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Titanium dioxide nanoparticles exacerbate pneumonia in respiratory syncytial virus (RSV)-infected mice

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ABSTRACT

To reveal the effects of TiO_2 nanoparticles, used in cosmetics and building materials, on the immune response, a respiratory syncytial virus (RSV) infection mouse model was used. BALB/c mice were exposed once intranasally to TiO_2 at 0.5 mg/kg and infected intranasally with RSV five days later. The levels of IFN-γ and chemokine CCL5, representative markers of pneumonia, in the bronchoalveolar lavage fluids of RSV-infected mice had increased significantly in TiO_2-exposed mice compared with the control on day 5 post-infection, but not in uninfected mice. While pulmonary viral titers were not affected by TiO_2 exposure, an increase in the infiltration of lymphocytes into the alveolar septa in lung tissues was observed. Immunohistochemical analysis revealed aggregation of TiO_2 nanoparticles near inflammatory cells in the severely affected region. Thus, a single exposure to TiO_2 nanoparticles affected the immune system and exacerbated pneumonia in RSV-infected mice.

Abbreviations: TiO_2, titanium dioxide; RSV, respiratory syncytial virus; IFN-γ, interferon-gamma; BALF, bronchoalveolar lavage fluids; BFRs, brominated flame retardants; DBDE, decabrominated diphenyl ether; TBBPA, tetrabromobisphenol A; PBS, phosphate-buffered saline; IL, interleukin; PFU, plaque-forming units; ELISA, enzyme-linked immunosorbent assay; LPS, lipopolysaccharide.

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Abstract

To reveal the effects of TiO_2 nanoparticles, used in cosmetics and building materials, on the immune response, a respiratory syncytial virus (RSV) infection mouse model was used. BALB/c mice were exposed once intranasally to TiO_2 at 0.5 mg/kg and infected intranasally with RSV five days later. The levels of IFN-γ and chemokine CCL5, representative markers of pneumonia, in the bronchoalveolar lavage fluids of RSV-infected mice had increased significantly in TiO_2-exposed mice compared with the control on day 5 post-infection, but not in uninfected mice. While pulmonary viral titers were not affected by TiO_2 exposure, an

increase in the infiltration of lymphocytes into the alveolar septa in lung tissues was observed. Immunohistochemical analysis revealed aggregation of TiO_2 nanoparticles near inflammatory cells in the severely affected region. Thus, a single exposure to TiO_2 nanoparticles affected the immune system and exacerbated pneumonia in RSV-infected mice.

1. Introduction

Nanomaterials are engineered structures with at least one dimension of 100 nanometers or less (Nel et al., 2006). Various kinds of nanomaterials are known and have a wide range of applications (Maidalawieh et al., 2014; Nel et al., 2006; Stamatoiu et al., 2012). Titanium dioxide (TiO_2) nanoparticles are used in cosmetics and building materials because they are chemically and thermally stable. When research focused on TiO_2 nanoparticles were in drug delivery systems (Zhang et al., 2012) several findings of toxicity due to exposure to TiO_2 nanoparticles were reported, such as carcinogenesis of the lung in rats (Xu et al., 2010), induction of strong oxidative stress and mitochondrial damage in glial cells (Huerta-Garcia et al., 2014), and inflammatory disorder on the cardiovascular system in ApoE knockout mice (Chen et al., 2013). Although we are exposed to TiO_2 nanoparticles in daily life, the safety of TiO_2 nanoparticles for human health is poorly known.

Human respiratory syncytial virus (RSV), a member of the *Paramyxoviridae* family, is a prevalent infectious agent of acute lower respiratory illness in infants and young children (MacDonald et al., 1982). An initial RSV infection is frequent during the first few years of life, and most children have been infected by 24 months of age (Collins et al., 2001). Clinically severe RSV infection is seen primarily in infants and young children with naïve immune systems and/or genetic predispositions (Holberg et al., 1991) and patients with suppressed

T-cell immunity (MacDonald et al., 1982). RSV reinfects adults at a rate of approximately 5% to 10% per year (Falsey, 2007), and is an important cause of morbidity and mortality in the elderly (Falsey et al., 2005). Thus, because the severity of RSV infection reflects the condition of the host immunity, we established a novel assay system for evaluation of the immunotoxicity of the brominated flame retardants (BFRs) using a murine model of RSV infection (Watanabe et al., 2008a). We subsequently demonstrated that decabrominated diphenyl ether (DBDE) (Watanabe et al., 2008b; Watanabe et al., 2010a) and tetrabromobisphenol A (TBBPA) (Takeshita et al., 2013; Watanabe et al., 2010b) caused developmental immunotoxicity and irregular production of cytokines in RSV-infected mice, respectively. In addition, we also revealed that perinatal exposure to methamidophos, a representative organophosphate insecticide, suppressed the production of proinflammatory cytokines using this model (Watanabe et al., 2013).

In the present study, we adopted the RSV infection mouse model to evaluate the effects of TiO_2 nanoparticles on the immunotoxicity after a single exposure. Then we investigated the effects of TiO_2 nanoparticles on pneumonia in RSV infection by focusing on the variations in of cytokine and chemokine levels in bronchoalveolar lavage fluid (BALF) and the exacerbation of pneumonia in lung tissues by histopathological assay.

2 Materials and methods

2.1 Animals

Female (5 weeks old) BALB/c mice were purchased from Kyudo Animal Laboratory (Kumamoto, Japan) and housed at 25±2°C. The mice were allowed free access to the

conventional solid diet CRF-1 (Oriental Yeast Co., Chiba, Japan) and water and used in this experiment after 7 d acclimation. The animal experimentation guideline of the Kyushu University of Health and Welfare were followed in the animal studies.

2.2 Cell and virus

The A2 strain of RSV was obtained from American Type Culture Collection (ATCC, Rockville, MD) and grown in HEp-2 cell (human epidermoid carcinoma, ATCC CCL-23) cultures. Viral titers of HEp-2 cells were measured by the plaque method (Watanabe et al., 2008a) and expressed as plaque-forming units per milliliter (PFU/mL).

2.3 Chemical compound

TiO_2 nanoparticles were kindly provided by Tayca Corp. (Osaka, Japan). The particles form ultra-fine rutile crystals primarily 35 nm in diameter. TiO_2 nanoparticles readily aggregate to form microparticles in phosphate-buffered saline (PBS). To avoid aggregation, the suspension of TiO_2 nanoparticles in PBS was dispersed using a portable ultrasonic disruptor just before treatment of mice. Then the mean secondary diameter of the particles was 913 nm, ranging from 804-1,022 nm, as measured by a Zetasizer Nano (Malvern Instruments, Worcestershire, UK).

2.4 Animal tests

Six-week-old mice were intranasally administered 0.1 mL of a suspension of TiO_2 nanoparticles at 0.25 or 2.5 mg/kg of body weight one time under anesthesia for histological assays or 0.5 mg/kg for measuring cytokines in BALF and pulmonary viral titers. In control

group, mice were given PBS intranasally under anesthesia.

The RSV infection test was performed as reported previously (Watanabe et al., 2008a). Briefly, 5 d after from TiO_2 exposure, mice were infected intranasally with 3.5 x 10^5 PFU of the A2 strain of RSV under anesthesia. In a mock-infected group, mice were given PBS intranasally. On day 5 after infection, blood samples were prepared from RSV-infected mice under anesthesia and BALF was obtained from the mice under anesthesia by instilling 0.8 mL of cold PBS into the lungs and aspirating it from the trachea using a tracheal cannula. Following the acquisition of BALF, the lungs were removed, immediately frozen in liquid N_2, and stored at -80°C until virus titration. Ice-cold BALF was centrifuged at 160 x g at 4°C for 10 min. After centrifugation, the supernatant was stored at -80°C until to use. The cell pellet was suspended in 0.3 mL of cellbanker-1 (Nippon Zenyaku Kogyo Co., Ltd., Koriyama, Japan) as bronchoalveolar lavage cells, and then stored at -80°C prior to use. Frozen lung tissue was homogenized with cold quartz sand in a homogenizer. After centrifugation at 480 x g at 4°C for 15 min, the supernatants of the homogenates were used for a plaque assay. Viral titers in lungs of mice were expressed as PFU/mL.

2.5 ELISA

Interleukin (IL)–2, IL-4, IL-10, and interferon (IFN)–γ levels in BALF were measured using specific ELISA kits (Ready-set-go, eBioscience Inc., San Diego, CA) according to the manufacturer's instructions. Levels of CCL5 (RANTES) in BALF and serum and CCL3 (MIP-1α) in BALF and the culture supernatant of bronchoalveolar lavage cells were measured using specific ELISA kits (Quantikine, R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions. The lower limits of detection of the kits are 2 (pg/mL) for IL-2, 4

(pg/mL) for IL-4, 8 (pg/mL) for IL-10, 15 (pg/mL) for IFN–γ, 2 (pg/mL) for CCL5, and 1.5 (pg/mL) for CCL3. The intra- and interassay coefficients of variation for the ELISA results were less than 10%.

2.6 Flow cytometric analysis of bronchoalveolar lavage cells

Flow cytometric analysis was performed according to our previous report (Takeda et al., 2014). Briefly, bronchoalveolar lavage cells were stimulated with BD GolgiStop (BD PharMingen, San Diego, CA) at 1 μL/mL for 6 h at 37°C. After incubation, the cells were washed twice and stained for intracellular IFN-γ (FITS Rat Anti-Mouse IFN-γ, BD PharMingen, San Diego) and IL-4 (PE Rat Anti-Mouse IFN-γ, BD PharMingen, San Diego), according to the manufacturer's instructions. The cells were washed twice and analyzed on an FACS Calibur 35 flow cytometer (Becton Dicknson, Sunnyvale, CA).

2.7 Histological methods and evaluation

For histological examination of RSV-infected lungs, 3 to 5 mice per group of infected mice were sacrificed by cervical dislocation on day 5 after infection, and the lungs were removed and placed in buffered formalin for a minimum of 24 h. The tissue was then embedded in low-melting point paraffin, sectioned at a thickness of 5μm, and stained with hematoxylin and eosin. After taking two pictures randomly of each pulmonary lobe using a microscope (x100), the pictures were analyzed for the proportion of alveolar septa and infiltration of the inflammatory cells into the tissues per unit area by Adobe Photoshop (Adobe Systems, Inc., San Jose, CA).

2.8 Immunohistochemical evaluation

The lung tissue sections were deparaffinized and hydrated through xylenes and graded alcohols. After washing with water, they were incubated in unmasking solution (Vector Laboratories, Inc., Burlingame, CA) at 90°C for 30 min. Then, the sections were incubated in the 0.3% H_2O_2 in PBS for 30 min to quench the endogenous peroxidase activity and treated with blocking serum (Vector Laboratories, Inc.) for 30 min. The lung tissues were stained with a goat polyclonal antibody against RSV protein (1:250, Acris Antibodies GmbH, Inc., San Diego, CA) for 90 min. Then, RSV proteins were detected using a VECTASTAIN ABC kit (Vector Laboratories, Inc.) according to the manufacturer's instructions. The sections were faintly counterstained with hematoxylin.

2.9 Culture of bronchoalveolar lavage cells

Culture of bronchoalveolar lavage cells obtained from RSV-infected mice on day 1 post-infection was performed according to our previous report (Watanabe et al., 2010a). Briefly, two hundred microliters of bronchoalveolar lavage cells suspension (2.5×10^5 cells/mL) was seeded on each well in a 96-well microtiter plate and incubated at 37°C for 24 h in a humidified air with 5% CO_2. After incubation, the culture medium was removed by aspiration and replaced in fresh RPMI medium with or without 0.1 mg/mL of TiO_2 nanoparticles. Following 24 h further incubation, the culture medium was removed by aspiration and replaced in fresh RPMI medium with or without 100 ng/mL of lipopolysaccharide (W E. coli O127: B8, Difco, Detroit, MI; LPS) for 24 h. The culture supernatant was harvested from each well and the amount of CCL3 was measured by ELISA.

2.10Statistical analysis

Comparisons between the pulmonary viral titers and the levels of cytokines and chemokines of the control and TiO_2-treated groups were carried out using Student's *t*-test. A *P* value of 0.05 or less was considered to be significant.

3. Results

3.1 Effects of TiO_2 nanoparticles on RSV infection in mice

To investigate the effects of TiO_2 nanoparticles on the immune response to RSV infection, six-week-old female BALB/c mice were exposed intranasally to 0.1 mL of TiO_2 suspension at 0.5 mg/kg of body weight under anesthesia. No abnormal behavior or dystrophy due to the stress of TiO_2 exposure was observed compared to the control (0 mg/kg) in the mice, and the mice were infected intranasally with the A2 strain of RSV at 3.5×10^5 PFU five days after TiO_2 exposure. The levels of IFN-γ, a representative marker of pneumonia in RSV infection, in BALF were measured on day 5 post-infection (Table 1). The IFN-γ levels of RSV-infected mice treated with TiO_2 were significantly ($P<0.05$) higher than those in the control. In mock-infected mice treated with or without TiO_2, the levels of IFN-γ in BALF were under the limit of detection. These results indicated that pneumonia in RSV-infected mice was exacerbated by TiO_2 exposure. To investigate further effects of exposure to TiO_2 on the immune system of RSV-infected mice, the levels of Th1 cytokines (IFN-γ and IL-2) and Th2 cytokines (IL-4 and IL-10) in BALF were also measured on day 5 after infection (Table 1). The levels of IL-10 in BALF were significantly ($P<0.05$) increased by approximately 92% compared with the control. No significant increase of IL-2 in BALF was found after TiO_2

treatment, and the levels of IL-4 in BALF were under the limit of detection. In mock-infected mice treated with or without TiO_2, the levels of cytokines in BALF were under the limit of detection. To reveal effects of exposure to TiO_2 on the Th1/2 immune balance of RSV-infected mice on day 5 post-infection, intracellular IFN-γ and IL-4 productions by the bronchoalveolar lavage cells were examined by flow cytometry (Table 2). There was not a significant change in the population of IFN-γ-positive cells and IL-4-positive cells due to TiO_2 treatment. These results suggested that TiO_2 exposure should affecte the immune response to RSV infection.

Chemokine CCL5 is a common marker of the severity of inflammation in the lungs due to RSV infection (Lambert et al., 2003) and chemokine CCL3 also is an inflammatory marker. Therefore, we measured the levels of CCL5 and CCL3 in BALF on day 5 after infection (Table 3). The levels of CCL5 in BALF were significantly ($P<0.05$) increased by approximately 36% compared with the control, but there was no significant increase of CCL3 levels in TiO_2-exposed mice. In mock-infected mice treated with or without TiO_2, the levels of chemokines in BALF were under the limit of detection. The levels of CCL5 in serum were significantly ($P<0.05$) increased by approximately 31% compared with the control (Table 3). Thus, these results strongly suggested that TiO_2 exposure exacerbated the pneumonia due to RSV infection.

To evaluate the effects of TiO_2 exposure on the growth of RSV in mice, pulmonary viral titers were measured by plaque assay (Fig. 1). Viral titers of mice exposed to TiO_2 were not elevated significantly compared with those of control. Thus, TiO_2 exposure did not enhance proliferation of RSV in mice.

3.2 Effects of TiO_2 nanoparticles on severity of pneumonia in RSV infection

To clarify the effects of TiO_2 nanoparticles on the severity of pneumonia in RSV infection, a histopathological assay was performed. In this experiment, three to five mice in each group were treated with TiO_2 as follows: a control group at 0 mg/kg, low-dose group at 0.25 mg/kg, and high-dose group at 2.5 mg/kg. These mice were infected with or without RSV 5 days after TiO_2 exposure. On day 5 post-infection, the mice were sacrificed, and their lung tissues were analyzed histopathologically. Representative results and changes in severity are presented in Figure 2 and Table 4, respectively. In mock-infected mice, no obvious change in the lung tissues due to TiO_2 exposure was observed compared with the control (Fig. 2A-a, -c, and -e). In RSV-infected mice, typical features of pneumonia due to RSV infection, such as degeneration of the bronchial epithelium and infiltration of lymphocytes and neutrophils, were observed in mice treated with or without TiO_2 (Fig. 2A-b, -d, and -f). Severity of pneumonia was assessed as the proportion of alveolar septum tissue in RSV-infected mice (Table 4). In the control group at 0 mg/kg, the proportion of alveolar septa of all mice was less than 60%. On the other hand, two mice in the TiO_2 (0.25 mg/kg)-treated group had more than 60% alveolar septa, and one mouse in the TiO_2 (2.5 mg/kg)-treated group had more than 70%. The unit area means were 51.8%, 60.6%, and 68.6% in each group, respectively. Thus, we confirmed exacerbation of the pneumonia due to RSV infection by TiO_2 exposure.

To investigate whether the distribution of RSV-infected cells was changed qualitatively due to TiO_2 (2.5 mg/kg) exposure, sections of the lung tissues of RSV-infected mice were stained immunohistochemically with a goat-polyclonal antibody against RSV protein (Fig. 2B). There was no significant change in the localization of RSV-positive cells, but the TiO_2 nanoparticles were not close to RSV-positive cells (Fig. 2B-b and –d). Similar results were observed for TiO_2 (0.25 mg/kg)-treated mice (data not shown). However, there was

aggregation of TiO_2 nanoparticles near inflammatory cells in the severe region (Fig. 2B-d). Because these results suggested that TiO_2 nanoparticles might influence the function of macrophage/monocyte in an early phase of RSV infection, bronchoalveolar lavage cells on day 1 post-infection from RSV-infected mice were incubated for 48 h with or without 0.1 mg/mL of TiO_2, corresponding to a dose of 0.5 mg/kg *in vivo*. After incubation, the levels of CCL3 in the culture supernatant of the cells were measured by ELISA (Table 5). Although the cells were stimulated with LPS, there was no significant change in the production of CCL3 from bronchoalveolar lavage cells due to TiO_2 treatment.

4. Discussion

We assessed the effects of TiO_2 nanoparticles on the immune response using a mouse model of RSV infection (Watanabe et al., 2008a) and found that prior exposure to TiO_2 nanoparticles exacerbated pneumonia in the lungs of mice.

Various studies concerning risk assessment of TiO_2 nanoparticles in murine models have been reported (Chen et al., 2013; Kwon et al., 2012; Lindberg et al., 2012; Xu et al., 2010). In many cases, TiO_2 exposure was performed at a high dosage and/or multiple times. For example, to evaluate the carcinogenesis of TiO_2 nanoparticles in lung tissues, female (10 weeks old) SD rats were subjected to intra-pulmonary spraying five times with 0.5 mL suspensions of TiO_2 nanoparticles at 500 μg/mL in saline (Xu et al., 2010). In our assay, female (6 weeks old) BALB/c mice were exposed once intranasally to 0.1 mL of a suspension of TiO_2 nanoparticles at 50 μg/mL or 100 μg/mL, corresponding to a dose of 0.25 mg/kg or

0.5 mg/kg of body weight. The levels of TiO_2 exposure in our assay are much lower than those in previous studies (Chen et al., 2013; Kwon et al., 2012; Lindberg et al., 2012; Xu et al., 2010), and suppression of body weight, abnormal behavior, or dystrophy due to the stress of TiO_2 exposure was not observed compared with the control mice (data not shown). Furthermore, in the TiO_2-treated mice without RSV infection, no significant enhancement of cytokines or chemokines in BALF and any obvious changes in lung tissues were observed (Tables 1, 3, Fig. 2A-a, -c, -e, 2B-a, and -c). Based on these experiments, we are confident that RSV infection is a useful tool for evaluation of the effects of low-level exposure to TiO_2 nanoparticles on the immune system, which was not reported previously.

The IFN-γ levels of RSV-infected mice treated with TiO_2 were enhanced significantly ($P<0.05$) compared with the control (Table 1). These results suggest that pneumonia in RSV-infected mice was exacerbated due to TiO_2 exposure. However, viral titers in lung tissues of RSV-infected mice exposed to TiO_2 were not elevated significantly compared with the control (Fig. 1), and there was no significant increase of the number of RSV-positive cells in lung tissues due to TiO_2 exposure (Fig. 2B). Previous studies concerning evaluation of immunotoxicity of BFRs such as DBDE and TBBPA have shown that these compounds clearly increased both the levels of IFN-γ and viral titers in RSV-infected mice, resulting in the exacerbation of pneumonia (Watanabe et al., 2008b; Watanabe et al., 2010a; Watanabe et al., 2010b). Therefore, we speculated that the mechanism of action of TiO_2 nanoparticles was different from that of BFRs on the immune system (Watanabe et al., 2008b; Watanabe et al., 2010a; Watanabe et al., 2010b). We investigated further effects of exposure to TiO_2 on the Th1/2 balance of RSV-infected mice. The levels of IL-10, a Th2 cytokine, in BALF were increased significantly ($P<0.05$) compared with the control (Table 1). IL-10 is produced

mainly by T cells and acts by inhibiting the production of pro-inflammatory cytokines (Cavalcanti et al., 2012). Remarkably, although enhancement of the IL-10 levels should alleviate immune response, exacerbation of pneumonia was conversely exhibited (Fig. 2A). On the other hand, enhancement of IL-4 production due to TiO_2 exposure was not observed (Table 1). However, the levels of chemokine CCL5, an inflammatory marker called RANTES produced mainly by T cells and basophils (Schall et al., 1988), in BALF were also increased significantly ($P<0.05$) compared with the control (Table 3). Thus, this irregular activation of T cells due to TiO_2 exposure might induce a Th1/2 imbalance. However, there was not a significant change in the population of IFN-γ-positive cells and IL-4-positive cells due to TiO_2 treatment by flow cytometric analysis (Table 2). These results suggested that TiO_2 exposure probably affected the secretion of the cytokines rather than differentiation of $CD4^+$ cells, responding to RSV infection.

In the histopathological analysis, the infiltration of lymphocytes in alveolar septa in RSV-infected mice tended to be increased by TiO_2 exposure, particularly in wide areas of lung tissues (Fig. 2A and Table 4). Because chemokine CCL5 promotes the migration of T cells and basophils (Schall et al., 1988), enhancement of it should contribute to the infiltration (Table 3). Moreover, in an immunohistochemical analysis, we observed TiO_2 nanoparticles explicitly because the sections were faintly counterstained with hematoxylin (Fig. 2B). It became evident that TiO_2 nanoparticles were aggregated by lymphocytes/macrophages but were not close to RSV-positive cells. It has been reported that TiO_2 nanoparticles were engulfed in alveolar macrophages and involved in the inflammation in lung tissues of the TiO_2-treated rodents (Kwon et al., 2012; Xu et al., 2010). Then, to evaluate whether TiO_2 exposure influence the function of macrophages/monocytes in lung tissue of RSV-infected

mice, the bronchoalveolar lavage cells on day 1 post-infection, consist mainly of the macrophage/monocyte-like cells (Watanabe et al., 2010a), were cultured with or without TiO_2 (Table 5). The *in vitro* experiment using a LPS showed that TiO_2 treatment did not affect the production of chemokine CCL3 from the bronchoalveolar lavage cells, although the ingestion of TiO_2 nanoparticles in the macrophage/monocyte-like cells was observed under a microscope. Therefore, further studies are needed to understand whether macrophages are involved in the immune response in an early phase of RSV infection.

TiO_2 nanoparticles are used in various products, and we may be exposed to them at various times of life. We have already confirmed that anatase crystals of TiO_2 nanoparticles used in building materials induced the elevation of CCL5 in RSV-infected mice (data not shown). In this study, we used TiO_2 nanoparticles that form rutile crystals and are used in cosmetics. Further studies are required to investigate the effects of TiO_2 nanoparticles of different forms and sizes on immune response. These studies should provide useful information to manage their effects on health, including methods of skin care.

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Figure legends

Figure 1

Effect of exposure to TiO_2 on pulmonary viral titers of RSV-infected mice on day 5 post-infection. The data represents mean±standard deviation of values of 7 control or 5 TiO_2 -treated mice. N.S., not significant.

Figure 2

Lungs of mice 5 days after RSV-infection. A) Hematoxylin and eosin staining (x100). (a) Control mouse with mock infection. (b) Control mouse with RSV infection. (c) TiO_2-treated (0.25 mg/kg) mouse with mock infection. (d) TiO_2-treated (0.25 mg/kg) mouse with RSV infection. (e) TiO_2-treated (2.5 mg/kg) mouse with mock infection. (f) TiO_2-treated (2.5 mg/kg) mouse with RSV infection. Arrows indicate infiltration of lymphocytes in alveolar septa. B) Immunostained with anti-RSV protein antibodies (1:250) and counterstained with hematoxylin (x400). (a) Control mouse with mock infection. (b) Control mouse with RSV infection. (c) TiO_2-treated (2.5 mg/kg) mouse with mock infection. (d) TiO_2-treated (2.5 mg/kg) mouse with

RSV infection. Closed arrows indicate RSV-positive cells, closed arrowheads indicate TiO_2 nanoparticles, and open arrowheads indicate aggregation of TiO_2 nanoparticles in inflammatory cells.

Table 1

Effects of TiO_2 on levels of cytokines in BALF of RSV-infected mice on day 5 post-infection.

TiO_2 exposure (mg/kg)	Concentration (ng/mL)[a]							
	RSV-infected				Mock-infected			
	IFN-γ	IL-2	IL-4	IL-10	IFN-γ	IL-2	IL-4	IL-10
0	8.59±3.44	0.02±0.01	<0.01	1.65±0.71	<0.01	<0.01	<0.01	<0.01
0.5	12.90±2.10*	0.03±0.01	<0.01	3.17±1.15*	<0.01	<0.01	<0.01	<0.01

* Statistically different from control at $P<0.05$ (Student's *t*-test).

[a] Concentration (ng/mL) of each cytokine in BALF from RSV-infected mice treated with or without TiO_2 (0.5 mg/kg) was measured by ELISA for each specific cytokine. Data represents mean values of 3-6 mice. Numbers in parentheses indicate standard deviation.

Table 2

Effects of TiO_2 on intracellular cytokine levels in bronchoalveolar lavage cells on day 5 post-infection from RSV-infected mice.

TiO_2 exposure	% of total population[a]			
(mg/kg)	IFN-γ^- IL-4^-	IFN-γ^+ IL-4^-	IFN-γ^- IL-4^+	IFN-γ^+ IL-4^+
0	98.7	1.1	0.1	0.1
0.5	98.9	0.8	0.1	0.2

[a]Bronchoalveolar lavage cells were collected from RSV-infected mice treated with or without TiO_2 (0.5 mg/kg) on day 5 post-infection. The pooled bronchoalveolar lavage cells were stimulated BD GolgiStop for 6 h at 37°C and stained intracellular IFN-γ and IL-4. The stained cells were analyzed by flow cytometry.

Table 3

Effects of TiO_2 on levels of chemokines in BALF and serum of RSV-infected mice on day 5 post-infection[a].

TiO_2 exposure (mg/kg)	Concentration in BALF (ng/mL)				Concentration in serum (ng/mL)	
	RSV-infected		Mock-infected		RSV-infected	Mock-infected
	CCL3	CCL5	CCL3	CCL5	CCL5	CCL5
0	0.10±0.04	0.24±0.06	<0.01	<0.01	0.14 ± 0.01	0.10 ± 0.01
0.5	0.11±0.03	0.32±0.07*	<0.01	<0.01	0.18 ± 0.03*	0.09 ± 0.03

* Statistically different from control at $P<0.05$ (Student's *t*-test).

[a] Concentration (ng/mL) of each chemokine in BALF and serum from RSV-infected mice treated with or without TiO_2 (0.5 mg/kg) was measured by ELISA for each specific chemokine. Data represents mean values of 3-6 mice. Numbers in parentheses indicate standard deviation.

Table 4

Effects of TiO_2 on proportion of tissue of alveolar septa in RSV-infected mice on day 5 post-infection.

TiO_2 exposure (mg/kg)	% of alveolar tissues[a]				% of mean alveolar tissues
	>40	>50	>60	>70	
0	2*	2	0	0	51.8 (3.7)
0.25	0	2	2	0	60.6 (1.6)
2.5	0	0	1	1	68.6 (3.1)

*Number of mice. Numbers in parenthesis indicate the standard error.

[a]The proportion of tissue in alveolar septa per unit area by Adobe Photoshop.

Table 5

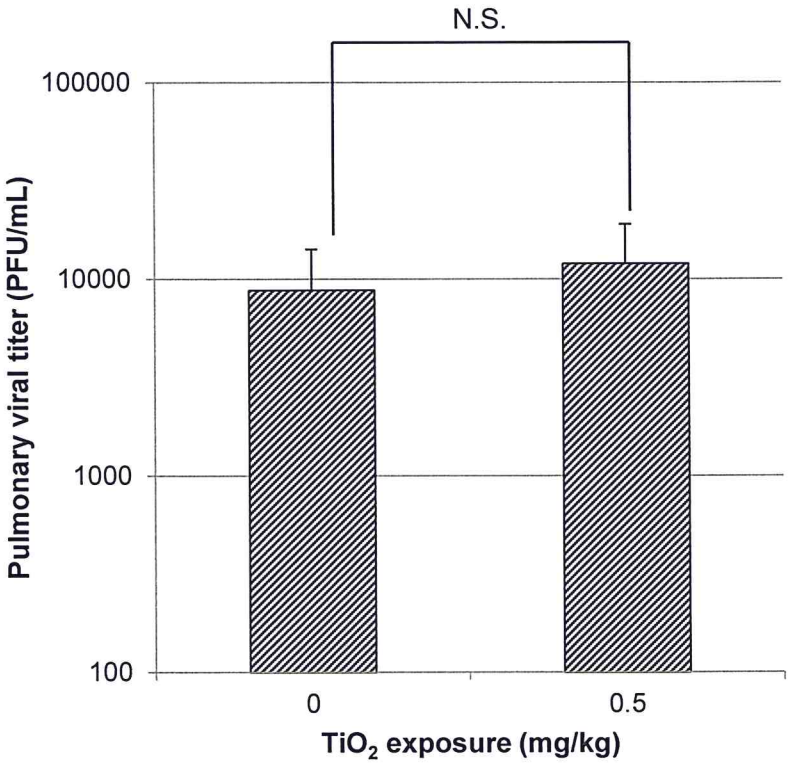
Effects of TiO_2 on CCL3 production from bronchoalveolar lavage cells on day 1 post-infection in RSV-infected mice.

TiO_2 exposure	CCL3 (ng/mL)[a]	
(mg/mL)	-LPS	+LPS
0	<0.01	0.17 (0.12-0.23)*
0.1	<0.01	0.15 (0.13-0.16)

*Data represent mean of values of two seprate experiments. Numbers in parentheses indicate the range of values.

[a]Bronchoalveolar lavage cells were collected from RSV-infected mice and cultured for 48 h with or without TiO_2 (0.1 mg/mL).

Figure 1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10000
1000
100
Pulmonary viral titer (PFU/mL)
0
0.5
TiO2 exposure (mg/kg)

Figure 2

(A)

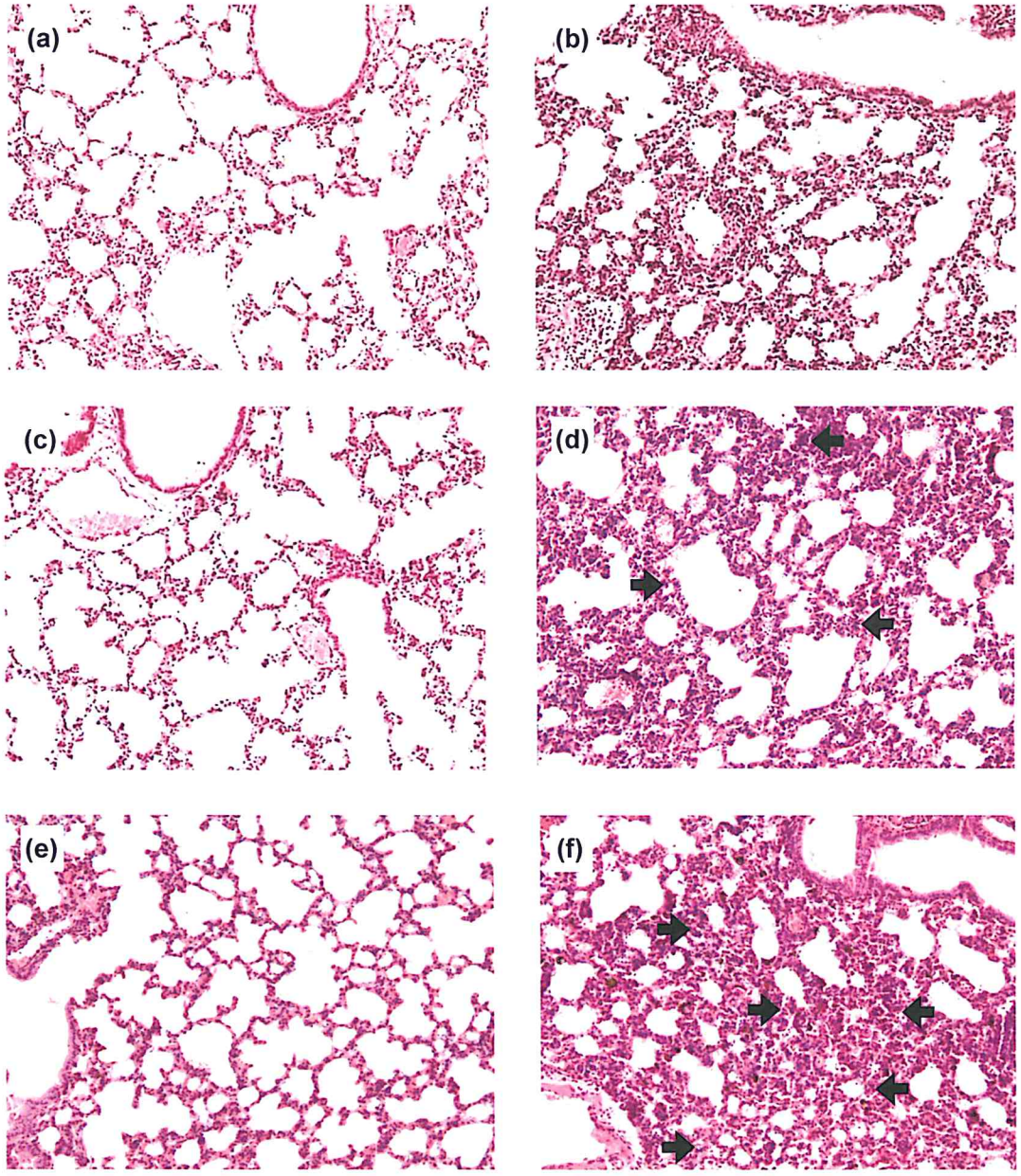
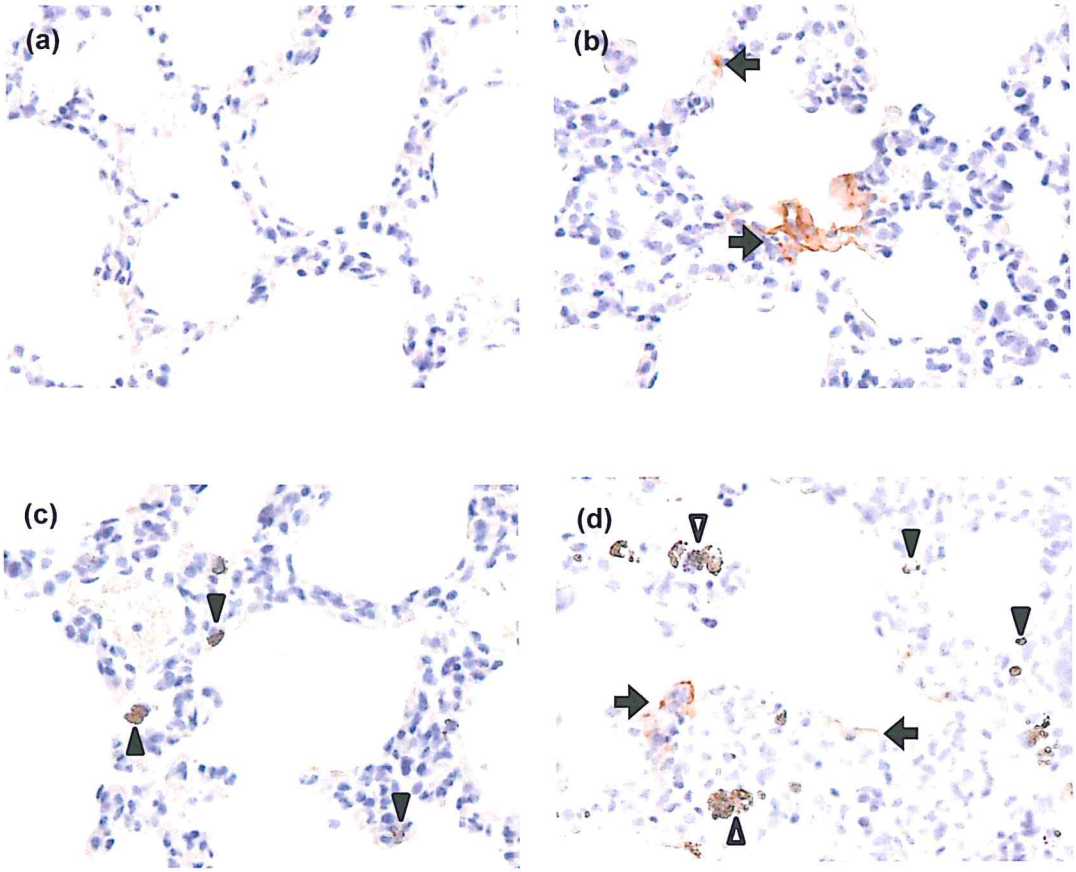


Figure 2

(B)



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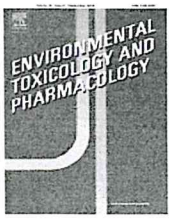

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