Titanium oxide nanoparticle instillation induces inflammation and inhibits lung development in mice

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1Department of Pediatrics, University of Alabama at Birmingham, Birmingham, Alabama; 2Department of Molecular and Cellular Pathology, University of Alabama at Birmingham, Birmingham, Alabama; 3Department of Cell, Developmental, and Integrative Biology, University of Alabama at Birmingham, Birmingham, Alabama; 4Department of Physics, University of Alabama at Birmingham, Birmingham, Alabama; 5Department of Medicine, University of Alabama at Birmingham, Birmingham, Alabama; 6Department of Anesthesiology, University of Alabama at Birmingham, Birmingham, Alabama; 7Department of Physiology and Biophysics, University of Alabama at Birmingham, Birmingham, Alabama; and 8Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama

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Ambalavanan N, Stanishevsky A, Bulger A, Halloran B, Steele C, Vohra Y, Matalon S. Titanium oxide nanoparticle instillation induces inflammation and inhibits lung development in mice. Am J Physiol Lung Cell Mol Physiol 304: L152–L161, 2013. First published December 7, 2012; doi:10.1152/ajplung.00013.2012.—Nanoparticles are used in an increasing number of biomedical, industrial, and food applications, but their safety profiles in developing organisms, including the human fetus and infant, have not been evaluated. Titanium oxide (TiO2) nanoparticles, which are commonly used in cosmetics, sunscreens, paints, and food, have been shown to induce emphysema and lung inflammation in adult mice. We hypothesized that exposure of newborn mice to TiO2 would induce lung inflammation and inhibit lung development. C57BL/6 mice were exposed to TiO2 (anatase; 8–10 nm) nanoparticles by intranasal instillation as a single dose on postnatal day 4 (P4) or as three doses on postnatal days 4, 7, and 10 (each dose = 1 µg/g body wt). Measurements of lung function (compliance and resistance), development (morphometry), inflammation (histology; multiplex analysis of bronchoalveolar lavage fluid for cytokines; PCR array and multiplex analysis of lung homogenates for cytokines) was performed on postnatal day 14. It was observed that a single dose of TiO2 nanoparticles led to inflammatory cell influx, and multiple doses led to increased inflammation and inhibition of lung development without significant effects on lung function. Macrophages were noted to take up the TiO2 nanoparticles, followed by polymorphonuclear infiltrate. Multiple cytokines and matrix metalloproteinase-9 were increased in lung homogenates, and VEGF was reduced. These results suggest that exposure of the developing lung to nanoparticles may lead to ineffective clearance by macrophages and persistent inflammation with resulting effects on lung development and may possibly impact the risk of respiratory disorders in later life.

Infant; newborn; inflammation; vascular endothelial growth factor (VEGF); cytokines; metalloproteinase-9

NANOPARTICLES (NPs) are used in an increasing number of biomedical, industrial, and food applications, leading to increasing concerns about their safety (27). Most nanotoxicology research has focused on in vitro systems, but recent in vivo studies have shown unique biodistribution, clearance, immune response, and metabolic responses, depending upon the physical and chemical properties of the NPs (44). A lack of correlation between in vitro and in vivo studies on NPs has also been demonstrated (34), indicating the need for more in vivo studies. Titanium oxide (TiO2) NPs are commonly used in cosmetics, paint, plastics, and multiple other applications and have served as the archetypal NP for evaluating pulmonary toxicity of NPs (21, 27). TiO2 NPs have been shown to induce an asthmatic response (19) and emphysema and lung inflammation in adult mice (3, 8, 31), and inhaled NPs have been shown to be distributed in various organs, including the liver, testis, spleen, and lung, and penetrate the blood-brain barrier into the brain (23). It has been shown that TiO2 NPs induce expression of multiple genes in lungs of pregnant but not control mice (22) and increase asthma susceptibility (14). Neurobehavioral alterations (18) have also been observed in mice exposed prenatally to TiO2 NPs, suggesting that pregnant animals and the developing fetus are vulnerable populations to NP exposure. In humans, the stage of alveolar development extends from about 32–36 wk of gestation through infancy and early childhood, with most alveoli developing after birth, and in general parallels lung development of mice in the first 2 postnatal wk (7, 37). We hypothesized that exposure of the developing lung to TiO2 NPs would induce lung injury and inflammation and result in inhibition of lung development.

MATERIALS AND METHODS

All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of University of Alabama at Birmingham (APN 100808884) and were consistent with the PHS policy on Humane Care and Use of Laboratory Animals (Office of Laboratory Animal Welfare, Aug. 2002) and the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, 1996). All experiments, unless otherwise specified, were done with a minimum of six mice from at least two litters for each experimental condition.

Development of a technique for NP administration to the newborn mouse lung. Because it is technically challenging to deliver NPs by inhalation to newborn mice, we developed a technique to deliver NPs by intranasal instillation for distal pulmonary delivery. Initially, 4-day-old mouse pups (P4) were held upright and different volumes (either 1, 3, 5, 10, 15, 20, 25, 30, 40, or 50 µl) of blue dye (Trypan blue) were applied to the nostrils. After the entire volume was aspirated, the pup was euthanized and the pharynx, thorax, and abdominal cavity were opened and inspected. It was observed that small volumes (<10 µl) were deposited in the nostrils and pharynx, whereas much larger volumes (40–50 µl) led to pulmonary and...
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Fig. 1. Qd705 nanoparticle instillation leads to pulmonary deposition with accumulation within macrophages. A: transmission electron microscopy images of Qd705 suspension indicating a shape and size similar to titanium oxide (TiO2) nanoparticles (×67,000; calibration bar = 20 nm) (B and C) postnatal day (P) mice were exposed to an application of either 5 μl of PBS (control) (B) or Qd705 (C) on the nostrils, followed by 25 μl of PBS. In vivo imaging was then performed 2 h later by excitation using white light, with emitted wavelengths captured via an infrared sensitive camera with a filter transmitting wavelengths beyond 690 nm. Control mice did not have any light emission, whereas Qd705 mice were observed to have light emission from the thorax, with trace emission from the stomach and nostrils. D and E: representative photomicrographs of unstained sections by fluorescence microscopy (Qd705: orange; tissue autofluorescence: green) 2 h after Qd705 instillation at ×100 (D); calibration bar = 250 μm, and at ×400 [E and F; hematoxylin and eosin (H&E) stain; calibration bars = 50 μm] demonstrated that most Qd705 is within alveolar macrophages, with rare tiny aggregates on epithelium. Br, bronchi; PA, pulmonary artery.

Table 1. Analysis of inflammatory cytokines and receptors from mice exposed to vehicle or TiO2

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene Symbol</th>
<th>Fold Change TiO2/Control</th>
<th>95% CI</th>
<th>Fold Up- or Downregulation of TiO2 vs. Control</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemokine (C-C motif) ligand 24</td>
<td>Cc24</td>
<td>2.19</td>
<td>(1.25, 3.13)</td>
<td>2.19</td>
<td>0.03</td>
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<tr>
<td>Chemokine (C-C motif) ligand 8</td>
<td>Cc8</td>
<td>4.39</td>
<td>(0.36, 8.42)</td>
<td>4.39</td>
<td>0.006</td>
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<tr>
<td>Chemokine (C-C motif) receptor 5</td>
<td>Ccr5</td>
<td>2.24</td>
<td>(1.40, 3.08)</td>
<td>2.24</td>
<td>0.01</td>
</tr>
<tr>
<td>Chemokine (C-X-C motif) ligand 7</td>
<td>Cxcl9</td>
<td>2.58</td>
<td>(1.81, 3.35)</td>
<td>2.58</td>
<td>0.002</td>
</tr>
<tr>
<td>Interleukin 11</td>
<td>Ili1</td>
<td>0.31</td>
<td>(0.20, 0.42)</td>
<td>0.31</td>
<td>0.02</td>
</tr>
<tr>
<td>Interleukin 1 receptor, type II</td>
<td>Ili2</td>
<td>2.46</td>
<td>(1.75, 3.17)</td>
<td>2.46</td>
<td>0.01</td>
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<tr>
<td>Integrin alpha M</td>
<td>Itgam</td>
<td>2.05</td>
<td>(1.41, 2.69)</td>
<td>2.05</td>
<td>0.02</td>
</tr>
<tr>
<td>Lymphotrocin B</td>
<td>Ltb</td>
<td>0.34</td>
<td>(0.12, 0.56)</td>
<td>0.34</td>
<td>0.01</td>
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<tr>
<td>Secreted phosphoprotein 1</td>
<td>Spp1</td>
<td>3.82</td>
<td>(2.50, 5.14)</td>
<td>3.82</td>
<td>0.002</td>
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<tr>
<td>Actin, beta (Housekeeping gene)</td>
<td>Actb</td>
<td>1</td>
<td>(1.00, 1.00)</td>
<td>1</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Real-time PCR microarray analysis of 84 key mouse inflammatory cytokines and receptors from lung homogenates of mice exposed to vehicle (Control) or 3 doses of titanium oxide (TiO2) (P4, P7, and P10) identified the following genes that demonstrated both a twofold change in gene expression and were statistically significant at P < 0.05 by t-test (n = 3 mice per group; β-actin used as housekeeping gene).

gastric deposition, with gasping and cyanosis, often accompanied by death of the pup. Volumes of 25–30 μl were well tolerated, and dye was seen in distal regions of both lungs, with minimal volumes in the stomach.

It is important to assess whether NPs delivered to the lungs are dispersed uniformly in the lung. To confirm that NPs administered by techniques similar to Trypan blue would reach the distal lung, we used fluorescent quantum dots (Qd705; Qtracker 705 nontargeted quantum dots; excitation/emission 405–665/705 nm; Q21061MP; Molecular Probes, Eugene, OR) to track distribution of the administered agent to the lungs. Because the TiO2 particles are NPs below the resolution of light microscopy, it is not possible to directly visualize them and evaluate distribution in the lung by light microscopy unless they are in large clumps. Therefore, we used Qd705, which are quantum dots of similar size [5–10 nm; based on transmission electron microscopy (transmission EM) measurements; Fig. 1] to the TiO2 NPs that we used. Five microliters of Qd705 were instilled, followed by 25 μl of PBS. Two hours after instillation, mice were subjected to in vivo imaging using excitation with a fiberoptic light source with white light, and the emission visualized using an infrared sensitive camera with a filter allowing only >690 nm (Fig. 1). Mice pups were then euthanized at either 2 h or 24 h after administration, and lungs were fixed in inflation for histology. Lung sections were visualized by fluorescence imaging, which confirmed that Qd705 was mostly present in lung macrophages by 2 h (with similar findings at 24 h), with a few particles in the epithelial surface (Fig. 1).

TiO2 NP preparation. Anatase TiO2 NPs (size ~6 nm) were prepared and characterized by Dr. Stanishevsky in the Laboratory for Nanoparticle Research in the Center for Nanoscale Materials and Biointegration, Department of Physics, at the University of Alabama at Birmingham. TiO2 NPs were prepared with 3:1 mixture of titanium isobutanol, Ti(Obu)4, and isopropanol as precursor. This mixture was slowly added to ultrapure water under stirring, and the molar ratio of water to Ti(Obu)4 was 150. Then the solution was kept under reflux condition at 75°C for 3 doses of titanium oxide (TiO2) (P4, P7, and P10) identified the following genes that demonstrated both a twofold change in gene expression and were statistically significant at P < 0.05 by t-test (n = 3 mice per group; β-actin used as housekeeping gene).
24 h. The resulting milky suspension was centrifuged and the supernatant was substituted with ultrapure bacteria-free water at least five times and then ultrasonicated for 1 h. This suspension was further diluted to desired concentrations and ultrasonicated again, leading to transparent stable colloid of primary anatase particles. We performed analysis for endotoxins using the ToxinSensor Chromogenic LAL Endotoxin Assay Kit (GenScript USA, Piscataway, NJ) on the NP suspension and did not find significant quantities of endotoxin.

Animal model. Timed pregnant C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA) or bred in-house. Litters of C57BL/6 mice were exposed to the NP suspension on postnatal day 4 (single-dose experiment) or postnatal days 4, 7, and 10 (multiple-dose experiment), and compared with mice exposed to vehicle (sterile PBS) on postnatal days 4, 7, and 10. The NP suspension was sonicated for 30 s just prior to instillation, and 5 μl of the suspension (dose equivalent to 1 μg/g body wt) were placed using a P10 pipette on the nostrils of the mouse pup that was held upright. After the suspension was aspirated over the next 20–30 s, 25 μl of PBS were then applied over the nostrils, which were then aspirated over the next minute. The dose that we used (1 μg/g in the newborn mouse, or 1 mg/kg) was similar to (or slightly lower than) those used in previous literature in adult rodent in vivo models for either intratracheal instillation (21, 36, 39, 41) or oropharyngeal instillation (19). It must be noted that the average adult in the United States is currently exposed to about 1 mg Ti per kilogram per day in food (by ingestion) and personal care products (toothpastes, sunscreens), with children having a higher exposure (42).

Analysis of lung function. At 14 days of age, the mice were sedated with ketamine/xylazine and pulmonary function was evaluated on a flexiVent as previously described (28–30). Briefly, a 24-gauge Angiocath (BD Angiocath-N Autogard; 24 GA 0.56 in or 0.7 × 14 mm, made of FEP polymer; Becton Dickinson Infusion Therapy Systems, Sandy, UT) was inserted into the trachea and fixed with a ligature of 3-0 silk. The flexiVent apparatus (SCIREQ, Montreal, Canada) equipped with a Module 1 was used to perform measurement maneuvers including perturbations (predefined pressure of volume waveforms) such as forced oscillations, using room air in the closed-chest animal. The tidal volume was set at 6 ml/kg, similar to that clinically used, with a respiratory rate of 150/min. Tube calibration was done with the tracheal cannula to be used on the flexiVent, before each experiment. It must be noted that the use of a 24-gauge catheter is not optimal for lung function measurement, but this is dictated by the small lumen of the mouse trachea at 2 wk of age. We carried out additional experiments on adult mice comparing 24-gauge plastic catheters to the standard 18-gauge cannulas for lung function measurement. Although the Rt (tube resistance) was obviously very different (18-gauge: 0.50 cmH2O·s/kg·L vs. 24-gauge: 1.97 cmH2O·s/kg·L), we obtained relatively consistent lung function data regardless of cannula size in all adult mice tested (±5–15% variation in compliance and resistance). Because mice at 14 days are too small to insert the 18-gauge cannula, we could not compare lung function data using the 24-gauge catheter to the standard 18-gauge cannula in mice at that age.

Measurements made included total resistance (R; which encompasses Rn, G, and chest wall resistance, which in the mouse is essentially zero), compliance (C), elastance (E), airway resistance (Rn or Raw; Newtonian resistance, which is primarily the resistance of the central or upper airways), tissue damping (G), and tissue elastance (H) (Rn, G, and H were calculated by fitting the constant-phase model to input impedance). Lung volumes were measured by volume displacement after completion of the flexiVent measurements. Mice were then euthanized and the lungs were inflation fixed for histology, or lung homogenates were prepared for RNA and protein analysis (1, 29).

Lung histology and morphometry. Lung alveolar and vascular morphometry was performed as previously described (1, 29) using the MetaMorph software (v.6.2r4, Universal Imaging) interfaced with a Nikon TE2000U microscope equipped with a QiCam Fast Cooled high resolution CCD camera.

Alveolar development was evaluated by mean linear intercepts (MLI; an estimate of alveolar size as increased development and septation is associated with smaller alveoli) and radial alveolar counts (RAC; an estimate of the number of alveolar septae from the terminal bronchiole to the nearest connective tissue septum). Images from six random ×100 lung fields were taken from each animal, with one image from the apex, middle, and base of each lung for MLI measurement, and six RAC measurements were performed on each animal.

Vascular morphometry was done on pulmonary arteries, defined as vascular structures that accompanied airways and were between 20 and 150 μm in external diameter. At least 20 pulmonary arteries from each section were evaluated. Vessels cut transversely were measured along both axes and the average wall thickness was obtained. Vessels cut obliquely or longitudinally were measured along the short axis. The wall thickness (%) of each artery was expressed as a percentage of the vessel diameter.

Fig. 2. TiO2 nanoparticle accumulation within the newborn mouse lung: A: TiO2 nanoparticles are noted in clumps within bronchiole and alveoli (white arrows) and as multiple granular opacities within macrophages (white arrowheads) at 1 h after administration (×400, H&E stain; calibration bar = 50 μm). B: TiO2 nanoparticles within macrophages (red arrow) in lung sections from P14 mice exposed to TiO2 nanoparticles on P4, P7, and P10 (×1,000, H&E stain; calibration bars = 20 μm).
Inflammatory cell infiltrate was qualitatively evaluated in CD68-stained sections (macrophage marker; rabbit polyclonal antibody at 1:100 from Abbiotec, San Diego, CA followed by DAKO EnVision+ System-HRP secondary antibody and staining kit, Carpinteria, CA) and with neutrophils identified by multilobar nuclear morphology in hematoxylin and eosin (H&E)-stained sections, by using autofluorescence of cytoplasm to highlight nuclear morphology (neutrophil-specific immunohistochemical staining in mice with multiple antibodies including MPO did not prove reliable). Additional lung sections were also made 1 h and 24 h following NP administration to observe initial distribution and uptake by cells. Transmission EM following inflation-fixation with EM-grade fixative (glutaraldehyde 6%-paraformaldehyde 2%) and staining with uranyl acetate and lead citrate was also done as previously described (29).

Hearts were sectioned transversely just below the level of the mitral leaflet, and the thickness of the free wall of the right ventricle (RV) compared with that of the left ventricle (LV) (RV/LV free wall thickness ratio) was determined as an index of RV hypertrophy secondary to pulmonary hypertension, as described previously (1, 29). Of note, this measure was used because the conventional RV/(LV + S) (where LV + S is weight of the LV + septum) weight ratio is not accurate in newborn mice.

Analysis of mRNA. Whole-lung tissue homogenates were used for real-time PCR microarray analysis of 84 key mouse inflammatory cytokines and receptors (PAMM-011, SABiosciences, Frederick, MD) (Table 1). RNA was extracted by using TRIzol (Invitrogen) from homogenized lung from mice at 14 days of age, treated with proteinase K and DNase I, and then quantified and reverse transcribed by use of the SYBR Green RT-PCR kit (Applied Biosystems). The real-time PCR microarray analysis was performed using the Bio-Rad iCycler System as described in the manufacturer’s literature, with controls for reverse transcription, positive PCR, and genomic DNA contamination, and normalization of gene expression values in each sample to that of β-actin in that sample. Validation of molecules identified by microarray was done by real-time RT-PCR to confirm the findings.

Analysis of protein. Newborn mouse lungs were homogenized in 1 ml of a tissue protein extraction reagent (T-PER, Pierce Biotechnology) containing complete proteinase inhibitor cocktail (Roche Diagnostics), centrifuged at 7,000 g for 5 min, and the supernatant frozen at −80°C until analysis. Protein concentrations were measured using the Bio-Rad Bradford Protein Assay (Bio-Rad). Twenty-five microliters of each sample were analyzed for 32 key mouse cytokines and chemokines by using the Milliplex MAP mouse cytokine/chemokine Premixed 32 Plex (Millipore, Billerica, MA) on a Luminex 200 platform (Luminex, Austin, TX). The standard curve range for this assay panel was 3.2–10,000 pg/ml, with interassay precision of 4–21% and intra-array precision of 3–23%. Matrix metalloproteinase-9 (MMP-9) was analyzed by gelatin zymography of lung homogenates as previously described (2).

Statistical analysis. Data were expressed as means ± SE. Data were analyzed by ANOVA to test for effects of single or multiple-dose TiO2 nanoparticles on P4, P7, and P10 (B–E) demonstrating patchy inflammatory foci primarily in peribronchiolar areas (arrows) in lung sections with TiO2 nanoparticle exposure. AD, alveolar duct.

Fig. 3. Patchy lung inflammation in mice exposed to TiO2 nanoparticles. A–E: representative photomicrographs at ×100 (A, B, C, D; H&E stain; calibration bars = 250 μm) and ×400 magnification (E; inset of D; H&E stain, calibration bar = 50 μm) from P14 mouse pups exposed to vehicle (Control) (A) or to TiO2 nanoparticles on P4, P7, and P10 (B–E) demonstrating patchy inflammatory foci primarily in peribronchiolar areas (arrows) in lung sections with TiO2 nanoparticle exposure. AD, alveolar duct.
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Administration of TiO$_2$ NPs was well tolerated, and no mortality was observed in the pups receiving either 1 or 3 doses of the NPs. No change in weight, tachypnea, cyanosis, or obvious illness was noted in the pups, compared with the vehicle controls.

NP administration causes inflammatory cell infiltrate and inhibits lung development. One hour after administration of NPs, accumulations of NPs were noted mainly within macrophages and with a few on the surface of the ciliated epithelium in airways (Fig. 2). On day 14, mice that received a single dose of TiO$_2$ NPs had patchy collections of inflammatory cells around airways and the accompanying arteries. These inflammatory cells consisted of macrophages containing accumulations of TiO$_2$ NPs surrounded by other inflammatory cells (polymorphonuclear and some mononuclear cells) (Fig. 3). Impairment of lung development was also noted in mice that received a single dose of TiO$_2$ NPs, with higher MLI and lower RAC (Fig. 4). Mice that had received three doses of TiO$_2$ NPs had similar-appearing but a larger number of inflammatory foci (Fig. 3) and also had a higher MLI and lower RAC (Fig. 4) and secondary septal crest density (data not shown) compared with vehicle-exposed mice, indicating inhibition of alveolar development.

CD68 staining confirmed that most of the inflammatory cells were macrophages (Fig. 5), and evaluation of nuclear morphology indicated that most of the rest of the infiltrating cells were polymorphonuclear leukocytes (neutrophils) (Fig. 5).

EM confirmed that the inclusions seen in the macrophages were agglomerations of TiO$_2$ NPs (Fig. 6).

NP administration does not significantly affect lung function or pulmonary vascular remodeling. Lung compliance (C) and total resistance (R) evaluated on day 14 did not significantly differ between the vehicle controls and the mice given three doses of TiO$_2$ NPs [C (ml/cmH$_2$O): vehicle 0.007 ± 0.0004 vs. TiO$_2$ 0.0065 ± 0.0005, P not significant (NS)]; R (cmH$_2$O·s$^{-1}$·ml$^{-1}$): vehicle 2.6 ± 0.2 vs. TiO$_2$ 2.4 ± 0.2, P NS]. Other parameters (E, Rn, G, H, Eta) were also not significantly different [E (cmH$_2$O/ml): 155 ± 20 vs. TiO$_2$ 174 ± 25, P NS; Rn (cmH$_2$O·s$^{-1}$·ml$^{-1}$): vehicle 0.4 ± 0.1 vs. TiO$_2$ 0.5 ± 0.15, P NS; G (cmH$_2$O/ml): vehicle 22 ± 5 vs. TiO$_2$ 26 ± 7, P NS; H (cmH$_2$O/ml): vehicle 154 ± 24 vs. TiO$_2$ 183 ± 44, P NS; H (cmH$_2$O/ml): vehicle 154 ± 24 vs. TiO$_2$ 183 ± 44, P NS]. Lung volumes were also similar [Vend (ml): vehicle 0.33 ± 0.01 vs. TiO$_2$ 0.33±0.02, P NS]; RV/LV thickness ratio (vehicle: 0.3 ± 0.04 vs. TiO$_2$ 0.3 ± 0.05, P NS) and pulmonary arterial wall thickness (vehicle: 10.2 ± 0.6 vs. TiO$_2$ 11.0 ± 0.8, P NS) did not significantly differ between vehicle controls and mice given three doses of TiO$_2$ NPs, indicating no significant pulmonary hypertension or vascular remodeling.

NP administration increases gene expression and protein amounts of specific cytokines in lung homogenates. The PCR array analysis indicated that Ccl8 (also known as monocyte chemoattractant protein or MCP-2), Spp1 (secreted phosphoprotein 1), Cxcl9 (also known as monokine induced by interferon-γ or MIG), IL1r2 (interleukin 1 receptor, type II), Ccr5 (C-C chemokine receptor 5), Ccl24 (also known as eotaxin-2), and Itgam (integrin, α M) were significantly increased in lungs of mice instilled with TiO$_2$ NPs, whereas IL11 (interleukin 11), Ltb (lymphotoxin β), and Ccr7 (C-C chemokine receptor 7) were reduced (Table 1). The results of the PCR array analysis were validated by real-time RT-PCR of these identified molecules, which confirmed that these changes were indeed present (data not shown).

The multiplex analysis of protein amounts of cytokines in lung homogenates indicated that multiple cytokines [e.g., eotaxin, G-CSF (granulocyte colony-stimulating factor), IL-1β (interleukin-1β), IL-2 (interleukin-2), IL-4 (interleukin-4), IL-9 (interleukin-9), IP-10 (interferon-γ-induced protein-10, also known as C-X-C motif chemokine 10), KC (chemokine C-X-C motif ligand 1), M-CSF (macrophage colony-stimulating factor), MIG (monokine induced by interferon-γ), MIP-1α (macrophage inflammatory protein-1α), MIP-1β (macrophage inflammatory protein-1 β), MIP-2 (macrophage inflammatory protein-2), and TNF-α (tumor necrosis factor-α)] were increased with TiO$_2$ NP exposure, whereas VEGF (vascular endothelial growth factor) was significantly decreased (Table 2).

Gelatin zymography indicated that MMP-9 (matrix metalloproteinase-9) was significantly increased in lung homogenates (Fig. 7).

Fig. 4. Mice exposed to TiO$_2$ nanoparticles have inhibition of alveolar development. Mean linear intercept (A) and radial alveolar count (B) of P14 mouse pups exposed to vehicle (Control) (A) or TiO$_2$ nanoparticles on P4, P7, and P10 demonstrating an increase in mean linear intercept and a reduction in radial alveolar count with TiO$_2$ nanoparticle exposure. Means ± SE; n = 6 mice/group; *P < 0.05 vs. vehicle.
DISCUSSION

The present study is the first to determine the effect of NP exposure on lung development and to determine alterations in the cytokine/chemokine milieu and the nature of the inflammatory cell infiltrate with NP exposure in the lungs of the newborn/juvenile mouse model. We observed that TiO2 NP administered intranasally to neonatal mice were dispersed into the lung and rapidly taken up by macrophages, which then induced influx of neutrophils and other inflammatory cells, leading to a patchy inflammatory cell infiltrate. Multiple doses of TiO2 NPs led to increased inflammation and inhibition of lung development. Specific alterations in inflammatory cytokines and chemokines were also observed.

Our study has multiple strengths. We exposed mice to TiO2 NPs during the critical phase of alveolar septation, which is postnatal in the mouse but begins before birth in humans and continues during early childhood (7, 37). Therefore, this model simulates human exposure during infancy and childhood, a period of rapid lung development. We evaluated single and multiple doses that were clinically relevant (1 mg/g body wt), corresponding to exposure to 10 mg of TiO2 by an infant weighing 10 kg. Our study demonstrates that inhalation, that may not be apparent, of even small amounts of NPs (of which TiO2 is the archetype) due to environmental contamination may have adverse long-term consequences. In addition to estimation of lung development and inflammatory infiltrate, other outcomes such as lung function, RV/LV ratio, and alterations in cytokines and chemokines by multiplex PCR or protein assays were also determined.

However, our study has limitations. Mouse models may not closely resemble human disease owing to interspecies differences. Intranasal TiO2 NP instillation in a suspension, although a reproducible and useful animal model, may not closely mimic inhalational exposure, since there may be differences with particle sizes and characteristics of the particles inhaled and deposited and the location of the deposition. Lung function measurements using a 24-gauge catheter in mice at 2 wk of age may not permit the detection of relatively small-magnitude changes in lung compliance or resistance. Experiments using whole lung homogenates do not permit the identification of changes in gene expression or protein synthesis in selected cell populations, such as airway epithelia, endothelial cells, or interstitial cells. Nevertheless, the upregulation of various inflammatory genes and cytokines as well as increased cellularity are clear signs that inhalation of TiO2 may damage the developing lungs.

Nanotechnology is involved with the manufacturing and manipulation of materials at the nanometer scale, and the properties of materials when in the “nano” range may be very different from those of larger particles of the same material (35). Substances that are normally harmless may be toxic due to altered chemical and physical properties when prepared as NPs (35). Much recent research has focused on respirable air pollution particles that are ultrafine (<100 nm), which have been shown to induce oxidative stress and lead to inflammation, resulting in exacerbations of preexisting respiratory and cardiovascular disease with increases in morbidity and mortality (35). TiO2, carbon black, and polystyrene bead particles are normally low solubility and low toxicity when larger but are more toxic and inflammogenic when ultrafine (NP range) (35). Most studies in nanotoxicology have so far focused on in vitro systems, but there is a strong rationale for in vivo animal studies to assess nanotoxicity as we have done, because a comparison of in vitro to in vivo nanotoxicity showed little correlation, indicating that much more development, standard-
ization, and validation (to in vivo effects) of in vitro studies is required (34), and in vivo systems are complicated as the interactions of the NPs (which may vary based on the exact size, shape, aggregation, and surface chemistry) with biological components, such as proteins and cells, may lead to unique biodistribution, clearance, immune response, and metabolism (15). In addition to accidental inhalation, nanocarrier systems are being evaluated for local or systemic pulmonary drug delivery.

Table 2. Analysis of protein amounts of mouse cytokines and chemokines from lung homogenates of mice exposed to vehicle (Control) or 3 doses of TiO₂

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Control (in pg/ml)</th>
<th>TiO₂ (in pg/ml)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eotaxin</td>
<td>914 ± 80</td>
<td>1156 ± 33</td>
<td>0.01</td>
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<tr>
<td>Granulocyte colony stimulating factor (G-CSF)</td>
<td>6.9 ± 1.4</td>
<td>11.4 ± 1.3</td>
<td>0.04</td>
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<tr>
<td>IL-1β</td>
<td>69.4 ± 7.7</td>
<td>95.1 ± 7.7</td>
<td>0.04</td>
</tr>
<tr>
<td>IL-2</td>
<td>9 ± 0.6</td>
<td>14.8 ± 1.9</td>
<td>0.04</td>
</tr>
<tr>
<td>IL-4</td>
<td>6.7 ± 1.6</td>
<td>21.1 ± 4.9</td>
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</tr>
<tr>
<td>IL-9</td>
<td>330 ± 33</td>
<td>432 ± 21</td>
<td>0.02</td>
</tr>
<tr>
<td>Interferon gamma-induced protein (IP)-10</td>
<td>107 ± 3</td>
<td>319 ± 58</td>
<td>0.02</td>
</tr>
<tr>
<td>Keratinocyte chemoattractant (KC)</td>
<td>47 ± 2</td>
<td>89.3 ± 5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Macrophage colony stimulating factor (M-CSF)</td>
<td>18 ± 2</td>
<td>32 ± 3</td>
<td>0.01</td>
</tr>
<tr>
<td>Monokine induced by interferon-gamma (MIG)</td>
<td>399 ± 32</td>
<td>1,024 ± 223</td>
<td>0.01</td>
</tr>
<tr>
<td>Macrophage inflammatory protein (MIP)-1α</td>
<td>70.9 ± 16</td>
<td>187 ± 17</td>
<td>0.002</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>24.9 ± 4</td>
<td>48.3 ± 4</td>
<td>0.003</td>
</tr>
<tr>
<td>MIP-2</td>
<td>74.9 ± 9</td>
<td>99.4 ± 3</td>
<td>0.01</td>
</tr>
<tr>
<td>Tumor necrosis factor (TNF)-α</td>
<td>4.1 ± 0.2</td>
<td>7.8 ± 0.7</td>
<td>0.003</td>
</tr>
<tr>
<td>Vascular endothelial growth factor (VEGF)</td>
<td>592 ± 24</td>
<td>457 ± 15</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Analysis of protein amounts of 32 key mouse cytokines and chemokines from lung homogenates of mice exposed to vehicle (Control) or 3 doses of TiO₂ (P4, P7, and P10) identified the following significant differences in cytokines (n = 4-6 mice per group; means ± SE).
livery (25), and the potential short-term and long-term toxicity of such methods of iatrogenic NP administration needs to be addressed by using in vivo models, especially since NPs often form aggregates and have different properties in vivo compared with in vitro models.

NPs administered intratracheally in adult mice are rapidly (<1 h) taken up by macrophages, and with repeated dosing there is cumulative accumulation of the NPs within the macrophages (33). Solitary NPs and smaller aggregates are taken up by alveolar macrophages with a very slow clearance (16). Adult rats exposed to low-toxicity dusts (such as TiO2) had impaired pulmonary clearance (particle retention half-times of 68 days for 5 mg/m3 TiO2 vs. 330 days for 250 mg/m3) and persistent inflammation through 6 mo postexposure (40).

The mechanism of inhibition of lung development induced by the macrophage accumulation of TiO2 and similar NPs may possibly be mediated by the alterations in local cytokines and growth factors. We observed that mRNA of Ccl8 (MCP-2), Spp1 (osteopontin), Cxcl9 (Mig), IL1r2, Ccr5, Ccl24, and Itgam (CD11b, Mac-1) were increased and IL11 and Ccr7 were decreased by TiO2 NPs. All these genes are probably relevant to the inflammation observed: Ccl8 is a well-known chemotactic agent for monocytes (32), Spp1 has a role in immune regulation and has Th1 cytokine functions (38), Cxcl9 is inducible in macrophages in response to interferon-γ and may play a role in T cell trafficking (24), IL1r2 inhibits IL-1 activity by acting as a decoy target for IL-1 (11) (suggesting that its increase may be compensatory rather than contributory to pathology), Ccr5 is a receptor for MIP-1α and -1β and is known to have a role in lung inflammation (5), Ccl24 is strongly chemotactic for eosinophils (43), and Itgam is important in neutrophil recruitment to the lung (26), whereas IL-11 (9) and Ccr7 (13), which were decreased, are known to have a protective role in lung injury and inflammation. We also observed that protein amounts of multiple proinflammatory cytokines (e.g., eotaxin, G-CSF, IL-1β, IL-2, IL-4, IP-10, M-CSF, MIP-1α, MIP-1β, MIP-2, TNF-α) were increased with TiO2 NP exposure, whereas VEGF was decreased. These results are consistent with our observations that TiO2 exposure resulted in macrophage and neutrophil recruitment, although it is not certain from which cell type each of these cytokines are produced or released. Overexpression of proinflammatory cytokines such as IL-1β is known to impair lung alveolarization (6, 17, 20). VEGF is important for normal lung development, and a reduction in VEGF such as that we noted may contribute to the impairment of alveolarization (4). We also observed that MMP-9 was increased in TiO2-exposed lung, and this metalloprotease is known to be involved in lung injury and inhibition of development in neonatal animal models and preterm human infants (10, 12). Therefore, TiO2 NP exposure by increasing various proinflammatory mediators and reducing growth factors such as VEGF may inhibit lung development (characterized by a reduction in alveolar septation, i.e., reduced RAC, and increased alveolar size, i.e., increased MLI), in a manner similar to bronchopulmonary dysplasia (20).

In this study, we observed that exposure of newborn mice to TiO2 NPs induced macrophage accumulation with subsequent neutrophil accumulation, inflammation, and inhibition of lung development, associated with a proinflammatory milieu. Chen et al. (8) have demonstrated that exposure of adult mice to TiO2 NPs induced macrophage accumulation with pulmonary emphysema due to extensive disruption of alveolar septae, epithelial cell apoptosis, and type II pneumocyte hyperplasia within three days of intratracheal instillation, and which was persistent at 2 wk after exposure. Microarray analyses using a homemade cDNA microarray indicated that TiO2 NPs induced differential expression of hundreds of genes, and in particular increased placental growth factor (PiGF) and other chemokines (Cxcl1, Cxcl5, and Ccl3) (8). Park et al. (31) have also shown that a single intratracheal instillation of TiO2 NPs in mice led to sustained lung inflammation associated with increases in inflammatory cytokines. Bermudez et al. (3) in a multispecies subchronic (13 wk) inhalation study exposed rats, mice, and hamsters to 0.5, 2.0, or 10 mg/m3 of TiO2 NPs, and found that the higher dose (10 mg/m3) led to pulmonary particle overload with an inflammatory response with a greater magnitude of histological changes (epithelial proliferation, metaplasia) in rats compared with mice or hamsters. Similar to our observations, mice exposed to TiO2 NPs in this study had aggregations of particle-laden macrophages in central lobar centracinar sites that over time became localized in the interstitium around blood vessels and bronchioles (3). The major point of difference between our study and the studies in adult mice is that lung development is essentially complete in adult mice, as alveolar septation in mice occurs mostly in the first 2 postnatal wk (7, 37). Our study therefore simulates neonatal and pediatric human exposure (alveolar septation in humans initiates in late infancy) (7, 37).

No differences in lung compliance, total resistance, or other parameters of lung function were observed. This may probably be because NP exposure leads to patchy inflammation at sites...
of macrophage accumulation of NPs and relative preservation of lung function and oxygenation. The impairment in lung development was relatively moderate. It is possible that a larger doses, more frequent dosing, or a later time point (e.g., in older mice) may demonstrate effects on lung function, or more subtle changes detectable by methacholine challenge.

Identification of potential toxicity of NPs to the developing fetus and neonate is of great public health significance, because such effects may lead to life-long effects with NPs that may persist in tissues for long periods of time, cause epigenetic effects, or impair development permanently by perturbing organ development/maturaton during critical developmental windows. Our study adds to the literature by demonstrating that early childhood exposures to even relatively low concentrations of NPs may induce lung inflammation and inhibit lung development.

DISCLAIMERS

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


