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Effects of Th1 and Th2 cells balance in pulmonary injury induced by nano titanium dioxide

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ABSTRACT

To explore the potential immunoregulatory mechanisms linking nano TiO_2 and pulmonary injury, Sprague Dawley rats were exposed by intra-tracheal instillation to nano TiO_2 with the individual doses of 0.5, 4.0 and 32 mg/kg b.w., micro TiO_2 with 32 mg/kg b.w. and 0.9% NaCl, respectively. The exposure was conducted twice a week, for four consecutive weeks. The results of lung histology demonstrated increased macrophages accumulation, extensive disruption of alveolar septa, slight alveolar thickness and expansion hyperemia. Mitochondria tumefaction organelles dissolution, endoplasmic reticulum expansion and the gap of nuclear broadening were shown. The changes of IFN-γ and IL-4 level showed no statistical difference. The mRNA expression of GATA-3 was up-regulated, whereas T-bet was significantly down-regulated. The protein expression of T-bet decreased and there were significant differences in nano 4 and 32 mg/kg groups. The imbalance of Th1/Th2 cytokines might be one of the mechanisms of immunotoxicity of respiratory system induced by nano TiO_2 particles.

1. Introduction

The essence of nanotechnology is the production of nanoparticles (NPs) with unique physicochemical properties allowing worldwide application in new structures, materials, and devices (Iavicoli et al., 2012). These properties offer great opportunities for the development of new NP industrial applications increasing their worldwide distribution and enhancing the likelihood of environmental and human exposure (Borm et al., 2006). The consequently increasing human exposure to NPs has raised concerns regarding their health and safety profiles (Pietroiusti, 2012). TiO_2 is an example of a fine, white, crystalline, odorless, low-solubility powder which was considered to exhibit relatively low toxicity (Sager et al., 2008). TiO_2 is a naturally occurring mineral that can exist in three crystalline forms, known as rutile, anatase, and brookite, and in an amorphous form. In 2006, the International Agency for Research on Cancer (IARC), classified and in 2010 reassessed TiO_2 as "possibly carcinogenic to humans" on the basis of the sufficient evidence of carcinogenicity in experimental animals and inadequate evidence in humans (Group 2B) (IARC, 2010).

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TiO_2 is a versatile compound that has broadly been used in nanoparticulate form. According to the National Nanotechnology Initiative of America, nano TiO_2 are one of the most highly manufactured global nanomaterials. Nano TiO_2, a non-combustible and odorless powder, is an important material widely used in paints, waste water treatment, cosmetics, food additives, sterilization, biomedical ceramic and implanted biomaterials and so on. With extensive application, more and more nano TiO_2 are entering into the environment. Therefore, the potential adverse effects of nano TiO_2 particles on human health have attracted particular attention (Liang et al., 2009). Commercial production of nano TiO_2 between 2006 and 2010 has been estimated at 5000 metric tons per year, more than 10,000 metric tons per year between 2011 and 2014 and approximately 2.5 million metric tons by 2025 (Menard et al., 2011). With the increasing applications of nano TiO_2 in the industry and our daily life, an increasing number of studies have investigated their potential toxicity and shown that nano TiO_2 may have negative effects on the respiratory system or metabolic circle system of organisms (Zhang et al., 2011). Despite concerted, nano materials research in recent years, we are still at the very early stages of assessing the potential hazards and human health risks posed by nano sized particles (Thomas et al., 2010).

Immune system safeguards the host from various foreign assaults and malignancies. The process involves recognition of foreign particles followed by finely orchestrated and regulated immune attack, eventually destroying or disposing off the particle. In vitro and animal studies are showing that nanoparticles are capable of activating proinflammatory cytokines, chemokines and adhesion molecules, with recruitment of inflammatory cells including basophils, macrophages, dendritic cells, T cells, neutrophils and eosinophils. These changes may have an impact on immune defense, but also on the Th1/Th2 balance, and even on non-immunologic function (Chang, 2010). Owing to their small size, nanoparticles may escape the phagocytic activity of macrophages, bind to serum proteins and act as haptens, activate complement cascades, disturb the Th1/Th2 balance (Dwivedi et al., 2011). Nanoparticles can imbalance both the innate and the adaptive immune system, but mechanistically functions on how nanoparticles modulate the immune system and the diseases resulting from this imbalance remain unclear (Hussain et al., 2012). Protective immunity depends on the proper balance between Th1 and Th2 cells. Th1/Th2 cells associated with a series of immune and inflammatory diseases. Th1 cells selectively produce large amounts of IFN-γ which provides protection against intracellular bacteria, fungi and viruses and are involved in some autoimmune responses (Zhao et al., 2012). Th2 cells selectively produce IL-4 which is particularly strong in driving B cells to generate IgE-secreting cells. Th2 cells mediate protection against extracellular parasites but may also cause harmful allergic responsiveness. In addition, the transcription factors GATA-3 have been verified to play a relevant role in Th2 cell commitment (Zhang et al., 1997). The most important role of GATA-3 is to establish transcriptional competence for the Th2 cytokine gene cluster through chromatin remodeling (Lee et al., 2000). Besides, T-box transcription factor T-bet plays a crucial role in Th1 cell differentiation by promoting chromatin remodeling of IFN-γ locus (Szabo et al., 2000). Along with other molecules, these cytokines and their receptors are believed to be essential for inflammatory cell recruitment (Kim, 2004). A recent study demonstrated that, in ovalbumin sensitized mice, silica, coated rutile nano TiO_2 inhalation decreased TNF-α and IL-13 expression in spleen cells (Rossi et al., 2010). Reduction in total plenocyte, $CD4^+$ and $CD8^+$ T-lymphocyte number, retardation in B-lymphocyte development, and reduction in LPS stimulate d NK cells were reported after exposure to nano TiO_2 (Moon et al., 2011). Scarino et al. found that exposure to nano TiO_2 significantly decreased BAL leukocytes compared to OVA-challenged controls. Plasma and BAL IL-4, IL-6, and INF-γ levels were also decreased in the nano TiO_2 group (Scarino et al., 2012). Fish exposed to nano TiO_2 for 48 h in vivo had significantly increased expression of interleukin 11, macrophage stimulating factor 1, and neutrophil cytosolic factor 2. Nano TiO_2 has potential to interfere with the evolutionary conserved innate immune system responses, as evidenced with observed changes in gene expression and neutrophil function (Jovanovic et al., 2011). However, the limited number of studies investigating the immunological effects of nano TiO_2 do not allow comparison and extrapolation of certain conclusions (Gioacchino et al., 2011; Iavicoli et al., 2012). Further mechanistic studies investigating particle immunomodulatory effects are required to improve our understanding of the physicochemical parameters of nanoparticles that define their effects on the immune system (Zolnik et al., 2010).

Consequently, our study hypothesizes that the imbalance of Th1 and Th2 cytokines is a potential mechanism of the effects of nano TiO_2 particles on pulmonary injury. The aim of the study is to determine whether experimental short-term exposure to nano TiO_2 particles which entered into lungs produce a detectable pro-inflammatory and immune response in lung tissues of rat, meanwhile, the study is to explore whether the immune mechanism linking nano TiO_2 and pulmonary injury is mediated by the imbalance of Th1 and Th2 cells.

2. Materials and methods

2.1. *Preparation and characterization of TiO_2 nanoparticles*

Nano-TiO_2 of size 21 nm was purchased from Degussa Corporation (Hanau, Germany). Micro-TiO_2 of size 1–2 μm was purchased from Beijing DK nano technology Co., LTD (Beijing, China). The TiO_2 samples were characterized by Malvern Instruments Zetasizer Nano ZS90 (Worcestershire WR, UK) and transmission electron microscopy (TEM, JEM-2100, JEOL Ltd., Tokyo, Japan).

Particles were heated to 123 ± 2 °C for 20 min to reduce the risk of bacteria contamination. TiO_2 powder was dispersed into an aqueous solution buffered with 0.9% NaCl solution to sufficiently disperse particles for sizing analyses. Solutions containing TiO_2 particles were treated by ultrasound for 15–20 min and mechanically vibrated for 5 min.

2.2. Animals and treatment

Sprague Dawley (SD) rats (40 males, 200–220 g) were purchased from the Liao Ning Chang Sheng Biotechnology Co., Ltd (Liao Ning, China). The animal room was maintained at 20 ± 2 °C, 60 ± 10% relative humidity and a 12-h light/dark cycle. Distilled water and sterilized food for mice were available ad libitum. They were acclimated to this environment for 5 days prior to dosing. All the animal experiments were performed in compliance with the local ethics committee. All experimental procedures conformed to the guide for the care and use of laboratory animals. Forty rats were randomly divided into five groups: control group and four experimental groups. Experimental groups were instilled (intra-tracheal) with nano TiO_2 (0.5, 4, and 32 mg/kg b.w.) and bulk TiO_2 (32 mg/kg b.w.) twice a week, for four consecutive weeks, respectively. The control group was treated with 0.9%NaCl. After rats were anesthetized via inhalation with ether, TiO_2 suspension was given via intratracheal instillation. The volume of intratracheal instillation is 0.1 mL/100 g b.w. Four weeks later, the body weight of all animals was weighed accurately. After being anesthetized by ether, the rats were sacrificed by exsanguination via the abdominal aorta.

2.3. Histopathologic examination

All histopathological tests were performed using standard laboratory procedures. The lung tissue samples were excised carefully and part of the tissues were in flated at a pressure of approximately 20 cm H_2O at one week with 4% formalin and then fixed similarly by immersion. Then, the tissues were embedded in paraffin blocks, and then sectioned into 3–5 μm slices and mounted onto the glass slides. After traditional histological hematoxylin–eosin (HE) stain, the slides were observed by optical microscopy (CX21FS1, Olympus, Japan).

2.4. Electron microscopy

According to the results of histopathological examinations, tissues of rats chosen were excised and the tissue samples were cut into <1 mm^3 cubes, immersed in 4% glutaraldehyde at 4 °C. After being washed several times with phosphate-buffered saline, they were fixed with 1% osmium tetroxide, dehydrated with serial acetone, embedded in Epon 618, and polymerized for 36 h at 60 °C. 50–80 nm ultrathin sections were obtained and the sections were visualized using a transmission electron microscope (TEM, JEM-1010, Japan).

2.5. Th1 and Th2 related cytokines analyses

Tissue samples were obtained from lung of SD rats. Samples were snap frozen in liquid nitrogen and stored at −80 °C until analysis. Samples were weighed and 150 mg tissue was homogenized and centrifuged at 4000 × *g* for 10 min at 4 °C. Supernatants were collected and immediately frozen at −80 °C for ELISA. IFN-γand IL-4 were measured by ELISA kits for rats according to the manufacturers' instructions (eBioscience).

Table 1 – Primers sequences used for RT-PCR reactions.

Genes	Primer sequences (5′ → 3′)
β-Actin	Reverse: TGTTGGCATAGAGGTCTTTACGG Forward: TGGGTATGGAATCCTGTGGCA
T-bet	Reverse: GAGCAGAGGGTAGGAATGTGGG Forward: CAGCCGTTTCTACCCTGACCTT
GATA-3	Reverse: GTAGAGGTTGCCCCGCAGTT Forward: CCATTACCACCTATCCGCCCTA

2.6. Real-time quantitative RT-PCR analysis

Total RNA was extracted using TRIzol reagent (Invitrogen, NY). 1 μg of RNA was used to synthesize cDNA using the Reverse Transcription System (TaKaRa). Real-time quantitative PCR (RT-PCR) was performed using the SYBR®Premix Ex TaqTM (Perfect Real Time) (TaKaRa) in an ABI 7300 detection system (Applied Biosystems) according to the instructions of the manufacturer. For all reactions, annealing was carried out at 67 °C, and 40 cycles of amplification were performed. Primers were designed using Primer Express Software according to guidelines (Table 1). Data were normalized using β-actin as a reference gene. The relative levels of expression of target gene among different samples were analyzed using the $2^{-\Delta\Delta CT}$ relative quantitation method (Livak and Schmittgen, 2001).

2.7. Immunohistochemical analysis

Five-micrometer-thick sections cutting from paraffin-embedded blocks were deparaffinized in Xylene and rehydrated in graded alcohol and distilled water. Sections were boiled in 10 mM citrate buffer for 20 min and then washed in phosphate-buffered saline. Subsequently, 6% H_2O_2 in methanol was used to block endogenous peroxidase activity. For antigen recovery, the cuts were incubated in a citrate tampon solution in a pressure cooker for 3 min. The slides were then incubated overnight with primary antibodies for T-bet (Santa Cruz, CA) and GATA-3 (Abcam). Incubation with secondary antibody and product visualization was performed with diaminobenzidine (DAB) substrate chromogen according to the manufacturer's instructions. A final counterstaining was performed with Mayer's hematoxylin. Slides were seen by a dermatopathologist who was not involved in the study. Five randomly selected areas were examined. Positive staining was detected as a brown coloration of the tissues. T-bet and GATA-3 were located in the nucleus (Zhu et al., 2010).

Following immunostaining, the slides were first analyzed manually. Any nuclear staining for T-bet or GATA-3 was considered positive. The immunostaining results were evaluated by defining a threshold of positive staining for all sections before automated processing. Briefly, the threshold of positive signal was defined for each antibody for all the sections following different treatments. Color signal above the threshold for each antibody defined was deemed to be positive, whereas any signal below the threshold was regarded as negative. The density was averaged from ten fields of view. This was performed using Image-Pro Plus (IPP) 4.5 (Media Cybernetics, Silver Spring, MD). All images analyzed with IPP 4.5 were counter checked by a histopathologist (Chua et al., 2010, 2009).

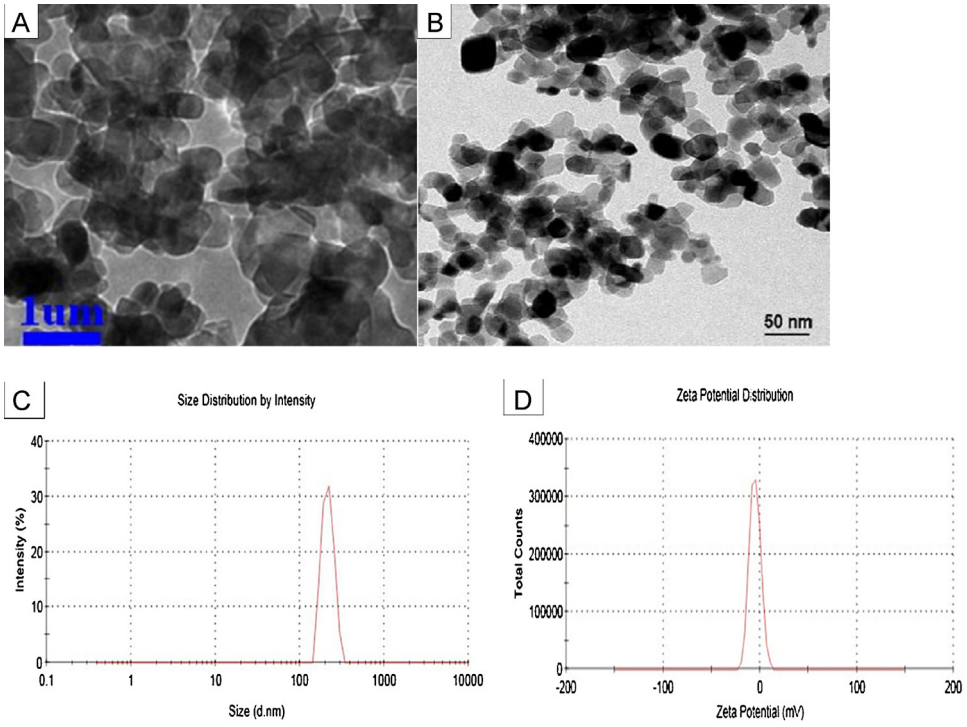

Fig. 1 – Characteristics of TiO_2 particles: A, TEM image of micro TiO_2; B, TEM image of nano TiO_2; C, particle size distribution of nano TiO_2 in NaCl suspension; and D, zeta potential of TiO_2.

2.8. *Statistical analyses*

Statistical analyses were done using SPSS17.0 software. Data were expressed as mean ± SD. One-way analysis of variance (ANOVA) was carried out to compare the differences of means among multi-group data. LSD test was carried out when each group of experimental data was compared with control data. Statistical significance for all tests was judged at a probability level of 0.05.

3. Results

3.1. *Particle characterization*

Particle sizes of the two groups of TiO_2 were approximately 21 nm and 1–2 μm (Table 2 and Fig. 1). As shown in the TEM images (Fig. 1A and B), the TiO_2 particles were uniform in size and shape. However, as measured by laser diffraction particle analyzer at the time of intratracheal instillation, the average diameter of nano TiO_2 suspended in 0.9% NaCl was 195.2 nm (Fig. 1C). The results of diameter show that rats were exposed to agglomerates of TiO_2 that were larger than the primary nanoparticles. The Zeta potential value was −4.95, which suggests the TiO_2 is stable in the solution (Fig. 1D).

Table 2 – Physical properties of TiO_2.

Particle size	Crystalline phase	Surface area (m^2/g)	Purity (%)
21 nm	80% anatase/20% rutile	50	>99.5
1–2 μm	Anatase	18	>99.9

3.2. *General effects of toxicity and gross observation*

After administration of TiO_2, no abnormal behavior and clinical symptoms were found in experimental groups. The changes of body weight are presented in Fig. 2. With the dose increasing, the change of body weight decreased and a significant difference was observed in the nano TiO_2 32 mg/kg group compared to NaCl and micro TiO_2 32 mg/kg groups.

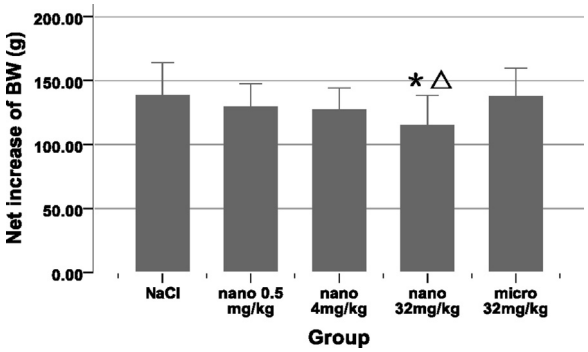

Fig. 2 – The changes of body weight in different groups. Data are expressed as means ± standard deviation, $N = 8$. *Significant difference between control and nano groups, $P < 0.05$. △ Significant difference between nano and micro groups, $P < 0.05$.

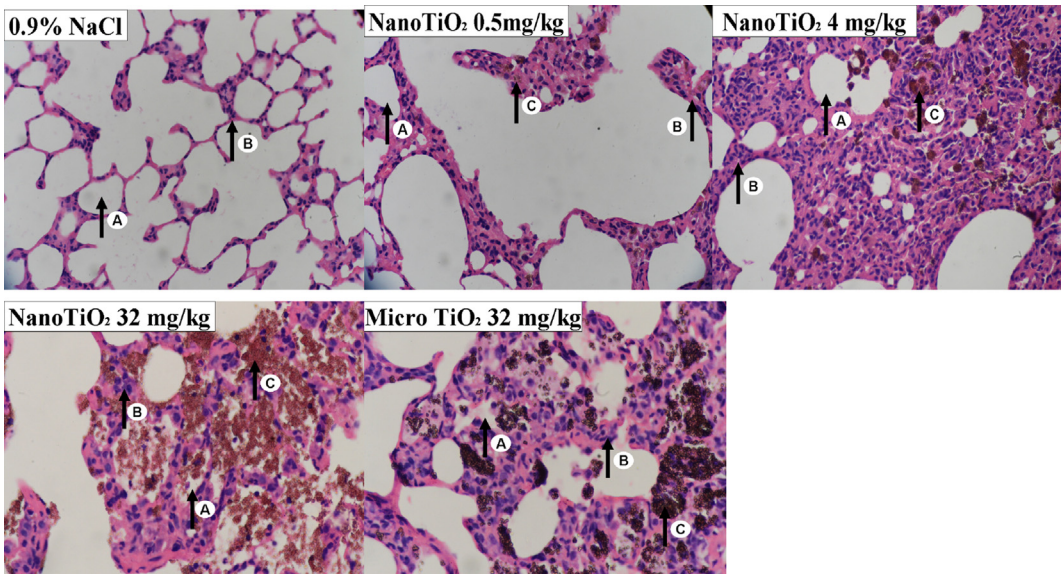

Fig. 3 – The histopathological changes following intra-tracheal instillation of 0.9%NaCl (control) and different dose TiO_2 (×400), A: alveolus; B: alveolar septa; and C: TiO_2 deposition.

3.3. *Histopathology*

Histopathological observations of tissues were conducted (Fig. 3). Control group shows the representative normal architecture of lung tissue sections of controls, including the terminal bronchiole, and the corresponding alveolar duct and alveolus. We found that slight aggregations of lymphocytes and macrophages were revealed and induced pulmonary emphysema, macrophages accumulation, extensive disruption of alveolar septa, from lung tissues exposed to 0.5 mg/kg nano TiO_2 particles. In 4 mg/kg nano TiO_2 groups, slight inflammation was present and aggregations of lymphocytes and macrophages were revealed and thickening of the alveolar wall, collapse of terminal bronchioles, and interstitial thickening were observed and the field shown is accurately representative across animals. After 4 weeks of exposure to 32 mg/kg nano TiO_2, massive particulate deposition was observed in gaps and the alveolar cavity of lung tissue. The observed lesions included macrophages accumulation, extensive disruption of alveolar septa, slight alveolar thickness and expansion hyperemia in comparison to saline treatment. In the micro TiO_2 32 mg/kg group, most of the brown refractive particles gathered into lumps can be found in the alveolar space and interval. Slightly infiltration of inflammatory cells was present on lung cells.

3.4. *TEM inspection*

The ultrastructure of the tissues was examined by TEM. Fig. 4 shows the micrographs of the lungs after four weeks exposure to intratracheally instilled nano TiO_2 particles. Type II cells have prominent lamellar bodies and the damage of lamellar bodies were observed. Mitochondria tumefaction organelles dissolution, endoplasmic reticulum expansion and the gap of nuclear broadening were shown. The degree of nano 32 mg/kg group damage was more serious than nano 4 mg/kg group.

3.5. *Inflammatory cytokines in pulmonary tissues*

The effects of nano TiO_2 were evaluated on the production of cytokines associated with Th1 and Th2 cells by ELISA. No significant changes of IFN-γ and IL-4 expression were observed throughout the experimental period (Fig. 5).

3.6. *T-bet and GATA-3 mRNA relative expression evaluation*

We used semiquantitative RT-PCR to measure the expression levels of T-bet and GATA-3 mRNA in lung tissue from different treatment groups. With the dose increasing, the relative expression of T-bet mRNA decreased and there were significant differences in nano 4 and 32 mg/kg groups compared to control group (Fig. 6). As shown in Fig. 6, the nano 32 mg/kg group expressed higher levels of GATA-3 mRNA than of NaCl controls. The level of T-bet and GATA-3 showed no statistical difference between nano and micro 32 mg/kg groups.

3.7. *T-bet and GATA-3 protein expression evaluation*

The protein expression of T-bet and GATA-3 was also evaluated immunohistochemically. Positive staining for T-bet and GATA-3 was detected in the lymphocyte nucleus of lung lesions of rats. With the dose increasing, the density levels of T-bet decreased and there were significant differences in nano 4 and 32 mg/kg groups compared to control group (Fig. 7). There was no statistical difference in GATA-3 level between nano group and control or micro groups (Fig. 7). The protein level of T-bet and GATA-3 showed no statistical difference between nano and micro 32 mg/kg groups.

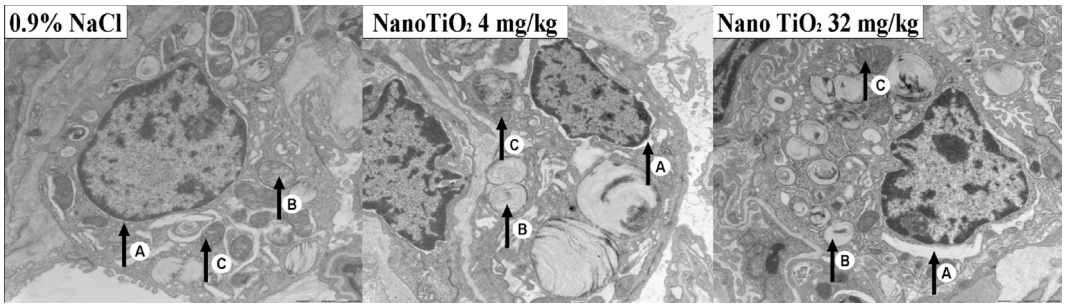

Fig. 4 – TEM image of the lung after intratracheal instillation of nano TiO_2 (×15,000), A: the gap of nuclear; B: lamellar bodies; and C: Mitochondria.

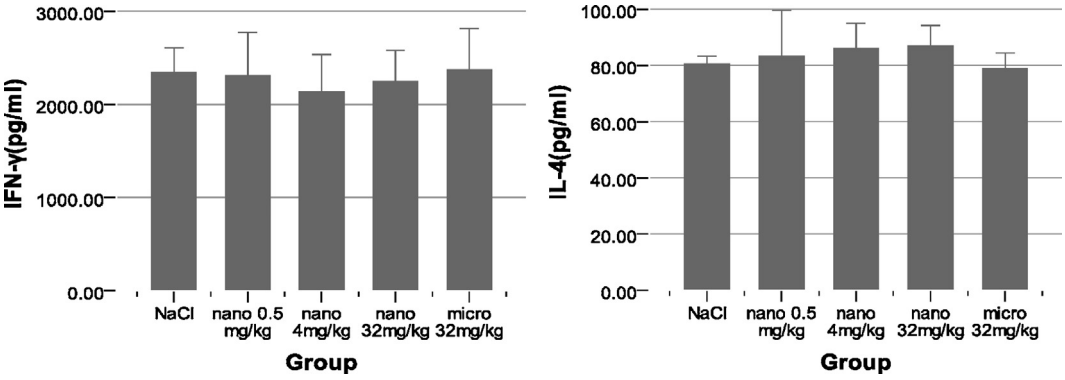

Fig. 5 – The level of IFN-γ and IL-4. Data are expressed as means ± standard deviation, N = 8.

4. Discussion

The ex vivo isolated perfused rat lung model affords discrete examination of lung translocation kinetics without the confounding issue of whole body disposition. It is an acute model only but one that allows exact dosing via intra-tracheal administration with >95% of administered CNTs deposited in the airways of the pulmonary parenchymal regions which are fully perfused in this model (Matthews et al., 2013). To explore the potential immunoregulatory mechanisms, Sprague Dawley rats were exposed by intra-tracheal instillation to nano TiO_2. The histopathology results showed that the lesions of lung tissue included macrophages accumulation, extensive disruption of alveolar septa, slight alveolar thickness and expansion hyperemia and expansion hyperemia in rats after exposure to nano TiO_2, consisting with other studies. Liu (Liu et al., 2009) found that in 0.5 mg/kg and 5.0 mg/kg TiO_2 groups, slight inflammation was present and aggregations of lymphocytes and macrophages were revealed. Thickening of the alveolar wall, collapse of terminal bronchioles, and interstitial thickening were observed in some rats exposed to high-dose TiO_2 nanoparticles. Tang (Tang et al., 2011) results showed that after 7 days of exposure to 5 nm TiO_2 particles, massive particulate deposition was observed in gaps and the alveolar cavity of lung tissue. The observed lesions included increased proliferation

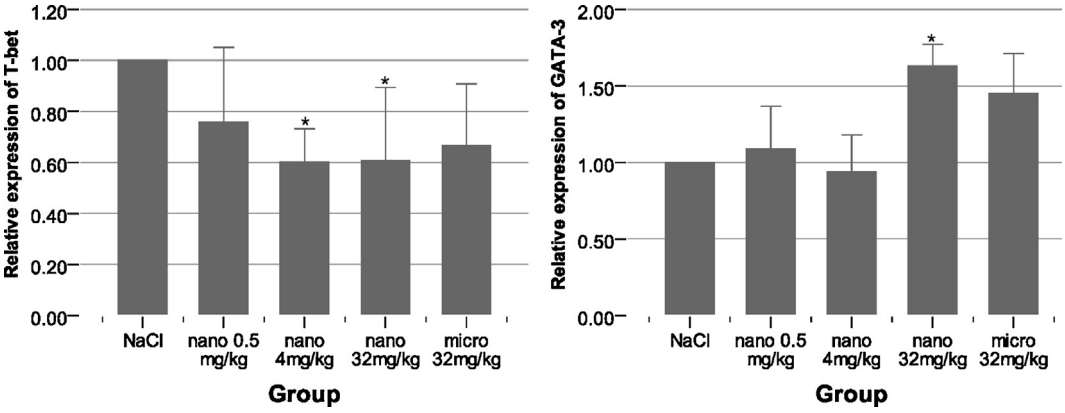

Fig. 6 – The mRNA expression of T-bet and GATA-3. Data are expressed as means ± standard deviation, N = 8. *Significant difference between NaCl and nano groups, $P < 0.05$.

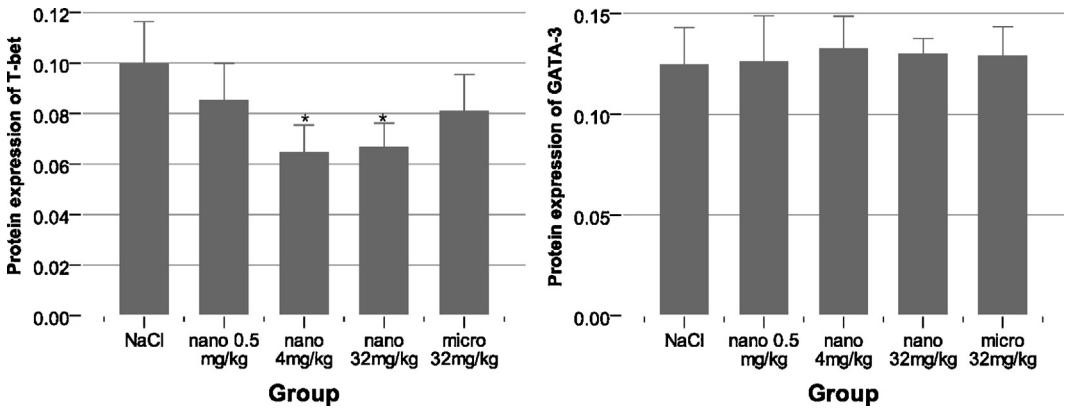

Fig. 7 – Protein expression of T-bet and GATA-3. Data are expressed as means ± standard deviation, N = 8. *Significant difference between NaCl and nano groups, $P < 0.05$.

of tissue macrophages, expansion of lung gaps, slight alveolar thickness and expansion hyperemia in comparison to saline treatment. Mitochondria tumefaction organelles dissolution, endoplasmic reticulum expansion and the gap of nuclear broadening were shown by TEM. The results indicated that nano TiO_2 could induce inflammation and massive particulate deposition in pulmonary tissues and pulmonary injury.

Immune system plays a vital role during the progress of inflammation. The balance of Th1 and Th2 are important in regulating immune function and inflammatory response. The Th1/Th2 paradigm provides a useful model system for investigating the cellular and molecular mechanisms that mediate protective as well as harmful immune responses. Fish exposure to nano TiO_2 resulted in significant changes in innate immune function at the levels of gene expression and cellular function. The immune toxic effects have been observed with concentrations near the estimated environmental concentration values (Jovanovic et al., 2011). Scarino et al. found that allergic pulmonary inflammation is not up-regulated by inhalation of the pollutants ethanol and nano TiO_2. On the contrary, nano TiO_2 decreases lung inflammation in asthmatic rats (Scarino et al., 2012). Gustafsson et al. study results detected an early cytokine expression at Days 1–2 (IL-2, IL-4, IL-6, and IFN-γ), indicating systemic late-phase effects in addition to the local response in airways. These data demonstrate a dynamic response to TiO_2 nanoparticles in the lungs of DA rats, beginning with an innate immune activation of eosinophils, neutrophils, dendritic cells, and NK cells, followed by a long-lasting activation of lymphocytes involved in adaptive immunity (Gustafsson et al., 2011). However, studies on the path-ways of Th1/Th2-related cytokines regulating the inflammatory response and immune function in nano TiO_2 induced disease remain limited. In this study, we analyzed the typical type 1 cytokines (IFN-γ) and typical type 2 cytokines (IL-4). But no significant changes of IFN-γ and IL-4 expression were observed throughout the experimental period.

In addition, there is growing evidence to suggest that two transcription factors, T-bet and GATA-3, are the determining factors of Th cell differentiations and the relative expression of T-bet and GATA-3 has been implicated in a number of immunological diseases (Finotto, 2008). The study showed that cytokines IFN-γ and IL-4 activated the central Th1/Th2 differentiation programs leading to the down-regulation of T-bet and up-regulation of transcription factors GATA-3 exposure to ambient fine particles (Zhao et al., 2012). However, as far as we know, no studies have been carried out dealing with the expression of T-bet/GATA-3 in lung lesions and their relationship with Th1/Th2 cytokines production after exposure to nano TiO_2. As a member of the Tbr1 subfamily of T-box genes, T-bet plays a key role in Th1 differentiation and has been confirmed as a unique Th1-specific transcription factor. T-bet is a Th1 specific transcription factor, which promotes Th1 lineage commitment and maintains a Th1 immune response by an auto-regulatory positive feedback loop with interferon-gamma (Lighvani et al., 2001). It not only controls the expression of the Th1-specific cytokine IFN-γ, but also restrains the generation of IL-4 of the Th2-specific cell factor (Zhu et al., 2010). Moreover, it also induces the shift of Th2 cell dominance toward the opposite Th1 cell dominance. We found that the expression levels of T-bet mRNA in lung tissue from different treatment groups decreased and there were significant differences in nano 4 and 32 mg/kg groups compared to NaCl group. Positive staining for T-bet was detected in the lymphocyte nucleus of lung lesions in rats. With the dose increasing, the density levels of T-bet decreased and there were significant differences in nano 4 and 32 mg/kg groups compared to control group. On the other hand, GATA-3, a member of the GATA family of zinc-finger transcription factors, has been identified as a key regulator of Th2 development (Zheng and Flavell, 1997). GATA-3 could promote Th2 differentiation and induce Th2 cytokine production in an analogous way to T-bet (Lantelme et al., 2001). Blocking of GATA-3 with a dominant-negative construct or antisense DNA prevents Th2 cytokine activation and eosinophilia in allergen-challenge mouse models (Finotto et al., 2001). The relative expression of GATA-3 mRNA level increased at nano TiO_2 32 mg/kg compared to control group. When exposure dose was 4 mg/kg, GATA-3 had slightly high but no statistical differences compared to control group. However, there was no statistical difference in GATA-3 protein level between nano group and control or micro groups. Taken together, exposure to nano TiO_2, the expression of GATA-3 was up-regulated,

whereas T-bet was significantly down-regulated, especially at nano 32 mg/kg. This might be one of the mechanisms linking nano TiO_2 and lung injury.

From a toxicology perspective, fine sized titanium dioxide particulates are often utilized as negative control reference particle-types (Hext et al., 2005). The limited toxicological database demonstrates that P25 ultrafine anatase/rutile TiO_2 particles (henceforth referred to as uf-3) are, on a mass basis, significantly more potent than fine-sized TiO_2 particles in producing adverse lung effects (Warheit and Frame, 2006). The results of shorter-term pulmonary toxicity studies with ultrafine-TiO_2 particles in rats have supported the notion of enhanced lung inflammatory potency of the ultrafine particles when compared to exposures of fine-sized particulates of similar composition. This was considered to be associated with a greater surface area of the ultrafine particles when compared to fine-sized particles (Bermudez et al., 2004; Warheit et al., 2007). In present study, there have the expression changes of IL-4, IFN-γ, GATA-3 and T-bet between the nano TiO_2 32 mg/kg and micro TiO_2 32 mg/kg but no statistical differences.

In conclusion, while this manuscript seeks to investigate Th1 vs. Th2 signatures with an eye toward causally implicating these immune programs in lung pathology, it is essentially a 'negative' manuscript in current form. No changes in Th cytokines were demonstrated, but the changes of histopathological observations can be shown. The mechanism between Th-related cytokines and lung pathology need further study. Our study also provided some clues about the imbalance of Th1/Th2 and lung injury. The dose used in this study was higher than the relevancy of this exposure in the real world exposure scenarios. Maybe in vivo subacute study, we focus far more on the injury effect of higher dose and the injury mechanism. The discrepancy between our study and others is most likely attributed to use of hosts of different susceptibility and differences in the treatment times. Further research is necessary to confirm these results and to shed light on the roles of NP characteristics inducing the alterations described above.

Conflict of interest statement

The authors declare that they have no conflict of interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.etap.2013.12.001.

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