

Effects of physicochemical properties of TiO₂ nanomaterials for pulmonary inflammation, acute phase response and alveolar proteinosis in intratracheally exposed mice

Pernille Høgh Danielsen^a, Kristina Bram Knudsen^a, Janez Štrancar^b, Polona Umek^b, Tilen Koklič^b, Maja Garvas^b, Esa Vanhala^c, Sauli Savukoski^c, Yaobo Ding^d, Anne Mette Madsen^a, Nicklas Raun Jacobsen^a, Ingrid Konow Weydahl^a, Trine Berthing^a, Sarah Søs Poulsen^a, Otmar Schmid^d, Henrik Wolff^{c,e}, Ulla Vogel^{a,f,*}

^a National Research Centre for the Working Environment, Copenhagen Ø, Denmark

^b Jožef Stefan Institute, Ljubljana, Slovenia

^c Finnish Institute of Occupational Health, Helsinki, Finland

^d Institute of Lung Biology and Disease, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich, Germany

^e Helsinki University, Department of Pathology, Helsinki, Finland

^f DTU Health Tech, Technical University of Denmark, Kgs. Lyngby, Denmark

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ABSTRACT

Nanomaterial (NM) characteristics may affect the pulmonary toxicity and inflammatory response, including specific surface area, size, shape, crystal phase or other surface characteristics. Grouping of TiO₂ in hazard assessment might be challenging because of variation in physicochemical properties. We exposed C57BL/6 J mice to a single dose of four anatase TiO₂ NMs with various sizes and shapes by intratracheal instillation and assessed the pulmonary toxicity 1, 3, 28, 90 or 180 days post-exposure. The quartz DQ12 was included as benchmark particle. Pulmonary responses were evaluated by histopathology, electron microscopy, bronchoalveolar lavage (BAL) fluid cell composition and acute phase response. Genotoxicity was evaluated by DNA strand break levels in BAL cells, lung and liver in the comet assay. Multiple regression analyses were applied to identify specific TiO₂ NMs properties important for the pulmonary inflammation and acute phase response. The TiO₂ NMs induced similar inflammatory responses when surface area was used as dose metrics, although inflammatory and acute phase response was greatest and more persistent for the TiO₂ tube. Similar histopathological changes were observed for the TiO₂ tube and DQ12 including pulmonary alveolar proteinosis indicating profound effects related to the tube shape. Comparison with previously published data on rutile TiO₂ NMs indicated that rutile TiO₂ NMs were more inflammogenic in terms of neutrophil influx than anatase TiO₂ NMs when normalized to total deposited surface area. Overall, the results suggest that specific surface area, crystal phase and shape of TiO₂ NMs are important predictors for the observed pulmonary effects of TiO₂ NMs.

1. Introduction

The global increase in production and application of titanium dioxide nanomaterials (TiO₂ NMs) in a wide range of industrial and consumer products leads to potential exposure-related adverse health effects for workers. In the workplace, exposure is most likely to occur via inhalation and thus cause lung inflammation as well as systemic effects. TiO₂ NMs are poorly soluble and have been considered as low toxicity particles. However, The International Agency for Research on

Cancer (IARC) has classified TiO₂ as a Group 2B carcinogen (possibly carcinogenic to humans) based on sufficient evidence in animal experiments (IARC, 2010) as lung tumors developed in rats after two years of chronic exposure to 250 mg/m³ of fine-sized rutile TiO₂ (Lee et al., 1985) and to 10 mg/m³ of nano-sized TiO₂ P25 (Heinrich et al., 1995). A large number of rodent studies have reported increased pulmonary inflammation, including neutrophil influx in bronchoalveolar lavage (BAL) fluid, after acute, sub-acute and sub-chronic exposure to TiO₂ NMs by either inhalation or intratracheal (i.t.) instillation (Shi

* Corresponding author at: National Research Centre for the Working Environment, Copenhagen Ø, Denmark.
E-mail address: ubv@nrcwe.dk (U. Vogel).

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et al., 2013; Hadrup et al., 2017). Neutrophil influx in BAL fluid has been shown to correlate closely with pulmonary acute phase response in terms of increased serum amyloid A 3 (*Saa3*) mRNA levels in lung tissue of mice exposed to TiO₂ NMs by i.t. instillation and by inhalation (Halappanavar et al., 2011; Saber et al., 2013). It has been suggested that the SAA produced in the lungs enters systemic circulation as part of high density lipoprotein molecules, causing reversal of the cholesterol flow leading to increased formation of foam cells and plaque progression, and that this contributes significantly to the pathogenesis of cardiovascular disease (Saber et al., 2014). NM characteristics may affect the pulmonary toxicity and inflammatory response, including specific surface area, size, shape, crystal phase, chemical composition, charge or other surface characteristics. The importance of particle size has been discussed widely in the past as the smaller sized NMs and accompanying increased specific surface area entails increased toxicity and translocation, greater lung retention and slower pulmonary clearance (Ferin et al., 1992; Stone et al., 2017). A larger specific surface area provides a larger interface for molecular and chemical interactions with biological fluids and tissues, potentially increasing the reactivity of NMs. Numerous in vivo studies on particle-induced acute pulmonary toxicity in animal models have confirmed that the most biological relevant dose metric for predicting adverse outcomes, such as inflammation, is likely the specific surface area rather than mass, as shown in a retrospective analysis by Schmid & Stoeger (Schmid and Stoeger, 2016). The crystal phase of TiO₂ NMs has likewise been suggested to be important for the toxic response (Johnston et al., 2009), but relatively few studies have investigated this, and with ambiguous results. Grouping of TiO₂ in hazard assessment might be challenging because of the variation in physicochemical properties. In the present study, the TiO₂ NMs are of various sizes and shapes of the anatase crystal phase. We will also compare the results with previous studies on TiO₂ NMs of the rutile crystal phase. The shape of NMs can have an effect on the specific surface area and in some cases, as in case of fibers, it also seems to have a qualitative effect on the toxic response. This has been the foundation of the “fiber paradigm” stating that fibers meeting specific requirements represent a health hazard (Donaldson et al., 2010). In the present study on pulmonary toxicity, we have included an elongated form of anatase TiO₂ NM, which however do not fulfill the requirements of the fiber paradigm.

The main objectives of the present study was to assess pulmonary toxicity in terms of influx of inflammatory cells into the lung, pulmonary acute phase response, genotoxicity, and lung histopathology in mice exposed to four anatase TiO₂ NMs with different physicochemical properties (a large and a small NM, and a cube and a tube shaped NM) by i.t. instillation. In addition, we assess systemic toxicity in terms of hepatic acute phase response and genotoxicity. We compare the data on anatase TiO₂ NMs with previously published data on five rutile TiO₂ NMs in order to discuss the importance of the crystal phase on pulmonary inflammation. In addition, we discuss the significance of BET surface area, crystal phase and shape as possible predictors of pulmonary toxicity (neutrophil influx, acute phase response) by using multiple regression analyses.

2. Materials and methods

2.1. Nanomaterials and characterization

Four anatase TiO₂ NMs with varying physicochemical properties were included in the present study. The TiO₂ NMs with declared sizes of 15 nm, and 100 nm were obtained from MK Nano, Canada (MKN-A015 and MKN-A100, denoted TiO₂ NM-1 and TiO₂ NM-2, respectively). The TiO₂ NMs with shapes like a tube and a cube (denoted TiO₂ tube and TiO₂ cube, respectively) were obtained from partners in the SmartNanoTox project (Jožef Stefan Institute, Ljubljana, Slovenia). The quartz (DQ12) was included as benchmark particle (kindly provided from Craig Poland, University of Edinburgh, United Kingdom). CB,

Printex 90, was used as internal reference material (a gift from Degussa-Hüls, Germany). The synthesis of the TiO₂ tube has been published elsewhere (Garvas et al., 2015). The description of the TiO₂ cube and TiO₂ tube synthesis is provided in the Supplementary material.

Morphology of the four anatase TiO₂ NMs and DQ12 was investigated with TEM (Jeol 2100, 200 kV). The specimens were prepared by probe sonication of NMs (TiO₂ NM-1, TiO₂ NM-2 and TiO₂ tube) in the suspension used for i.t. instillation (method described below) while DQ12 and TiO₂ cube NMs were dispersed ultrasonically in MeOH for 20 min. Then one drop of dispersion was deposited on a lacey carbon film supported by a copper grid.

The phase composition of the samples was determined with X-ray powder diffraction (XRD), using a D4 Endeavor, Bruker AXS diffractometer with Cu K α radiation ($\lambda = 1.5406 \text{ \AA}$) and a Sol-X energy-dispersive detector. Diffractograms were measured in the 2θ angular range between 5 and 80° with the step size of 0.02°/s and the collection time of 3 s.

Specific surface areas were determined by the Brunauer, Emmett and Teller method (referred to as the BET method) by Quantachrome Instruments (Boynton Beach, FL, USA). In brief, nitrogen adsorption at 77.4 K was used for the determination of the specific surface. At the statistic-volumetric method, a specific amount of measuring gas is dosed onto the temperatured sample, which is arranged in vacuum. The samples were prepared at 250 °C, under vacuum, for 2 h and analysis were performed on a QUANTACHROME Quadrasorb.

2.2. Acellular oxidation potential

The DCFH₂-DA assay is one of the most used assays to measure reactive oxygen species (ROS) formation (Møller et al., 2010). In cellular assays, cells are loaded with the compound DCFH₂-DA, which is hydrolyzed intracellularly by endogenous esterases, whereas chemical deacetylation in NaOH is required when performing acellular assays. Here we measure the acellular oxidation potential of NMs as previously described (Bengtson et al., 2016). In brief, particle suspensions in Hanks buffered saline solution (HBSS) were probe sonicated on ice, for 16 min with 10% amplitude without pause, using a Branson Sonifier S-450D (Branson Ultrasonics Corp., Danbury, CT, USA) equipped with a disruptor horn (model number 101–147-037). DCFH₂-DA was hydrolyzed to 2',7'-dichlorodihydro fluorescein (DCFH₂) by NaOH and diluted in HBSS. The particle suspensions with concentrations ranging from 0 to 200 $\mu\text{g/ml}$ and DCFH₂ (0.01 mM) was added to 96-well plates and incubated for 3 h. The level of DCF was measured at excitation and emission wavelengths of 490 and 520 nm, respectively (Victor Wallac-21,420, Perkin Elmer, Denmark).

2.3. Preparation of instillation suspensions and characterization

The TiO₂ NMs and DQ12 were suspended in Nanopure water added 2% v/v C57BL/6 mouse serum to a final concentration of 3.24 mg/ml and probe sonicated on ice, for 16 min with 10% amplitude without pause, using a Branson Sonifier S-450D (Branson Ultrasonics Corp., Danbury, CT, USA) equipped with a disruptor horn (model number 101–147-037). The final concentration of 3.24 mg/ml corresponds to the highest dose of 162 μg per 50 μl , which were the instillation volume per mice. The instillation suspensions were further diluted three-fold times to obtain 54 and 18 μg per 50 μl , respectively and sonicated for 2 min. The vehicle of Nanopure water added 2% v/v C57BL/6 mouse serum was similarly sonicated as just described.

The average hydrodynamic particle size of the anatase TiO₂ NMs in instillation suspensions (3.24 mg/ml) were determined by Dynamic Light Scattering (DLS); Malvern Nano Zetasizer equipment mounted with a 633 nm red laser) as described previously (Saber et al., 2016; Modrzyńska et al., 2018a, 2018b). Data were obtained from six repeated analyses of the same sample.

Endotoxin levels were measured using the Kinetic-QCL™ Kinetic

Table 1
Main physicochemical parameters of the NMs.

Name	NM-type	Source	Size (range) ^c	Morphology ^c	BET (m ² /g)	Endotoxin EU/ml ^d
TiO ₂ NM-1	Anatase TiO ₂ ^a	MK Nano	12–50 nm	rectangular/spherical	84.6	0.117
TiO ₂ NM-2	Anatase TiO ₂ ^a	MK Nano	16–28 nm	rectangular/spherical	73.5	0.156
TiO ₂ tube	Anatase TiO ₂	this study	length: 40–500 nm diameter: 6–11 nm	tube	154	0.095
TiO ₂ cube	Anatase TiO ₂	this study	a = 11–17 nm b = 15–27 nm	cube/cube-like	96.9	0.112
DQ12	Quartz	IOM ^b	50–400 nm	different	10.1	0.094

^a from XRD patterns an amount of rutile is present in TiO₂ NM-1 and TiO₂ NM-2 (11.5 wt% and 5.6 wt%, respectively).

^b kindly provided by Dr. Craig Poland, Institute of Occupational Medicine, Edinburgh, Scotland.

^c based on TEM analysis.

^d the level of endotoxin in Nanopure water with 2% mouse serum was 0.112 EU/ml.

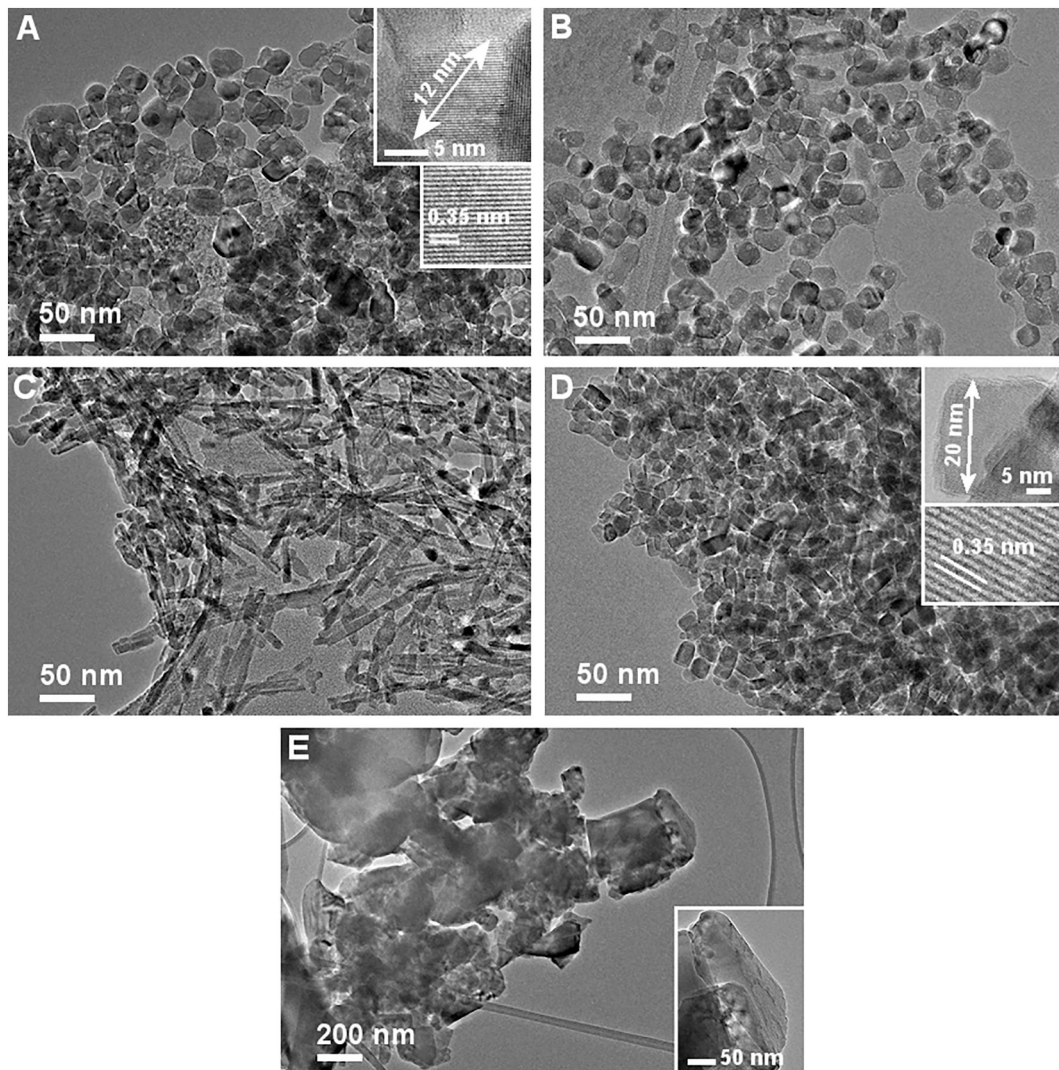


Fig. 1. TEM images of TiO₂ NM-1 (A), TiO₂ NM-2 (B), TiO₂ tube (C), TiO₂ cube (D) and DQ12 (E).

Chromogenic Limulus Amebocyte Lysate Assay Kit (Lonza, Walkersville, MD, USA) as described previously (Saber et al., 2012a).

The doses 18, 54 and 162 µg/mouse are equivalent to 1.5, 5 and 15 working days at the 8-h time-weighted average occupational exposure limit for TiO₂ by Danish Regulations (6.0 mg/m³ TiO₂) (Jackson et al., 2013a).

2.4. Animal handling and exposure

Seven-week-old female C57BL/6jBomTac mice (Taconic, Ejby, Denmark) were randomized to either polypropylene cages containing animals for NM exposure ($N = 7$ mice/group for BAL and tissue collection and $N = 5$ mice/group for histology) or to cages containing vehicle controls ($N = 2–4$ mice/group). The animal experiments were performed over several weeks and vehicle controls were included on each exposure day. The vehicle controls were combined for each post-

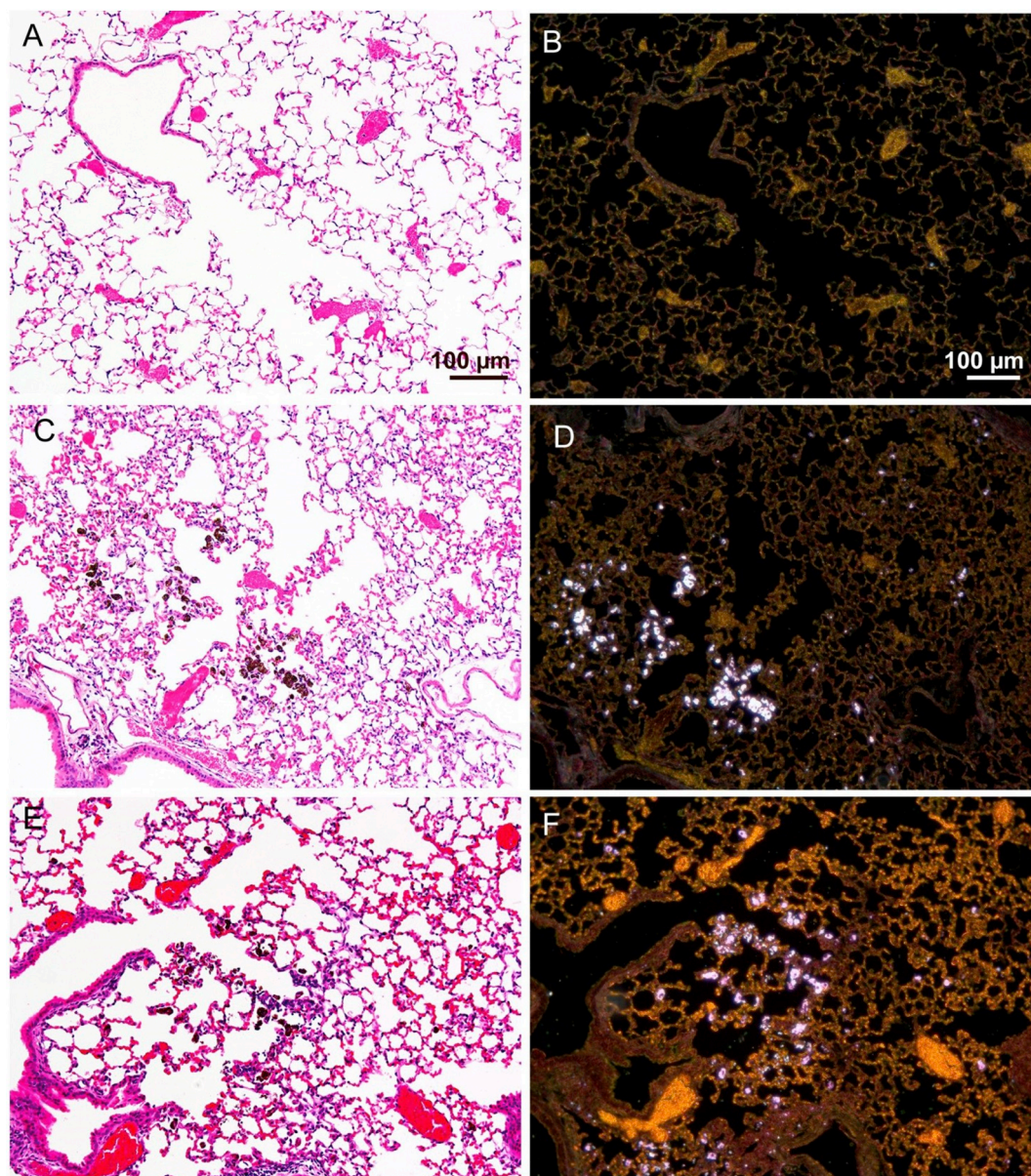


Fig. 2. Mouse lung histopathology and particle distribution 28 days post-exposure to (A, B) vehicle control, (C, D) TiO₂ NM-1 and (E, F) TiO₂ NM-2. (C, E, brightfield) Minor macrophage activity and (D, F, darkfield) focal distribution of nanomaterial (white) in the alveolar region. Brightfield and enhanced darkfield microscopy, H&E stain, scale bar 100 μm applies to all.

exposure day; $N = 15$ (day 1 and 3) and $N = 10$ (day 28, 90, 180) per dose group. The mice had access to food (Altromin 1324) and water ad libitum. The housing conditions have been described in detail elsewhere (Hadrup et al., 2017). At 8 weeks of age the mice were anesthetized and exposed to 50 μl anatase TiO₂ NMs, DQ12 or vehicle by single i.t. instillation. Mice for 180 days exposure only received the highest dose of 162 μg. For histology doses of 500 and 1000 μg/mouse DQ12 were included. The instillation procedure has been described in detail previously (Jackson et al., 2010; Saber et al., 2012a; Hadrup et al., 2017). In brief, the mice were placed on their backs on a 40° slope. A diode light was placed touching the larynx and the trachea was intubated using a catheter. The 50 μl NM suspension was instilled followed by 150 ml air. The catheter was removed and the mouse transferred to a vertical hanging position with the head up. This ensures that the administered material is maintained in the lung and breathing was observed to assure that airways were not blocked (Jackson et al., 2010).

Animal experiments were performed according to EC Directive 2010/63/UE in compliance with the handling guidelines established by

the Danish government and permits from the Experimental Animal Inspectorate (no. 2015-15-0201-00465). Prior to the study, the experimental protocols were approved by the local Animal Ethics Council.

2.5. Preparation of BAL and tissue collection

One, 3, 28, 90 or 180 days post-exposure mice were anesthetized and necropsied. The BAL collection procedure has been described in detail previously (Saber et al., 2012a; Hadrup et al., 2017). In brief, mice were anesthetized and lungs were flushed twice with sterile 0.9% sodium chloride through the trachea to obtain BAL fluid. The BAL fluid was kept on ice until separation of fluid and cells by centrifugation at 400 $\times g$ for 10 min at 4 °C. Small pieces of lung and liver tissue were snap frozen in cryotubes in liquid nitrogen and stored at 80 °C until isolation of RNA for mRNA expression analysis and sample preparation for the comet assay.

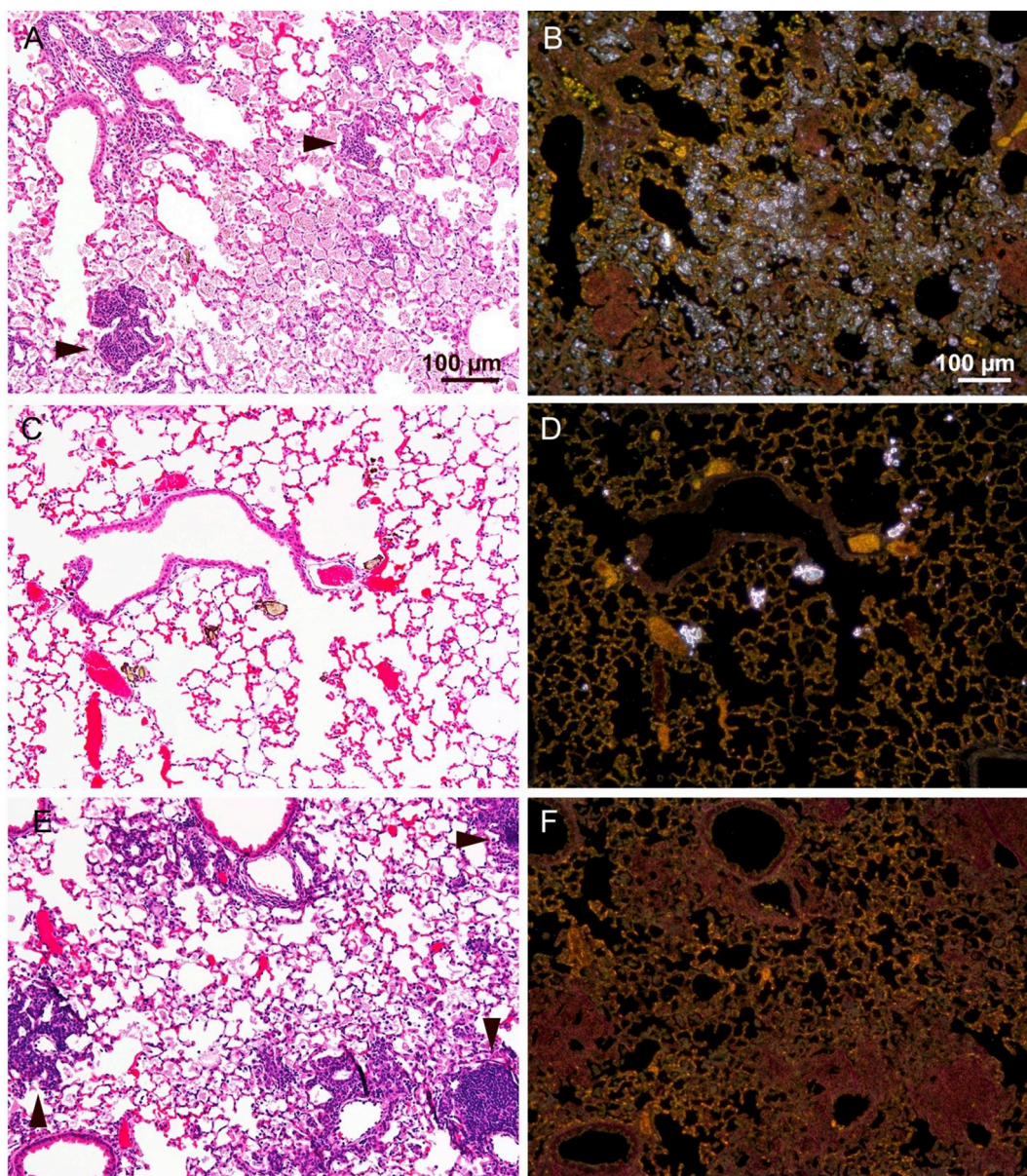


Fig. 3. Mouse lung histopathology and particle distribution 28 days post-exposure to (A, B) TiO₂ tube and (C, D) TiO₂ cube and 180 days post-exposure to (E) DQ12. (A and E) Lymphocytic infiltrates (arrow heads) and proteinosis in alveolar spaces in mice exposed to TiO₂ tube and DQ12. (B) Alveoli full of micron-sized agglomerates of TiO₂ tube (white). (D) TiO₂ cube (white) appeared mainly as scattered large aggregates. (F) DQ12 cannot be detected by darkfield microscopy. Brightfield and enhanced darkfield microscopy, H&E stain, scale bars 100 μm applies to all.

2.6. BAL cell differential counting

The separated BAL cells were re-suspended in 100 μl HAMF12 medium with 10% fetal bovine serum. 40 μl of the cell suspension was mixed with 160 μl medium containing 10% dimethyl sulfoxide (DMSO) and stored at -80 °C for later analysis in the comet assay. The total number of cells and of dead cells was determined from 20 μl diluted cell suspension by NucleoCounter NC-200 (Chemometec, Allerød, Denmark). 40 μl of the fresh cell suspension was collected on microscope slides by centrifugation at 60 xg for 4 min and then the cells were fixed with 96% ethanol and stained with May-Grünwald-Giemsa stain. Differential counts of macrophages, neutrophils, lymphocytes, eosinophils, and epithelial cells were determined by counting 200 cells/sample under light microscope (100× magnification).

2.7. mRNA expression of *Saa*

Pulmonary and hepatic acute phase response was assessed by the measurement of *Saa3* and *Saa1* mRNA expression levels, respectively, as described previously (Saber et al., 2009; Wallin et al., 2017). In brief, total RNA was isolated from frozen liver and lung tissue using Maxwell® 16 LEV simply RNA Tissue Kit (AS1280, Promega, USA) according to the manufacturer's protocol. Complementary DNA (cDNA) was prepared using TaqMan® reverse transcription reagents (Applied Biosystems, USA) as described by manufacturer's protocol. Total RNA and cDNA concentrations were measured on NanoDrop 2000c (ThermoFisher, USA). The *Saa3* and *Saa1* gene expression was determined by real-time reverse transcriptase polymerase chain reaction (RT-PCR) using TaqMan pre-developed reagents (Applied Biosystems, USA) and 18S as a reference gene. The samples were run in triplicates using ViiA7 Real-Time PCR detector (Applied Biosystems, USA). The relative expression was measured by the comparative C_T method. Negative

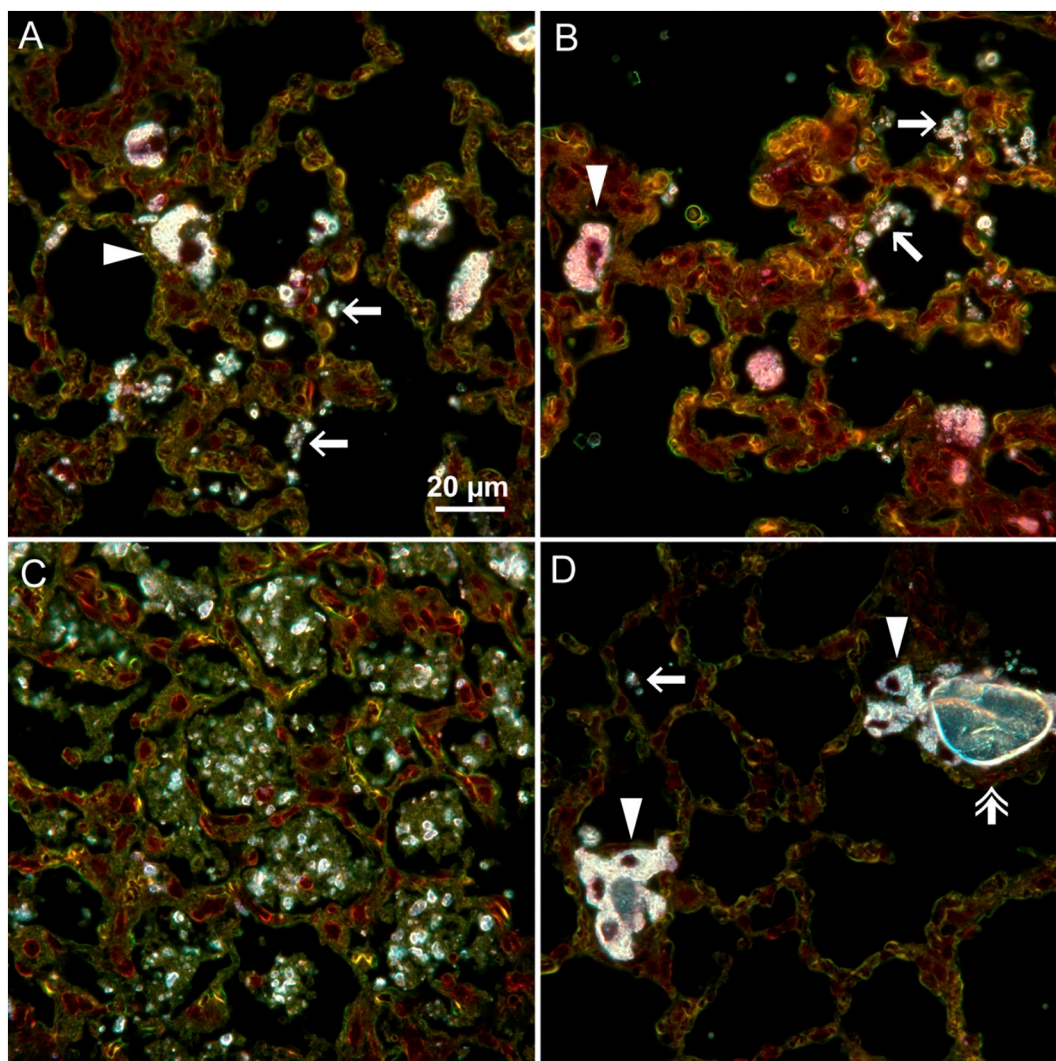


Fig. 4. Distribution and appearance of TiO₂ NM-1 (A), TiO₂ NM-2 (B), TiO₂ tube (C) and TiO₂ cube (D) in mouse lung 28 days post-exposure. All four TiO₂ NMs (white) appeared as micron-sized agglomerates in alveolar macrophages (arrow heads) or free in the alveolar lumen (arrows). (C) Alveoli full of micron-sized agglomerates of TiO₂ tube (white) and organic debris (green/grey). (D) TiO₂ cube appeared mainly as scattered large aggregates (double arrow). Enhanced darkfield microscopy, H&E stain, scale bar 20 μm applies to all. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

controls were included in each run of the analysis. The day-to-day variation for the plate control in the analyses was 17.5% and 4% for *Saa3* and *Saa1*, respectively.

2.8. Comet assay

DNA strand breaks were determined by the comet assay on BAL cells, lung and liver tissue as described in (Jackson et al., 2013b; Modrzynska et al., 2018a, 2018b). In brief, single-cell suspensions were obtained by homogenization of frozen liver and lung pieces in ice-cold Merchant's buffer through a stainless steel mesh. The BAL cells were thawed in a 37 °C water bath before diluting with Merchant's buffer. The cell suspensions were embedded in agarose (0.7%) on microscope Trevigen 20-Well CometSlides™. The slides were immersed in cold lysing solution and stored overnight at 4 °C. Samples were treated with alkaline buffer and electrophoresis with circulating ice-cold electrophoresis buffer was performed (25 min, 38 V, 70-77A). Thereafter, slides were washed in neutralization buffer (0.4 M Tris, pH 7.5), fixed with 96% ethanol and stained with SYBRGreen®. DNA strand breaks were quantified as percentage of DNA in the comet tail (%TDNA) and as the comet tail length (TL). The comets were scored by the fully

automated PathFinder™ system (IMSTAR, France). In order to control the day-to-day variation both negative (A549 human lung epithelial cell line treated with PBS for 30 min at 4 °C) and positive (A549 human lung epithelial cell line treated with 60 μM H₂O₂ for 30 min at 4 °C) controls were included. The samples were run over eight experiment days. The day-to-day variation for the negative and positive controls was 9% and 18% for TL, respectively.

2.9. Histopathology

At 28, 90 or 180 days post-exposure mice were weighed and anesthetized. Lungs were filled slowly with 4% formalin under 30 cm water column pressure. A knot was made around the trachea to secure formaldehyde in lungs to fixate tissue in “inflated state”. Lungs were then removed and placed in 4% neutral buffered formaldehyde solution for 24 h as previously described (Poulsen et al., 2016). After fixation the samples were trimmed, dehydrated on a Leica ASP300S (Leica Systems) and embedded in paraffin. Sections were cut at 3 μm on a Microm HM 355S Microtome (Thermo Scientific™). Sections for light microscopic examinations were stained with Haematoxylin and Eosin (H&E-staining) or Sirius Red staining. The sections were examined by light

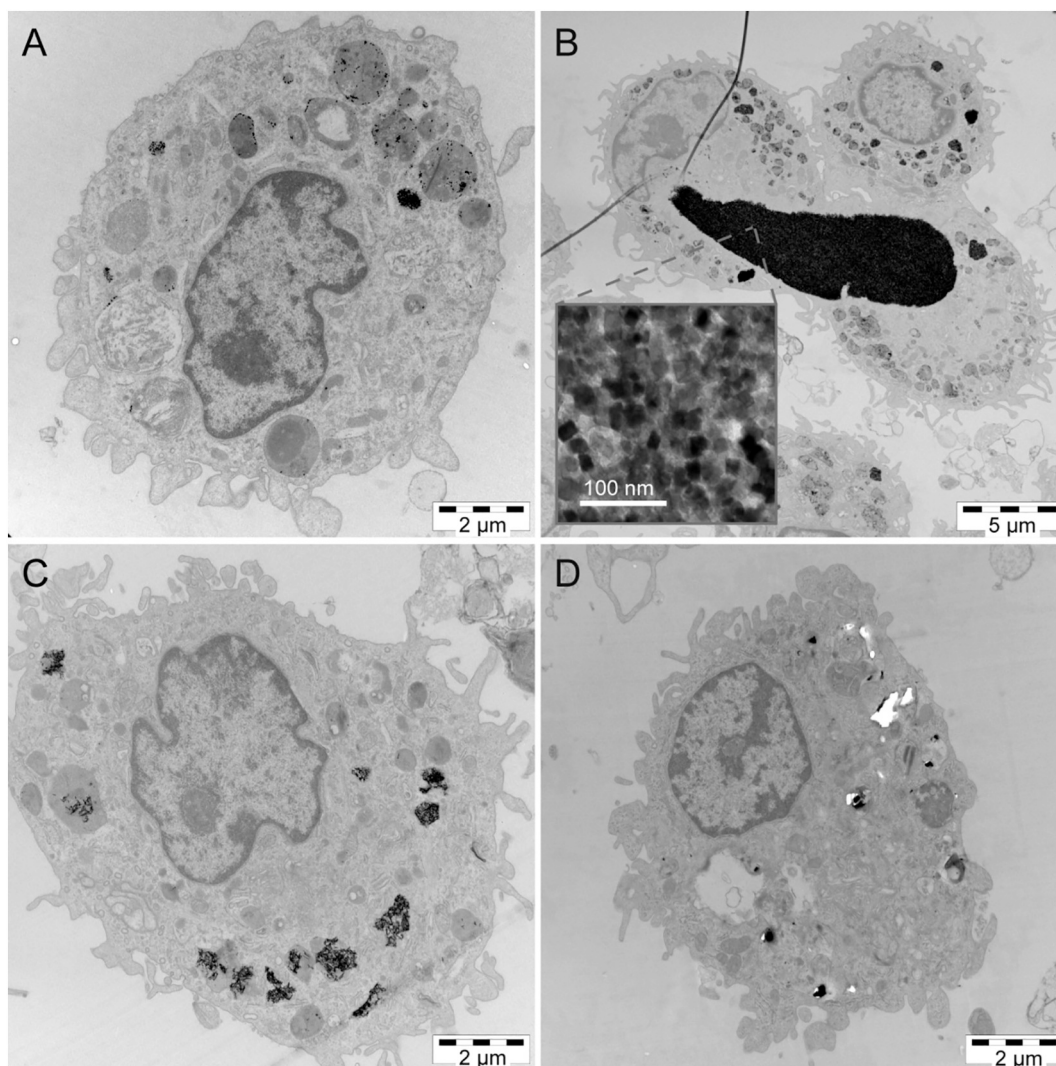


Fig. 5. Electron micrographs of subcellular distribution of (A, B) TiO_2 cube, (C) TiO_2 tube and (D) DQ12 in alveolar macrophages in bronchoalveolar lavage 90 days post-exposure. Insert in (B) is a high power image of the large aggregate of TiO_2 cubes.

microscopy using a Leica DM 4000B microscope equipped with a Leica DFC 480 camera and an Olympus BX43 microscope with a Nikon DS-Fi2 camera. Lymphocytic and macrophage infiltrates were defined as areas of tissue where the density of lymphocytes and macrophages were higher than the background. The minimum requirement was that they should contain 20 or more lymphocytes or 5 or more macrophages. The proteinaceous debris in the alveolar spaces was compatible with that seen in pulmonary alveolar proteinosis (PAP). The number refers to percentage of afflicted alveoli in the lobe. Granuloma formations refer to foreign body type granulomas with material surrounded by at least a few adherent macrophages and exclude giant cells.

2.10. Darkfield microscopy

Standard darkfield and Cytoviva enhanced darkfield hyperspectral system (Auburn, AL, USA) was used to detect particles in lung tissue by scanning histological sections at $10\times$ and $40\times$ magnification. Images were acquired at $10\times$ on a Leitz Laborlux K microscope with a Leica MC170 HD camera and a $100\times$ using an Olympus BX 43 microscope with a Qimaging Retiga4000R camera.

2.11. Electron microscopy

Samples were fixed in 2.5% glutaraldehyde and post fixed in 1%

osmium tetroxide, dehydrated and embedded in LX-112. Thin sections were collected on carbon coated copper grids, stained with uranyl acetate and lead citrate. Grids were analyzed with a transmission electron microscope operated at 100 kV (JEM-1220, Jeol Ltd, Japan, Tokyo) and photographed with Veleta TEM CCD camera (Olympus Soft Imaging Solutions GmbH, Germany). Preparation of TEM samples were done in Electron Microscopy Unit, University of Helsinki.

2.12. Statistical analysis

The data sets of BAL cells differential counting, mRNA expression, DNA strand breaks and histopathology were analyzed using the software package Graph Pad Prism 8.1.2. (Graph Pad Software Inc., La Jolla, CA, USA). All data are expressed as mean \pm standard deviation. Data were tested for normality using the Shapiro-Wilks test and for variance homogeneity using the Bartlett's test. It was not possible to fulfill the normality and variance homogeneity criteria for most data by neither logarithmic nor cube root transformations. Therefore, the data were analyzed by the nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison method as post hoc to test the differences between the test groups. P -value $\leq .05$ was considered significant.

To identify important TiO_2 NMs properties multiple regression analyses were conducted on the following endpoints: neutrophil influx in the BAL fluid and pulmonary *Saa3* expression. The four anatase and

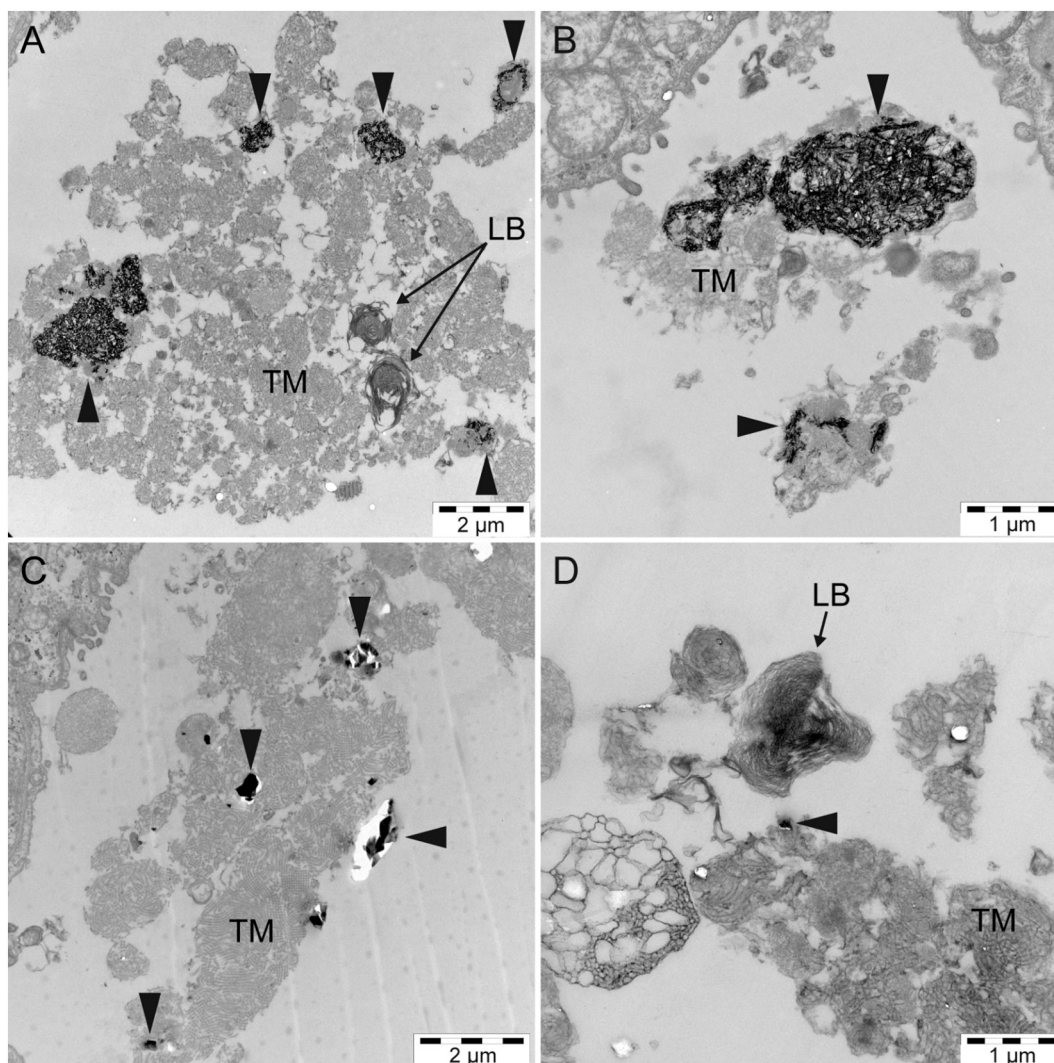


Fig. 6. Electron micrographs of alveolar proteinosis in mouse lung 90 days after exposure to (A, B) TiO₂ tube and (C, D) DQ12. Acellular organic debris (arrow heads), tubular myelin (TM) and lamellar bodies (LB).

the five rutile TiO₂ NMs were all included in the analyses. Selected physicochemical properties were: BET surface area, anatase phase, tube shape, and surface modification (NRCWE-002 and UV-Titan L181). Initially, a Pearson Correlation analysis was used to investigate the pairwise associations between physicochemical parameters (BET surface area, Anatase, Tube and Modified). No correlations between parameters were observed (results not shown), and all parameters were included as independent variables in the following multiple regression analyses. Multiple regression analyses investigating the relationship between physicochemical properties (BET surface area, Anatase, Tube and Modified) and neutrophil influx in the BAL fluid (all time points) or pulmonary *Saa3* expression (day 1 and 3 only), were performed. BET surface area was transformed using $\log(\text{BET})/\log(1.25)$, so the estimated effect corresponded to a 25% increase in BET. Statistical significance was determined at the 0.01 level in the multiple regression analyses, since no other correction for mass-significance was performed. The Pearson Correlations and all multiple regression analyses were performed in SAS version 9.4 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Physicochemical properties

The main characteristics of the anatase TiO₂ NMs and DQ12 are

summarized in [Table 1](#). Anatase TiO₂ NMs, labeled as TiO₂ NM-1, TiO₂ NM-2, TiO₂ tube, TiO₂ cube, and DQ12 were investigated with transmission electron microscopy (TEM) to determine particle morphology and size ([Table 1](#)). According to the supplier, TiO₂ NM-1 and TiO₂ NM-2 should have diameters of 15 and 100 nm, respectively. However, TEM of the anatase TiO₂ NMs ([Fig. 1](#)) revealed that the average diameter of the TiO₂ NM-1 particles ([Fig. 1A](#)) were around 30 nm and displayed a more rectangular than spherical shape. Only small amounts of the TiO₂ NM-1 particles had diameters around 10 nm. In addition, tiny particles with no certain shape and sizes below 10 nm were observed. The average diameter of the TiO₂ NM-2 particles ([Fig. 1B](#)) was 16 to 32 nm, instead of 100 nm which was the size reported by the manufacturer. These particles had a rectangular to spherical shape. The largest TiO₂ NM-2 particles were about 50 nm in diameter. However, these were not spherical, but rather elongated in one direction. The BET surface area of TiO₂ NM-1 and TiO₂ NM-2 was 85 and 74 m²/g, respectively, indicating similar particle diameter sizes as confirmed by TEM. The TiO₂ tube NMs showed distinctive aggregates composed of tubes, while individual tubes was rarely observed ([Fig. 1C](#)). The outer diameter of the TiO₂ tube NMs varied from 6 to 11 nm and the length were up to 500 nm. The BET surface area was 154 m²/g. The TiO₂ cube NMs had a rectangular shape (cube and cube-like (elongated in one direction)). The average size of the shorter side was about 17 nm and the longer side up to 26 nm. The particles were crystalline ([Fig. 1D](#)) and the BET surface

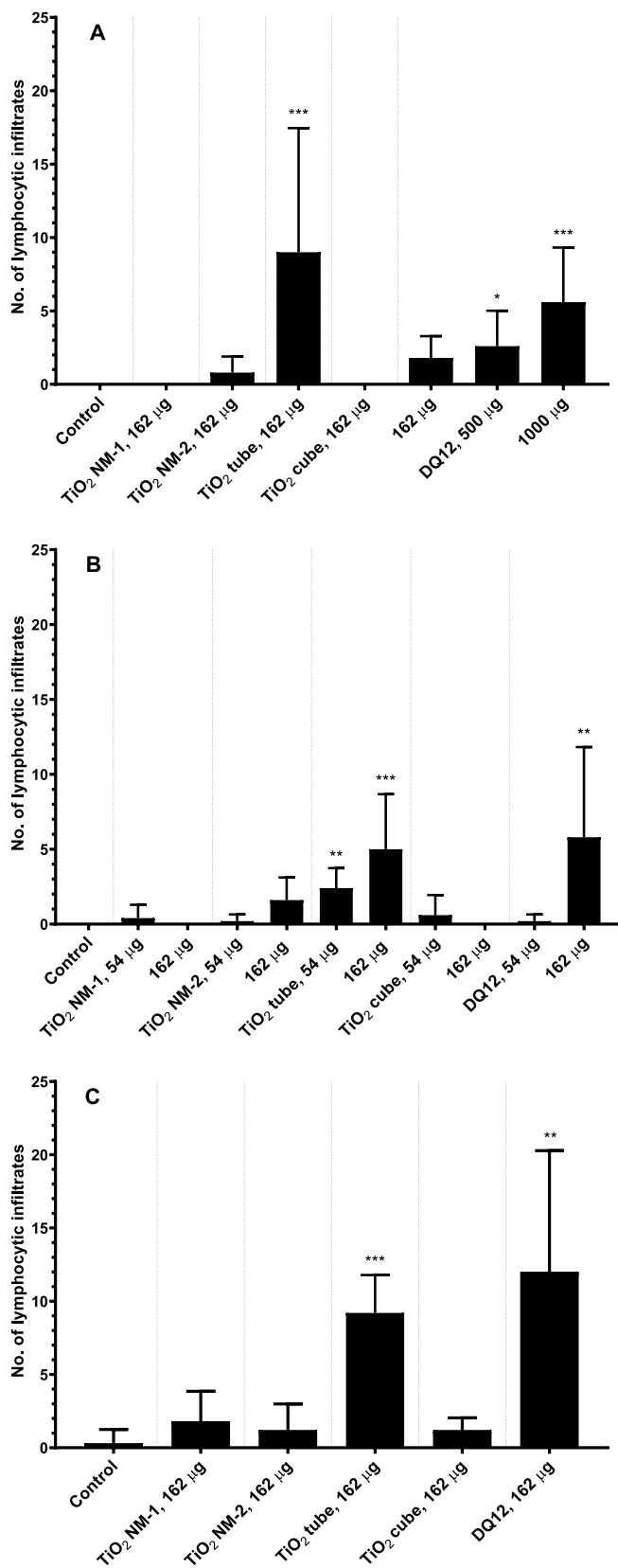


Fig. 7. Number of lymphocytic infiltrates based on histopathological evaluation of the lung tissue (A) 28 days, (B) 90 days (C) 180 days after a single i.t. instillation of TiO₂ NMs and DQ12.

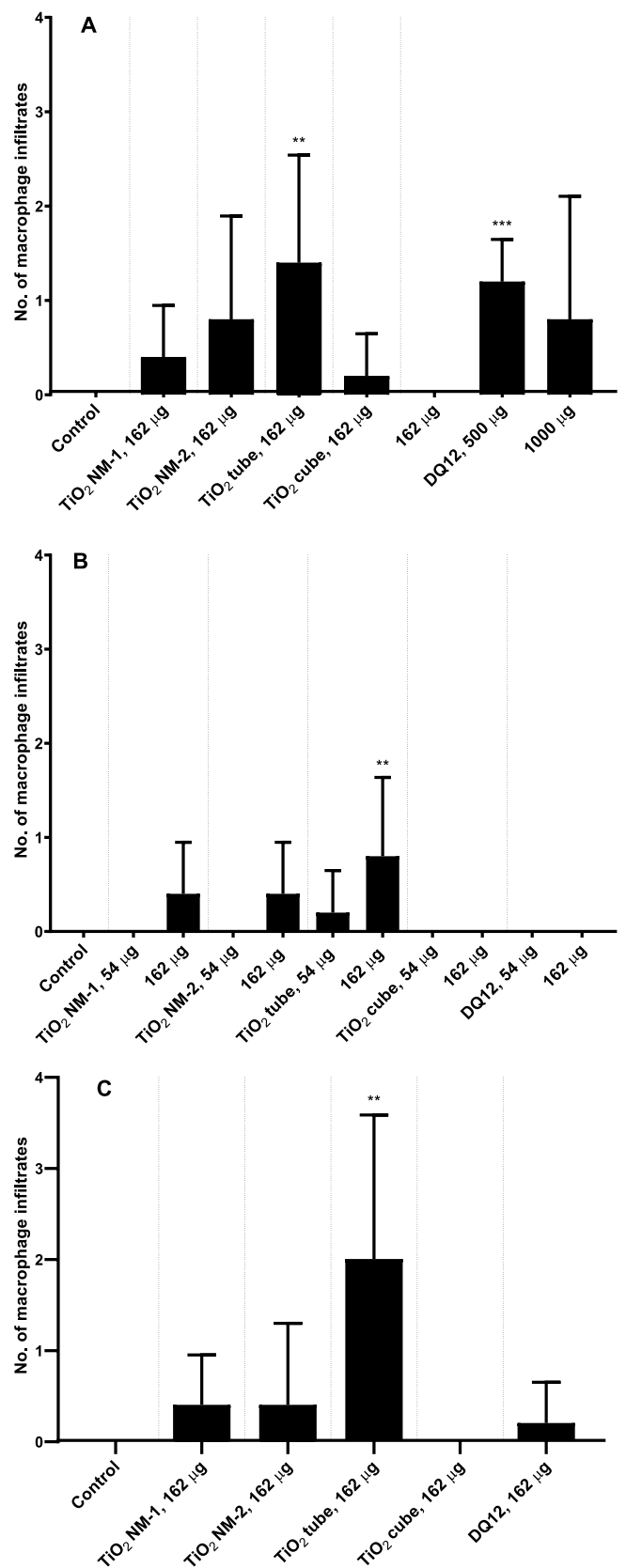


Fig. 8. Number of macrophage infiltrates based on histopathological evaluation of the lung tissue (A) 28 days, (B) 90 days (C) 180 days after a single i.t. instillation of TiO₂ NMs and DQ12.

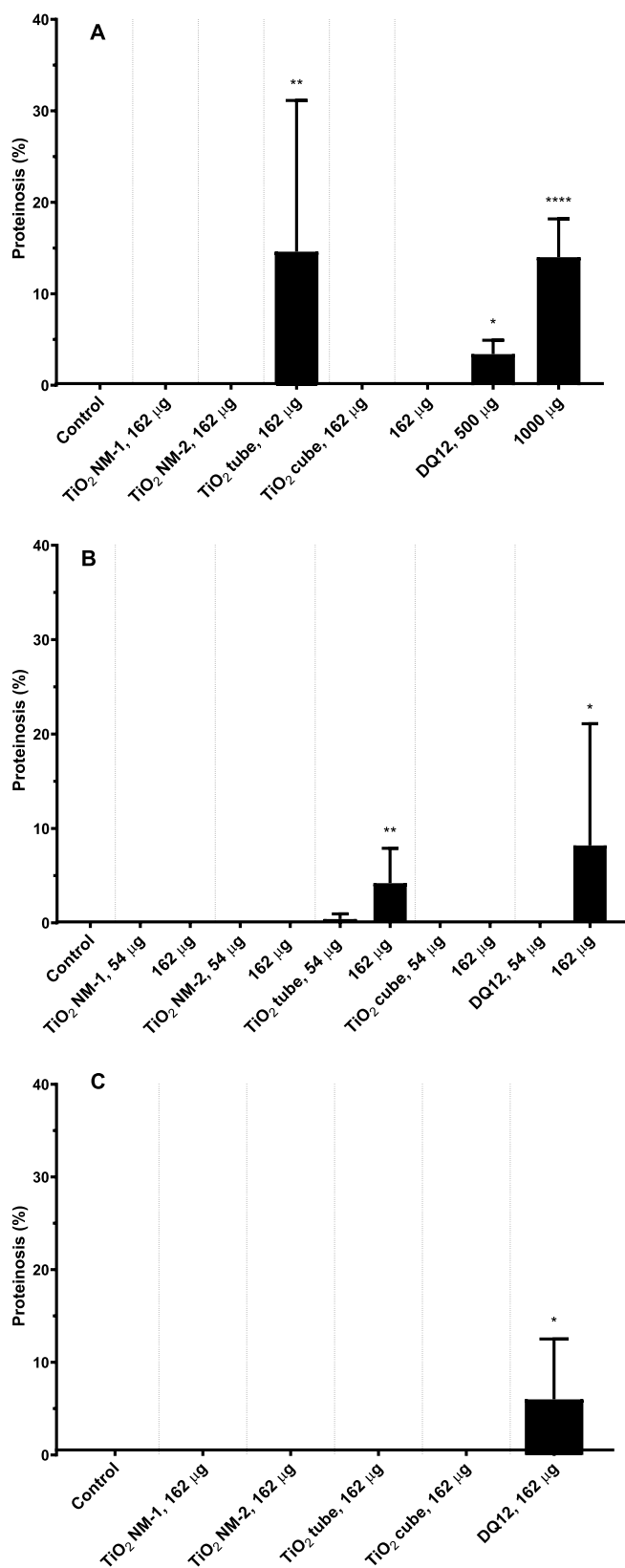


Fig. 9. Amount of protein debris (in percentage) based on histopathological evaluation of the alveolar spaces (A) 28 days, (B) 90 days (C) 180 days after a single i.t. instillation of TiO₂ NMs and DQ12.

area was 96.9 m²/g. TEM of the DQ12 sample showed that the particles had different shapes and were unequally sized. The size ranged from 50 nm to 400 nm and aggregates, exceeding few micrometers, were observed. There was no presence of micropores as calculated by the t-Plot-method for any of the anatase TiO₂ NMs.

X-ray Diffraction (XRD) patterns of TiO₂ NMs (Supplementary material, Fig. S1) showed that TiO₂ NM-1 and TiO₂ NM-2 contained a small amount of rutile. The amount of rutile calculated using Spurr's formula was 11.5 wt% and 5.6 wt%, for NM-1 and NM-2 respectively. TiO₂ NM-2 also contained some unidentified impurities (arrows in Supplementary material, Fig. S1). For TiO₂ tube and TiO₂ cube all diffraction peaks corresponded to anatase structure (Supplementary material, Fig. S1).

Dynamic Light Scattering (DLS) was used to determine the hydrodynamic number-based size distributions of TiO₂ NMs and DQ12 in the instillation suspensions (3.24 mg/ml in Nanopure water with 2% mouse serum). The NM suspensions were generally well-dispersed and the size distribution showed unimodal peaks for TiO₂ NM-1, TiO₂ cube and DQ12 at 68 nm, 38 nm and 210 nm, respectively (Supplementary material, Fig. S2). TiO₂ NM-2 was bimodally distributed with a major peak at 50 nm and a minor broad peak at 18–38 nm. In addition, TiO₂ tube was bimodally distributed with a major peak at 60 nm and a narrow peak at 21 nm (Supplementary material, Fig. S2). The intensity-based z-average size and polydispersity index (PI) are shown in the Supplementary material, Table S1. The PI was between 0.4 and 0.7 indicating some polydispersity in the suspensions.

The endotoxin contents were measured using the *Limulus* Amebocyte lysate enzyme kit (LAL) and low levels were found in all NM suspensions (Table 1).

Characterization data on the five rutile TiO₂ NMs; NRCWE-001, NRCWE-002, NRCWE-025, NRCWE-030 and UV-Titan L181, were published previously (Saber et al., 2012b; Kermanizadeh et al., 2013; Gomez et al., 2014; Halappanavar et al., 2015) and summarized in the Supplementary material, Table S2.

3.2. Acellular oxidation potential

The ability to generate reactive oxygen species (ROS) was determined using the acellular 2',7'-dichlorodihydrofluorescein diacetate DCFH₂-DA assay as described previously (Jacobsen et al., 2008). All TiO₂ NMs and DQ12 generated ROS in a dose-dependent manner (Supplementary material, Fig. S3). There were no clear differences between rutile and anatase TiO₂ NMs, except that the rutile TiO₂ NM (NRCWE-002) induced a 7-fold larger ROS level than the others did.

3.3. Visualization of TiO₂ particles in the lung tissue

To assess the distribution and persistence of the NMs in the lung, histological sections of lung tissues were imaged using enhanced darkfield microscopy (Figs. 2, 3 and 4). TiO₂ NMs show intense light scattering in enhanced darkfield and are easily detected in tissues. DQ12 does not give an optical fingerprint in neither darkfield nor brightfield and therefore cannot be detected by optical microscopy (Fig. 3F). Darkfield microscopy of lung tissues 28 days post-exposure showed foreign material in the alveolar region for the four TiO₂ NMs (Figs. 2 and 3). TiO₂ NM-1 and NM-2 had a similar focal distribution (Figs. 2D, F) and a similar appearance as micron-sized agglomerates phagocytized by macrophages or scattered in the alveolar lumen (Figs. 4A, B). For the TiO₂ tube, there were areas of the lung tissue with alveoli full of micron-sized agglomerates and acellular organic debris (Figs. 3B and 4C), whereas the TiO₂ cube primarily appeared as scattered large aggregates (Figs. 3D and 4D). The TiO₂ tube and cube were also detected as micron-sized agglomerates in macrophages (only shown for TiO₂ cube in Fig. 4D). All four TiO₂ NMs were detected in lung tissue up to 180 days post-exposure (not shown).

Table 2BAL fluid cell counts in mice 1, 3, 28 and 90 days post-exposure to 18, 54 and 162 µg TiO₂ NMs, 162 µg DQ12 and control mice.

	Control		TiO ₂ NM-1		TiO ₂ NM-2		
	0 µg	18 µg	54 µg	162 µg	18 µg	54 µg	162 µg
1 day							
Neutrophils (× 10 ³)	8.01 ± 5.95	6.57 ± 5.25	15.7 ± 10.5	49.6 ± 18.0**	9.15 ± 12.7	14.6 ± 9.66	50.2 ± 34.3**
Macrophages (× 10 ³)	53.5 ± 17.7	51.4 ± 18.8	68.2 ± 17.1	44.6 ± 8.66	45.6 ± 29.7	57.3 ± 17.6	49.3 ± 16.3
Eosinophils (× 10 ³)	0.96 ± 2.21	0.54 ± 0.80	0.56 ± 0.83	7.39 ± 7.55*	0.45 ± 1.05	0.29 ± 0.47	1.96 ± 1.70
Lymphocytes (× 10 ³)	0.75 ± 0.56	0.64 ± 0.72	1.22 ± 1.49	1.88 ± 1.96	0.37 ± 0.18	1.11 ± 0.77	1.17 ± 0.49
Epithelial (× 10 ³)	8.18 ± 4.23	10.9 ± 15.3	7.92 ± 3.64	5.38 ± 2.08	7.05 ± 7.08	9.49 ± 3.37	8.54 ± 4.86
Total BAL cells (× 10 ³)	71.4 ± 20.1	70.2 ± 34.6	93.6 ± 23.3	109 ± 31.2	60.1 ± 40.9	82.8 ± 22.1	111 ± 25.1
3 days							
Neutrophils (× 10 ³)	0.94 ± 1.25	0.64 ± 0.50	1.08 ± 0.87	11.4 ± 5.75**	0.69 ± 0.52	2.09 ± 1.87	5.53 ± 2.45*
Macrophages (× 10 ³)	52.9 ± 8.72	56.4 ± 20.3	62.8 ± 17.1	62.4 ± 16.1	57.9 ± 10.9	64.5 ± 12.5	67.4 ± 28.9
Eosinophils (× 10 ³)	0.05 ± 0.18	0.53 ± 0.42	3.89 ± 4.47	13.7 ± 13.3 [□]	0.70 ± 1.20	0.62 ± 0.66	9.89 ± 7.16 [□]
Lymphocytes (× 10 ³)	0.90 ± 0.65	0.59 ± 0.70	1.93 ± 1.36	7.93 ± 8.64*	0.52 ± 0.38	1.06 ± 0.82	3.16 ± 2.20
Epithelial (× 10 ³)	8.93 ± 9.08	8.72 ± 5.89	5.17 ± 3.28	5.49 ± 1.92	8.59 ± 3.80	13.9 ± 18.1	9.31 ± 9.50
Total BAL cells (× 10 ³)	63.9 ± 13.1	66.9 ± 24.7	74.9 ± 19.1	101 ± 29.8	68.4 ± 13.6	82.1 ± 27.2	95.3 ± 41.4
28 days							
Neutrophils (× 10 ³)	1.83 ± 2.41	1.74 ± 1.15	0.98 ± 0.67	2.82 ± 1.77	0.48 ± 0.59	1.28 ± 1.03	1.14 ± 0.75
Macrophages (× 10 ³)	49.5 ± 10.6	60.6 ± 25.5	62.0 ± 8.51	52.9 ± 12.2	55.0 ± 14.9	45.6 ± 22.2	46.2 ± 18.6
Eosinophils (× 10 ³)	0.47 ± 1.00	3.19 ± 4.29	0.59 ± 1.55	0.25 ± 0.51	0.17 ± 0.35	1.13 ± 2.56	0.08 ± 0.21
Lymphocytes (× 10 ³)	1.88 ± 2.47	4.28 ± 3.13	2.91 ± 2.75	5.32 ± 3.56	0.90 ± 0.49	0.98 ± 0.75	2.46 ± 2.56
Epithelial (× 10 ³)	7.85 ± 6.56	14.8 ± 10.9	6.74 ± 4.31	5.10 ± 4.08	6.43 ± 3.31	10.5 ± 14.9	6.99 ± 3.20
Total BAL cells (× 10 ³)	61.5 ± 17.1	84.7 ± 26.5	73.2 ± 7.57	66.4 ± 13.3	63.0 ± 18.0	59.4 ± 26.7	56.9 ± 21.9
TiO₂ tube							
	18 µg	54 µg	162 µg	TiO₂ cube			
				18 µg	54 µg	162 µg	DQ12
							162 µg
1 day							
Neutrophils (× 10 ³)	17.9 ± 6.05	115 ± 38.1#	186 ± 74.3 [□]	7.45 ± 4.17	4.66 ± 5.14	22.5 ± 15.1	116 ± 69.2#
Macrophages (× 10 ³)	55.6 ± 6.93	31.7 ± 10.2	22.8 ± 13.1*	54.1 ± 9.40	50.4 ± 22.2	55.9 ± 27.9	39.4 ± 10.3
Eosinophils (× 10 ³)	1.92 ± 3.70	6.08 ± 5.24*	10.3 ± 7.93**	0.04 ± 0.11	1.76 ± 3.22	2.00 ± 2.30	19.2 ± 30.2*
Lymphocytes (× 10 ³)	0.83 ± 0.73	1.85 ± 1.70	2.05 ± 1.26	0.98 ± 0.48	1.22 ± 1.21	1.52 ± 1.20	2.55 ± 3.10
Epithelial (× 10 ³)	7.89 ± 2.30	5.53 ± 2.72	4.56 ± 2.61	5.62 ± 2.38	8.95 ± 14.6	7.90 ± 3.23	5.84 ± 3.81
Total BAL cells (× 10 ³)	84.2 ± 15.6	160 ± 38.1**	226 ± 93.6 [□]	68.2 ± 8.29	67.0 ± 39.7	89.8 ± 31.5	183 ± 97.2**
3 days							
Neutrophils (× 10 ³)	1.40 ± 1.06	26.9 ± 13.8 [□]	120 ± 28.7#	0.46 ± 0.40	0.26 ± 0.39	1.98 ± 1.41	18.2 ± 7.25**
Macrophages (× 10 ³)	79.9 ± 12.8*	69.7 ± 31.3	81.1 ± 21.3*	57.0 ± 14.0	70.6 ± 14.3	86.4 ± 26.2*	79.1 ± 27.4
Eosinophils (× 10 ³)	6.97 ± 9.00*	52.8 ± 22.9#	43.7 ± 28.7#	0.18 ± 0.27	0.17 ± 0.22	3.34 ± 4.06	28.0 ± 12.8#
Lymphocytes (× 10 ³)	1.73 ± 1.76	9.70 ± 8.00**	13.5 ± 4.12 [□]	0.84 ± 0.42	1.60 ± 0.87	1.47 ± 1.46	9.68 ± 8.83**
Epithelial (× 10 ³)	5.75 ± 2.20	10.7 ± 9.14	8.24 ± 4.03	4.51 ± 2.25	5.50 ± 3.24	7.89 ± 4.19	9.17 ± 9.13
Total BAL cells (× 10 ³)	91.2 ± 22.7	170 ± 59.4 [□]	267 ± 32.0#	63.0 ± 15.2	78.1 ± 15.2	101 ± 31.4	144 ± 41.9 [□]
28 days							
Neutrophils (× 10 ³)	5.66 ± 5.13	8.38 ± 3.83*	37.1 ± 16.5 [□]	0.56 ± 0.51	0.97 ± 1.31	0.41 ± 0.27	4.06 ± 3.07
Macrophages (× 10 ³)	63.9 ± 14.8	75.0 ± 18.9	133 ± 48.7**	50.9 ± 16.4	47.8 ± 17.4	59.8 ± 12.4	50.4 ± 22.9
Eosinophils (× 10 ³)	2.52 ± 2.65	0.46 ± 0.98	1.76 ± 2.86	1.51 ± 2.46	4.19 ± 9.13	0.93 ± 2.45	0.18 ± 0.34
Lymphocytes (× 10 ³)	5.04 ± 3.82	18.3 ± 13.9	55.5 ± 27.5	3.81 ± 5.39	2.46 ± 3.87	0.95 ± 0.73	13.9 ± 14.9
Epithelial (× 10 ³)	7.24 ± 6.90	8.40 ± 4.14	13.0 ± 8.02	4.07 ± 1.72	5.04 ± 2.70	5.62 ± 3.62	4.09 ± 2.85
Total BAL cells (× 10 ³)	82.8 ± 8.05	111 ± 25.0*	240 ± 88.8 [□]	60.8 ± 23.3	61.4 ± 17.0	67.7 ± 15.6	72.7 ± 35.5

All values are presented as mean ± SD. A symbol (*) denotes P ≤ .05, (**) P ≤ .01, (□) P ≤ .001, (#) P ≤ .0001 compared to vehicle control.

3.4. Electron microscopy

TEM revealed that the NMs in the lungs were almost exclusively present in macrophages and mostly seen in phagosomal vesicles (Figs. 5A, C, D). As also noted in bright- and darkfield microscopy the TiO₂ cube formed large aggregates (Fig. 5B). The material in the aggregates appeared to be unchanged (Fig. 5B, insert).

The acellular organic debris associated with TiO₂ tube and DQ12 exposure visualized in darkfield microscopy was quite similar when using TEM (Fig. 6). Structures consistent with tubular myelin and lamellar bodies were identified. The findings in Fig. 6 conform to that seen in association with pulmonary alveolar proteinosis after quartz exposure (Corrin and King, 1970; Seymour and Presneill, 2002).

3.5. Histopathological analysis

The TiO₂ tube caused statistically significant increased inflammatory changes in lung tissue as defined by numbers of lymphocytic and macrophage infiltrates in the same manner as DQ12 compared to the control (Figs. 7 and 8). The other anatase TiO₂ NMs caused moderate inflammatory changes in the lung tissue. Macrophage infiltrations were not observed for DQ12 at 162 µg, in contrast to increased levels for TiO₂ tube at the same dose (Fig. 8). In addition to this a proteinaceous material was observed in the alveolar spaces of TiO₂ tube-exposed mice at day 28 post-exposure which was still present but at lower levels at day 90 post-exposure (Fig. 9). The same type of proteinaceous material was observed after exposure to DQ12, however the time course was different than for TiO₂ tube. For DQ12, at the 162 µg dose, the changes appeared later and persisted longer (Fig. 9). Increasing the dose of DQ12 to 500 µg or 1000 µg resulted in more

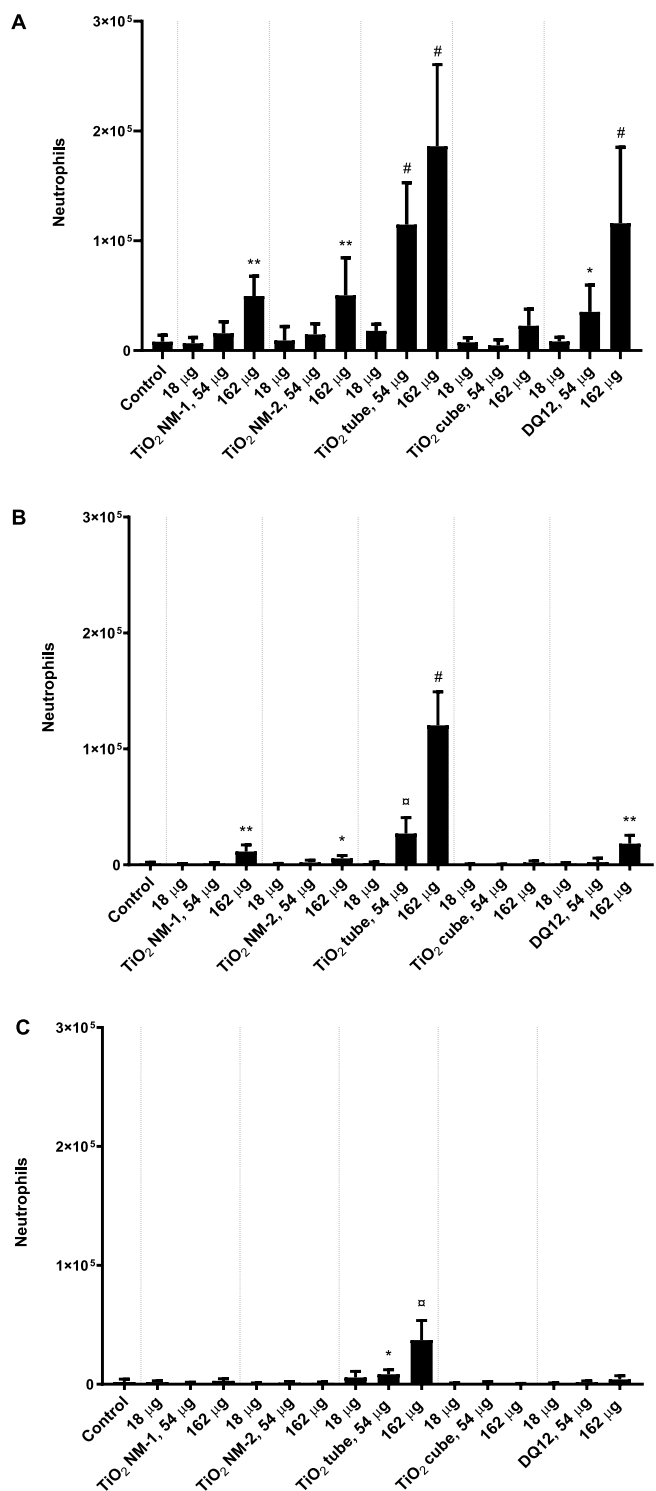


Fig. 10. Number of neutrophils detected in BAL fluid following a single i.t. instillation of 18, 54 or 162 µg of TiO₂ NMs and DQ12 measured (A) 1 day, (B) 3 days, and (C) 28 days post-exposure. All values are presented as mean ± SD. A symbol (*) denotes $P \leq .05$, (**) $P \leq .01$, (♠) $P \leq .001$, (#) $P \leq .0001$ of neutrophil levels in exposed groups versus vehicle control.

changes at day 28 post-exposure (Fig. 9A). Proteinaceous material in the alveolar spaces in quartz-exposed animals and humans are a well-known effect and are termed pulmonary alveolar proteinosis (PAP) (Corrin and King, 1970; Seymour and Presneill, 2002). No granulomas were observed in the present study (data not shown). No signs of fibrosis were observed (data not shown).

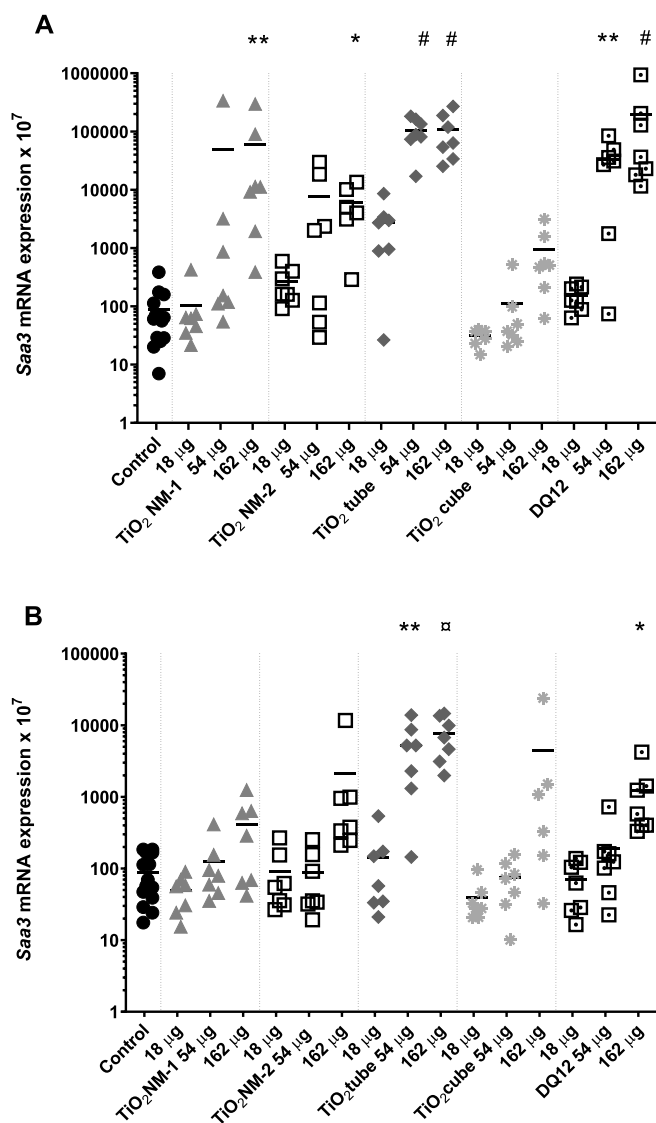


Fig. 11. Pulmonary mRNA expression levels of *Saa3* (A) 1 day and (B) 3 days after a single i.t. instillation of 18, 54 or 162 µg TiO₂ NMs and DQ12. All values are presented as mean ± SD at a logarithmic scale. A marker (*) denotes $P \leq .05$, (**) $P \leq .01$, (♠) $P \leq .001$, (#) $P \leq .0001$ compared to vehicle control.

3.6. Cell composition in bronchoalveolar lavage fluid

Recruitment of inflammatory cells in BAL fluid was assessed 1, 3, 28, 90 and 180 days post-exposure as a marker of the pulmonary inflammatory response. The distributions of total number of cells and the number of macrophages, neutrophils, eosinophils, epithelial and lymphocytes were determined (Table 2 (1, 3 and 28 days post-exposure) and Supplementary material, Table S3 (90 and 180 days post-exposure)).

Dose-dependent neutrophil influx was observed at day 1 and 3 post-exposure for DQ12 and all the anatase TiO₂ NMs with exception of TiO₂ cube (Fig. 10). The number of neutrophils decreased over time after exposure. For the TiO₂ tube, the number of neutrophils, macrophages, lymphocytes and total BAL cells was statistically significant increased at day 28 post-exposure, which were the only observed inflammatory responses at that time-point. No statistically significant inflammatory responses were observed at the lowest dose of 18 µg or at 90 days post-exposure, except for a borderline statistically significant increase in the number of lymphocytes for TiO₂ tube 90 days post-exposure (162 µg, P

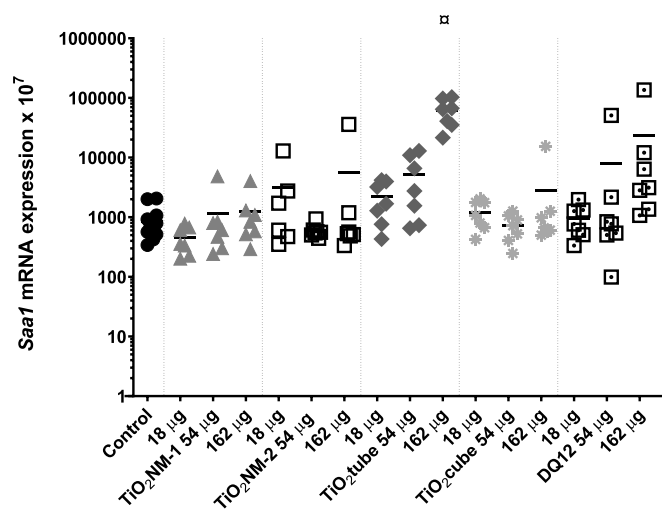


Fig. 12. Hepatic mRNA expression levels of *Saa1* 1 day after a single i.t. instillation of 18, 54 or 162 μg TiO_2 NMs and DQ12. All values are presented as mean \pm SD at a logarithmic scale. A marker (*) denotes $P \leq .05$, (**) $P \leq .01$, (#) $P \leq .001$, (#) $P \leq .0001$ compared to vehicle control.

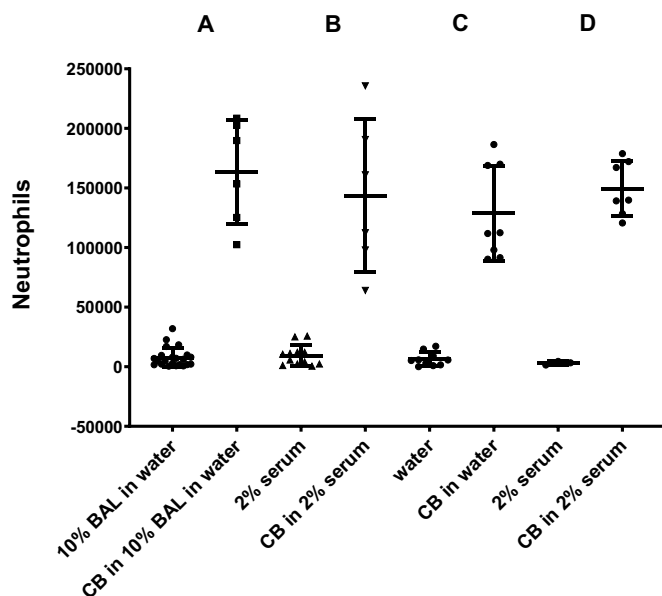


Fig. 13. Carbon black (Printex 90) was included (162 $\mu\text{g}/\text{mouse}$) in the previous studies on rutile TiO_2 NMs. A: (Saber et al., 2012a, 2012b) (UV-Titan L181); B: Saber et al. 2018 (NRCWE-025 and -030); C: Wallin et al., 2017 (NRCWE-001 and -002) and D: this study on anatase TiO_2 NMs.

value: 0.068). However, statistically significant increased levels of neutrophils (7-fold) and lymphocytes (6-fold) was seen for TiO_2 tube 180 day post exposure to 162 μg . Overall, the TiO_2 tube generated a higher inflammatory response than the other TiO_2 NMs, whereas TiO_2 cube did not generate an inflammatory response.

3.7. Acute phase response

Pulmonary and hepatic acute phase response was assessed using *Saa3* and *Saa1* mRNA expression levels as biomarkers of pulmonary and hepatic acute phase response, respectively (Poulsen et al., 2017). The *Saa3* gene expression was statistically significantly increased 1-day post-exposure compared to vehicle control at the highest dose (162 μg) for DQ12 and all anatase TiO_2 NMs except for TiO_2 cube (Fig. 11). The TiO_2 tube and DQ12 also induced statistically significantly increased

Saa3 mRNA levels at the middle dose (54 μg) 1-day post-exposure. The significant increase of *Saa3* mRNA expression was persistent at day 3 post-exposure for TiO_2 tube (54 and 162 μg) and for DQ12 (162 μg), though the level was 10 times lower than observed at day 1. A strong correlation was observed for *Saa3* mRNA expression and neutrophil influx day 1 post-exposure (Supplementary material, Fig. S4).

One day post-exposure hepatic *Saa1* mRNA expression was statistically significant increased only for TiO_2 tube at the highest exposure dose (70-fold) (Fig. 12). *Saa1* expression levels at post-exposure day 3 were not analyzed.

3.8. Comet assay

DNA strand break levels were assessed in BAL cells, lung and liver tissue by the comet assay (Supplementary material, Table S4). There were only few significant increases in DNA strand break levels. However, DNA strand break levels were also observed to be statistically significantly decreased for some of the TiO_2 NMs, especially in the lung tissue, at different time points. The observed changes were in general not dose-dependent and considered as chance findings.

3.9. Correlations between neutrophils and BET surface area

The toxicological response to anatase TiO_2 NMs was compared to previously published studies on rutile TiO_2 NMs (Supplementary material, Table S2, (Jacobsen et al., 2009; Bourdon et al., 2012; Saber et al., 2012b). Carbon black (CB), (Printex 90), was included as reference material to allow comparisons across studies. CB (162 $\mu\text{g}/\text{mouse}$) used in the previous studies on rutile TiO_2 NMs resulted in similar levels of neutrophil influx as observed in the present study on anatase TiO_2 NMs (Fig. 13).

This comparison of anatase and rutile TiO_2 NMs was performed for acute inflammation as measured by neutrophil influx into the lung (BAL fluid) adhering to the method described by Schmid and Stoeger, 2016 (Schmid and Stoeger, 2016). This approach calls for normalization of the neutrophil number to the total number of cells in the BAL (PMN in %) and normalization of the instilled NM surface area dose to the average weight of mouse lungs (0.18 g). We found strong logistic correlations between surface area dose and level of neutrophil influx 1-day post-exposure for anatase TiO_2 NMs without TiO_2 tube ($R^2 = 0.95$) and rutile TiO_2 NMs ($R^2 = 0.99$), Fig. 14A. Clearly, all anatase and all rutile TiO_2 NMs represent two distinct dose-response curves with rutile showing somewhat enhanced surface-specific inflammogenicity. Interestingly, the anatase TiO_2 tubes ($R^2 = 0.99$) show enhanced inflammogenicity matching the (spherical) rutile rather than the (spherical, cubic) anatase TiO_2 NMs toxicity. Again referring to the approach presented by Schmid and Stoeger (Schmid and Stoeger, 2016) this can be quantified by considering the equivalent doses inducing 30% PMN influx into the lung ($dose_{30\%PMN}$), which represents a severe inflammation. As seen from Fig. 14B, the inflammogenicity of anatase TiO_2 tubes ($dose_{30\%PMN} = 200 \text{ cm}^2/\text{g}$) agree within experimental uncertainties (95% confidence level, CL = ± 1.5 -fold $dose_{30\%PMN}$) with that of rutile (spherical) TiO_2 NMs (170 cm^2/g ; 110–260 cm^2/g (95% CL)), while spherical (and cubic) anatase TiO_2 NMs show a 3.2-fold less inflammogenicity ($dose_{30\%PMN} = 550$ (370–830) cm^2/g). Interestingly, also Printex 90 (P90) shows the same surface-specific inflammogenicity as rutile TiO_2 NMs ($dose_{30\%PMN} = 220 \text{ cm}^2/\text{g}$).

3.10. Multiple regression analyses

With the aim of identifying TiO_2 NMs properties important for their toxicity, the effect of dose, BET surface area, crystal phase and shape on neutrophil influx and *Saa3* expression after i.t. exposure to both anatase and rutile TiO_2 NMs was analyzed by multiple regression (Tables 3 and 4). Mass dose significantly predicted neutrophil influx on all post-exposure days. Increasing BET surface area significantly predicted

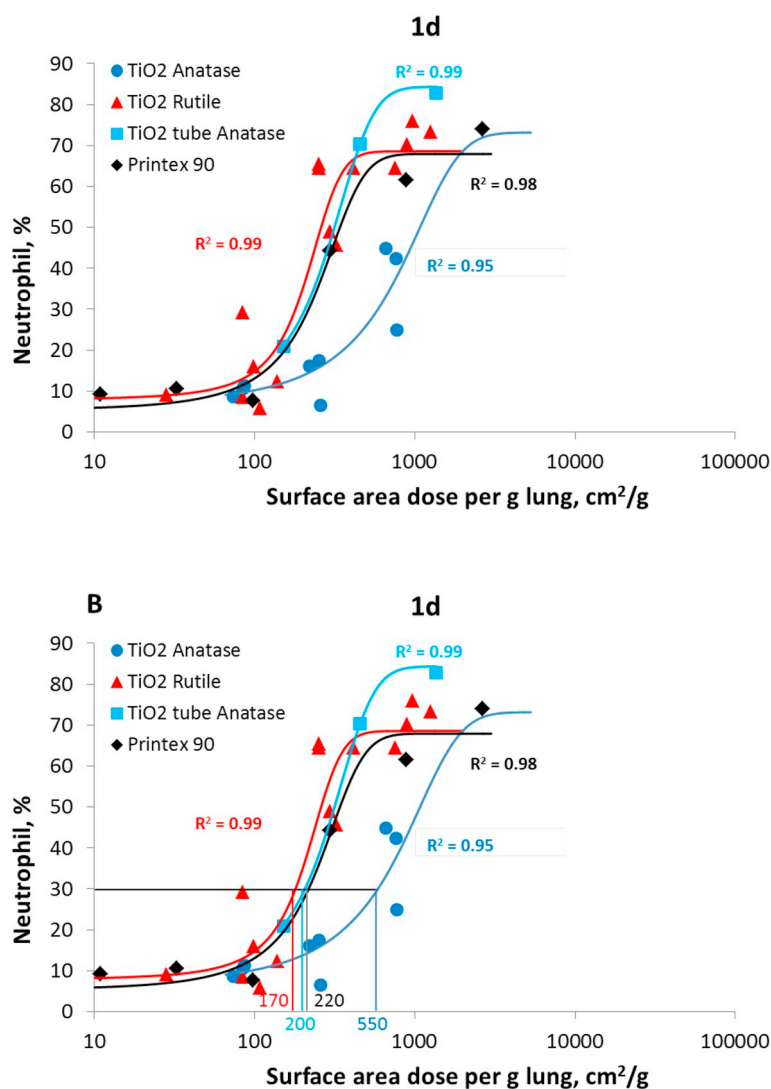


Fig. 14. Correlations between surface area dose and neutrophil influx for: A) rutile and anatase TiO₂ NMs; B) rutile and anatase TiO₂ with TiO₂ tube shown separately; C) TiO₂ tube and CNTs.

enhanced neutrophil influx 3 and 28 days post-exposure. The anatase phase predicted lower neutrophil influx 1 and 3 days after exposure. The tube shape significantly predicted increased neutrophil influx on all post-exposure days. When tube shape was included as a variable, the effect of BET surface area disappeared, suggesting that the crystal phase anatase/rutile and shape (tube) might be more important predictors than the BET surface area.

Mass dose and BET surface area (i.e. surface area dose) significantly predicted *Saa3* expression on all post-exposure days (1 and 3 days). The tube shape significantly predicted increased *Saa3* expression at both post-exposure days. When tube shape was included as a variable, again the effect of BET surface area disappeared. At 3 days post-exposure the anatase phase predicted lower neutrophil influx.

There was no effect of surface modifications (rutile NRCWE-002 and rutile UV-Titan L181), data not shown.

4. Discussion

Inhalation exposure is the gold standard for risk assessment. However, the i.t. instillation technique used in the present study is generally well-accepted for hazard ranking of different NMs (Warheit et al., 2005; Baisch et al., 2014). The i.t. instillation technique has shown an even distribution of NMs including MWCNT across most lung-

tissue sections (Mikkelsen et al., 2011; Poulsen et al., 2016). However, compared to inhalation the i.t. instillation results in a more patchy distribution as shown using fluorescent nanoparticles (Yang et al., 2019). Recently, a study concludes that neutrophil influx from i.t.- and inhalation-exposed rats correlated with the estimated pulmonary deposited surface area across both types MWCNT and types of exposure at two different time points (Gate et al., 2019). Additionally, it has been shown that global pulmonary transcriptomic pattern following i.t. instillation and inhalation of TiO₂ NMs in mice is remarkably comparable (Halappanavar et al., 2011; Husain et al., 2013), suggesting common biological responses between administration methods. The doses used in the present study reflect occupationally relevant exposure levels and allow comparison with previously performed animal studies using the same dose levels (e.g. the rutile TiO₂).

The study was designed to cover a wide NM size range. Unexpectedly, the BETs for the two TiO₂ NMs with a small and large diameter size as reported by the manufacturer (TiO₂ NM-1 and TiO₂ NM-2) showed to be similar. This was confirmed by TEM. Thus, the optimal study design having a wide BET range with larger surface areas was compromised.

The greatest overall inflammatory response was seen for neutrophil influx 1 and 3 day post-exposure, especially for the TiO₂ tube and DQ12. In addition, the total number of cells, lymphocytes and

Table 3
Multiple regression analyses.

		Neutrophil influx			
	Exposure Variable	Multiplicative Effect	Lower CL	Upper CL	Probt
1 day	Dose	1.016	1.011	1.020	< 0.0001
	Per 25% difference in BET	1.052	0.910	1.216	0.491
3 days	Anatase	0.307	0.173	0.544	< 0.0001
	Dose	1.030	1.023	1.038	< 0.0001
28 days	Per 25% difference in BET	1.370	1.094	1.716	0.0065
	Anatase	0.154	0.063	0.374	< 0.0001
	Dose	1.017	1.011	1.023	< 0.0001
	Per 25% difference in BET	1.289	1.065	1.559	0.0094
	Anatase	0.697	0.328	1.484	0.3477

		Neutrophil influx			
	Exposure Variable	Multiplicative Effect	Lower CL	Upper CL	Probt
1 day	Dose	1.016	1.011	1.020	< 0.0001
	Per 25% difference in BET	0.912	0.785	1.060	0.230
3 days	Anatase	0.191	0.107	0.342	< 0.0001
	Tube	10.23	3.783	27.65	< 0.0001
28 days	Dose	1.030	1.023	1.037	< 0.0001
	Per 25% difference in BET	1.125	0.888	1.424	0.328
	Anatase	0.082	0.033	0.202	< 0.0001
	Tube	26.36	5.439	127.7	< 0.0001
28 days	Dose	1.017	1.011	1.023	< 0.0001
	Per 25% difference in BET	1.083	0.888	1.321	0.431
	Anatase	0.389	0.180	0.839	0.016
	Tube	18.36	4.815	70.01	< 0.0001

Physiochemical parameters and the influence on neutrophil influx after exposure to anatase and rutile TiO₂ NMs in multiple regression analyses. BET surface area was transformed using log(BET)/log(1.25), so the estimated effect corresponded to a 25% increase. Significant *p*-values (*P* < .01) are highlighted in bold.

Table 4
Multiple regression analyses.

		Saa3 mRNA			
	Exposure Variable	Multiplicative Effect	Lower CL	Upper CL	Probt
1 day	Dose	1.028	1.023	1.033	< 0.0001
	Per 25% difference in BET	1.506	1.289	1.758	0.0001
3 days	Anatase	0.871	0.456	1.663	0.675
	Dose	1.017	1.013	1.020	< 0.0001
28 days	Per 25% difference in BET	1.163	1.045	1.294	0.0001
	Anatase	0.789	0.511	1.218	0.283

		Saa3 mRNA			
	Exposure Variable	Multiplicative Effect	Lower CL	Upper CL	Probt
1 day	Dose	1.028	1.023	1.033	< 0.0001
	Per 25% difference in BET	1.217	1.047	1.413	0.0107
3 days	Anatase	0.454	0.249	0.827	0.0102
	Tube	29.73	11.16	79.16	< 0.0001
28 days	Dose	1.017	1.013	1.020	< 0.0001
	Per 25% difference in BET	1.008	0.908	1.119	0.879
	Anatase	0.500	0.331	0.754	0.001
	Tube	9.928	4.983	19.78	< 0.0001

Physiochemical parameters and the influence on *Saa3* mRNA levels after exposure to anatase and rutile TiO₂ NMs in multiple regression analyses. BET surface area was transformed using log(BET)/log(1.25), so the estimated effect corresponded to a 25% increase. Significant *p*-values (*P* < .01) are highlighted in bold.

eosinophils were significantly increased for the TiO₂ tube. In general, the other anatase TiO₂ NMs were somewhat less responsive, including the TiO₂ cube, which induced less inflammation than TiO₂ NM-1 and TiO₂ NM-2 despite its 20–30% larger specific surface area. The TiO₂ cube NMs had a peculiar tendency to form large aggregates in the lungs, which could be related to the relatively modest inflammatory response. DLS data for TiO₂ cube confirms a homogeneous instillation suspension

without bigger aggregates, suggesting that this phenomenon happened in the lung. Persistent dose-dependent increases in the influx of inflammatory cells, including neutrophils, were observed up to 28 and 180 days post-exposure only for the TiO₂ tube suggesting that the difference in shape may be an important predictor for chronic lung inflammation.

We used *Saa3* mRNA levels in the lung as biomarker of the

pulmonary acute phase response and *Saa1* mRNA levels as biomarker for the hepatic acute phase response. Previous studies reported significant correlations between *Saa3* mRNA and protein levels in mice after MWCNTs exposure (Saber et al., 2014; Poulsen et al., 2015a; Poulsen et al., 2017). Consistent with the observed pulmonary inflammation the *Saa3* mRNA levels were dose-dependently increased 1 and 3 day post-exposure, again especially for the TiO₂ tube and DQ12. As in the present study, we have previously shown that pulmonary exposure to different NMs induce a pulmonary acute phase in parallel with the pulmonary inflammatory response (Bourdon et al., 2012; Saber et al., 2013; Poulsen et al., 2015a; Husain et al., 2013; Poulsen et al., 2017; Hadrup et al., 2019a; Hadrup et al., 2019b; Barford et al., 2020).

The strong pulmonary inflammation of TiO₂ tube was accompanied by a hepatic acute phase response measured as increased *Saa1* mRNA levels at the highest dose 1-day post-exposure (70-fold increase). In previous studies, instillation of MWCNTs led to increased levels of both *Saa1* mRNA and SAA1 protein (Poulsen et al., 2017), whereas CB and rutile UV-Titan L181 TiO₂ did not (Saber et al., 2009; Halappanavar et al., 2011). The hepatic acute phase response may reflect the stronger surface area-dependent pulmonary inflammation induced by the TiO₂ tube, as pulmonary exposure to NRCWE-030 (spherical, rutile TiO₂) with a similarly large BET surface area (139 m²/g) also induced hepatic *Saa1* mRNA levels (Modrzynska et al., 2018a, 2018b). The stronger response by the TiO₂ tube might be explained by a stronger adsorption of the TiO₂ tube onto cell membranes leading to the formation of membrane-wrapped nanoparticles allowing their relocation and diffusion into systemic circulation as recently shown (Urbancic et al., 2018).

DNA damage has been suggested to be caused by particle mediated ROS production and/or as a secondary consequence of an inflammatory response (Møller et al., 2010). The standard comet assay has been used in TiO₂ genotoxicity studies in cell cultures and animal models with mixed results, which might be due to physicochemical differences (Møller et al., 2017). The few changes (both increases and decreases) in DNA damage levels in BAL cells, lung and liver observed in the present study were considered as chance findings. Contrary, the rutile NMs have been able to induce DNA strand breaks in our previous animal studies (Saber et al., 2012b; Wallin et al., 2017). The acellular oxidation potential (DCFH₂-DA assay) of anatase and rutile TiO₂ NMs were in general similar, and cannot solely explain the difference in genotoxicity, although the rutile NRCWE-002 with the highest level of 2',7'-dichlorofluorescein (DCF) also generated the highest level of DNA damage (Wallin et al., 2017).

Mice exposed to TiO₂ tube and the positive control DQ12 showed increased levels of macrophage and lymphocytic infiltrations as compared to the other NMs. None of the studied particles, including DQ12, induced fibrotic changes. However, both DQ12 and TiO₂ tube exposure resulted in accumulation of alveolar matter that was compatible with pulmonary alveolar proteinosis (PAP) (Corrin and King, 1970; Seymour and Presneill, 2002). Electron microscopy confirmed that the alveolar matter for both TiO₂ tube and DQ12 exposure was similar and that it conforms to the description of PAP in the literature (Crouch et al., 1991). In humans, PAP is a rare condition of mostly unknown etiology, but hereditary forms of this condition are known to exist (Seymour and Presneill, 2002). The secondary type of PAP is known to occur after exposure to crystalline quartz but it has also been associated with exposures to other mineral particles (talc, cement and kaolin) and metal particles (aluminum, titanium and indium) (Borie et al., 2011). Interestingly at the dose of 162 µg TiO₂ tube, there was a substantial amount of proteinosis 28 days post-exposure. However, this reaction subsided during time and had disappeared at 180 days post-exposure. In contrast, DQ12 at the same mass dose did not cause accumulation of alveolar matter until 90 days post-exposure and there was still substantial amounts 180 days post-exposure suggesting that the effect of DQ12 is more persistent than that of TiO₂ tube. The persistence of quartz-induced inflammation has been noted previously (Brown et al., 1991).

However, it has to be noted that the BET surface area of TiO₂ tube is 15-fold larger than that of DQ12, i.e. for equivalent surface area dosing 2400 µg of DQ12 would have had to be dosed.

The length of the TiO₂ tube is not of the magnitude that it could be expected to cause asbestos-like effects as do some MWCNT. It is interesting that the effect of the TiO₂ tube shows similarities to DQ12 in terms of lymphocytic tissue inflammation and the ability to cause PAP. The effects of quartz have been linked to phagosomal destabilization (Hornung et al., 2008) and it would seem possible that the short fiber shape of the tube affects the lysosomes in a similar way (Köbler et al., 2015). The materials in this study definitely end up in lysosome/phagosome structures of macrophages as seen in the EM pictures. We have previously observed PAP in rats i.t.-exposed to a short CNT (NM-403) of similar dimensions as the TiO₂ tube (Gate et al., 2019).

Prolonged pulmonary effects 28-day post exposure have been shown for various CNTs (Poulsen et al., 2013; Poulsen et al., 2015b; Poulsen et al., 2016; Poulsen et al., 2017), in addition to long retention time in the lungs 28 and 90 days post-exposure (Poulsen et al., 2016a). We recently detected MWCNTs in the lungs of mice one year after a single exposure to 54 µg MWCNT by i.t. instillation suggesting that MWCNTs are biopersistent (Knudsen et al., 2019) as also concluded in review of CNT biodistribution (Jacobsen et al., 2017). Previous mice studies have shown dose-dependent deposition and sustained retention of TiO₂ in the lungs over 28 days (Husain et al., 2013) as well as in the liver 180 day after i.t exposure (Modrzynska et al., 2018a; Modrzynska et al., 2018b). Whether the long-lasting inflammation is due to a longer retention time is unclear, but all four anatase TiO₂ NMs were still present in lungs 180 days post exposure. However, it is not possible to apply a quantitative approach, as it would require a very accurate sectioning to compare the exact same lung region across the samples.

The crystal phase of TiO₂ has been suggested to be important for the toxicological response, where rutile TiO₂ has been considered as an inert form, whereas anatase has been considered as a more active form of TiO₂ (Johnston et al., 2009). Our results show that rutile TiO₂ NMs induces a higher inflammatory response, in terms of neutrophil influx, than similar surface area doses of anatase TiO₂ NMs. A review of in vitro and in vivo TiO₂ toxicity studies concluded that anatase TiO₂ NMs generally were more toxic in terms of cytotoxicity, cell damage, ROS production and inflammation than rutile TiO₂ NMs (Johnston et al., 2009). This was based on relatively few studies investigating the importance of crystallinity. In contrast, a recent toxicogenomic study demonstrated that the overall inflammatory and transcriptional response of mice exposed to anatase TiO₂ NMs was less compared with rutile TiO₂ NMs (Rahman et al., 2017). However, the underlying mechanisms of the different responses related to crystalline structure are unknown.

In the present study, neutrophil influx correlated closely with surface area dose for both anatase (excluding the TiO₂ tube) and rutile TiO₂ NMs, but rutile showed 3.2-fold more neutrophil influx by surface area. CB has been included in our animal studies, as an internal reference particle, to allow comparison of results across studies. CB (162 µg/mouse) used in the previous studies on rutile TiO₂ NMs resulted in similar levels of neutrophil influx as observed in the present study on anatase TiO₂ NM, despite differences in vehicle composition. We are therefore confident in doing such comparisons. We have previously reported dose-dependent neutrophil influx that correlate with BET surface area of both fine and nano-sized TiO₂ and CB (Saber et al., 2018; Saber et al., 2012b). A recent paper retrospectively analyzed animal data from mice and rats on the pulmonary toxicity of i.t. instilled NMs and concluded that the BET surface area was the biologically most effective dose metric for acute pulmonary inflammation and that so-called low solubility, low toxicity (LSLT) NMs have an equivalent 30% PMN (neutrophil) influx dose range of 175 [85–405] cm²/g lung (Schmid and Stoeger, 2016). Consequently, the neutrophil data presented here indicate that both CB as well as rutile and anatase TiO₂ NMs belong to the LSLT class of NMs.

The inflammatory response of the TiO₂ tube clearly clusters more

with rutile than anatase TiO₂ NMs, suggesting that the tube shape is a driver for the effect. In addition, the effect of BET surface area disappeared when the tube was included as a variable in the multiple regression analysis. In light of the substantial evidence for surface area being a strong predictor of inflammation for TiO₂ and carbonaceous NMs (Stoeger et al., 2007), this may indicate that the current data set has too limited variation in specific surface area (74–97 m²/g), with the TiO₂ tube as the only anatase NM with a substantially larger BET surface area (154 m²/g), and thus, more studies are needed to clarify this. However, both a previous (Rahman et al., 2017) and the present study suggest that in general anatase induces less inflammation than rutile NMs when normalized to surface area, however outliers like TiO₂ tube, which do not fit into the crystallinity paradigm, are possible.

In conclusion, anatase TiO₂ NMs with varying physicochemical properties induced pulmonary inflammation and pulmonary acute phase response, but no genotoxicity in mice after i.t. exposure. All four anatase TiO₂ NMs induced similar inflammatory responses when surface area was used as dose metrics, although inflammatory and acute phase response was greatest and more persistent for the TiO₂ tube. Lowest response was observed for the TiO₂ cube, which might be due to the formation of large aggregates in the lungs. Histopathological changes were observed for both TiO₂ tube and DQ12 and interestingly the effect of the TiO₂ tube was more similar to DQ12 than the other anatase TiO₂ NMs in terms of persistence and the ability to cause PAP, indicating a qualitative difference related to the tube shape. Comparison with previously published data on rutile TiO₂ NMs indicated that the rutile TiO₂ NMs were more inflammatory in terms of neutrophil influx than anatase TiO₂ NMs when normalized to total deposited surface area. BET surface area strongly correlated with neutrophil influx for both crystal phases. Multiple regression analyses indicated that BET surface area, crystalline structure and tube shape are potentially important predictors for pulmonary inflammation and acute phase response. Overall, the results suggest that specific surface area, crystal phase and shape of TiO₂ NMs are important predictors for the observed pulmonary effects of TiO₂ NMs.

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Authors' contribution

UV and KBK designed the study. KBK collected the in vivo data. JS, PU, TK and MG synthesized TiO₂ tube and TiO₂ cube and performed physicochemical characterization of the particles (TEM, XRD). HW, EV and SS analyzed and interpreted the histopathology data (light and electron microscopy). TB analyzed and interpreted the histopathology and particle distribution (light and enhanced darkfield microscopy). AMM did the endotoxin measurements. NRJ and IKW did the acellular ROS measurements. SSP performed the multiple regression analyses. PHD, KBK, UV, HW, OS and YD analyzed and interpret the data. PHD drafted the manuscript. All authors contributed to the writing of the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

All authors declare no competing interest.

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Appendix A. Supplementary data

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