiO ₂ day 33	No	Yes	day 33
OVA	Yes	Yes	No
OVA day 28	Yes	Yes	day 28
OVA day 33	Yes	Yes	day 33
Repeated exposure			
Naïve	No	No	No
Naïve + TiO ₂	No	No	Yes
OVA control	No	Yes	No
OVA	Yes	Yes	No
OVA Gr. 1	Yes	Yes	Yes
OVA Gr. 2	Yes	Yes	Yes
OVA Gr. 3	Yes	Yes	Yes

OVA-specific antibodies in serum

After respiratory mechanics, blood was sampled through an orbital sinus venipuncture technique immediately after the mice had been sacrificed. The blood was centrifuged and serum was stored at –80 °C until analyses. Both OVA-specific immunoglobulin E (IgE) and immunoglobulin G (IgG₁) were analyzed using enzyme-linked immunosorbent assay (ELISA) to confirm the OVA sensitization.

For the measurements of OVA-specific IgE, 96-well plates were coated with polyclonal anti-mouse IgE (rat-anti mouse IgE, PharMingen, clone R35-72, BD Biosciences, Stockholm, Sweden) at a concentration of 16 µg/ml in coating buffer. After incubation overnight at 4 °C, the plates were washed with washing buffer (PBS + Tween20) and thereafter blocked with blocking buffer (1% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO) in PBS + 0.05% Tween20)

during 1 h in room temperature (RT). After blocking buffer, the plates were washed with washing buffer. Serum samples were diluted 1:4 in blocking buffer and added to the plates in duplicate. Standards were added to the plates (Mouse anti-OVA [2C6] antibody, GeneTex (MediQip), Irvine, CA), also in duplicate. The readings were referenced to this standard (ng/ml). The plate was incubated for 2.5 h at RT on a rocking platform and thereafter washed (five times) with washing buffer. After washing, OVA-biotin 12 µg/ml solved in blocking buffer was added and the plates were incubated 1 h on a rocking platform in RT. After incubation, the plates were washed with washing buffer. Streptavidin-POD-conjugate (500U, Roche) diluted 1:1000 in blocking buffer was added to the plates and the plates were incubated for 0.5 h on a rocking platform in the dark in RT. The plates were washed with washing buffer. Please see below for further instructions.

For OVA-specific IgG₁ measurement, the plates were coated with OVA at a concentration of 50 µg/ml in NaHCO₃ buffer (pH 9.6). After overnight incubation at 4 °C, the plates were washed with washing buffer and thereafter blocked with blocking buffer (1% BSA in PBS + 0.05% Tween20) during 1 h in RT. The serum samples were diluted 1:10 000 in blocking buffer and added to the plates in duplicate. Standards were added to the plates (Mouse IgG₁ anti-OVA antibody [2D11], ab17290, Abcam, Cambridge, MA), also in duplicate. The readings were referenced to this standard (ng/ml). The plate was incubated for 2 h at RT on a rocking platform and thereafter washed with washing buffer. After washing, goat-anti-mouse IgG₁ (H/L) HRP-conjugated, 10 µg/ml (0300-0108P, AbD, Serotec, Oxford, UK) was added to the plates and the plates were incubated 1 h on a rocking platform in RT in aluminum foil and thereafter washed with washing buffer.

To both IgE and IgG₁ ELISA plates, TMB (3',3',5'-tetramethylbenzidine liquid substrate system, Sigma Aldrich,

St. Louis, MO) was added on a rocking table from 1 to 60 min and covered with aluminum foil. The reaction was stopped with 2 M H₂SO₄. The plates were then read using an ELISA reader (Thermo Scientific Multiskan FC, Thermo Fischer Scientific Oy, Vantaa, Finland) at 450 nm. The analysis of ELISA data was performed with the software program for the ELISA reader (SkanIt for Multiskan FC 3.1. Ink), referring to the standards that were added to each plate.

Measurement of respiratory mechanics

On the day of assessment of respiratory mechanics, the animals were weighed and anesthetized with pentobarbital sodium (90 mg/kg, i.p., purchased from local suppliers). The mice were tracheostomized with an 18-gauge cannula and mechanically ventilated in a quasi-sinusoidal fashion with a small animal ventilator (flexiVent™, SCIREQ) at a frequency of 3 Hz and a tidal volume (V_T) of 12 ml/kg body weight. The mice were paralyzed with pancuronium (0.1 mg/kg, i.p., purchased from local suppliers). This made strict respiratory mechanics measurement possible. A positive end-expiratory pressure of 3 cmH₂O was applied. A warming pad prevented cooling of the animal. Four sigh maneuvers at $3 \times V_T$ were performed at the beginning of the experiment to establish stable baseline respiratory mechanics and to ensure a similar volume history before the experiments. The mice were then allowed a 2-min resting period before the experiment began. Dynamic respiratory mechanics were measured by applying a sinusoidal standardized breath 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}) (Irvin & Bates, 2003). More thorough evaluations of respiratory mechanics of the peripheral components were made using FOT (Gomes et al., 2000; Hantos et al., 1992; Jonasson et al., 2009a). During the forced oscillatory maneuver, the ventilator piston delivers 13 superimposed sinusoidal frequencies, ranging from 1.0 to 20.5 Hz, during 2 s (Quick-Prime 2, impedance (Zrs)). The primary parameters obtained are tissue resistance (G) and tissue elastance (H) (Bates & Irvin, 2003; Irvin & Bates, 2003). All parameters were measured continuously using a standardized script and the maximum response to a given concentration of methacholine (MCh, acetyl- β -methylcholine chloride, Sigma-Aldrich, St. Louis, MO) is reported. MCh was diluted in saline and a volume of 20 μ l was given over 10 s with an aerosol with a volume diameter between 4 and 6 μ m (Aeroneb™, SCIREQ, Montreal, Canada). Incremental doses of MCh (25–70 mg/ml) were given at 5-min intervals and each solution was aerosolized without any interference with the ventilation pattern. Between each dose of saline and MCh, R_{RS} was allowed to return to the baseline level before the next MCh dose was administered.

Inflammatory cells in bronchoalveolar lavage

The lungs were lavaged four times via the tracheal tube with a total volume of 1 ml + 3×1 ml Ca²⁺/Mg²⁺-free Hanks' balanced salt solution (HBSS, Sigma-Aldrich, München, Germany). The BAL fluid was centrifuged (10 min, 4°C, 1500 rpm). After removing the supernatant, the cell pellet was

resuspended and then diluted with 1 ml PBS. The first 1 ml of supernatant was saved for further analyses. Leukocytes were counted manually in a hemocytometer so that 30 000 cells could be loaded onto cytospin slides and centrifuged using a Cytospin® centrifuge (Shandon® cytospin 3 cyto-centrifuge, Runcorn, UK). Cyto-centrifuged preparations were stained with May–Grünwald-Giemsa reagent and differential cell counts of pulmonary inflammatory cells (macrophages, neutrophils, lymphocytes, and eosinophils) were made in duplicates using standard morphological criteria and counting 300 cells per cytospin preparation. BAL samples were also used for FACS analyses.

Inflammatory mediators in bronchoalveolar lavage and serum

Inflammatory mediators in BAL and serum were analyzed for the presence of interleukin (IL)1A, IL1B, IL2, IL3, IL4, IL5, IL6, IL9, IL10, IL12B, IL12A, IL13, IL17, CSF3, CSF2, IFNG, CXCL1, CCL2, CCL3, CCL4, CCL11, CCL5 and TNFA. All cytokine analyses were performed with a multiplex kit (Bio-Plex™ Pro Mouse Cytokine 23-plex panel) according to the manufacturer's instructions (Bio-Rad, Hercules, CA) and analyzed on a Bio-Plex™ system (Luminex Bio-Plex™ 200 System (Bio-Rad, Hercules, CA)). Inflammatory mediators were analyzed in BAL from sensitized mice exposed to a single exposure to TiO₂, as well as in BAL and serum from both naïve mice and OVA-challenged animals repeatedly exposed to TiO₂.

Flow cytometry analysis of cells from bronchoalveolar lavage and lung tissue

Leukocytes from BAL and lung homogenates were analyzed with flow cytometry using a BD FACSort™ (Becton Dickinson, San Jose, CA). Cells from BAL were resuspended in PBS as described above. The whole lung was minced with scissors and the cell suspension was extracted by enzymatic digestion. Briefly, the lung pieces were digested in HBSS containing 1 mg/ml collagenase XI (Sigma) and 10% fetal calf serum (FCS, Hyclone, Perbio Science, Aalst, Belgium) at a temperature of 37°C for 0.5 h. The lung was thereafter gently meshed through a 100 μ m cell strainer. The cells were lysed in RBC lysis buffer (Red blood Cell lysis buffer); containing 8.26 g NH₄Cl, 1 g KHCO₃ and 0.037 g EDTA per liter ddH₂O and thereafter centrifuged and resuspended in PBS and stored on ice until antibody staining. Antibody staining was performed in 96-well plates with 2.0×10^5 cells/sample (note: BAL specimens containing a lower number of cells were pooled). Cells were pre-incubated with FcR blocking Ab (anti-CD16/CD32; clone 2.4G2) to reduce nonspecific binding. The mAbs used to identify subtypes of T cells were: CD3-FITC (clone 17A2), CD4-PE (clone H129.19), and CD8a-PE-Cy5 (clone 53-6.7). For NK-cell identification NK1.1-PE (clone PK136) was used. Isotype-matched antibodies were used as a negative control. Flow cytometry was performed using a BD FACSort™ (Becton Dickinson, San Jose, CA) according to standard procedure and analyzed with BD FACSDiva Software. All antibodies were originated from BD Sciences Pharmingen (San Diego, CA) except for NK1.1

which came from Serotec (Oxford, UK). T cells were defined as CD3⁺ and NK cells as CD3⁻ NK1.1⁺.

Analysis of fibrinogen in serum

Fibrinogen analyses were performed in OVA-sensitized mice that had been exposed for repeated exposures of TiO₂, using ELISA. The analysis was performed using a kit for murine fibrinogen (MFG-EIA, Enzyme Research Laboratories, Swansea, UK) according to the manufacturer's instructions (Enzyme Research Laboratories, Swansea, UK) and analyzed using an ELISA reader (Thermo Scientific Multiskan FC, Thermo Fischer Scientific Oy, Vantaa, Finland). Analysis of ELISA data was performed using the software program for the ELISA reader (SkanIt for Multiskan FC 3.1. Inc., Thermo Fischer Scientific Oy, Vantaa, Finland).

Statistical analysis

The results are presented as the mean $\pm$ standard error of mean (SEM). Statistical significance was assessed by parametric methods using a two-way analysis of variance (ANOVA) to determine differences between groups, followed by a Bonferroni post hoc test. When appropriate, a one-way ANOVA followed by a Dunnett's post hoc test or Student's unpaired t-test was used. A statistical result with $p < 0.05$ was considered as significant. The statistical analyses were carried out and graphs were prepared with GraphPad Prism program (version 4.03 GraphPad software Inc., San Diego, CA).

Results

Particle characterization and deposition in the lung

The droplet size of the aerosol was determined to $2.60 \pm 0.01 \mu\text{m}$ ($n = 8$). The hydrodynamic diameter of the TiO₂ particles in largest fraction (85% of total particles) within the droplet was $1.52 \pm 0.16 \mu\text{m}$ ($n = 5$). The other 15% of the total particles had a diameter between 100 and 200 nm. The total lung deposition of TiO₂ was calculated to $32 \pm 1 \mu\text{g}$ TiO₂/lung after one single exposure and $193 \pm 8 \mu\text{g}$ TiO₂/lung after eight repeated exposures ($n = 5$).

Animal status

All animals were weighed on the experiment day. Animals in Group 3 had lower final weight ($16.2 \pm 0.5 \text{ g}$) than OVA control mice ($20.2 \pm 0.5 \text{ g}$, $p < 0.001$). Mice in other groups did not have any significant weight change compared to unexposed mice (Table 2). Together with body-weight loss, mice in Group 3 showed signs of lethargy and ruffled fur indicating a decline in general health condition.

OVA-specific antibodies in serum

OVA-specific IgE and IgG₁ levels in serum were increased in mice sensitized and challenged with OVA when compared to non-sensitized mice (Table 3). TiO₂ exposure before or during OVA-challenge period did not further affect the levels of OVA-specific antibodies (Groups 2 and 3). TiO₂ exposure during the sensitization

Table 2. Mouse mean weight (g).

Naïve	20.0 $\pm$ 0.5
Naïve + TiO ₂ repeated	19.1 $\pm$ 0.4
OVA control	20.2 $\pm$ 0.4
OVA	20.1 $\pm$ 0.4
Gr. 1	19.4 $\pm$ 0.6
Gr. 2	19.1 $\pm$ 0.3
Gr. 3	16.2 $\pm$ 0.5***

All animals were weighed on the experiment day. Values indicate means $\pm$ SEM, *** $p < 0.001$ versus OVA alone.

Table 3. The optical density values for specific OVA IgE and IgG₁.

	OVA IgE	OVA IgG ₁
OVA control	0.0 $\pm$ 0.0***	0.06 $\pm$ 0.00***
OVA	1.35 $\pm$ 0.10	0.75 $\pm$ 0.05
Gr. 1	0.16 $\pm$ 0.04***	0.34 $\pm$ 0.04***
Gr. 2	1.14 $\pm$ 0.04	0.81 $\pm$ 0.03
Gr. 3	1.05 $\pm$ 0.12	0.71 $\pm$ 0.04

Values indicate means $\pm$ SEM, *** $p < 0.001$ versus OVA alone.

phase (Group 1) inhibited the antibody response resulting in a significant decrease of both IgE and IgG₁ levels ($p < 0.001$) compared to mice sensitized and challenged with OVA (OVA group).

Single exposures

Respiratory mechanics

A single exposure to TiO₂ alone did not have any effect on the central (R_{RS} and C_{RS}) or peripheral (G and H) airway compartments compared to unexposed control mice. Animals sensitized with OVA had significantly higher R_{RS}, G and H and lower C_{RS} than unsensitized controls (data not shown). A single exposure of TiO₂ on day 28 affected the peripheral airways compared to unexposed OVA mice as indicated by further decreased C_{RS}, and increased G and H (Figure 2). Mice receiving TiO₂ on day 33 did not display any change in respiratory mechanics compared to unexposed OVA mice (data not shown).

Inflammatory cells in BAL

Unsensitized mice exposed to TiO₂ at day 33 only developed an airway inflammation dominated by macrophages and neutrophils two days post exposure (Figure 3a). After seven days post exposure (day 28) there was no effect on the airway inflammatory cells when BAL cells were analyzed.

Animals sensitized with OVA showed a markedly increased total BAL cell count compared to unsensitized mice (Figure 3b) after three OVA-challenges. In OVA-sensitized mice, differential cell counts of BAL confirmed an inflammatory profile with an increased number of eosinophils. OVA-sensitized mice subjected to a single exposure of TiO₂ on day 28 or 33 developed airway inflammation with an increased total inflammatory cell count compared to unexposed OVA animals. Neutrophils, macrophages and eosinophils were all significantly increased

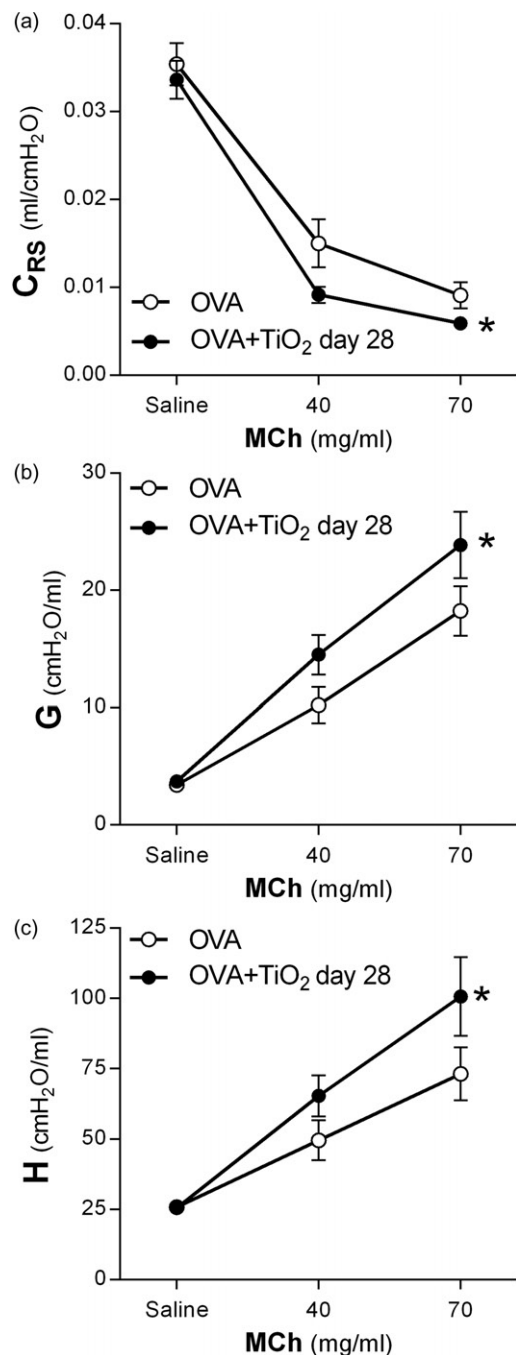


Figure 2. Analysis of respiratory mechanics in mice with OVA induced airway inflammation following a single inhalation exposure to TiO₂ on day 28. Data show measurements of methacholine (MCh)-induced (a) respiratory compliance; C_{RS}, (b) tissue resistance; G and (c) tissue elastance; H. There was no observed effect on respiratory function after TiO₂ exposure on day 33 (data not shown). Values indicate means $\pm$ SEM, * p < 0.05 versus unexposed OVA-sensitized mice.

after TiO₂ exposure, on both days 28 and 33, respectively compared to unexposed OVA animals. TiO₂ exposure on day 33 also induced an increased number of lymphocytes in BAL compared to unexposed allergic animals. Unsensitized and unexposed control mice showed no airway inflammation.

Inflammatory mediators in BAL and serum

OVA exposure alone in sensitized animals significantly induced all inflammatory cytokines tested in BAL compared

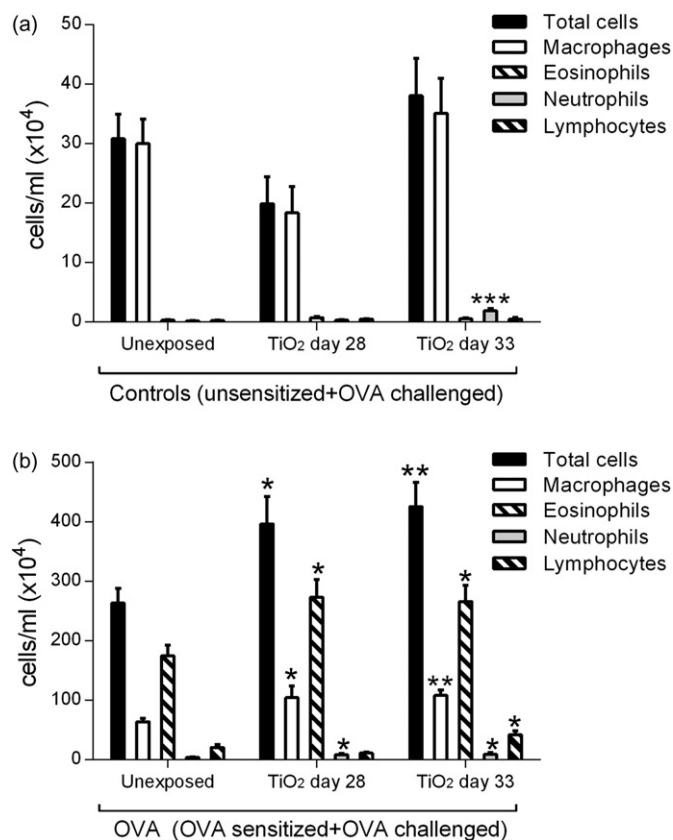


Figure 3. Analysis of inflammatory cells in BAL fluid following single exposures to TiO₂. Data show total and differential cell counts in BALF from (a) control animals (unsensitized and OVA-challenged) and (b) mice with OVA induced airway inflammation in animals with or without a single TiO₂ exposure on day 28 or 33. Values indicate means $\pm$ SEM, * p < 0.05, ** p < 0.01, *** p < 0.001 versus unexposed animals.

to control mice (Table 4). Cytokine expressions in BAL were not affected by a single dose of TiO₂. In serum, sensitized and challenged mice displayed increased levels of IL1B, IL3, IL5, IL6, IL12B, IL17, CSF3, CCL2, CCL3, CCL4, CCL5, and TNFA (Table 5). Some cytokines did not reach above detection level in BAL (CCL11, IL1A, and IL12A) or in serum (CCL11).

Repeated exposures

Respiratory mechanics

Naïve mice exposed to repeated TiO₂ exposure resulted in significantly higher maximum G and H compared to unexposed naïve mice (Figure 4a,b). TiO₂ exposure also resulted in significantly decreased C_{RS} while there were no significant effects on R_{RS} compared to unexposed naïve mice (Figure 4c,d).

TiO₂-exposure of OVA-sensitized mice before the challenge period (Group 2) caused no change in MCh-induced AHR in central or peripheral airways compared to unexposed OVA mice while repeated exposure to TiO₂ during sensitization phase (Group 1) and exposure during the challenge period (Group 3) of OVA mice caused a decrease in both R_{RS} and C_{RS} (Figure 5). Animals in Group 1 also showed decreased H compared to the

Table 4. Inflammatory mediators in BAL after repeated exposure of TiO₂ (pg/ml).

	Naïve	Naïve + TiO ₂	OVA control	OVA	Gr. 1	Gr. 2	Gr. 3
IL1B	10 ± 1	20 ± 2**	10 ± 10***	40 ± 3	20 ± 10***	50 ± 7	40 ± 4
IL2	30 ± 2	30 ± 4	35 ± 4**	55 ± 4	70 ± 10	65 ± 4	55 ± 4
IL3	4 ± 1	2 ± 1	4 ± 2***	60 ± 4	30 ± 4**	30 ± 6**	20 ± 4***
IL4	10 ± 3	4 ± 2*	20 ± 5***	1800 ± 300	670 ± 220**	940 ± 110*	1000 ± 180*
IL5	5 ± 1	5 ± 1	20 ± 7***	17 700 ± 2200	1400 ± 450***	4600 ± 1400***	2300 ± 720***
IL6	0 ± 0	1 ± 1	4 ± 2***	80 ± 20	15 ± 4**	20 ± 5**	3 ± 2***
IL9	9 ± 1	10 ± 2	10 ± 2**	20 ± 2	30 ± 10	20 ± 3	20 ± 4
IL10	3 ± 1	0 ± 0**	4 ± 1***	110 ± 20	40 ± 10**	10 ± 4***	30 ± 10***
IL12B	480 ± 80	2100 ± 440**	460 ± 70***	3900 ± 720	2700 ± 400	2400 ± 120	1100 ± 60***
IL13	10 ± 1	20 ± 2	20 ± 3***	460 ± 100	50 ± 10***	140 ± 30***	50 ± 10***
IL17	30 ± 3	30 ± 5	30 ± 2***	80 ± 10	80 ± 10	70 ± 10	70 ± 20
CSF3	50 ± 10	260 ± 40***	290 ± 30*	400 ± 40	300 ± 30	320 ± 50	160 ± 10***
CSF2	8 ± 1	10 ± 3	10 ± 2***	180 ± 30	40 ± 10***	50 ± 10***	20 ± 6***
IFNG	0 ± 0	0 ± 0	4 ± 4**	20 ± 7	6 ± 5**	0 ± 0***	0 ± 0***
CXCL1	1990 ± 270	9670 ± 890***	3460 ± 290***	13 100 ± 1000	14 000 ± 1800	13 900 ± 900	8800 ± 920*
CCL2	4 ± 1	10 ± 4	4 ± 1***	200 ± 40	30 ± 2***	70 ± 20**	60 ± 10***
CCL3	33 ± 10	390 ± 110**	20 ± 10***	900 ± 80	370 ± 70***	530 ± 50**	460 ± 90***
CCL4	11 ± 1	50 ± 7*	20 ± 2***	100 ± 10	50 ± 4**	60 ± 10**	70 ± 10
CCL5	30 ± 10	30 ± 10	30 ± 10***	1000 ± 130	400 ± 60***	600 ± 50**	500 ± 60***
TNFA	30 ± 3	30 ± 6	30 ± 3***	70 ± 10	80 ± 20	50 ± 4	40 ± 4**

Values indicate means ± SEM. Gr. 1, Gr. 2, Gr. 3 versus OVA alone or naïve + TiO₂ versus naïve. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

Table 5. Inflammatory mediators in serum after repeated exposure of TiO₂ (pg/ml).

	Naïve	Naïve + TiO ₂	OVA control	OVA	Gr. 1	Gr. 2	Gr. 3
IL1A	410 ± 40	350 ± 10	710 ± 120	790 ± 50	620 ± 50	690 ± 90	600 ± 150
IL1B	50 ± 6	40 ± 5	60 ± 10**	900 ± 260	72 ± 20*	436 ± 100	280 ± 100*
IL2	210 ± 20	220 ± 10	190 ± 30	220 ± 10	190 ± 10	200 ± 20	190 ± 10
IL3	150 ± 20	120 ± 10	130 ± 20*	3400 ± 1200	160 ± 10	1300 ± 310	1070 ± 350
IL4	90 ± 20	80 ± 3	80 ± 10	100 ± 10	60 ± 10	90 ± 10	80 ± 10
IL5	130 ± 10	90 ± 10**	100 ± 10***	1400 ± 280	210 ± 40**	600 ± 80*	230 ± 30***
IL6	170 ± 10	130 ± 10*	140 ± 20**	1900 ± 600	200 ± 20	1050 ± 320	530 ± 150
IL9	150 ± 17	100 ± 10*	110 ± 10	160 ± 20	60 ± 10**	110 ± 20	100 ± 10
IL10	80 ± 10	70 ± 10	60 ± 10	510 ± 200	110 ± 30	270 ± 70	340 ± 110
IL12B	1590 ± 120	960 ± 60***	1420 ± 240**	3040 ± 450	2400 ± 50	3600 ± 450	1900 ± 320
IL12A	330 ± 30	220 ± 30***	250 ± 30	230 ± 30	130 ± 50	180 ± 30	180 ± 30
IL13	180 ± 10	140 ± 20*	150 ± 20	190 ± 20	100 ± 20*	150 ± 20	140 ± 20
IL17	670 ± 50	370 ± 60**	500 ± 60*	1400 ± 300	320 ± 50	830 ± 200	660 ± 160
CSF3	820 ± 80	810 ± 92	680 ± 80**	4340 ± 1060	1280 ± 340	1280 ± 340	2300 ± 500
CSF2	120 ± 10	100 ± 10	100 ± 10	160 ± 20	60 ± 10*	100 ± 20	90 ± 10*
IFNG	270 ± 20	170 ± 24**	180 ± 30	310 ± 40	120 ± 30*	210 ± 30	180 ± 20*
CXCL1	380 ± 10	340 ± 40	760 ± 120	900 ± 150	1440 ± 200	810 ± 80	600 ± 60
CCL2	240 ± 40	170 ± 30	120 ± 10**	220 ± 30	140 ± 10	170 ± 10	140 ± 20
CCL3	150 ± 30	100 ± 10	180 ± 10**	360 ± 50	120 ± 30**	220 ± 30	200 ± 30**
CCL4	170 ± 10	110 ± 10**	140 ± 20***	1300 ± 290	170 ± 30*	880 ± 220	440 ± 100*
CCL5	570 ± 60	510 ± 60	890 ± 130**	3900 ± 740	900 ± 130*	2700 ± 480	1960 ± 460
TNFA	300 ± 20	190 ± 20**	240 ± 30***	2350 ± 510	340 ± 70*	1430 ± 340	780 ± 230*

Values indicate means ± SEM. Gr. 1, Gr. 2, Gr. 3 versus OVA alone or naïve + TiO₂ versus naïve. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

unexposed OVA group whereas G was unchanged. Significantly decreased MCh-induced AHR in peripheral airways (G and H) was seen in Group 3 compared to OVA but were still more reactive than non-sensitized control animals (Figure 5c,d).

Inflammatory cells in BAL

Naïve mice subjected to repeated TiO₂ exposure developed airway inflammation with an increased total inflammatory cell count in BAL compared to unexposed mice, with a dominance of macrophages and neutrophils (Figure 4e).

Repeated exposures of TiO₂ did not increase the total inflammatory cell count in any of the three groups of mice sensitized to OVA (Figure 6). On the contrary, animals in both

Groups 1 and 3 had significantly lower total cell count compared to the unexposed OVA group (Figure 6a). TiO₂ exposure significantly decreased the number of eosinophils in all groups compared to the OVA group (Figure 6). The numbers of macrophages were not significantly changed in any of the TiO₂-exposed groups while Groups 1 and 3 displayed significantly lower lymphocyte numbers and the number of neutrophils was significantly increased in Group 3 compared to the OVA group (Figure 6c–e). Control mice showed no airway inflammation.

Inflammatory mediators in BAL

In naïve mice, TiO₂ exposure significantly increased the levels of IL1B, IL12B, CSF3, CXCL1, CCL3, CCL4 and

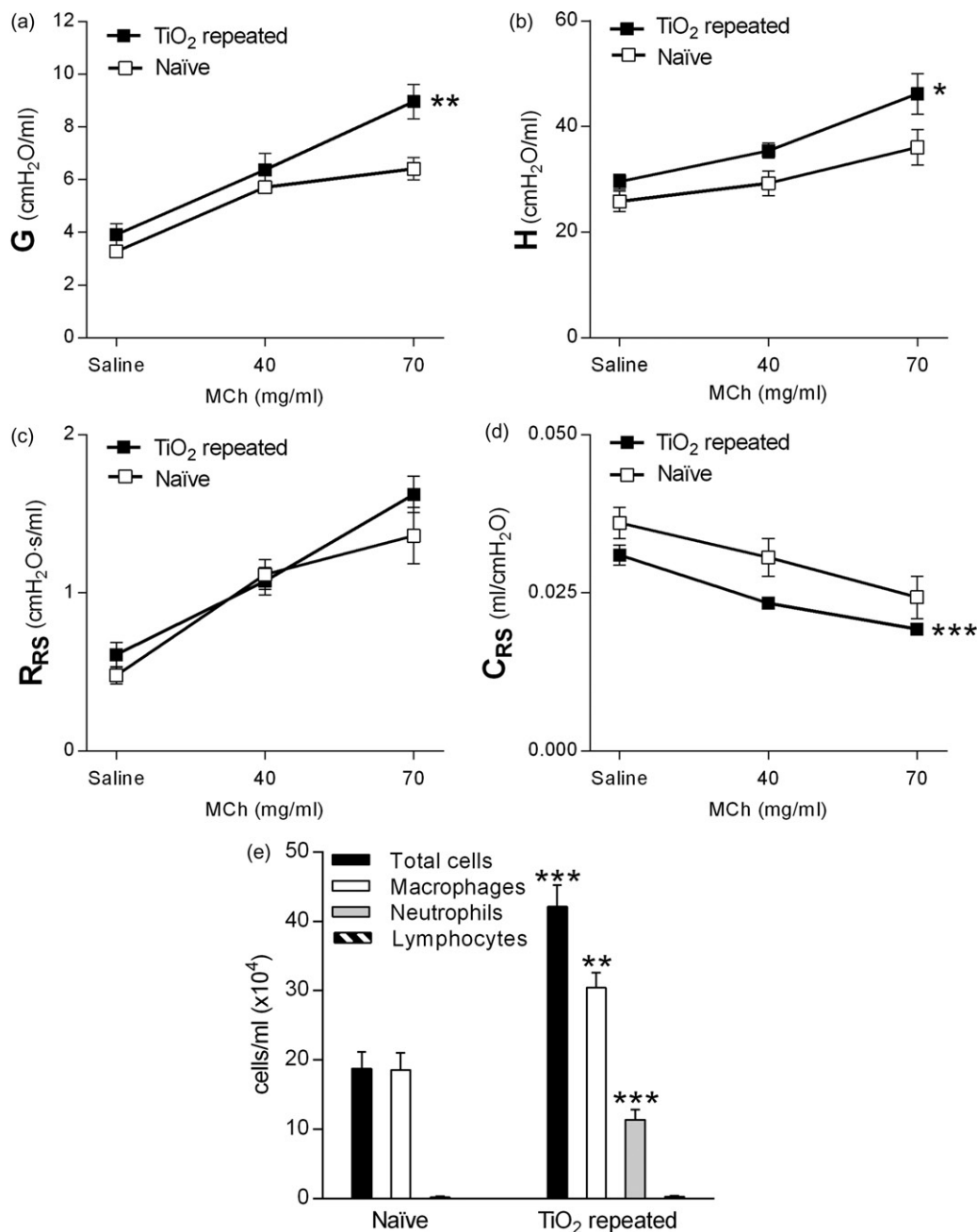


Figure 4. Analysis of respiratory mechanics and inflammatory responses in naïve mice exposed to repeated doses of TiO₂. Data show measurement of methacholine (MCh)-induced (a) tissue resistance; G, and (b) tissue elastance; H, which were performed with the FOT (Zrs measurements, Prime-2). Respiratory (c) resistance; R_{RS}, and (d) compliance; C_{RS}, were performed with the single compartment model. (e) Analysis of total and differential cell counts in BAL from naïve mice with and without repeated exposures to TiO₂. Values indicate means ± SEM, **p* < 0.05, ***p* < 0.01, ****p* < 0.001 versus naïve.

decreased the levels of IL4 in BAL. There were no other significant differences between the cytokines analyzed (Table 4). TiO₂ exposure significantly decreased the levels of several inflammatory mediators IL3, IL4, IL5, IL6, IL13, IFNG, CCL2, CCL3, CCL4, and CCL5 in all OVA-sensitized groups compared to the unexposed OVA-sensitized group. Only IL2, IL9, and IL17 were not affected by the TiO₂ exposure (Table 4).

Inflammatory mediators in serum

In naïve mice, TiO₂ exposure significantly decreased the level of IL5, IL6, IL9, IL12A, IL12B, IL13, IL17, CCL4, TNFA,

and IFNG in serum compared to unexposed naïve mice. All other cytokines remained unchanged (Table 5).

OVA-exposure alone significantly induced almost all inflammatory mediators in serum from sensitized mice compared to control (unsensitized) mice (IL1B, IL3, IL5, IL6, IL9, IL10, IL12B, IL17, CSF3, CCL2, CCL3, CCL4, CCL5, and TNFA). In Group 1, 8 out of 23 cytokines were decreased compared to mice treated with OVA alone (IL1B, IL5, IL9, IL13, CSF2, IFNG, CCL3, and CCL5). In Group 2, TiO₂ exposure only resulted in a decline of IL5. In Group 3, we observed decreased levels of 7 out of 23 cytokines (IL1B, IL5, CSF3, CCL3, CCL4 and TNFA).

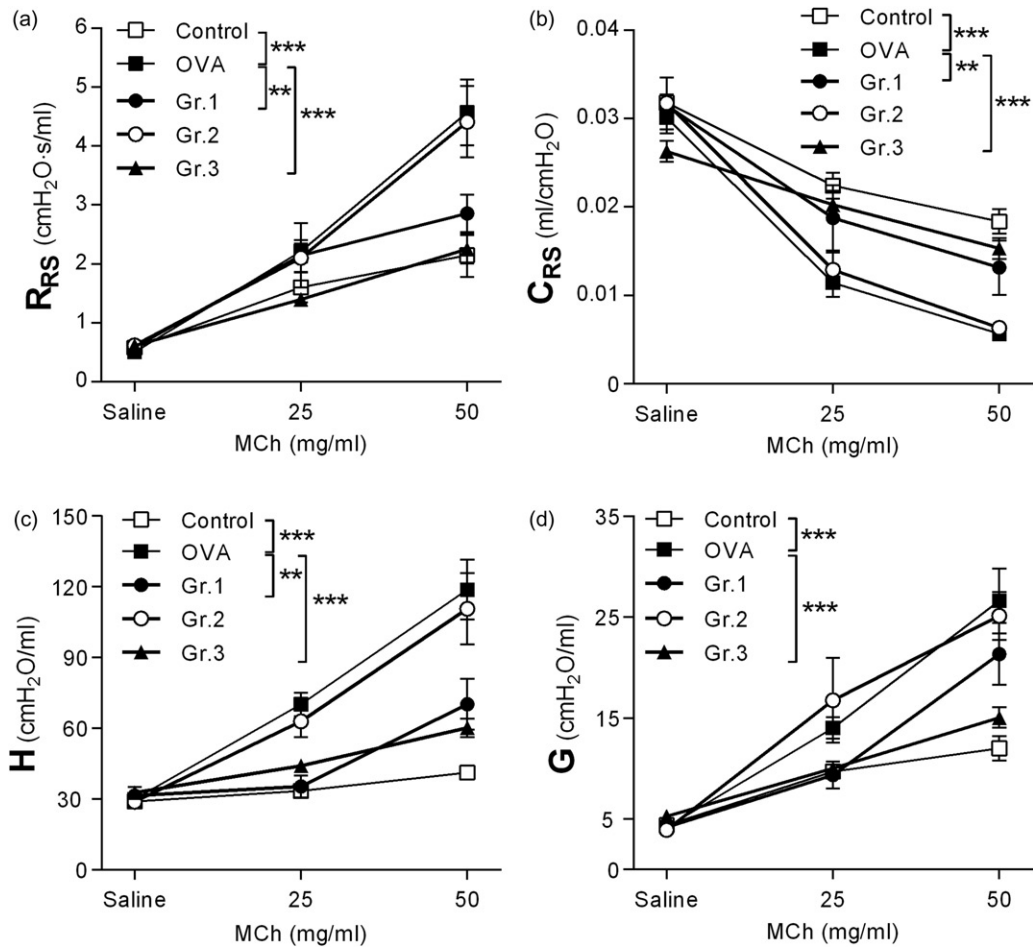


Figure 5. Analysis of respiratory mechanics in OVA-sensitized mice exposed to repeated doses to TiO₂ (Gr.1, Gr. 2, and Gr. 3). Data show measurements of methacholine (MCh)-induced respiratory (a) resistance; R_{RS} , and (b) compliance; C_{RS} , were performed with the single compartment model. (c) Tissue elastance; H and (d) tissue resistance; G, were performed with the FOT (Zrs measurements, Prime-2). Values indicate means $\pm$ SEM, ** p < 0.01, *** p < 0.001 versus OVA group.

Table 6. Flow cytometry analysis of cells from BAL.

	OVA control	OVA	Gr. 1	Gr. 2	Gr. 3
CD3 cells	37 300 $\pm$ 10 500***	576 200 $\pm$ 66 800	145 900 $\pm$ 14 200**	496 300 $\pm$ 68 200	180 600 $\pm$ 23 700***
CD4 cells	19 100 $\pm$ 9500***	402 700 $\pm$ 47 700	74 200 $\pm$ 13 800***	312 200 $\pm$ 40 400	125 000 $\pm$ 13 600***
CD8 cells	5000 $\pm$ 1300***	60 900 $\pm$ 6000	29 200 $\pm$ 2500*	39 800 $\pm$ 5600	12 800 $\pm$ 1700***
CD4:CD8 ratio	2.9 $\pm$ 0.6***	6.7 $\pm$ 0.4	2.6 $\pm$ 0.5**	7.9 $\pm$ 0.3	10.1 $\pm$ 1.0**

Values indicate means $\pm$ SEM, * p < 0.05, ** p < 0.01, *** p < 0.001 versus OVA alone.

Flow cytometry analysis of cells from BAL and lung tissue

In BAL, there were significant differences between (unsensitized) OVA control mice and OVA-sensitized mice in all the cell types analyzed. The number of T cells (CD3⁺) was significantly lower in Groups 1 and 3 compared to the group exposed to OVA alone (Table 6). The numbers of CD8⁺ cells, and CD4⁺ cells were also significantly lower in both Groups 1 and 3. The ratio of T helper and cytotoxic T cells (CD4:CD8) was significantly increased in Group 3 and significantly decreased in Group 1 compared to OVA group.

In lung tissue, it was also noted that TiO₂ exposure increased the number of NK cells significantly in Group 3 compared to the OVA group (OVA control; 1010 $\pm$ 120 cells/ml, OVA; 730 $\pm$ 90 cells/ml, Group 1; 900 $\pm$ 50 cells/ml, Group 2; 800 $\pm$ 90 cells/ml and Group 3; 1660 $\pm$ 510 cells/ml).

Fibrinogen in serum

There was no significant increase of fibrinogen in serum after exposure to TiO₂ in sensitized OVA mice in any of the exposure groups compared to unexposed OVA mice and OVA controls. (OVA control; 830 $\pm$ 190 ng/ml, OVA;

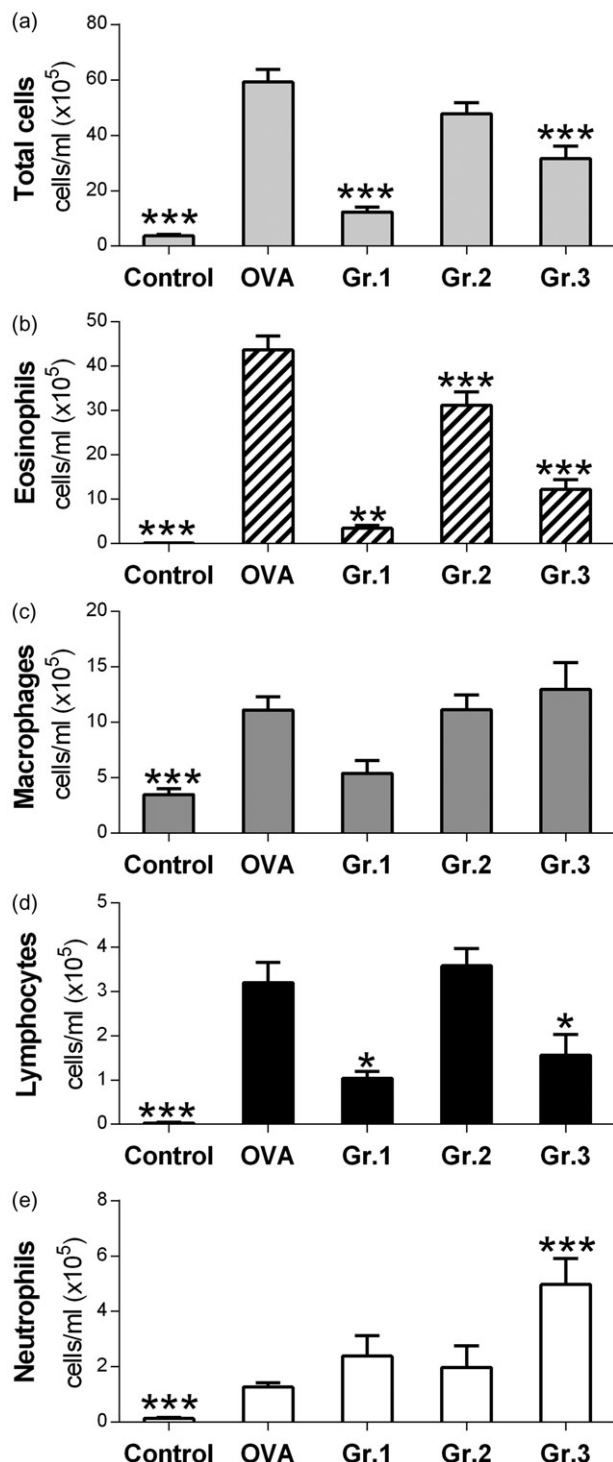


Figure 6. Analysis of inflammatory response in OVA-sensitized mice repeatedly exposed to TiO₂ (Gr. 1, Gr. 2, and Gr. 3). Data show total and differential cell counts in BAL fluid, indicating the number of: (a) total cell counts, (b) eosinophils, (c) macrophages, (d) lymphocytes and (e) neutrophils. Values indicate means $\pm$ SEM, * p < 0.05, ** p < 0.01, *** p < 0.001 versus OVA group.

830 $\pm$ 180 ng/ml, Group 1; 700 $\pm$ 25 ng/ml, Group 2; 870 $\pm$ 200 ng/ml and Group 3; 560 $\pm$ 110 ng/ml).

Discussion

The aim of this study was to examine whether inhalation of manufactured TiO₂ with a primary size of NPs aggravates

respiratory and systemic symptoms in a murine model of allergic airway inflammation. The animals with inflamed airways and AHR represent more sensitive populations, for example, individuals with allergic asthma. We compared the influence of a single exposure of TiO₂ with that of repeated exposures, and investigated whether effects of inhaled NPs during the sensitization phase differ from exposure applied before or during the phase of allergen challenge. We examined both local inflammatory processes in airways and in blood circulation by performing detailed analyses of inflammatory cells recruited to airspaces in combination with a comprehensive analysis of cytokine expression in BAL and serum. A thorough examination of effects on respiratory mechanics following MCh challenge was performed using a small animal ventilator allowing recording of multiple respiratory parameters. Our most important results in this study are: (1) in naïve mice; repeated exposures of TiO₂ induced a marked increased reactivity in peripheral airways together with an inflammatory response dominated by neutrophils and pro-inflammatory cytokines. (2) Single exposure to TiO₂ before OVA challenge in sensitized mice enhanced the AHR in peripheral airways and increased the inflammatory response locally in the lung. (3) Repeated exposure to TiO₂ during OVA sensitization inhibited the allergen-induced AHR, as well as local and systemic inflammatory responses. (4) Concomitant exposure to repeated TiO₂ during the OVA challenge period resulted in diminished local allergic symptoms in airways, but in the same animals an increased neutrophilic response and a decline in general health condition which was demonstrated by weight loss.

We demonstrated that single aerosol exposure to TiO₂ NPs in unsensitized mice resulted in an influx of macrophages and neutrophils into the airways two days after exposure but did not cause any detectable effect on respiratory mechanics. Previously, Grassian et al. (2007) and Hussain et al. (2011) reported a mild cellular inflammation in TiO₂ (anatase) NP-exposed naïve mice comprising macrophages but not neutrophils. Hussain et al. (2011) also reported that naïve mice exposed to a single dose of TiO₂ NPs did not have any significant effect on lung physiology measurements. Grassian et al. (2007) hypothesized that the reason for the low response in their study was due to an insufficient dose of NPs to reach the threshold for the inflammatory response which is consistent with our findings. On the contrary to single exposure, repeated inhalation exposures of TiO₂ activated a significant T_H1 type of inflammatory response in naïve mice as indicated by the activation of CXCL1, IL1B, IL12, CSF3, CCL3, and CCL4 as well as a neutrophil inflammation in the airways together with AHR in the peripheral airways. The induction of the inflammatory response is in agreement with previous studies by Park et al. (2009) and Sager et al. (2008). In naïve mice, the complex cascade of events that activate IL1B and involves NLRP3 inflammasomes has been shown to be activated by TiO₂ and SiO₂ NPs, as well as by asbestos or alum (Palomaki et al., 2011; Petrilli et al., 2007b; Yazdi et al., 2010). In the present study, the induction of chemokines and cytokines in naïve mice exposed to aerosolized TiO₂ P25 further support the involvement of inflammasomes in the recruitment of neutrophils to the airways. As in naïve mice,

repeated TiO₂ exposure in OVA-challenged mice most likely also resulted in the activation of inflammasomes although it appears from our data that the levels of IL1B detected in mice with allergic airway inflammation was not further increased by TiO₂ exposure. Dysregulated IL1B signaling has previously been shown to play a role in monogenic auto-inflammatory diseases (Goldbach-Mansky & Kastner, 2009). We and others have previously suggested that these inflammasomes can be triggered further by reactive oxygen species involved in airway inflammation (Petrilli et al., 2007a; Jonasson et al., 2009b) and that oxidative stress is induced by TiO₂ NPs in cultured lung epithelial cells (Andersson et al., 2011; Ekstrand-Hammarstrom et al., 2011).

In the present study, we demonstrated that a single exposure to TiO₂ NPs before OVA challenge in sensitized mice was sufficient to increase the infiltration of inflammatory cells into the lung and aggravate AHR in small airways, but not to induce any detectable inflammatory effects in BAL. On the contrary to single TiO₂ exposure, repeated exposure to TiO₂ before allergen challenge did not have any significant effect on MCh-induced central and peripheral respiratory mechanics. While cautiously interpreting responses based on the body plethysmography technique and refraining from directly comparing enhanced pause system with respiratory resistance (Adler et al., 2004; Bates & Irvin, 2003), Rossi et al. (2010) show an inhibition of MCh-induced airway reactivity after repeated exposure to TiO₂ NPs (SiO₂ coated), which resembles our findings. Their results are also in line with our data indicating that repeated exposure to TiO₂ particles throughout the OVA-protocol diminished allergic symptoms both locally and systemically in the mouse model of allergic inflammation (Rossi et al., 2010).

Mice exposed to TiO₂ during the sensitization with OVA did not induce an OVA-specific IgE and IgG₁ response in contrast to the two groups receiving TiO₂ after the sensitization period. These data indicate that one mechanism for inhibition of allergic airway responses by TiO₂ might interfere with the immunologic sensitization reaction provided that animals are exposed before the immune response is established. However, our data on reduced OVA-specific IgG₁ response along with the decline in specific IgE antibodies argue for a general immune suppression rather than a T_H1 immune deviation. Different from our study, other investigators have previously reported an adjuvant effect of TiO₂ resulting in the promotion of the allergic sensitization in mice (de Haar et al., 2006; Granum et al., 2001; Larsen et al., 2010). From our data on cytokine expression, we concluded that TiO₂ exposure during sensitization significantly inhibited the T_H2-mediated immune activation as indicated by the decreased levels of IL4, IL5, and IL13 together with lower IgE antibodies in serum, but in our experimental setup this was not associated with a detectable induction of a T_H1-mediated immune response. Notably, this effect is evident even though TiO₂ and OVA were administered by different routes of exposure (inhalation versus i.p.), implicating the systemic component of the immune suppression induced by TiO₂ exposure.

We have previously demonstrated that TiO₂ P25 NPs induce a T_H1-associated inflammatory response in human bronchial epithelial cells (Ekstrand-Hammarstrom et al., 2011)

and that lung exposure to a high-dose of P25 NPs results in a strong innate immune response together with a neutrophilic inflammation in airways of rats exposed to TiO₂ NPs (Gustafsson et al., 2011). This was followed by a secondary lymphocyte activation and increase of T_H1-associated cytokines in the circulation. Such immune response supports a scenario of diminished pro-allergic T_H2-associated immune response in mice exposed to TiO₂ particles during sensitization. The deviating results in previous studies are most likely due to differences in experimental design, where choice of administration routes, exposure doses, particle structures, size of the particles and animal species may strongly influence the impact on the immune activation.

We observed diminished symptoms of airway eosinophilia and AHR in mice exposed to TiO₂ particles during the period of OVA challenge. This was associated with general lower levels of detectable cytokines in BAL except for IL17, IL9, IL2, CCL4, and IL1B measured on day 35. Despite the apparent reduction in pro-inflammatory cytokines and symptoms of asthma, these animals displayed a marked increase of neutrophils in airways as a result of the response to TiO₂, and a significant decline in general health condition as indicated by body weight loss together with signs of lethargy and ruffled fur. In association with the airway neutrophilia and declined general health condition of sensitized mice challenged for both the allergen and TiO₂, we observed a deviation in the distribution of T helper (CD4⁺) and cytotoxic (CD8⁺) T cell subsets toward a more pronounced predominance of CD4⁺ cells (high CD4:CD8 ratio). From our data and several previous studies, it is clear that this subset of T cells is expanded in the allergic airway inflammation along with the eosinophils and that T helper cells play a major role in the cell-mediated immune response to respiratory allergens. It is conceivable that T helper cells triggered by the allergic reaction in airways contribute to the severe decline in general health condition in concert with neutrophils recruited to the lungs due to the TiO₂ exposure. Despite depressed numbers of total inflammatory cells in the airways of mice with a concomitant exposure to respiratory allergens and NPs, it can be hypothesized that the systemic health impact is a result of harmful neutrophil/T cell interplay in airways leading to a local lung injury. In the same group, it was also noted that TiO₂ exposure increased the number of NK cells significantly in lung tissue. The systemic health impact has further been discussed in a study by Rossi et al. (2010) that their results point to the similar conclusion with TiO₂ (SiO₂ coated) exposure in an OVA-induced asthma mouse model. In support for this harmful neutrophil/T cell interplay it is previously reported that TiO₂ repeated exposure can induce emphysema-like lung injury in mice (Chen et al., 2006) but the specific mechanism for the contribution of T cells to this response is still unclear.

In addition to the direct effects in the airways, it is possible that inhalation of TiO₂ NPs affects blood circulation and non-respiratory tissues secondarily. This can be accomplished indirectly by release of signaling transmitters from the lung epithelium to the blood and lymphatic vessels or by direct translocation of the particles to the blood and draining lymph nodes along with particle deposition in the nasal cavity. In our study, we observed increased expression of several

T_H1-associated inflammatory cytokines in BAL fluid of naïve mice following repeated exposure to TiO₂, indicating that the repeated inhalation of NPs may also provoke a systemic T_H1-associated inflammatory response. This systemic response was, however, not superimposed on top of the cytokine expression induced in the blood circulation in mice with allergic inflammation. Instead, a suppressed cytokine expression was noted in the serum of mice with concomitant challenge for respiratory allergen and TiO₂ NPs when compared to mice only exposed for the sensitizing immunogen.

In this study, we exposed the animals to TiO₂ through inhalation of aerosol droplets using a nose-only exposure chamber. This administration method provides a more natural exposure than intratracheal instillation but has a general drawback in the difficulty to determine the exposure dose. Other studies investigating the effects of intratracheal instillation of TiO₂ NPs, have reported a more severe inflammation and lung injury than after nose-only inhalation (Chen et al., 2006; Kobayashi et al., 2009) which might be explained by differences in total deposition and distribution of particles in the airways by the two different exposure methods.

The deposition of particles in the respiratory tract, delivered through inhalation, depends on particle characteristics, airway flow properties and airway structure (IARC, 2010). To determine the deposition, the lungs were removed from the animals after termination of the single or repeated TiO₂ exposure experiments, and thereafter the amount of titanium deposited in the lung was analyzed by ICP-SFMS. Thereby, it was possible to determine the total lung deposition to 32 µg TiO₂ after a single exposure and 193 µg TiO₂ after eight repeated exposures. These results imply a clearance, of about 25%, of particles in the lung. This clearance indicates that most of the particles have been deposited in the central larger airways, where the ciliated- and mucous secreting cells are located. These cells transport the inhaled particles up toward the oropharynx, where it is swallowed (Miller, 2000). The results of the particle characterization and the droplet size measured also contribute to the assumption that these particles deposit in the larger central airways (ICRP, 1994). In addition to lung clearance, a fraction of these inhaled particles could also have been translocated from the lungs to other tissues through the blood circulation and lymphatic vessels (Miller, 2000). The extent of deposition and retention of NPs in non-respiratory tissues is, however, difficult to estimate in general terms due to the differences in surface chemistry and agglomeration properties of individual NPs leading to a high variability in systemic distribution. Clearly, properties such as size, shape, crystal structure, and surface binding to biomolecules play a crucial role in both cellular uptake and biological reactivity (Card et al., 2008). For TiO₂ NPs it is specifically shown by us (Andersson et al., 2011; Ekstrand-Hammarstrom et al., 2011; Gustafsson et al., 2011) and others that biological reactivity linked to potential adverse health effects depends on the crystal structure and size of the particles (Kobayashi et al., 2009; Madl & Pinkerton, 2009; Warheit & Frame, 2006; Warheit et al., 1997, 2007). Although TiO₂ NPs are regarded to possess relatively low toxicity when compared to other NPs such as nickel, cobalt, or carbon black (Dick et al., 2003), it is shown

that the agglomerated P25 mixture of anatase and rutile used in the present study has a high potency to induce oxidative stress and pro-inflammatory responses in isolated lung epithelial cells in relation to other types of TiO₂ NPs (Ekstrand-Hammarstrom et al., 2011).

Several human studies suggest that one characteristic indication of severe asthma besides eosinophilic inflammation is the neutrophils which may contribute to the severe inflammation (Nakagome et al., 2012; Wenzel, 2012). Our present results point that TiO₂ exposure during OVA challenge has an adjuvant effect and that TiO₂ particles together with OVA may be considered to contribute to worsening of asthma symptoms, e.g. inducing accumulation of neutrophils into the airways. In contrast to healthy animals exposed to TiO₂, inhalation exposure to TiO₂ during the OVA challenge period caused a significant severe weight loss in animals with airway inflammation, suggesting that susceptible individuals have a greater risk of adverse health effects than the general population. Future work is necessary to further elucidate a mechanism for the decline in general health condition.

Conclusion

We have investigated whether a single or repeated exposure to TiO₂ before or during challenge of aerosolized allergen could aggravate inflammatory responses and respiratory dysfunction in mice with allergic airway disease. It was demonstrated that a single inhalation exposure to TiO₂ performed before allergen challenge induces a moderate neutrophil response in airways and enhances the allergic airway reaction measured as increased eosinophilia and AHR in small airways. Increasing the total deposition dose to TiO₂ to eight repeated inhalation exposures during 10 days induced a prominent inflammatory response dominated by neutrophils and pro-inflammatory cytokines together with a marked reactivity in peripheral airways of naïve mice. Inhalation exposure to repeated TiO₂ during the OVA challenge period resulted in diminished local allergic symptoms in airways, but in the same animals an increased neutrophilic response and a decline in general health condition was evident.

We conclude that inhalation of TiO₂ may aggravate allergic airway disease and that the adverse health effects of NPs are highly dependent on the dose and timing of exposure. Our data imply that inhalation of NPs may increase the risk for individuals with allergic airway disease to develop symptoms similar to that of severe asthma.

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Declaration of interest

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