Lung inflammation caused by long-term exposure to titanium dioxide in mice involving in NF-κB signalling pathway

Dong Liu[a], *Jie-Lu Zhou*[b], *Fashui Hong*[c, d*], *Yu-Qing Zhang*[a §]

a) Department of Applied biology, School of Basic Medical and Biological Sciences, Soochow University; RM702-2303, Renai Road No. 199, Dushuhu Higher Edu. Town, Suzhou 215123; P R China;

b) Department of Scientific and Educational Affairs, Suzhou Kowloon Hospital Affiliated with Shanghai Jiao Tong University School of Medicine, Suzhou 215021, People's Republic of China;

c) Jiangsu Collaborative Innovation Center of Regional Modern Agriculture & Environmental Protection, Huaiyin Normal University, Huaian 223300, China;

d) Jiangsu Key Laboratory for Eco-Agricultural Biotechnology around Hongze Lake, Huaiyin Normal University, Huaian 223300, China

*Corresponding author: 086-0517-83525992(Tel/Fax); E-mail: hongfsh_cn@sina.com

§Corresponding author: 086-0512-65880181(Tel/Fax); E-mail: sericult@suda.edu.cn;

Abstract

Titanium dioxide nanoparticles (TiO_2 NPs) are used in many fields, such as paints, medicine additives, food additives, sunscreens and agriculture. The aim of this study was to investgate the mechanism behind the formation of inflammation induced by TiO_2 NPs. ICR mice were exposed to TiO_2 NPs through intragastric administration at 2.5, 5 and 10 mg/kg body weight every day for 90 consecutive days. The experiment suggested that long-term exposure to

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1002/jbm.a.35945

TiO_2 NPs resulted in an obvious inflammatory response in mice lung tissues, which led to a thickened alveoli septum, lung hyperaemia, and titanium accumulation. Furthermore, our results show that TiO_2 NPs exposure remarkably altered the expression of inflammation-related cytokines, with increases in pro-inflammatory cytokines, such as nucleic factor-κB, interferon-α, interferon-β, interleukin-1β, interleukin-6, cyclo-oxygen-ase, interleukin-8, interferon-inducible protein-10 and platelet derived growth factor AB and decreases in anti-inflammatory cytokines, such as inhibitor of NF-κB suppressor of cytokine signalling 1, endothelin 1, peroxisome proliferators-activated receptors-γ and peroxisome proliferators-activated receptors coactivator-1α. This finding indicated that TiO_2 NPs cause lung inflammation in mice after intragastric administration, primarily through the NF-κB signalling pathways. Therefore, more attention should be placed on the application of TiO_2NPs and their potential long-term effects, especially in human beings.

Keywords: titanium dioxide nanoparticles; long-term exposure; lung inflammation; mice; NF-κB signalling pathway;

Introduction

Due to their special physicochemical properties, such as high stability, anticorrosion and photocatalytic properties, TiO_2 nanoparticles (TiO_2 NPs) are widely used in paints[1], medicine additives[2], plastics[3], food additives[4], cosmetics[5], sunscreens[6], and agriculture [7,8]. Food-grade titanium dioxide, known as E171 additive, is also widely used in the field food additives. But the differences are that TiO_2 NPs has strong UV shielding effect, good dispersion, and good weather resistance and is more expensive compared to food-grade titanium dioxide. Generally, it was accepted that TiO_2 NPs are biologically inactive in experimental animals and humans, but recent researches suggested that long-term exposure to TiO_2 NPs may have negative biological effects in mice [9,10, 11, 12]. Studies have unequivocally showed that TiO_2 NP exposure can induce injury in the spleen [13,14], brain [15,16], heart [17,18], liver [19,20], kidney [21,22,23] and lung [24,25,26] of mice.

Importantly, previous studies demonstrate that the administration of TiO_2 NPs could induce alterations in inflammatory-related cytokines and oxidative stress in mice lung and cause damage to lung cells in vitro. For example, it was reported that TiO_2 NPs may impair lung mitochondrial function in vitro [27], which forces cells to perform anaerobic respiration promoting changes in the cellular phenotype and severe side effects on metabolism. Besides, it was found that TiO_2 NPs induced oxidative stress in human lung epithelial cells in a concentration- and time-dependent manner [28]. Further more, it was reported that TiO_2 NPs are genotoxic, and

exposure of human hepatoma HepG2 cells to TiO_2 NPs induced changes in the mRNA expression of tumor protein p53 and its downstream regulated DNA damage response genes, p21, gadd45α and mdm2 [29]. However, little is known about the mechanism how TiO_2 NPs exposure can induce lung inflammation in mice.

Therefore, the present article was performed to investigate the issue by comprehensive methods. We designed an experiment to study the changes in lung indices, the net increase in the body weight of mice and investigate histopathological changes in lung inflammatory levels in mice following exposure to 0-10 mg/kg body weight TiO_2 NPs by intragastric administration for 90 consecutive days. Furthermore, the expressions of inflammation-related cytokines were also examined by Western Blotting and ELISA. The primary purpose of our findings was to benefit the understanding of intragastric administration of TiO_2 NP -induced effects in lung, and thereby, to arouse the attention of the effects of TiO_2 NPs exposure.

Materials and Methods

Chemicals and preparation

Anatase TiO_2 NPs were prepared via controlled hydrolysis of titanium tetrabutoxide. The details of the synthesis and characterization of TiO_2 NPs were described in our previous reports [30,31]. Anatase TiO_2 NPs is pure and does not has any biological (endotoxin) contamination. The average particle size of powdered TiO_2 NPs suspended in 0.5% (w/v) hydroxyl propylmethyl cellulose (HPMC) K4 M (Sigma–Aldrich, St. Louis, MO, USA) solvent prepared with deionized and distilled water was 5–6 nm after both a 12 and 24 h incubation, and the surface area of the

sample was 174.8 m^2/g. HPMC is a common suspending agent, which can reduce the solid-liquid interfacial tension and reduce the surface free energy of solid particles, so that forming a heterogeneous dispersed system stabilized to promote the dissolution of refractory material. The mean hydrodynamic diameter of the TiO_2 NPs in HPMC solvent was 294 nm (range, 208–330 nm), and the zeta potentials after 12 and 24 h incubations were 7.57 and 9.28 mV, respectively [32].

Animals and treatment

One hundred sixty CD-1 (ICR) female mice (24±2g) were purchased from the Animal Center of Soochow University (China). The mice were housed in stainless steel cages in a ventilated animal room. The room temperature in the housing facility was maintained at 24±2°C, with a relative humidity of 60±10% and a 12-h light/dark cycle. Distilled water and sterilized food were available ad libitum. Before treatment, the mice were acclimated to this environment for five days. All animals were handled in accordance with the guidelines and protocols approved by the Care and Use of Animals Committee of Soochow University (China). TiO_2 NP powder was dispersed onto the surface of 0.5% (w/v) HPMC, and the suspensions containing TiO_2 NPs were treated ultrasonically for 30 min and mechanically vibrated for 5 min. The mice were randomly divided into four groups (n = 40 each), including a control group treated with 0.5% (w/v) HPMC and three experimental groups treated with 2.5, 5, and 10 mg/kg body weight (BW) TiO_2 NPs. For dose selection, we consulted the World Health Organization report of 1969. According to that report, the LD50 of orally administered TiO_2 in rats is >12 g/kg BW. In addition, the quantity of TiO_2 NP cannot exceed 1% of the consumed food by weight according

to US Federal Regulations. In the present study, we selected the doses of 0, 2.5, 5, and 10 mg/kg TiO_2 NPs, which were intragastrically administered to the mice daily. These doses were approximately equal to 0.15-0.7 g TiO_2 NP exposure in humans with 60–70 kg body weight, which is considered a relatively safe dose range. The mice were weighed, the volume of the TiO_2 NP suspensions was calculated for each mouse, and the fresh TiO_2 NP suspensions were administered to the mice intragastrically daily for 90 days. Any symptoms, including growth state, eating, drinking and activity as well as mortality were carefully observed and recorded every day during the 90-day treatment period.

Net increase in body weight and lung indices

After weighing the body and lung, the net increase in body weight and lung indices was calculated as the mean ratio of lung weight (mg) to body weight (g).

Histopathological evaluation of lung

All histopathological examinations were performed using standard laboratory procedures. Five lung tissue samples from the lungs of each mouse were embedded in paraffin blocks, sliced to 5μm thickness, and placed on separate glass slides (five slices for each lung). After hematoxylin-eosin staining, the sections were evaluated by a histopathologist who was blinded to the treatments using an optical microscope (U-III Multi-point Sensor System; Nikon, Tokyo, Japan).

Western blotting

Lung tissues were lysed using protein lysis buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 50 mM Tris HCl, pH 7.4), and protein concentration was determined using

a standard BCA protein assay kit (Genmed Scientifics Inc.). Forty micrograms of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were blocked for 2 h at 37°C with 5% skim milk in Tris buffered saline containing 0.05% Tween-20 (TBST) and were then incubated overnight at 4°C with the following primary antibodies: anti-nucleic factor-κB (NF-κB), anti-interferon-α (IFN-α), anti-interferon-β (IFN-β), anti-interleukin-1β (IL-1β), anti-interleukin-6 (IL-6), anti-inhibitor of NF-κB (IκB) and anti-suppressor of cytokine signalling 1 (SOCS1). After washing the membrane with TBST three times, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000) at room temperature for 2h, followed by detection using enhanced chemiluminescence (ECL, Millipore). Immunoreactive bands were visualized using X-ray films. For quantitative analysis, the bands were evaluated with Image J software and were normalized to β-actin.

ELISA Assay

To determine the concentrations of endothelin 1 (ET-1), peroxisome proliferators-activated receptors-γ(PPAR-γ), peroxisome proliferators-activated receptors coactivator-1α (PGC-1α), interleukin-6 (IL-6), cyclo-oxygen-ase (COX), interleukin-8(IL-8), interferon-inducible protein-10 (IP-10) and platelet derived growth factor AB (PDG-AB) in mouse lung tissues, enzyme linked immunosorbent assays (ELISAs) were performed using commercial kits that were selective for each protein (R&D Systems, USA) following the manufacturer's instructions. The absorbance was measured on a microplate reader at 450 nm (Varioskan Flash, Thermo Electron,

Finland), and the concentrations of ET-1, PPAR-γ, PGC-1α, IL-6, COX, IL-8, NGAL, IP-10 and PDG-AB were calculated from the standard curve for each sample (n = 5 each).

Statistical analysis

All results are expressed as means ± SD. One-way analysis of variance (ANOVA) was performed to compare the differences in the means among the multi-group data using SPSS 19 software (SPSS, Inc., Chicago, IL, USA). Dunnett's test was performed to compare each dataset with the solvent control data. A probability level of 0.05 ($P < 0.05$) was considered to indicate statistical significance for all tests.

Results

Net increase in body weight and lung indices

The net increases in body weight and lung indices caused by exposure to TiO_2 NPs for 90 consecutive days are presented in Fig. 1. As shown, with increased TiO_2 NPs doses, the net increase in body weight significantly decreased and the lung indices gradually increased. The lung indices increased significantly at TiO_2 NPs doses of 5 and 10 mg/kg BW. This observation suggests that long-term exposure to TiO_2 NPs may cause damage to the lungs of mice.

Lung tissue histopathological observation

Figure 2 presents the histopathological changes in the lungs of mice following exposure to TiO_2 NPs and, semi-quantifications for the macrophages in each sample are shown in Figure 3. The findings indicate that lung injury following TiO_2 NP exposure was dose-dependent. Long-term exposure to TiO_2 NPs led to a thickened alveoli septum, lung hyperaemia, and TiO_2

NPs deposition. Of these injuries, the most severe were in the 10 mg/kg group (Fig. 2d), followed by the 5 mg/kg groups (Fig. 2c) and the 2.5 mg/kg group (Fig. 2b). In addition, as shown in Fig. 3, the counts of macrophages increased with the increase of the TiO_2 NP doses, which suggests that TiO_2 NP may cause lung inflammation in mice in a dose manner.

Expression of pro-inflammatory and anti-inflammatory factors

The expressions of NF-κB signalling pathway-related proteins in mouse lung measured by western blotting are shown in Fig 4. As shown, the pro-inflammatory cytokines NF-κB, IFN-α, IFN-β, IL-1β, and IL-6 were up-regulated, while anti-inflammatory cytokines IκB and SOCS1 were down-regulated. The results suggest that TiO_2 NPs may cause inflammation in the lungs of mice.

Expression of proteins related to inflammation

To confirm expression of IFN signalling pathway–related cytokines, including ET-1, PPAR-γ, PGC-1α, IL-6, COX, IL-8, IP-10 and PDG-AB, in lung tissues, their protein levels were measured by ELISA, and they are presented in Table 1. The protein levels of ET-1, PPAR-γ, and PGC-1α in the lungs of exposed mice were lower than those in the unexposed mice (Table 1), while the protein levels of COX, IL-8, IP-10 and PDG-AB were significantly elevated with increasing TiO_2 NP dose.

Discussion

An increasing number of consumer products based on nanotechnology are already on the market, and it is estimated that millions of workers and consumers will directly contact nanomaterials in the near future. Therefore, it is crucial to evaluate the possible negative effects

of these novel nanomaterials. It was reported that mice may result in spleen injury and reduction of immune capacity after intragastric administration of TiO_2 NPs, and TiO_2 NP-induced injury in spleen may result from alteration of inflammatory and apoptotic cytokines expression [14]. In addition, study has demonstrated that intragastric administration of TiO_2 NPs could cause overproliferation of spongiocytes, hemorrhage and oxidative damage in the mouse brain via the p38-Nrf-2 signaling pathway [15]. Study also has demonstrated that intragastric administration of TiO_2 NPs could result in obvious titanium accumulation in heart, in turn led to sparse cardiac muscle fibers, inflammatory response, cell necrosis, and cardiac biochemical dysfunction [17]. More over, it was also reported that the signaling pathway of hepatitis in mice after intragastric administration of TiO_2 NPs might occur via activation of TLRs, NIK, IκB kinase, NF-κB, TNF-α, in return led to inflammation, apoptosis and liver injury[19]. And TiO_2 NPs were accumulated in the kidney of mice, resulting in nephric inflammation, cell necrosis and dysfunction after intragastric administration of TiO_2 NPs [21], and might occur via Nrf2 signaling pathway [22]. Numerous studies demonstrated that TiO_2NPs can reach pulmonary tissue [33,34,35]. However, numerous studies have been conducted mainly focused on the toxicology of lung cell following exposure to TiO_2 NP in vitro [36,37,38,39], instead of lung tissue after intragastric administration of TiO_2 NPs.

Therefore, the inflammatory effects on lung by intragastric administration of TiO_2 NPs were studied in mice. TiO_2 NP is an inert and poorly soluble matter, so hydroxyl propylmethyl cellulose (HPMC) was used in order to make TiO_2 NP have a better solubility. A potential exposure route for general population is the oral ingestion because TiO_2 NP is used as a food

additive in toothpaste, capsule, cachou, and so on. Therefore, we chose intragastric administration as the exposure route. There are two main types of nanodelivery systems, liquid and solid. Liquid nanodelivery systems include nanoemulsions, nanoliposomes, and nanopolymersomes. Solid nanodelivery systems include nanocrystals, lipid nanoparticles, and polymeric nanoparticles [40]. In addition, it was reported that liquid nanodelivery has a better bioavailability than olid nanodelivery system [41]. In the present experiment, the average particle size of powdered TiO_2 NPs suspended in 0.5% (w/v) HPMC K4 M solvent prepared with deionized and distilled water was 5–6 nm after both a 12 and 24 h incubation, and the surface area of the sample was 174.8m^2/g. Study has demonstrated that nano particle with less than 100nm particle size and no charge has higher absorption and longer circulation in mice [40]. To understand the underlying mechanism for the inflammation of mice caused by long-term exposure to TiO_2 NPs, the present study was designed to investigate the changes of splenic morphology, immunity and expression of relevant genes and relevant proteins in the mouse lung.

After intragastric administration of 2.5, 5, and 10 mg/kg BW TiO_2NPs for 90 consecutive days, significant reductions in the net increase in body weight and increases in lung indices (Fig. 1) were observed. Based on the change in the net increase in body weight and lung indices, we can observe that the net increase in body weight significantly decreased and the lung indices gradually increased with the dose of TiO_2 NPs increasing, which implied that long-term exposure of TiO_2 NPs may damage lung tissues in mice. To investigate the damage caused by TiO_2 NPs, histopathological observations of lung tissues of mice were performed, and are presented in Fig. 2 and Fig. 3. The results suggested that long-term exposure to TiO_2 NPs could increase

macrophages and, cause inflammation, hyperaemia, fibrotic thickening, inflammatory cell infiltration and even potential TiO_2 NPs deposition.

Furthermore, we evaluated the effects of TiO_2NPs exposure on the NF-κB signalling pathway (Fig. 5), which is very important in the inflammatory response. TNF-α is known to be a major pro-inflammatory cytokine that is especially important in the induction phase of the inflammatory response. Kim et al reported that TNF-α suppresses arthritic inflammation in rats [42]. TNF-α and IL-1β activate the NF-κB signalling pathway by binding to the cell surface receptor binding domain TNFR and IL-1β Receptor respectively, resulting in the recruitment and activation of the IκB kinase complex. Then, the IκB kinase complex phosphorylates IκB, causing the release of NF-κB, which then alters the expression of downstream genes [43]. Shin et al. also suggested that TiO_2 NP exposure induces TNF-α expression and augments NF-κB binding activity, promoting an exaggerated neuroinflammatory response by enhancing microglial activation in the pre-inflamed brain of mice [44]. Furthermore, PPAR-γ was reported to inhibit NF-κB activity by directly interacting with the p65 subunit of NF-κB or increasing the synthesis of the NF-κB inhibitor IκB [45]. Fig. 4 and Table 1 show that TiO_2NPs exposure increased the protein expression of IL-1β, NF-κB and PPAR-γ and decreased the protein expression of IκB.

PPAR-γ has a coactivator, PGC-1α. Both PPAR-γ and PGC-1α are reduced simultaneously in patients with metastatic disease [46]. Table 1 shows that exposure to TiO_2 NPs caused decreases in PPAR-γ and PGC-1α protein levels, which suggests that TiO_2 NPs accumulation causes pulmonary inflammation in mice.

Furthermore, exposure to TiO_2 NPs can also cause lung inflammation in mice through the

IFN signalling pathway. SOCS1 is a protein that is known to function as a negative regulator of cytokine signalling and also has a critical role in the regulation of IFN signalling [47]. IFN-α and IFN-β are produced by fibroblasts and monocytes, and excessive or dysfunctional secretion of IFN-α and IFN-β in specific tissue microenvironments can induce inappropriate T-cell survival and contribute to the development of chronic inflammation [48]. In the present study, TiO_2 NP exposure led to downregulation of SOCS1 expression and upregulation of IFN-α and IFN-β expressions in mouse lung (Fig.4), which may aggravate pulmonary inflammation in mice.

Finally, we also evaluated the expression of other proteins related to inflammation, such as COX, IL-8 and IP-10. COX is a necessary enzyme before the compound of PGs, which are involved in the inflammatory response and can decrease inflammation. IL-8 is a chemokine that has been identified as a neutrophil chemotactic polypeptide and is normally produced in the presence of inflammatory stimuli, such as IL-1 and TNF [49]. IP-10 is a recently described potent angiostatic factor that regulates lung cancer-derived angiogenesis, tumour growth, and spontaneous metastasis. Arenberg et al [50] found significantly elevated levels of IP-10 in freshly isolated human lung cancer tissues. Our results presented in Table 1 show that exposure to TiO_2NPs can increase the expression of COX, IL-8 and IP-10, which may be involved in the induction of lung inflammation in mice and may even can cause lung cancer.

Conclusions

The present mouse study found that 90 days of TiO_2 NPs exposure resulted in serious lung inflammation involving in the NF-κB signalling pathways, in addition to significantly decrease

the expression of IκB, SOCS1, ET-1, PPAR-γ, PGC-1α and significantly increase the expression of NF-κB, IFN-α, IFN-β, IL-1β, IL-6, COX, IL-8, NGAL, IP-10 and PDG-AB. This research indicates that TiO_2 NPs possesses biological toxicity and that more attention should be placed on determining the proper and safe usage of TiO_2 NPs.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant No. 81473007, 81273036, 30901218, 31671033). This work was supported by the National Natural Science Foundation of Jiangsu Province (grant No. BK20161306). The authors gratefully acknowledge the earmarked fund (CARS-22-ZJ0504) from China Agriculture Research System (CARS) and a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions, P. R. China.

Statement: All animals were handled in accordance with the guidelines and protocols approved by the Care and Use of Animals Committee of Soochow University (China).

Author Contributions

Yu-Qing Zhang and Fashui Hong conceived this study; Dong Liu and Jie-Lu Zhou performed the experiment and constructed the database; Dong Liu and Jie-Lu Zhou performed the statistical analysis and wrote the paper. Dong Liu and Jie-Lu Zhou contributed equally to this work. Yu-Qing Zhang revised the manuscript.

Additional Information

Competing financial interests: The authors declare no competing financial interests.

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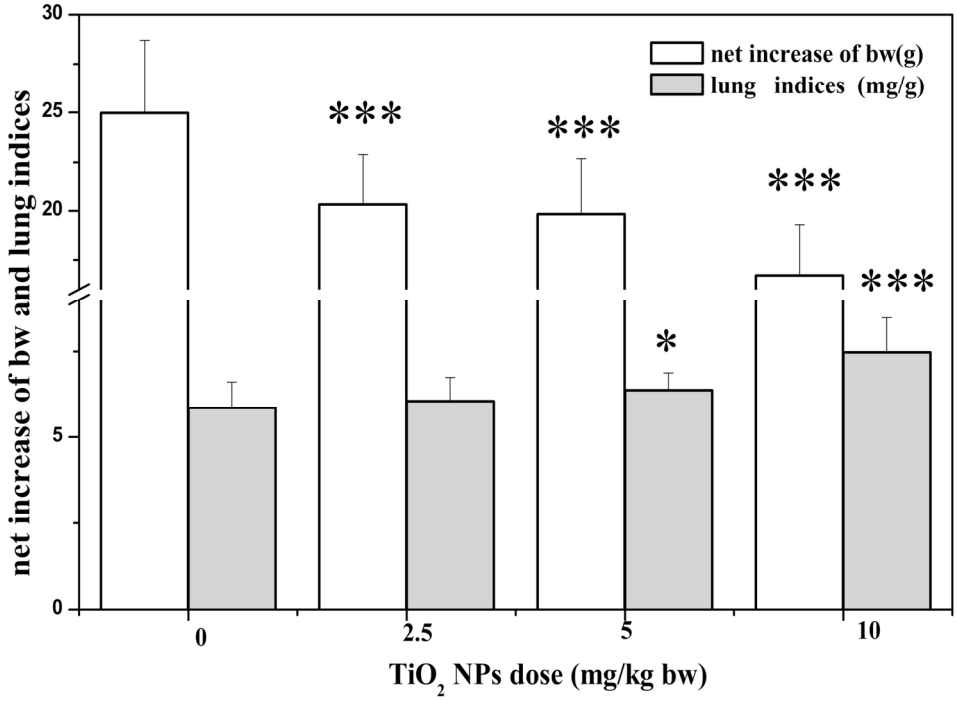

Figure 1. Net increase in body weight and lung indices in mice caused following intragastric administration of TiO2 NPs for 90 consecutive days. Bars marked with a single double or triple asterisk indicate significant differences from control (unexposed mice) at 5%, 1%, and 0.1% confidence levels, respectively. Values are presented as means±SD (n=15).
Fig. 1.
76x57mm (600 x 600 DPI)

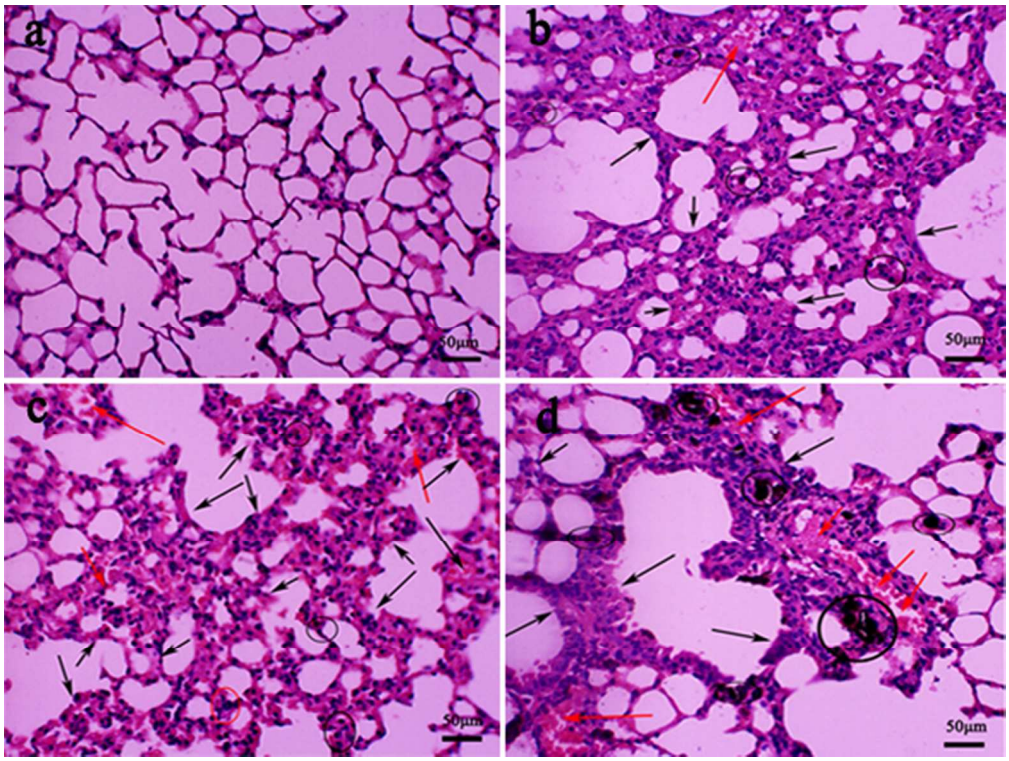

Figure 2. Histopathological observation of lung tissues of mice after intragastric administration of TiO2 NPs for three consecutive months (N=15). Pictures a, b, c and d are tissues of mice exposed to TiO2 NPs doses of 0, 2.5, 5 and 10 mg/kg BW, respectively. Red short arrows indicate hyperaemia, black short arrows indicate fibrotic thickening, and the circles encompass areas with inflammatory cell infiltration which may suggest TiO2 NPs deposition. (a) Control group (400×) has normal lung architecture; (b) 2.5 mg/kg BW TiO2 NPs group (400×); (c) 5 mg/kg BW TiO2 NPs group (400×) (d) 10 mg/kg BW TiO2 NPs group (400×). The sections were stained with HE and examined by light microscopy.
Fig. 2.
76x57mm (300 x 300 DPI)

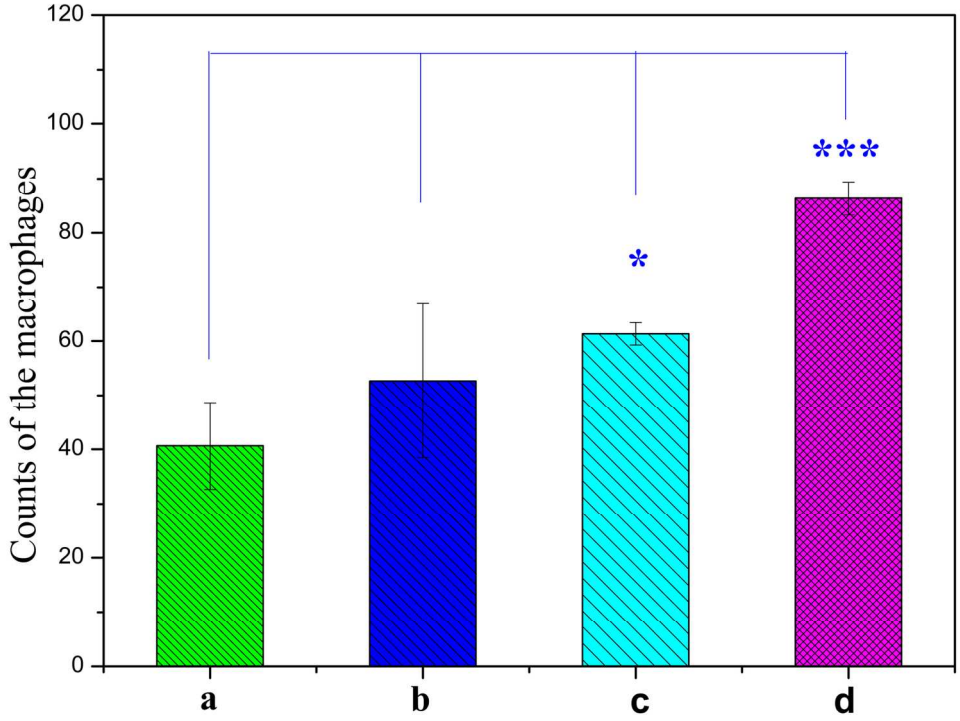

Figure 3. Semi-quantifications for macrophages in each sample.

Fig. 3
76x57mm (600 x 600 DPI)

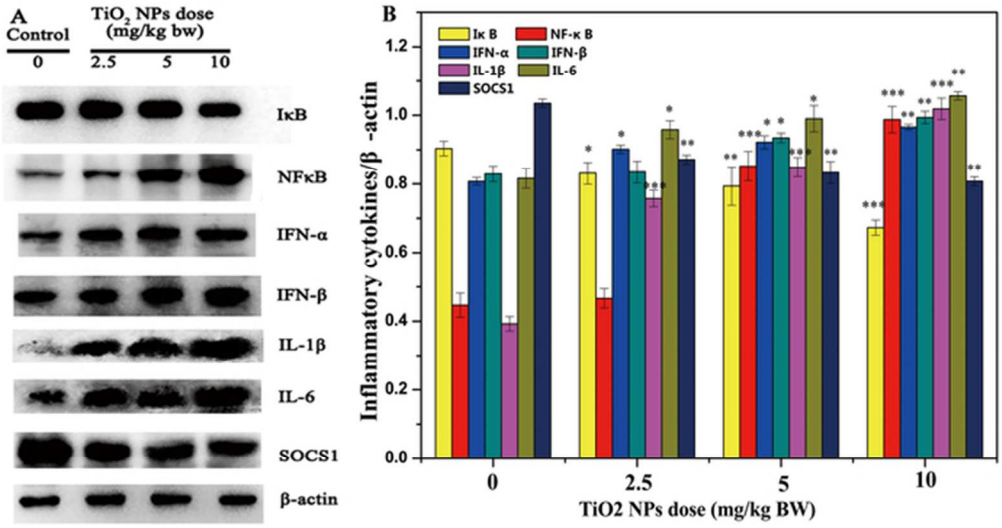

Figure 4. Protein expressions of pro-inflammatory factors in mouse lung measured by Western blotting
Fig. 4
58x33mm (300 x 300 DPI)

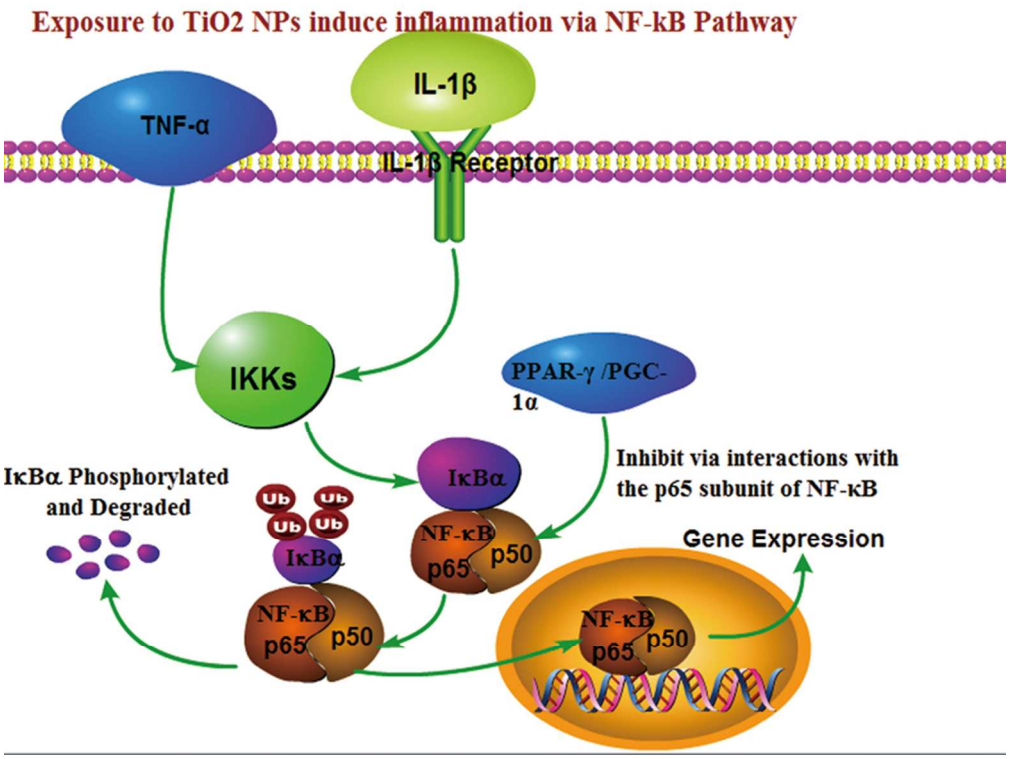

Figure 5. Exposure to NPs induces inflammation via NF-κB pathway.
Fig. 5
76x57mm (300 x 300 DPI)

Table 1 Effects of TiO_2NP exposure on protein expression in lung in vivo

Protein expression	TiO_2 NP dose (mg/kg)			
(ng/g tissue)	0	2.5	5.0	10.0
PPAR-γ	280.18±35.45	250.90±62.53	240.97±26.41	227.56±18.53*
PGC-1α	420.88±54.84	289.44±35.85**	243.96±25.58**	231.82±42.96***
COX	520.45±42.83	530.90±44.08	559.74±108.04	592.01±17.67**
IL-8	3.75±0.41	4.10±0.82	4.47±0.40 *	4.76±0.48**
IP-10	5.41±0.44	7.08±0.97 **	8.79±4.17 **	14.23±2.06***
PDG-AB	16.66±2.98	25.26±2.99**	28.23±4.92**	40.49±9.51***
ET-1	5.34±0.94	5.07±0.32	4.70±0.48	4.43±0.37

*p<0.05, **p<0.01, ***p<0.001 Values are means ± SE (n = 5).