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Th2 Factors May Be Involved in TiO_2 NP-Induced Hepatic Inflammation

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Supporting Information

ABSTRACT: TiO_2 nanoparticles (NPs) are used in the food industry but have potential toxic effects in humans and animals. The resulting immune response is driven by the production of Th2 cytokines IL-4 and IL-5, which contribute to the development of hepatic inflammation. However, TiO_2 NPs have been demonstrated to impair liver function and cause liver inflammation in animal models, which may be associated with activation of Th2 factor-mediated pathways. Mice were administered a gavage instillation of 2.5, 5, or 10 mg/kg body weight TiO_2 NPs for six consecutive months. We investigated whether TiO_2 NPs activate the Th2 factor-mediated signaling pathway under TiO_2 NP-induced hepatic toxicity. The results showed that mice exhibited an accumulation of titanium in the liver, which in turn led to reductions in body weight, increases in liver indices, liver dysfunction, infiltration of inflammatory cells, and hepatocyte apoptosis or necrosis. Furthermore, hepatic inflammation was accompanied by increased (0.67 ± 0.09- to 2.14 ± 0.19-fold) IL-4 expression and up-regulation of its target genes including IL-5 (0.1 ± 0.06- to 0.69 ± 0.12-fold), IL-12 (0.08 ± 0.03- to 0.83 ± 0.21-fold), IFN-γ (0.17 ± 0.09- to 0.87 ± 0.15-fold), GATA3 (0.05 ± 0.02- to 1.29 ± 0.18-fold), GATA4 (0.04 ± 0.01- to 0.87 ± 0.13-fold), T-bet (0.3 ± 0.06- to 0.93 ± 0.15-fold), RORγt (0.32 ± 0.11- to 1.67 ± 0.17-fold), STAt3 (0.16 ± 0.06- to 2.14 ± 0.23-fold), STAT6 (0.2 ± 0.05- to 0.63 ± 0.12-fold), eotaxin (0.53 ± 0.13- to 1.49 ± 0.21-fold), MCP-1 (0.5 ± 0.11- to 0.74 ± 0.18-fold), and MIP-2 (0.27 ± 0.07- to 0.71 ± 0.18-fold) and significant down-regulation of its target gene STAT1 (−0.15 ± 0.05 to −0.81 ± 0.11-fold). Taken together, the alteration of Th2 factor expression may be involved in the control of hepatic inflammation induced by chronic TiO_2 NP toxicity.

KEYWORDS: *titanium dioxide nanoparticles, liver, inflammation, Th2 factors, IL-4-mediated pathway*

INTRODUCTION

Titanium dioxide nanoparticles (TiO_2 NPs) have broadly been used in various areas including food additives, food packaging components, or as dietary supplements, cosmetics, and sunscreens due to their unique physical, chemical, and biological properties.[1] Recently, studies have suggested that TiO_2 NP exposure with 5 to 150 mg/kg body weight and for 14, 60, or 90 consecutive days induced liver inflammation in mice.[2−5] However, the liver toxicological effect of TiO_2 NPs for longer exposure duration and the lower dose is not well understood.

As for the immunotoxicity of TiO_2 NPs, the exposure of mice to TiO_2 NPs resulted in the induction of interleukin (IL)-2 and IL-4 production and the activation of the transcription factors NF-κB in the liver, kidney, spleen, and lung[2−10] and decreased the number of natural killer cells, T-lymphocyte subpopulations, and the number of B lymphocytes from the peripheral blood.[5,9] TiO_2 NP exposure had been demonstrated to result in inflammation of liver,[2−5,11,12] kidney,[6,13,14] spleen,[7,8] lung,[9,10,15] and brain[16−19] in mice and increased numbers of neutrophils and eosinophils in bronchoalveolar lavage of mice,[15] while eosinophils are generally induced by helper T lymphocyte (Th) 2 cytokines.[20] Therefore, we hypothesized that Th2 factors may be involved in the TiO_2 NP-induced liver inflammation.

As shown, T cell-regulated immune responses play important roles in the pathogenesis of various liver disorders.[20−22] The action of T cells in the liver is modulated via releasing multiple cytokines, which target hepatocytes and immunocytes by activating various immune regulatory factors, such as the signal transducers and activators of transcription factor (STAT) family members.[23] STAT6 is specifically activated by IL-4, which plays pivotal roles in Th2 differentiation, tissue adhesion, and inflammatory responses.[24,25] Th cells are subdivided into Th1, Th2, and Th17 subsets due to their unique production of cytokines and characteristic transcription factors. Th1 cells require "T-box expressed in T cells" (T-bet) and secrete interferon (IFN)-γ; Th2 cells require GATA-binding domain-3 (GATA-3) and generate IL-4, IL-5, and IL-13.[26,27] IL-4 and IL-5 can influence a variety of events involved in inflammation. IL-4 promotes IgE production and the development of mast cells, while IL-5 is closely associated with the development of eosinophils.[20] In Th cell-mediated liver injury, IL-4 promotes the productions of eotaxin-1 and IL-5, which in turn attract neutrophils and eosinophils into the liver, resulting in hepatitis.[25] We hypothesized that TiO_2 NP exposure may aggravate inflammatory responses through its effects on the Th2 factor-mediated pathway.

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Table 1. Real-Time PCR Primer Pairs. PCR Primers Used in the Gene Expression Analysis

gene name	description	primer sequence	primer size (bp)
Refer-actin	mactin-F	5′-GAGACCTTCAACACCCCAGC-3′	
	mactin-R	5′-ATGTCACGCACGATTTCCC-3′	263
IL-4	mIL-4-F	5′-TGTAGGGCTTCCAAGGTGCT-3′	
	mIL-4-R	5′-TGATGCTCTTTAGGCTTTCCAG-3′	199
IL-5	mIL-5-F	5′-GTGAAAGAGACCTTGACACAGCTG-3′	
	mIL-5-R	5′-CACACCAAGGAACTCTTGCAGGTA-3′	290
IL-12	mIL-12-F	5′-ACTCGGCTCCTCATGGACAT-3′	
	mIL-12-R	5′-TGCAACAGTCAGGCTCTT-3′	278
IFN-γ	mIFN-γ-F	5′-TGAAAGACAATCAGGCCATCA-3′	
	mIFN-γ-R	5′-CTGGACCTGTGGGTTGTTGA-3′	140
GATA3	mGATA3-F	5′-CCACGGGAGCCAGGTATG-3′	
	mGATA3-R	5′-CGGAGGGTAAACGGACAGAG-3′	169
GATA4	mGata4-F	5′-CCTGGAAGACACCCCAATCT-3′	
	mGata4-R	5′-GGTAGTGTCCCGTCCCATCT-3′	115
T-bet	mT-bet-F	5′-TGGACCCAACTGTCAACTGC-3′	
	mT-bet-R	5′-CTCGGAACTCCGCTTCATAAC-3′	173
STAT1	mSTAT1-F	5′-ACGCTGCCTATGATGTCTCG-3′	
	mSTAT1-R	5′-ACGGGATCTTCTTGGAAGTTATC-3′	163
STAt3	mSTAT3-F	5′-TGACCAATAACCCCAAGAACG-3′	
	mSTAT3-R	5′-TGACACCCTGAGTAGTTCACACC-3′	181
STAT6	mSTAT6-F	5′-AGCATCTTGCCGCACATCA-3′	
	mSTAT6-R	5′-GGCAGGTGGCGGAACTCT-3′	128
eotaxin	mEotaxin-F	5′-TGCTCACGGTCACTTCCTTC-3′	
	mEotaxin-R	5′-GGTGCTTTGTGGCATCCTG-3′	231
MCP-1	mMCP-1-F	5′-GCTGACCCCAAGAAGGAATG-3′	
	mMCP-1-R	5′-TTGAGGTGGTTGTGGAAAAGG-3′	184
MIP-2	mMIP-2F	5′-CACCAACCACCAGGCTACAG-3′	
	mMIP-2R	5′-GCTTCAGGGTCAAGGCAAAC-3′	189

TiO_2 is considered to be an inert and poorly soluble matter. As a common additive in many foods, TiO_2 is used for whitening and brightening foods, particularly for confectionaries, white sauces and dressings, and certain powdered foods.[28] It has been estimated that in the UK the dietary intake of TiO_2 is 5 mg per person per day.[29] The quantity of TiO_2 cannot exceed 1% by weight of the food according to the federal regulations of the U.S. government. In 1969, WHO reported that the LD_{50} of TiO_2 for rats is greater than 12 g kg^{-1} BW after an oral administration. Therefore, a potential exposure route for the general population is oral ingestion. The studies of both longer exposure duration and the lower dose of TiO_2 are of interest for risk assessors. In view of the above, therefore, the aim of the present study was stated to be the investigation of whether the liver inflammation observed in the earlier studies is mediated by Th2 factors in mice for longer exposure duration and the lower dose.

MATERIALS AND METHODS

Chemicals. The preparation and characteristics of TiO_2 NPs particles including the anatase structure, size, surface area, mean hydrodynamic diameter, and ζ potential have been described in our previously work.[30,31] X-ray-diffraction (XRD) was used to detect the anatase structure and size with a charge-coupled device (CCD) diffractometer (Mercury 3 Versatile CCD detector; Rigaku Corporation, Tokyo, Japan) using Ni-filtered Cu Kα radiation. The NP size was determined using a TecnaiG220 transmission electron microscope (TEM) (FEI Co., USA). The surface area of NPs was determined by Brunauer−Emmett−Teller (BET) adsorption measurements on a Micromeritics ASCORBIC ACIDP 2020M+C instrument (Micromeritics Co., USA). The average aggregate or agglomerate size and ζ potential of NPs were measured by dynamic light scattering (DLS) using a Zeta PALS + BI-90 Plus (Brookhaven Instruments Corp., USA). XRD measurements suggested that TiO_2 NPs showed an anatase structure. The average particle size of powdered TiO_2 NPs suspended in 0.5% w/v HPMC solvent after 24 h (5 mg/mL) incubation ranged from 5 to 6 nm, and the surface area was 174.8 m^2/g. The mean hydrodynamic diameter of TiO_2 NPs in HPMC solvent (5 mg/mL) ranged from 208 to 330 nm (mainly 294 nm), and the ζ potential after 24 h incubation was 9.28 mV.[31] The anatase structure, size, surface area, mean hydrodynamic diameter, and ζ potential have been described in the Supporting Information.

Animals and Treatment. 160 CD-1 (ICR) male mice (20 ± 2 g body weight) were purchased from the Animal Center of Soochow University (China). All mice were housed in stainless steel cages in a ventilated animal room. Room temperature of 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 for mice *ad libitum*. Prior to dosing, the mice were acclimated to this environment for 5 days. All procedures used in animal experiments conformed to the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals.

TiO_2 NP powder was dispersed onto the surface of 0.5% w/v HPMC, and the suspension containing TiO_2 NPs was treated ultrasonically for 30 min and mechanically vibrated for 5 min. The mice were randomly divided into four groups (n = 30 each), including a control group treated with 0.5% w/v HPMC and three experimental groups treated with 2.5, 5, or 10 mg/kg body weight (BW) TiO_2 NPs. The mice were weighed, the volume of TiO_2 NP suspensions was calculated for each mouse, and the fresh TiO_2 NP suspensions were gavaged to the mice by a gavage needle every day for six months. Any symptoms, growth state (weighing all animals every day), eating and drinking (quantitatively measuring food and water consumption every day), activity, and/or mortality were observed and recorded carefully daily during the six months.

Liver Indices. After six months, mice were weighed and then sacrificed after ether anesthesia. Blood samples were collected from the

eye vein by rapidly removing the eyeball, and serum was collected by centrifuging the blood samples at 1200*g* for 10 min. The livers of all animals were quickly removed and placed on ice. After weighing the body and livers, the liver indices were calculated as the ratio of liver (wet weight, mg) to body weight (g).

Titanium Content Analysis. The frozen liver tissues (n = 5 each) were thawed, and approximately 0.3 g samples were weighed, digested, and analyzed for titanium content. Prior to elemental analysis, the liver tissues were digested overnight with nitric acid (ultrapure grade), combined with 0.5 mL of H_2O_2, and incubated at 160 °C in high-pressure reaction containers in an oven until the samples were completely digested. The solutions were incubated at 120 °C to remove any remaining nitric acid until the solutions were clear. Finally, the remaining solutions were diluted to 3 mL with 2% nitric acid. Inductively coupled plasma-mass spectrometry (ICP-MS; Thermo Elemental X7; Thermo Electron Co., Waltham, MA, USA) was used to determine the titanium concentration in the samples. Indium (20 ng/mL) was chosen as an internal standard element. Elemental titanium (isotopes ^{48}Ti or ^{49}Ti) was quantified using ICP-MS against titanium standards, which also contained the internal standard.

Biochemical Analysis of Liver Functions. Serum biochemical functions including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) concentrations (n = 5 each) were determined by ELISA (R&D Systems, Minneapolis, MN, USA). All biochemical assays were performed using a clinical automatic chemistry analyzer (type 7170A, Hitachi, Japan).

Histopathological Examination of the Liver. All histopathological examinations were performed using standard laboratory procedures. Five sets of liver tissues from each dose group were embedded in paraffin blocks, sliced to 5 μm thickness, and placed on separate glass slides (five slices from each kidney). After hematoxylin−eosin staining, the sections were evaluated by a histopathologist unaware of the treatments, using an optical microscope (U−III Multipoint Sensor System; Nikon, Tokyo, Japan).

Observation of Hepatocyte Ultrastructure. Livers (n = 5 each) were fixed in a fresh solution of 0.1 M sodium cacodylate buffer containing 2.5% glutaraldehyde and 2% formaldehyde followed by a 2 h fixation period at 4 °C with 1% osmium tetroxide in 50 mM sodium cacodylate (pH 7.2−7.4). Staining was performed overnight with 0.5% aqueous uranyl acetate. The specimens were dehydrated in a graded series of ethanol (75, 85, 95, and 100%) and embedded in Epon 812. Ultrathin sections were obtained, contrasted with uranyl acetate and lead citrate, and observed with a Hitachi H600 TEM (Hitachi Co., Japan). Liver apoptosis was determined based on the changes in nuclear morphology (e.g., chromatin condensation and fragmentation).

Assay of Cytokine Expression. The levels of mRNA expression of *IL-4*, *IL-5*, *IL-12*, *FN-γ*, *GATA3*, *GATA 4*, *T-bet*, *STAT1*, *STAt3*, *STAT6*, *eotaxin*, *MCP-1*, and *MIP-2* in the livers (n = 5 each) were determined using real-time quantitative (q)RT-PCR, as described previously.[32] Synthesized cDNA was generated by qRT-PCR with primers designed with Primer Express Software (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's guidelines, and the sequences are listed in Table 1. The right livers from mice with or without TiO_2 NP treatment were homogenized using QIAzol lysis reagent with a TissueRuptor (Roche, USA). Total RNA from the homogenates was isolated using Tripure isolation reagent (Roche, USA) according to the manufacturer's instructions. The RT reagent (Shinegene, China) of 30 μL was prepared by mixing 15 μL of 2× RT buffer, 1 μL of random primer in a concentration of 100 pmol·μL^{-1}, 1 μL of RTase, 5 μL of RNA, and 8 μL of DEPC water together. The reaction condition was 25 °C for 10 min, 40 °C for 60 min, and 70 °C for 10 min. The internal reference gene was actin3. qRT-PCR was performed using the 7500 real-time PCR system (ABI) with SYBR Premix Ex Taq (Takara) according to the manufacturer's instructions. The gene expression analysis and experimental system evaluation were performed according to the standard curve and quantitation reports.

To determine IL-4, IL-5, IL-12, IFN-γ, GATA3, GATA4, T-bet, STAT1, STAt3, STAT6, eotaxin, MCP-1, and MIP-2 levels in the mouse liver tissues, ELISAs were performed using commercial kits specific for each protein (R&D Systems, Minneapolis, MN, 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 IL-4, IL-5, IL-12, IFN-γ, GATA3, GATA4, T-bet, STAT1, STAt3, STAT6, eotaxin, MCP-1, and MIP-2 were calculated from a standard curve for each sample.

Statistical Analysis. All results are expressed as means ± standard error (SE). One-way analysis of variance (ANOVA) was carried out to compare the differences of means among the multigroup data using SPSS 19 software (SPSS, Inc., Chicago, IL, USA). Dunnett's test was performed when each data set was compared with the solvent control data. Statistical significance for all tests was judged with a probability level of 0.05 (p < 0.05).

RESULTS

Body Weight, Liver Indices, and Titanium Accumulation. During the treatment, all mice were all at growth state. The daily behaviors such as food consumption, drinking, and activity in TiO_2 NP-treated groups were not significantly different from those of the control group. Figure 1 shows the net increase in body weight, liver weight, and liver indices caused by TiO_2 NP exposure. TiO_2 NP exposure resulted in significant reductions in the net increase of body weight and increases in liver indices as compared with the controls (p < 0.05), but liver weight did not exhibit differences among the four groups (p > 0.05). Furthermore, there was significant titanium accumulation with increased TiO_2 NP dose (Figure 2, p < 0.001). The decreased body weight and increased liver indices caused by TiO_2 NP exposure may be related to liver dysfunction and tissue injury, which were confirmed by the biochemical assays and histopathological observations of mouse livers.

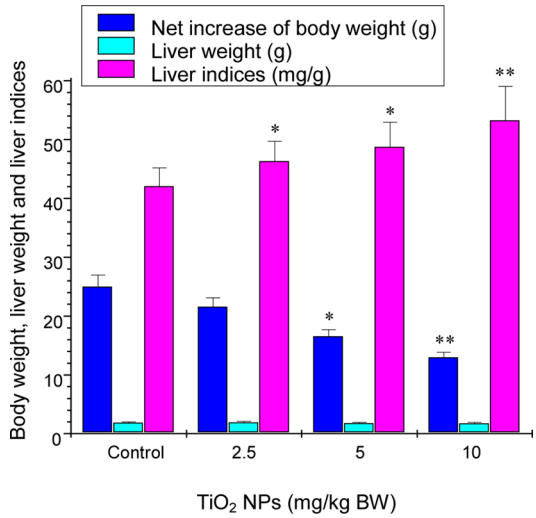

Figure 1. Net increase of body weight, liver weight, and liver indices of male mice after gavage administration of TiO_2 NPs for six consecutive months. *p < 0.05, and **p < 0.01. Values represent means ± SE (n = 40).

Histopathological Evaluation. The histological changes in the liver specimens are shown in Figure 3. Unexposed liver samples exhibited normal architecture including intact hepatic lobule and normal blood sinusoid opening (Figure 3), while those from five mice of each group exposed to increasing TiO_2 NP concentrations exhibited severe pathological changes,

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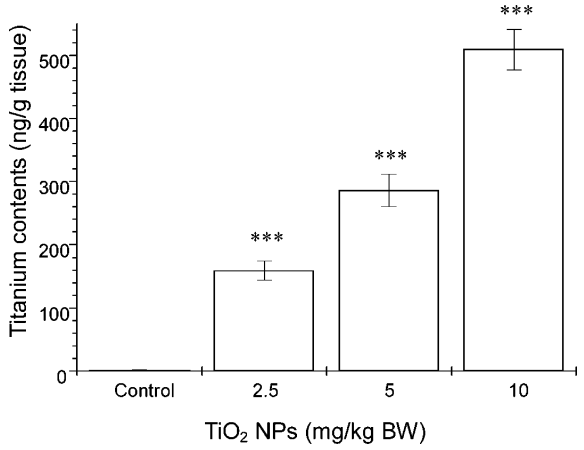

Figure 2. Titanium contents in mouse livers after gavage administration of TiO_2 NPs for six consecutive months. ***p < 0.001. Values represent means ± SE (n = 5).

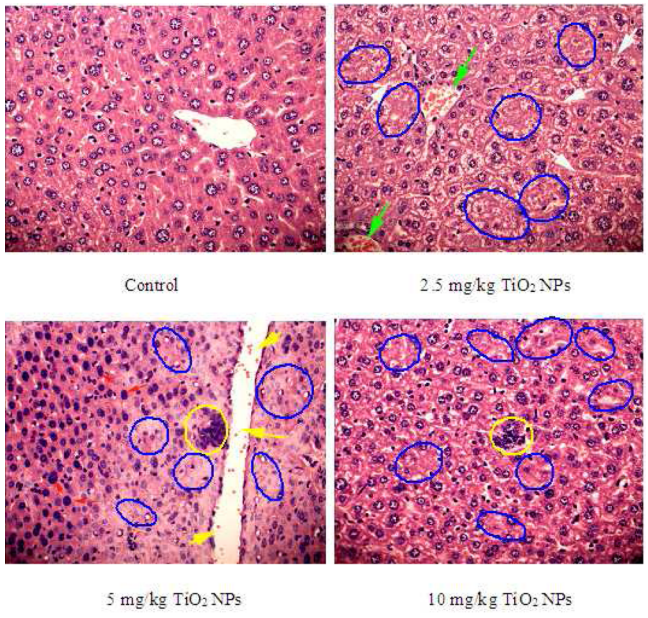

Figure 3. Histopathological observation of livers after gavage administration of TiO_2 NPs for six consecutive months (n = 5). The control group (unexposed mice) showed intact hepatic lobule and normal blood sinusoid opening (400×). The 2.5 mg/kg TiO_2 NP group presented with vein congestion (green arrows), angiectasis and hyperemia (white arrows), and neorobiosis of hepatocytes (blue circles) (400×). The 5 mg/kg TiO_2 NP group presented with inflammatory cell infiltration (yellow circle), macrophages (red arrows)), hepatic tissue crevice (yellow arrows), and neorobiosis of hepatocytes (blue circles) (400×). The 10 mg/kg TiO_2 NP group presented with inflammatory cell infiltration (yellow circle) and neorobiosis of hepatocytes (blue circles) (400×).

including angiectasis and hyperemia, infiltration of inflammatory cells, macrophage aggregation, hepatic tissue crevice, and hepatocyte necrosis (Figure 3). The results suggested that chronic exposure to TiO_2 NPs resulted in significant pathological changes in the livers, which may be related to cytokine expression.

Observation of Hepatocyte Ultrastructure. Changes to hepatocyte ultrastructure in the mouse liver samples are presented in Figure 4. As shown, hepatocytes of the control group contained elliptical nuclei with homogeneous chromatin

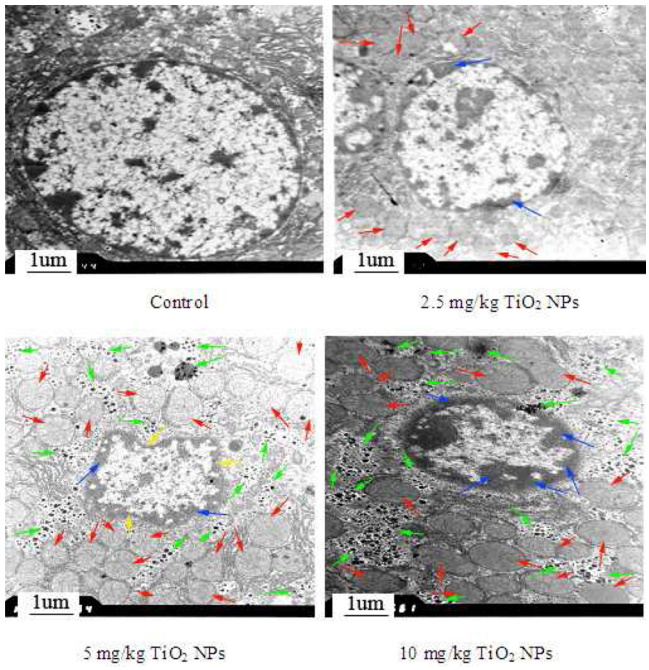

Figure 4. Ultrastructure observation of hepatocytes in mouse liver after gavage administration of TiO_2 NPs for six consecutive months (n = 5). The control showed elliptical nuclei with homogeneous chromatin (10,000×). The 2.5 mg/kg TiO_2 NP exposure group exhibited light mitochondrial swelling (red arrows) and chromatin marginalization (blue arrows) (10,000×). The 5 mg/kg TiO_2 NP exposure group exhibited mitochondrial swelling (red arrows), nuclear membrane collapse (yellow arrows), chromatin marginalization (blue arrows), and TiO_2 NP deposition in the cytoplasm (green arrows) and/or mitochondria (green arrows) (10,000×). (d) The 10 mg/kg TiO_2 NP exposure group exhibited severe mitochondrial swelling (red arrows), chromatin marginalization (blue arrows), and significant TiO_2 NP deposition in the cytoplasm and/or on nuclear membrane (green arrows) (10,000×).

(Figure 4); however, ultrastructure of hepatocytes in the liver treated with TiO_2 NPs indicated a typical apoptosis, including significant mitochondrial swelling, nuclear membrane collapse, and chromatin marginalization (Figure 4). In addition, we also significantly observed black particle agglomerates in the cytoplasm, in mitochondria, and/or on nuclear membranes in the TiO_2 NP-exposed hepatocytes (Figure 4), further confirming that TiO_2 NPs were deposited in the mouse liver.

Liver Function. The changes in the serum biochemical parameters induced by TiO_2 NP exposure are presented in Table 2. With increased TiO_2 NP dose, the levels of ALT, AST, ALP, and LDH were gradually increased, respectively (p < 0.05 or 0.01), suggesting that chronic exposure to TiO_2 NPs resulted in hepatic dysfunction.

Th2 Factor Expression. Mice with TiO_2 NP-induced hepatopathy presented with a significant, dose-dependent increase in Th2-type cytokines IL-4 and IL-5 and Th1-type cytokine IL-12 expression in the liver tissue (Tables 3, 4) (p < 0.05). This was associated with marked up-regulation of the measured IL-4 and IL-12 target genes, including IFN-γ, GATA3, GATA4, T-bet, STAt3, STAT6, eotaxin, MCP-1, and MIP-2, and significant down-regulation of STAT1 expression in the mouse liver under conditions of TiO_2 NP-induced hepatopathy (Tables 3, 4) (p < 0.05). These findings pointed to the promoted activation of the IL-4-mediated pathway in mice following exposure to TiO_2 NPs.

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Table 2. Changes of Biochemical Parameters in the Blood Serum of Mice after Gavage Administration of TiO_2 NPs for Six Consecutive Months

	TiO_2 NPs (mg/kg BW)			
index	control	2.5	5	10
ALT (U/L)	27.44 ± 1.58	28.61 ± 1.63	38.95 ± 2.04[a]	41.43 ± 2.27[a]
AST (U/L)	103.26 ± 5.56	110.75 ± 5.78	142.81 ± 7.69[a]	175.96 ± 9.06[b]
ALP (U/L)	125.48 ± 6.48	132.78 ± 6.89	161.27 ± 8.36[a]	176.78 ± 9.18[b]
LDH (U/L)	876.74 ± 45.84	903.08 ± 46.25	1272.91 ± 65.68[a]	1352.65 ± 68.66[b]
T-Bil (μmol/L)	1.75 ± 0.11	1.68 ± 0.11	1.25 ± 0.08[a]	1.31 ± 0.07[b]
TChol (mmol/L)	2.65 ± 0.23	2.98 ± 0.29	3.64 ± 0.32[b]	4.08 ± 0.41[b]
TG (mmol/L)	2.01 ± 0.21	2.53 ± 0.25[a]	3.49 ± 0.31[b]	4.14 ± 0.46[c]

[a]$p < 0.05$. [b]$p < 0.01$. [c]$p < 0.001$. Values represent means ± SE ($n = 5$).

Table 3. qRT-PCR Assay of mRNA Expression in Mouse Livers after Gavage Administration of TiO_2 NPs for Six Consecutive Months

	TiO_2 NPs (mg/kg BW)			
ratio of gene/actin	control	2.5	5	10
IL-4	0.65 ± 0.12	1.09 ± 0.12[a]	1.88 ± 0.16[b]	2.42 ± 0.21[c]
IL-5	2.48 ± 0.21	2.74 ± 0.22	3.01 ± 0.25[a]	4.18 ± 0.32[b]
IL-12	1.56 ± 0.13	1.68 ± 0.12	2.07 ± 0.18[a]	2.86 ± 0.25[b]
IFN-γ	2.61 ± 0.23	3.06 ± 0.23	3.27 ± 0.35[a]	4.89 ± 0.45[b]
GATA3	1.56 ± 0.12	1.64 ± 0.11	2.74 ± 0.24[a]	3.04 ± 0.35[b]
GATA 4	0.89 ± 0.11	0.93 ± 0.15	1.39 ± 0.12[a]	1.48 ± 0.12[a]
T-bet	1.19 ± 0.15	1.55 ± 0.14[a]	1.62 ± 0.17[a]	2.66 ± 0.22[b]
RORγt	2.21 ± 0.18	2.91 ± 0.22[a]	3.36 ± 0.37[b]	5.91 ± 0.41[c]
STAT1	6.62 ± 0.53	5.57 ± 0.45	4.33 ± 0.32[a]	3.66 ± 0.28[b]
STAt3	0.76 ± 0.08	0.88 ± 0.10	1.59 ± 0.13[b]	2.76 ± 0.25[c]
STAT6	2.45 ± 0.16	2.94 ± 0.19[a]	3.87 ± 0.26[b]	3.99 ± 0.29[c]
eotaxin	0.89 ± 0.06	1.36 ± 0.09[a]	1.51 ± 0.10[a]	2.27 ± 0.18[b]
MCP-1	1.32 ± 0.16	1.98 ± 0.18[a]	2.25 ± 0.20[a]	2.33 ± 0.22[a]
MIP-2	2.15 ± 0.21	2.74 ± 0.27[a]	3.03 ± 0.31[a]	3.57 ± 0.35[b]

[a]$p < 0.05$. [b]$p < 0.01$. [c]$p < 0.001$. Values represent means ± SE ($n = 5$).

Table 4. ELISA Assay of Protein Expression in Mouse Livers after Gavage Administration of TiO_2 NPs for Six Consecutive Months

	TiO_2 NPs (mg/kg BW)			
protein expression (ng/g tissue)	control	2.5	5	10
IL-4	176.15 ± 9.81	295.39 ± 15.67[a]	529.48 ± 27.45[b]	555.82 ± 28.89[b]
IL-5	672.08 ± 34.56	742.54 ± 39.55[a]	1115.71 ± 53.61[b]	1148.78 ± 57.66[b]
IL-12	422.76 ± 23.28	555.28 ± 29.67[a]	570.97 ± 31.62[a]	798.06 ± 45.32[b]
IFN-γ	707.31 ± 38.72	879.26 ± 46.73[a]	896.17 ± 48.99[a]	1341.19 ± 63.81[b]
GATA3	429.98 ± 23.53	648.46 ± 35.66[b]	752.54 ± 39.65[b]	993.82 ± 55.97[c]
GATA4	241.19 ± 13.42	352.03 ± 18.58[a]	396.69 ± 21.29[a]	465.11 ± 25.52[b]
T-bet	322.49 ± 18.22	420.05 ± 24.06[a]	439.02 ± 22.37[a]	630.86 ± 33.71[b]
RORγt	598.91 ± 32.86	788.61 ± 45.28[a]	1520.56 ± 79.31[b]	1611.61 ± 86.95[b]
STAT1	1794.02 ± 96.28	1709.47 ± 89.67	1283.43 ± 66.56[b]	991.86 ± 53.01[b]
STAt3	205.96 ± 13.32	218.48 ± 14.06	435.89 ± 23.42[b]	657.96 ± 35.18[c]
STAT6	663.95 ± 35.57	696.74 ± 37.11	1058.77 ± 55.02	1091.29 ± 56.09
eotaxin	247.39 ± 13.69	368.56 ± 19.55[a]	419.21 ± 23.76[a]	625.17 ± 32.09[b]
MCP-1	357.72 ± 18.87	536.58 ± 28.16[b]	619.75 ± 34.62[b]	631.43 ± 35.75[b]
MIP-2	582.65 ± 33.01	792.54 ± 42.51[a]	831.13 ± 45.73[a]	999.47 ± 53.35[b]

[a]$p < 0.05$. [b]$p < 0.01$. [c]$p < 0.001$. Values represent means ± SE ($n = 5$).

DISCUSSION

The results of the present study indicate that gavage administration of 2.5, 5, or 10 mg/kg of TiO_2 NPs for six consecutive months led to body weight reduction, increased liver indices (Figure 1), and titanium accumulation (Figure 2). This resulted in an inflammatory response, macrophage aggregation, and hepatocyte necrosis in the livers (Figure 3) and ultrastructure damages of hepatocytes (Figure 4). Our previous studies observed that TiO_2 NP exposures with 10–250 mg/kg BW for 14, 60, or 90 consecutive days led to liver histopathological changes, including congestion of vascellum, wide-bound basophilia, focal ischemia,[2] large overall fatty

degeneration, inflammatory cell infiltration, necrosis,[3] congestion of interstitial vessels,[4] focal inflammatory cell infiltration and edema,[5] and apoptosis.[33] ALT, AST, ALP, and LDH are cellular enzymes that indicate the presence of injury in the liver. Under healthy circumstances, ALT, AST, ALP, and LDH are contained within the cell. When cellular injury occurs, the enzymes are released from the cytoplasm into the bloodstream, suggesting liver damage.[34] In the current study, TiO_2 NP exposure significantly elevated the serum ALT, AST, ALP, and LDH levels (Table 2), which is consistent with previous reports in our laboratory experiments[2−4] and may be cellular injury in the mouse liver (Figure 4). The liver injury and dysfunction caused by exposure to TiO_2 NPs may be involved in the impairment of immune-mediated function in mice, such as alteration in Th2-mediated gene expression in the liver.

Our previous studies had demonstrated that TiO_2 NP-induced hepatic inflammation was associated with overexpression of nucleic factor (NF)-κB, IκB kinase, NF-κBP52, NF-κBP65, NF-κB-inducible kinase (NIK), Toll-like receptor (TLR)-2 and -4, and proinflammatory cytokines including macrophage migration inhibitory factor, tumor necrosis factor-α, IL-6, IL-1β, cross-reaction protein, IL-4, IL-10, and IL-2 expression in mice.[2,3] In this study, however, a relationship between TiO_2 NP-induced liver injury and immunological factors was demonstrated (Tables 3 and 4). Elevated IL-4 has been reported in human liver diseases such as chronic hepatitis C[35] and primary biliary cirrhosis.[36] In the current study, increased IL-4 expression was also demonstrated to be involved in the TiO_2 NP-induced liver injury for longer exposure duration and lower dose. The administration of 2.5, 5, or 10 mg/kg of TiO_2 NPs for six consecutive months significantly increased the expression of hepatic IL-5, IL-12, STAT3, STAT6, and eotaxin-1, whereas TiO_2 NPs decreased STAT1 expression (Tables 3 and 4). These results suggested that Th2-mediated factors could be involved in the TiO_2 NP-induced liver injury for longer exposure duration and lower dose. It has been reported that IL-4 activates STAT6, which induces IL-5 and eotaxins-1,[25] and induces SOCS1 and SOCS3, which inhibit the STAT1 activity.[37] STAT3 is a transcription factor that participates in many biological processes, especially those of cell survival and proliferation.[38,39] IL-12 induces the tyrosine phosphorylation and DNA binding of STAT3 and STAT4.[40] Serine phosphorylation has been noted to increase transcriptional activation of STAT proteins.[41] On the other hand, STAT6 is necessary for the normal development of Th2 cells.[24] Therefore, the increased expression in the STAT3 and STAT6 due to exposure to TiO_2 NPs may indicate the protection of lymphocytes against apoptosis induction and development of Th2 cells.

T-bet and GATA play early roles in Th cell development to regulate IFN-γ and IL-4/IL-5 gene expression, respectively. The cytokines IL-12 and IL-4/IL-5 secondarily act via STATs to promote cell growth and extinguish expression of either GATA or T-bet.[42] GATA transcription factors belong to the family of zinc finger DNA-binding proteins and play critical roles in cell growth and differentiation.[43] Studies have shown that GATA1, GATA2, and GATA3 are involved in hematopoietic cell differentiation, whereas GATA4, GATA5, and GATA6 control specification and differentiation of mesoderm- and endoderm-derived cell types.[44,45] Our data demonstrated that TiO_2 NP exposure significantly up-regulated T-bet, IFN-γ, GATA3, and GATA4 expression in the liver (Tables 3 and 4), implying that these Th2-related factors may be associated with the TiO_2 NP-induced liver injury due to its hematogenic function for longer exposure duration and the lower dose.

A number of chemokines are responsible for the recruitment of inflammatory cells into the liver during the development of injury. MCP-1 is a CCL-type chemokine responsible for the recruitment of monocytes to sites of inflammation.[46] MIP-2 belongs to the CXC-type chemokine family and acts on CXC receptor 2. The chemokine is synthesized by activated tissue macrophages. MIP-2 performs similar functions to increase neutrophil egress from the bone marrow and mediate transmigration of these cells into the peripheral tissues.[47,48] Eotaxin-1/CCL11 is a chemokine belonging to the CC family and has been shown to be a potent chemoattractant for eosinophils both *in vitro* and *in vivo*.[49] Increased production of eotaxin-1 has been associated with allergic diseases.[50−52] It has also been shown that eotaxin-1 neutralization in mice substantially reduced eosinophil recruitment after Ag challenge. IL-5 is thought to be infiltrating T cells or eosinophils, and its overexpression exacerbates Th2 immune responses.[53] Kay also suggested that eotaxin-1 and IL-5 are involved in inflammation.[20] Therefore, the present study showed that the overexpression of MCP-1, MIP-2, eotaxin-1, and IL-5 (Tables 3 and 4) may be involved in the TiO_2 NP-induced hepatic inflammation such as infiltration of inflammatory cells and macrophage aggregation (Figure 3). However, possible direct evidence that Th2 factor expression is involved in the control of hepatic inflammation induced by chronic TiO_2 NP toxicity should be further confirmed in future experiments.

In conclusion, hepatotoxicity is closely associated with increased expression of IL-4, IL-5, IL-12, IFN-γ, GATA3, GATA4, T-bet, STAt3, STAT6, eotaxin, MCP-1, and MIP-2 and decreased STAT1 expression due to TiO_2 NP exposure in the mouse liver. Therefore, TiO_2 NP-induced liver injury may be via alteration of Th2 cytokine expression and/or a possible IL-4-mediated pathway in mice. The present study provides new insights into the mechanisms of TiO_2 NP-induced liver injury.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

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Notes

The authors declare no competing financial interest.

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