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**To cite this article:** Chao Huang, Mayu Sun, Yang Yang, Feng Wang, Xueqi Ma, Jingquan Li, Yilong Wang, Qiurong Ding, Hao Ying, Haiyun Song, Yongning Wu, Yiguo Jiang, Xudong Jia, Qian Ba & Hui Wang (2017) Titanium dioxide nanoparticles prime a specific activation state of macrophages, *Nanotoxicology*, 11:6, 737-750, DOI: [10.1080/17435390.2017.1349202](https://doi.org/10.1080/17435390.2017.1349202)

**To link to this article:** <https://doi.org/10.1080/17435390.2017.1349202>



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Accepted author version posted online: 03  
Jul 2017.  
Published online: 15 Jul 2017.



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ORIGINAL ARTICLE



## Titanium dioxide nanoparticles prime a specific activation state of macrophages

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### ABSTRACT

Titanium dioxide nanoparticles (TiO<sub>2</sub> NPs) are widely used in foods, cosmetics, and medicine. Although the inhalation toxicity of TiO<sub>2</sub> NPs has been studied, the potential adverse effects of oral exposure of low-dose TiO<sub>2</sub> NPs are largely unclear. Herein, with macrophage cell lines, primary cells, and mouse models, we show that TiO<sub>2</sub> NPs prime macrophages into a specific activation state characterized by excessive inflammation and suppressed innate immune function. After a month of dietary exposure in mice or exposure *in vitro* to TiO<sub>2</sub> NPs (10 and 50 nm), the expressions of pro-inflammatory genes in macrophages were increased, and the expressions of anti-inflammatory genes were decreased. In addition, for macrophages exposed to TiO<sub>2</sub> NPs *in vitro* and *in vivo*, their chemotactic, phagocytic, and bactericidal activities were lower. This imbalance in the immune system could enhance the susceptibility to infections. In mice, after a month of dietary exposure to low doses of TiO<sub>2</sub> NPs, an aggravated septic shock occurred in response to lipopolysaccharide challenge, leading to elevated levels of inflammatory cytokines in serum and reduced overall survival. Moreover, TLR4-deficient mice and primary macrophages, or TLR4-independent stimuli, showed less response to TiO<sub>2</sub> NPs. These results demonstrate that TiO<sub>2</sub> NPs induce an abnormal state of macrophages characterized by excessive inflammation and suppressed innate immune function in a TLR4-dependent manner, which may suggest a potential health risk, particularly for those with additional complications, such as bacterial infections.

### ARTICLE HISTORY

Received 21 July 2016

Revised 20 June 2017

Accepted 26 June 2017

### KEYWORDS

TiO<sub>2</sub> NPs; dietary exposure; macrophage activation; innate immunity

### Introduction

Engineered nanoparticles (NPs, 1–100 nm in at least one dimension) have been widely used and provided considerable benefits in various fields (Chen et al., 2014; Youns et al., 2011; Zhang et al., 2008). Compared with the fine-size analogs, NPs possess distinctive physicochemical properties, including the smaller sizes, larger surface areas, distinct crystalline structures, and enhanced reactivity (Shi et al., 2013). Their higher biological activity raises the concerns about the potential side effects of NPs on human health (Duncan, 2011). Health risks come from the occupational exposure of manufacturing workers (mostly inhalation at high dosage), as well as from everyday contact of the general population (low doses of oral or dermal exposure) (Kaur et al., 2014; Qiu, 2012). Most safety studies, however, have focused on pulmonary toxicity induced by inhalation or intratracheal exposure of NPs (Ho et al., 2017; Morimoto et al., 2016; Presume et al., 2016; Sisler et al., 2016), which are not conclusive for illustrating the effects of NPs exposure at lower concentrations in pharmaceutical and food products containing NP additives.

Titanium dioxide nanoparticles (TiO<sub>2</sub> NPs), one of the most produced NPs in the world, are commonly present in foods, sunscreens, cosmetics, and medicines (Mahler et al., 2012; Mishra et al., 2016). TiO<sub>2</sub> are used for the photodynamic tumor therapy due to the photocatalytic properties (Ackroyd et al., 2001), and

mostly as a brightening agent for food products, especially candies, powdered food, and white sauces (Rashidi & Khosravi-Darani, 2011). Due to its high content in candies and sweets, children are the population most exposed to TiO<sub>2</sub> (Weir et al., 2012). To improve the quality, TiO<sub>2</sub> particles with smaller sizes tend to be used (Hendren et al., 2011). The production of TiO<sub>2</sub> NPs is predicted to exceed that of TiO<sub>2</sub> microparticles (Robichaud et al., 2009). Approximately 10–36% of food-grade TiO<sub>2</sub> particles are prepared in nanoscale (Chen et al., 2013; Rompelberg et al., 2016; Yang et al., 2014). At present, there is no safety standard specialized for nano-sized TiO<sub>2</sub>. Although TiO<sub>2</sub> has been considered as an inert and low-toxicity material, TiO<sub>2</sub> NPs may present unusual properties and damage human health (Shi et al., 2013). Moreover, the International Agency for Research on Cancer (IARC) has classified TiO<sub>2</sub> as a possibly carcinogenic to humans (Group 2B), based on evidence obtained with experimental animals [Working Group on the Evaluation of Carcinogenic Risks to Humans (IARC), 2010]. Thus, it is necessary to comprehensively evaluate the biological effects of TiO<sub>2</sub> NPs, especially under the conditions of low doses and oral exposure.

Following oral exposure to TiO<sub>2</sub> NPs, there is minor absorption in the gastrointestinal tract, and titanium (Ti) was detected mainly in mesenteric lymph nodes (0.07 µg/g) with the slow elimination (Geraets et al., 2014). As an immune inductive site, mesenteric lymph nodes contain numerous immune cells such as T cells and

macrophages (Diehl et al., 2013). With multiple functions in development, homeostasis, tissue repair, and immunity, macrophages play the central role in proper functioning of the immune system (Ley et al., 2016; Zhang et al., 2015). Through sensing the surroundings, such as the state of tissue and pathogens, macrophages are capable of ingesting and digesting dead cells and foreign materials, as well as recruiting effector cells in response to inflammatory stimuli (Murray & Wynn, 2011). Furthermore, macrophages execute diverse functions through different activation states in response to various stimuli (Wynn et al., 2013; Zhang et al., 2015). In addition to classical activation (M1) and alternative activation (M2), a more extensive spectrum of macrophage activation is recognized recently (Murray et al., 2014; Zhang et al., 2015). Although some types of NPs could induce the pro-inflammatory effects of macrophages (Bianchi et al., 2015; Kodali et al., 2013; Shi et al., 2013; Yazdi et al., 2010), the comprehensive impacts on the activation state of macrophages by  $\text{TiO}_2$  NPs and the functional consequences are poorly understood.

The present study aimed to investigate the influence of  $\text{TiO}_2$  NPs on macrophage activation and their immunological functions *in vitro* and *in vivo*. We found that, through altering the inflammation regulatory status, chemotaxis, phagocytosis, and bactericidal activities of macrophages in a TLR4-dependent manner,  $\text{TiO}_2$  NPs primed specifically activated macrophages and impaired their functional response to external infection, such as bacteria, thus potentially exerting an adverse effect on health.

## Material and methods

### $\text{TiO}_2$ nanoparticles

$\text{TiO}_2$  NPs (anatase; 10 and 50 nm) were purchased from DK Nanotechnology Co. (Beijing, China). The particles were irradiated with ultraviolet light overnight, and stock solutions (1 mg/mL) were prepared in ultrapure water. For use, the stock solutions were serially diluted with cell culture medium, sonicated (285 W output) for 20 min to disperse the particles, and added to cells immediately. The mean size (Z-average), zeta potential, and polydispersity index (PDI) of  $\text{TiO}_2$  NPs in cell culture medium were measured by dynamic light scattering (DLS) with a Zetasizer Nano ZS90 (Malvern Co., Worcestershire, UK). The morphology of  $\text{TiO}_2$  NPs was observed using Tecnai G2 Spirit transmission electron microscope (TEM) (FEI, Hillsboro, OR). For TEM analysis of the engulfed  $\text{TiO}_2$  NPs in cells, macrophages were fixed in 2.5% glutaraldehyde and 2% osmium tetroxide, dehydrated with graded concentrations of ethanol, infiltrated with 33% Epon 812 resin, and embedded in 100% Epon 812 resin. After sectioning and staining, the slices were photographed by TEM.

### Preparation of macrophages

Raw264.7 and J774a.1 cell lines were cultured in complete DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 mg/mL penicillin and streptomycin, and maintained in an incubator with a humidified atmosphere of 5%  $\text{CO}_2$  at 37 °C. The primary macrophages were isolated from mice. Bone marrow-derived macrophages (BMDMs) were obtained by flushing the femurs of mice and culturing them for one week in medium containing 60% RPMI 1640, 30% L929-conditioned medium, and 10% inactivated FBS. Unattached cells were removed by changing the medium every 2 days. L929-conditioned medium was obtained by incubating L929 cells ( $1 \times 10^6$ ) in 55 ml medium (90% RPMI 1640, 10% FBS) for 7 days and harvesting and filtering the supernatant. To collect peritoneal macrophages, mice were injected

intraperitoneally (i.p.) with 1 mL 4% sterile thioglycollate (TG) medium (Sigma-Aldrich, St. Louis, MO). After 72 h, the peritoneal macrophages were harvested by rinsing the peritoneal cavity with 5 mL of cold PBS. After macrophages were attached, other cells were removed by changing the medium. For exposure of cells to  $\text{TiO}_2$  NPs, the culture media of the various macrophages were replaced with normal medium (0 µg/mL) or with media containing  $\text{TiO}_2$  NP10 or NP50 at concentrations of 0.1, 1, or 10 µg/mL and incubated for 48 h.

### Mice

TLR4-knockout ( $\text{TLR4}^{-/-}$ ) mice with a C57BL/6 background were kindly provided by Prof. Yingying Le (Pang et al., 2010), and wild-type C57BL/6J mice were purchased from Shanghai Laboratory Animal Research Center (Shanghai, China). Animals were housed in the specific pathogen-free (SPF) facility of the Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (SIBS, CAS) and fed with the standard laboratory rodent diet or with diets containing 0.1%  $\text{TiO}_2$  NP10 or NP50 ( $\text{TiO}_2$  NPs were added to the diet as an ingredient in the process of feed preparation) which were irradiated by cobalt-60 (SLRC Laboratory Animal Co. Ltd., Shanghai, China) and reverse-osmosis water *ad libitum*. All mice were treated humanely and with regard for alleviation of suffering according to the Biomedical Research Ethics Committee of the SIBS, CAS.

### Quantitative RT-PCR

Total RNA from macrophages was extracted with TRizol reagent (Invitrogen, Carlsbad, CA) and cDNA synthesis was accomplished with PrimeScript™ RT reagent Kit (Takara, Otsu, Japan). Quantitative RT-PCR was performed with an ABI 7900 Real-Time PCR System (Applied Biosystems, Waltham, MA). Dissociation curves were obtained to verify the specificity of the amplification. Relative mRNA expression was determined by the  $\Delta\Delta\text{C}_t$  method, with  $\beta$ -actin or ribosomal protein L32 as the internal control. Primer sequences used for PCR are provided in Supplementary Table S1.

### Analysis of cell surface proteins

To determine the levels of surface proteins, cells were washed with PBS, re-suspended in 100 mL of PBS, and incubated with fluorescein-conjugated antibodies for 40 min at 4 °C. After removal of the unbound antibodies, the stained cells were analyzed with a FACSAria III flow cytometer (BD Biosciences, Franklin Lakes, NJ). BV-anti-F4/80, PE/APC-anti-CD11b, PE-anti-CD86, PE-anti-CD80, PE-anti-CD16/32, FITC-anti-Mrc1, APC-anti-Cleac7a, PE-anti-TLR4, and the isotype control IgG were purchased from Biolegend (San Diego, CA).

### Macrophage chemotaxis assay

*In vitro.* After exposure to  $\text{TiO}_2$  NPs for 48 h, J774a.1 and Raw264.7 macrophages in serum-free medium were placed in upper Boyden chambers with 8-µm micropore membranes in 24-well plates. The lower compartments were filled with serum-free medium containing *Escherichia coli* (*E. coli*) (MOI = 10). After incubation for 5 h, cells on the upper surface of the membrane were removed; cells that migrated to the lower surface and across the filters were fixed and stained with crystal violet in methanol, and photographed under a microscope.

*In vivo.* Weaned mice were fed with the control diet or with diets containing 0.1%  $\text{TiO}_2$  NPs for 1 month and then injected i.p.

with 4% TG medium (1 mL). After 72 h, the recruited cells in the peritoneal cavity were collected by rinsing with PBS, and the numbers of macrophages ( $F4/80^+$  cells) were measured by flow cytometry.

#### Phagocytosis and bactericidal activity assays

The phagocytic and bactericidal activities of macrophages were determined by incubation of macrophages with *E. coli* tagged with green fluorescent protein (GFP). *Escherichia coli* strain Top10 carrying the GFP gene on plasmid Puc-19 was from Dr. Bao-Xue Ge (Kong et al., 2009). After exposure for 48 h, the  $TiO_2$  NPs were removed and macrophages were infected with GFP-*E. coli* for 45 min (MOI = 100), washed three times with PBS containing 12.5  $\mu$ g/mL gentamicin, and subjected to GFP analysis by flow cytometry to measure the level of phagocytosis. Alternatively,  $TiO_2$  NPs-exposed macrophages were infected with *E. coli* for 20 min (MOI = 10), and the medium was replaced with serum-free medium containing gentamicin (100  $\mu$ g/mL) for another 20 min to remove extracellular *E. coli*. These macrophages were then divided into two parts. One half (I) was lysed with 0.1% Triton X-100 and coated on ampicillin plates. And the other half (II) continued to be incubated in serum-free medium containing gentamicin (25  $\mu$ g/mL) for 90 min to digest intracellular *E. coli* (II) and then were lysed. CFU<sub>I</sub> was regarded as the phagocytosis level. The bactericidal rate was calculated by:  $100\% \times (CFU_I - CFU_{II})/CFU_I$ .

#### Nitric oxide (NO) production

After exposure to  $TiO_2$  NPs for 48 h, macrophages were washed with PBS and infected with heat-activated (95 °C, 30 min) *E. coli* (MOI = 50) or treated with TNF $\alpha$  (50 ng/mL, PeproTech, Rocky Hill, NJ) and IFN $\gamma$  (50 ng/mL, PeproTech) in FBS-free medium for 24 h. The culture medium was collected and centrifuged at 1500g for 5 min. The concentration of NO in the supernatant was determined with Nitric Oxide Assay Kit (Beyotime, Shanghai, China) according to the manufacturer's instruction.

#### Lipopolysaccharide (LPS) septic shock model

Weaned wild-type or  $TLR4^{-/-}$  mice were fed with the control diet or diets containing 0.1%  $TiO_2$  NPs for 1 month and then injected i.p. with LPS (Sigma-Aldrich, 10 mg/kg BW) to induce septic shock (Jiao et al., 2013). Wild-type mice in each group were divided into three parts, which were used for monitoring survival, collection of serum at 4 h, and collection of serum and peritoneal cells at 48 h.

#### Cecal ligation and puncture

To induce TLR4-unspecific septic shock, the cecal ligation and puncture (CLP) procedure was performed as described previously (Toscano et al., 2011). Briefly, wild-type mice were anesthetized with chloral hydrate (4%, 10  $\mu$ L/g). A midline incision was performed. The cecum was exteriorized and ligated at its base below the ileocecal valve (the length of ligated cecum was 0.5–0.75 cm). The ligated cecum was then perforated once using a 19-gauge needle and gently squeezed to extrude feces. The cecum was returned to the peritoneal cavity, and the incision was closed. Each mouse was injected subcutaneously with 1 mL of warm normal saline for fluid resuscitation and then placed on a heating pad for revival.

#### Analysis of mouse cytokines

Cytokines in serum or medium were measured by Cytometric Bead Arrays (BD Biosciences) performed following the

manufacturer's instructions. The samples were measured with a FACSaria III flow cytometer, and the data were analyzed by FCAP Array Software (BD Biosciences).

#### LPS measurement and elimination

The concentration of LPS in  $TiO_2$  NPs stock solutions was measured by ToxinSensor Chromogenic LAL Endotoxin Assay Kit (Genscript) with the limulus amebocyte lysate (LAL) assay. The inhibitor of LPS, polymyxin B (Cayman Chemical, 10  $\mu$ g/mL), was used in cell medium to exclude endotoxin priming.

#### Statistical analyses

Data are presented as mean  $\pm$  standard deviation (SD). The statistical significance of differences was examined with one-way or two-way analysis of variance (ANOVA) (multiple groups) and Student's *t*-test (two groups). For *in vivo* studies, the Mann-Whitney *U*-test was used. A value of  $p < .05$  was considered significant.

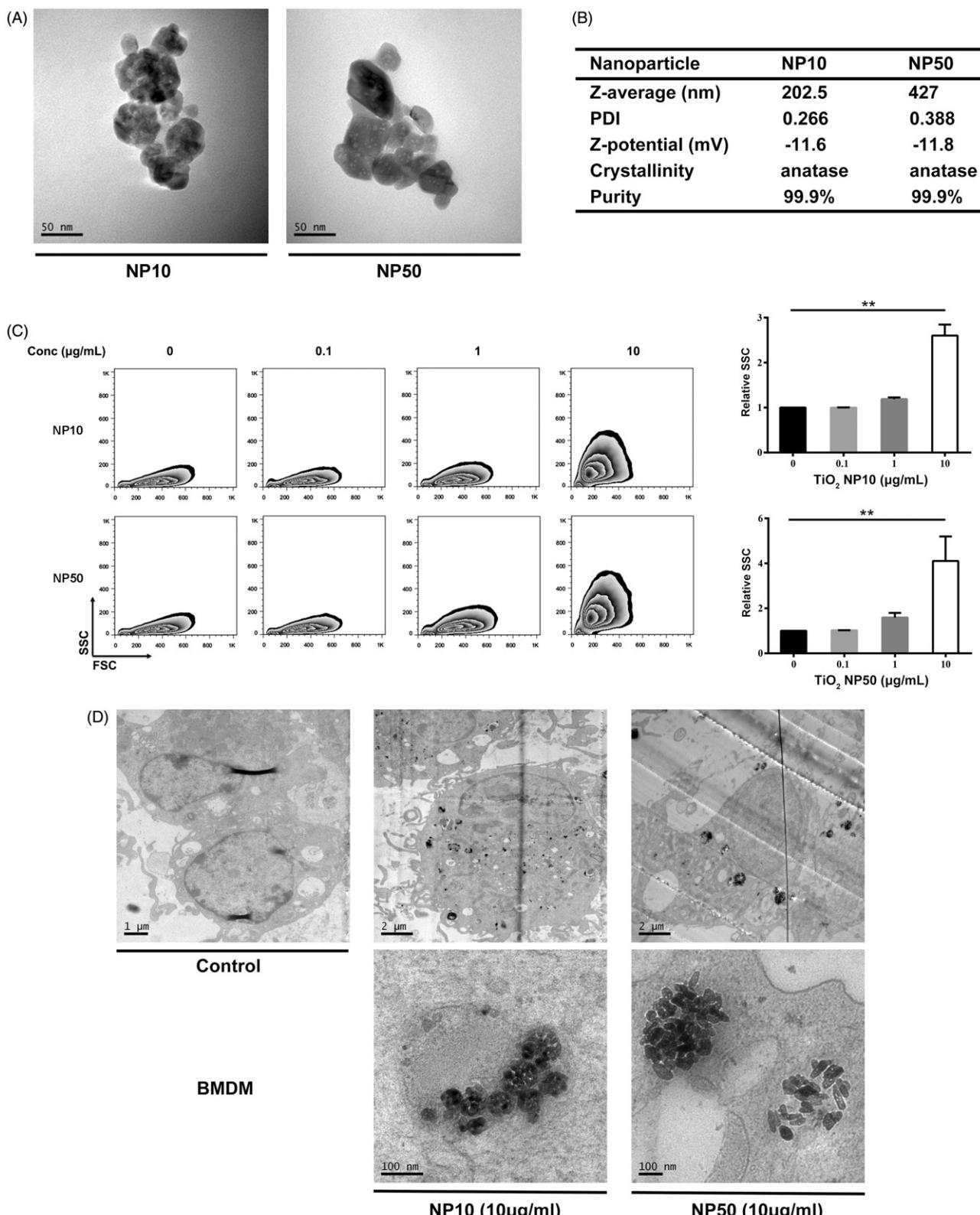
## Results

#### Characterization and bioavailability of nanoparticles

To evaluate the effects of nanosized  $TiO_2$  on the function of macrophages,  $TiO_2$  NPs with primary diameters of 10 nm (NP10) and 50 nm (NP50) were used. The shapes of both types of particles were observed by TEM (Figure 1(A)); the hydrodynamic diameters (202.5 and 427 nm) and zeta potentials (-11.6 and -11.8 mV) of NP10 and NP50 in cell culture medium with FBS were determined, respectively (Figure 1(B) and Supplementary Figure S1(A)). Their characteristics indicate that some aggregates were formed in cell culture medium. To determine the bioavailability of  $TiO_2$  NPs in macrophages, primary bone marrow-derived macrophages (BMDMs) and cultured macrophage cell lines were incubated with 10  $\mu$ g/mL of the  $TiO_2$  NP10 or NP50. In BMDMs exposed to either NP10 or NP50, the sideward scatter (SSC), which is related to cell granularity and can be used as a marker of particle uptake (Scherbart et al., 2011), was increased (Figure 1(C)), indicating an efficient internalization of  $TiO_2$  NPs by macrophages. Moreover, TEM analysis revealed that the internalized  $TiO_2$  NPs distributed mostly in the cytoplasm and showed fewer aggregates relative to those in cell culture medium (Figure 1(D), Supplementary Figure S1(B,C)). These results confirm the previous findings that  $TiO_2$  NPs can be ingested efficiently by macrophages (Xia et al., 2006) and suggest that  $TiO_2$  NPs might exert nanoscale-dependent biological effects in macrophages.

#### $TiO_2$ NPs primed macrophages activation in vivo

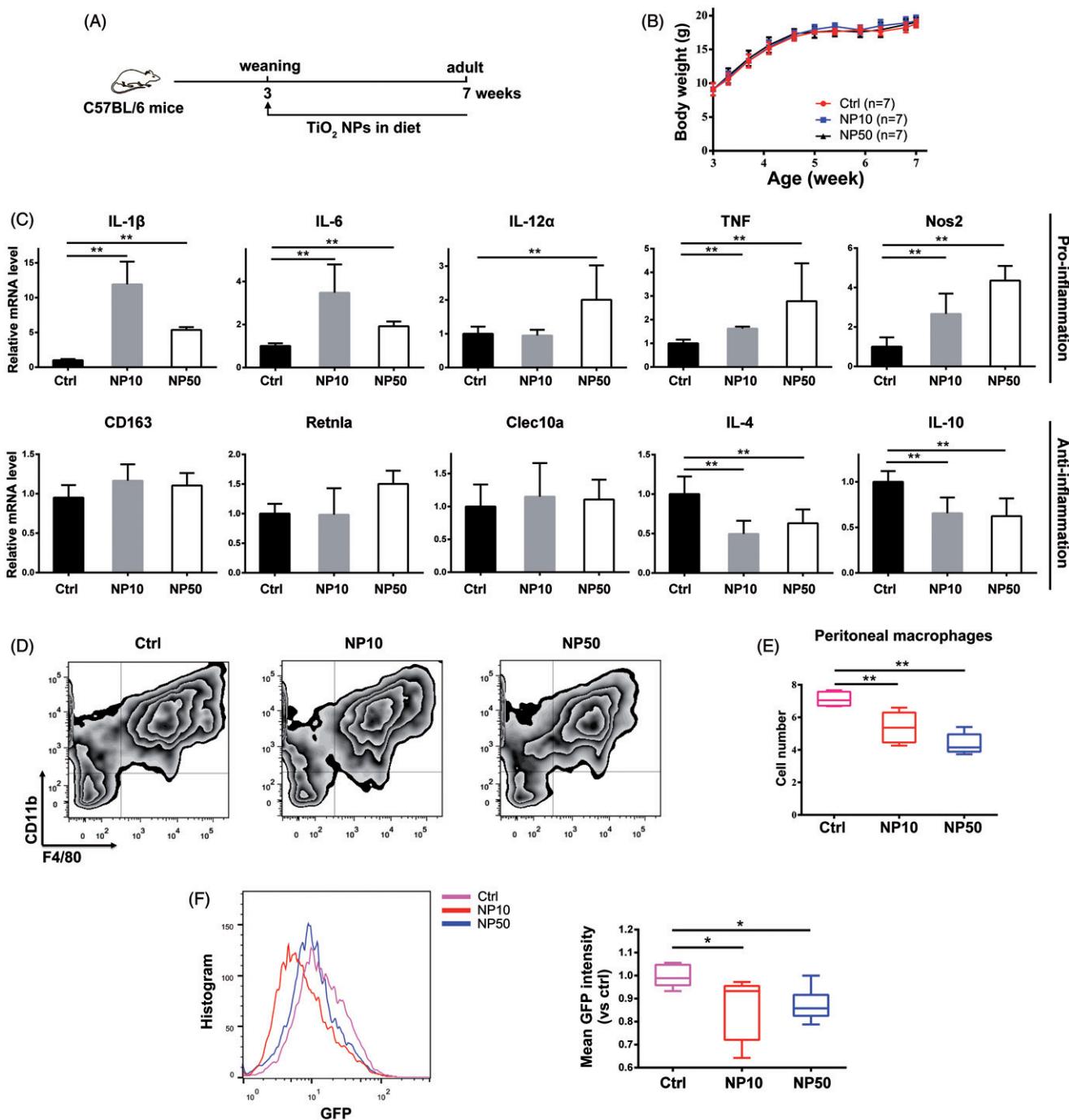
To investigate the effect of dietary  $TiO_2$  NPs on macrophages *in vivo*, we fed C57BL/6J mice with diets containing 0.1%  $TiO_2$  NPs, which is close to the real-world exposure level. It is approved by FDA that the allowing level of  $TiO_2$  in food is up to 1%. And  $\sim$ 10–36% of  $TiO_2$  particles in food products are in nanoscale (Peters et al., 2014; Weir et al., 2012; Yang et al., 2014). Weaned mice were used, because children have the highest oral exposure to  $TiO_2$  NPs (Weir et al., 2012). These mice were fed with control diet or diets containing 0.1%  $TiO_2$  NPs until adulthood ( $\sim$ 1 month) (Figure 2(A)); there were no body weight changes during the feeding period (Figure 2(B)). After that, sterile TG medium was injected i.p. to elicit the peritoneal macrophages.



**Figure 1.** Characterization of  $\text{TiO}_2$  NPs. (A) TEM images of the  $\text{TiO}_2$  NP10 and NP50 powders. (B) The average sizes, PDIs, crystallinity, purity, and zeta potential of  $\text{TiO}_2$  NP10 and NP50 in DMEM medium with 10% FBS. (C) Representative flow cytometry plots and relative mean SSCs of BMDMs exposed to  $\text{TiO}_2$  NPs (0, 0.1, 1, or 10  $\mu\text{g}/\text{mL}$ ) for 48 h. (D) TEM images of BMDMs with no treatment or with exposure to  $\text{TiO}_2$  NPs (10  $\mu\text{g}/\text{mL}$ ) for 48 h. The results are representative of three different experiments.  $^{**}p < .01$

In response to various stimuli, macrophages can be primed to different activation states (Wynn et al., 2013; Zhang et al., 2015), including the classical activated macrophages (M1) and alternative activated macrophages (M2) according to their plasticity and

polarization (Sica & Mantovani, 2012). We assessed the effect of TiO<sub>2</sub> NPs on the activation status of peritoneal macrophages *in vivo*. Both NP10 and NP50 elevated the levels of pro-inflammatory interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6), interleukin-12 $\gamma$  (IL-12 $\gamma$ )

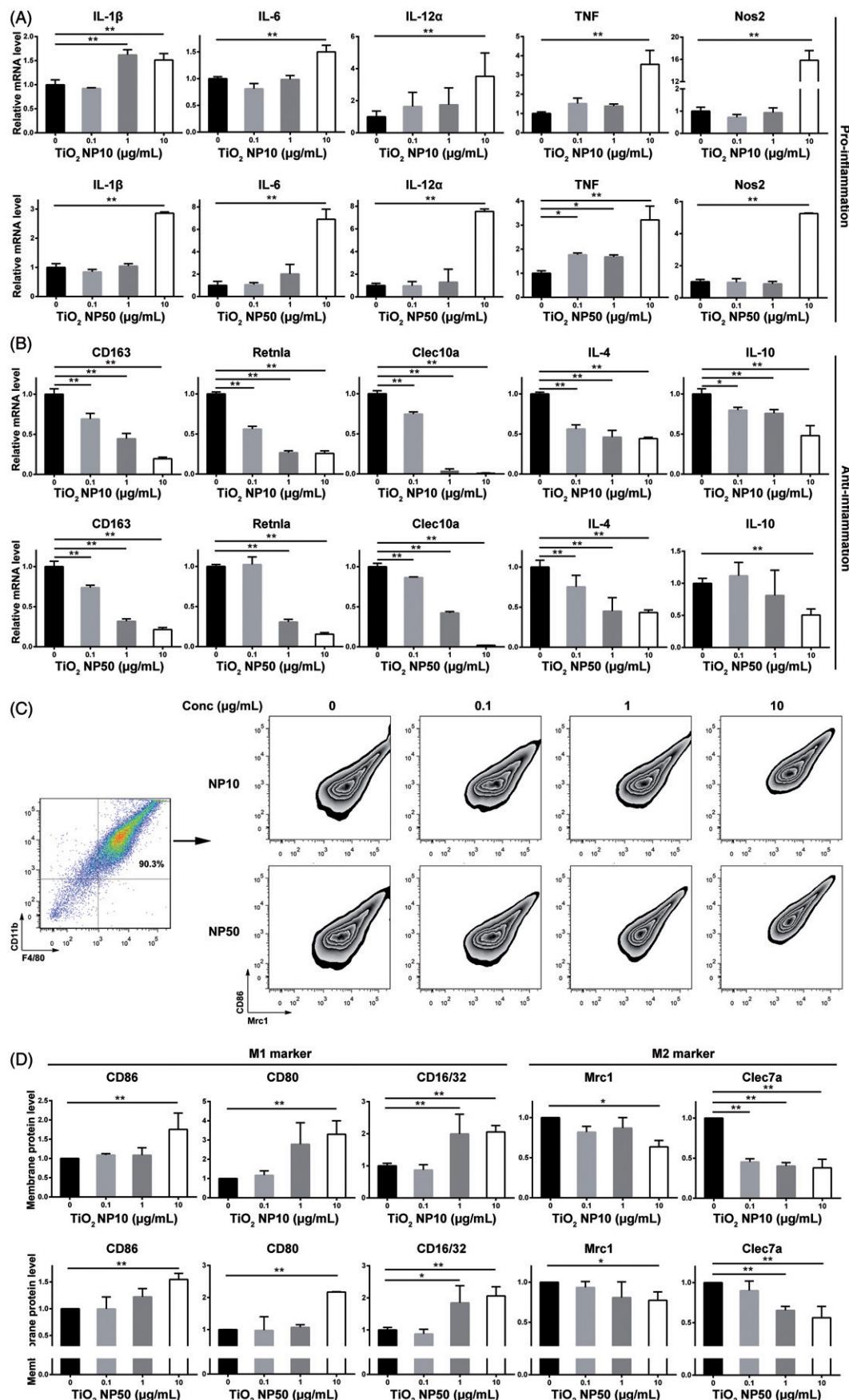


**Figure 2.**  $\text{TiO}_2$  NPs primed macrophage activation *in vivo*. (A) Experimental design. (B) Mice received normal diet or diets containing 0.1%  $\text{TiO}_2$  NPs for 1 month. The body weights of mice were monitored ( $n = 7$ ). (C) The expressions of genes in peritoneal macrophages isolated from mice with different diets were determined by quantitative RT-PCR ( $n = 4-6$ ). (D,E) Mice ( $n = 4-6$ ) fed with normal or  $\text{TiO}_2$  NPs-containing diets were injected i.p. with sterile TG medium. Peritoneal cells were collected at 72 h, and  $\text{CD11b}^+/\text{F4}/80^+$  cells were identified as macrophages. Representative flow cytometry plots (D) and the quantification results (E) are shown. (F) Peritoneal macrophages isolated from mice fed with different diets were infected with GFP-*E. coli* (MOI = 100) for 45 min, and the GFP-positive cells were analyzed by flow cytometry and normalized to the control group ( $n = 4-6$ ). \* $p < .05$ ; \*\* $p < .01$ .

tumor necrosis factor (TNF), and nitric oxide synthase-2 (Nos2) (Figure 2(C)). These changes are similar to M1 but not M2 activation. On the other hand, the expressions of anti-inflammatory factors, interleukin-4 (IL-4), and interleukin-10 (IL-10), were reduced in groups exposed to  $\text{TiO}_2$  NPs (Figure 2(C)), which is different from M1 polarization. These results suggest that, in mice dosed with  $\text{TiO}_2$  NPs, macrophages show a M1-like and dominant pro-inflammatory activation state.

Through various activation status, macrophages execute diverse functions. In addition to inflammation, macrophages are

also involved in innate immunity, with chemotactic, phagocytic, and bactericidal functions (Murray & Wynn, 2011). In mice dosed with NP10 or NP50, TG-induced macrophages in the peritoneal cavity were reduced relative to the control group (Figure 2(D,E)), indicating that the recruitment of macrophages in mice was suppressed. To determine the effect of  $\text{TiO}_2$  NPs on the phagocytosis of macrophage, TG-induced macrophages were infected with *E. coli* expressing GFP (Kong et al., 2009). The mean fluorescent intensity in macrophages was decreased (Figure 2(F)), suggesting an inhibitory effect of  $\text{TiO}_2$  NPs on the phagocytic activity of



**Figure 3.**  $\text{TiO}_2$  NPs induced a pro-inflammatory state in macrophages. BMDMs were exposed to  $\text{TiO}_2$  NPs (0, 0.1, 1, or 10  $\mu\text{g/mL}$ ) for 48 h. (A,B) The expressions of pro-inflammatory (A) and anti-inflammatory genes (B) were determined by quantitative RT-PCR. (C) BMDMs were gated by F4/80 and CD11b, and the double-positive cells were subjected to analysis for membrane CD86 and Mrc1. The variation is shown by zebra plots. (D) The mean membrane levels of M1 and M2 markers were quantified by flow cytometry. The results are representative of three different experiments.  $^*p < .05$ ;  $^{**}p < .01$ .

macrophages in mice. In sum, these findings demonstrate that  $\text{TiO}_2$  NPs induce an activation state of macrophages with pro-inflammation and impaired innate immunity functions.

#### ***TiO<sub>2</sub> NPs induced a special activation of macrophages with an altered inflammatory state***

To assess the effect of  $\text{TiO}_2$  NPs on macrophage polarization *in vitro*, mouse BMDMs were incubated with various concentrations of  $\text{TiO}_2$  NP10 or NP50 (0.1, 1, or 10  $\mu\text{g}/\text{mL}$ ) in culture medium. At these concentration, no evident cytotoxicity was observed (Supplementary Figure S2(A)). Consistent with the results from *in vivo* exposure, the expressions of pro-inflammatory genes were enhanced (Figure 3(A)) and Supplementary Figure S2(B)).  $\text{TiO}_2$  NPs also increased secretions of cytokines IL-1 $\beta$ , IL-6, and TNF (Supplementary Figure S2(C)). And the expression of the anti-inflammatory factors, CD163, Retnla, Clec10a, IL-4, and IL-10, were decreased (Figure 3(B)). Moreover, the cell surface markers for M1 and M2 macrophages varied after exposure of cells to  $\text{TiO}_2$  NPs. Both NP10 and NP50 increased the numbers of CD86-positive (M1 marker) cells; in contrast, the numbers of Mrc1-positive (M2 marker) cells were diminished (Figure 3(C)). In addition to CD86 and Mrc1, the membrane protein levels of M1 markers, CD80 (B7-1) and CD16/32 (Fc-Gamma Receptor III/II), were elevated; another M2 marker, Clec7a (Dectin-1), was also decreased by  $\text{TiO}_2$  NPs (Figure 3(D)) and Supplementary Figure S3). These results show that, in macrophages,  $\text{TiO}_2$  NPs induce a M1-like and dominant pro-inflammatory activation state.

#### ***The immune function of $\text{TiO}_2$ NPs-primed macrophages was impaired***

To investigate the immune function of macrophages which were exposed to  $\text{TiO}_2$  NPs *in vitro*,  $\text{TiO}_2$  NPs were removed and the primed BMDMs were subjected to a short-term *E. coli* challenge, which showed no evident cytotoxicity (Supplementary Figure S4(A)). Similar with the above results, the expressions of bacteria-induced pro-inflammatory genes were elevated, and the expression of anti-inflammatory genes were reduced (Supplementary Figure S4(B)), suggesting an excess inflammatory response to bacterial infection.

To examine the chemotaxis of macrophages, J774a.1 and RAW264.7 cells were pretreated with NP10 or NP50 and recruited with *E. coli* as the chemoattractant. In groups exposed to  $\text{TiO}_2$  NPs, the numbers of migrating macrophages decreased (Figure 4(A)), which established the inhibitory effect of  $\text{TiO}_2$  NPs on the capacity for chemotaxis. After infection with *E. coli* expressing GFP, the mean fluorescent intensity in macrophages (Figure 4(B)) and the proportion of GFP-positive cells (Figure 4(C)) were decreased in the NP10 and NP50 groups. The same trend was also observed for J774a.1 cells and for TG-induced peritoneal macrophages (Supplementary Figure S4(C,D)). Further, BMDMs were lysed and the phagocytosed *E. coli* were measured directly. Compared with the control group, after exposure to  $\text{TiO}_2$  NPs, macrophages showed fewer colony forming units (CFU) of bacteria (Figure 4(D)). These results demonstrate the decreased phagocytic activity of macrophages exposed to  $\text{TiO}_2$  NPs. We next examined the elimination of the captured bacteria in macrophages. With increasing concentrations of  $\text{TiO}_2$  NPs, the bactericidal effect of primed BMDMs was decreased (Figure 4(E)). Moreover, the production of nitric oxide (NO), an anti-microbial factor (Mougnau, et al., 2011), induced by heat-inactivated *E. coli*, was reduced in BMDMs, J774a.1 cells, and TG-induced peritoneal macrophages (Figure 4(F), Supplementary Figure S4(E,F)).

These findings consistent with the results *in vivo* and further demonstrate that  $\text{TiO}_2$  NPs exposure impairs innate immunity functions of macrophages.

#### ***TiO<sub>2</sub> NPs weakened the host defense of mice***

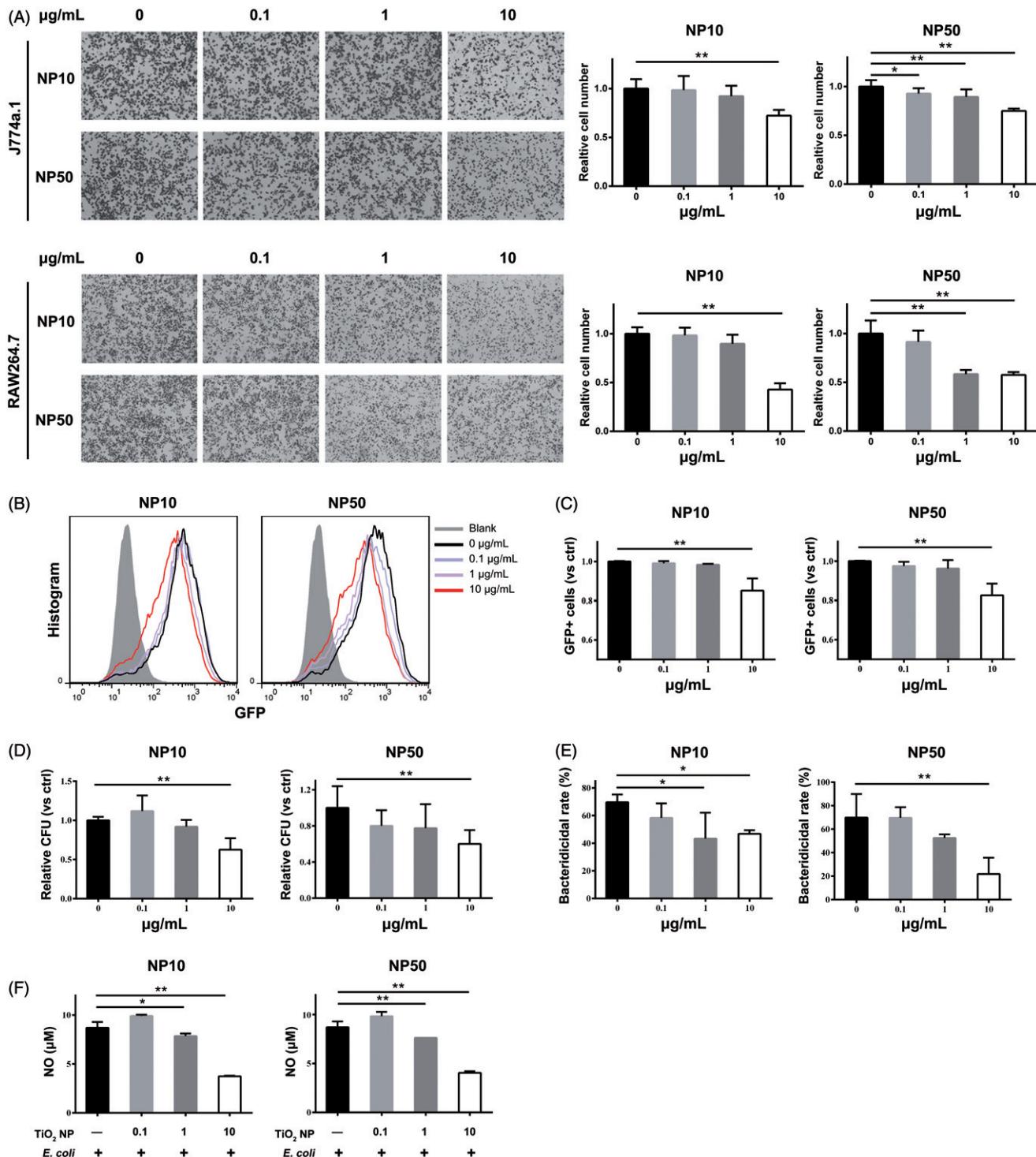
Since  $\text{TiO}_2$  NPs damaged the immuno-activity of macrophages, we determined their effects on host defense against a foreign invader. Mice were fed with control diet or diets containing 0.1%  $\text{TiO}_2$  NPs for 1 month and injected i.p. with LPS to introduce septic shock (Figure 5(A)). In this challenge, the survival of mice was lowered from 90% (control) to 50% (NP10) or 60% (NP50) (Figure 5(B)), suggesting an aggravated septic shock. From mice at 48 h after LPS challenge, peritoneal cells were collected and assessed by flow cytometry. These macrophages of mice dosed with  $\text{TiO}_2$  NPs were decreased (Figure 5(C)), which is consistent with the aforementioned outcome in relation to chemotaxis. Further, the serum concentrations of various pro-inflammatory factors were elevated in mice dosed with  $\text{TiO}_2$  NPs. The levels of IL-1 $\beta$  and IL-12 p70 were increased at 4 h after LPS challenge (Figure 5(D)); while the anti-inflammatory cytokine IL-10 was reduced in  $\text{TiO}_2$  NPs-treated mice (Figure 5(D)). After 48 h of LPS challenge, IL-1 $\beta$  and TNF recovered to basal levels (data not shown), whereas IL-6 and IL-12 p70 had higher serum concentrations in  $\text{TiO}_2$  NPs-treated groups compared with controls (Figure 5(E)). These findings indicate that exposure to  $\text{TiO}_2$  NPs could disrupt the homeostasis of immunity and aggravate the inflammatory responses to an external stimulus such as LPS.

#### ***TiO<sub>2</sub> NPs induced a TLR4-specific abnormality in macrophages***

Considering that macrophages recognize LPS from Gram-negative bacteria and undergo activation through the membrane receptor, TLR4 (Murray et al., 2014; Zanoni et al., 2011), we investigated the potential role of TLR4 in the macrophage abnormality induced by  $\text{TiO}_2$  NPs. No LPS was detected in the  $\text{TiO}_2$  NPs stock solutions (Figure 6(A)); and  $\text{TiO}_2$  NPs influenced the expression of inflammatory genes even in the presence of the LPS inhibitor, polymyxin B (Supplementary Figure S5), thus excluding the possibility of LPS contamination.

The involvement of TLR4 in the effects of  $\text{TiO}_2$  NPs on macrophages was then investigated. First, in BMDMs exposed to NP10 or NP50, the levels of cell surface TLR4 were reduced (Figure 6(B)), showing that TLR4 is affected by  $\text{TiO}_2$  NPs. Second, in BMDMs from  $TLR4^{-/-}$  mice,  $\text{TiO}_2$  NPs did not increase the expression of pro-inflammatory cytokine genes; the inhibitory effects of  $\text{TiO}_2$  NPs on the anti-inflammatory genes were also diminished (Figure 6(C,D)). Similarly, after exposure to  $\text{TiO}_2$  NPs,  $TLR4^{-/-}$  BMDMs showed little difference in phagocytosis (Figure 6(E)), indicating that  $\text{TiO}_2$  NPs affect inflammation and phagocytosis through TLR4. Third, due to the fact that  $TLR4^{-/-}$  BMDMs had no response to *E. coli* in the production of NO (Supplementary Figure S6(A)), a process mediated by TLR4, we employed an alternative TLR4-independent NO inducer, TNF $\alpha$  + IFN $\gamma$  (Supplementary Figure S6(A)) (Saha & Pahan, 2006), and found that  $\text{TiO}_2$  NPs did not affect the TLR4-independent induction of NO in wild-type or  $TLR4^{-/-}$  BMDMs (Figure 6(F)) and Supplementary Figure S6(B)). Finally, we used a TLR4-unspecific polymicrobial septic shock model, CLP (Toscano et al., 2011), and found that  $\text{TiO}_2$  NPs exposure did not exacerbate the CLP-induced septic shock (Figure 6(G)).

Consistently, in  $TLR4^{-/-}$  mice, no aggravated LPS septic shock induced by  $\text{TiO}_2$  NPs was observed in our results (Figure 7(A,B)) or previous report (Roger et al., 2009). Further, the peritoneal

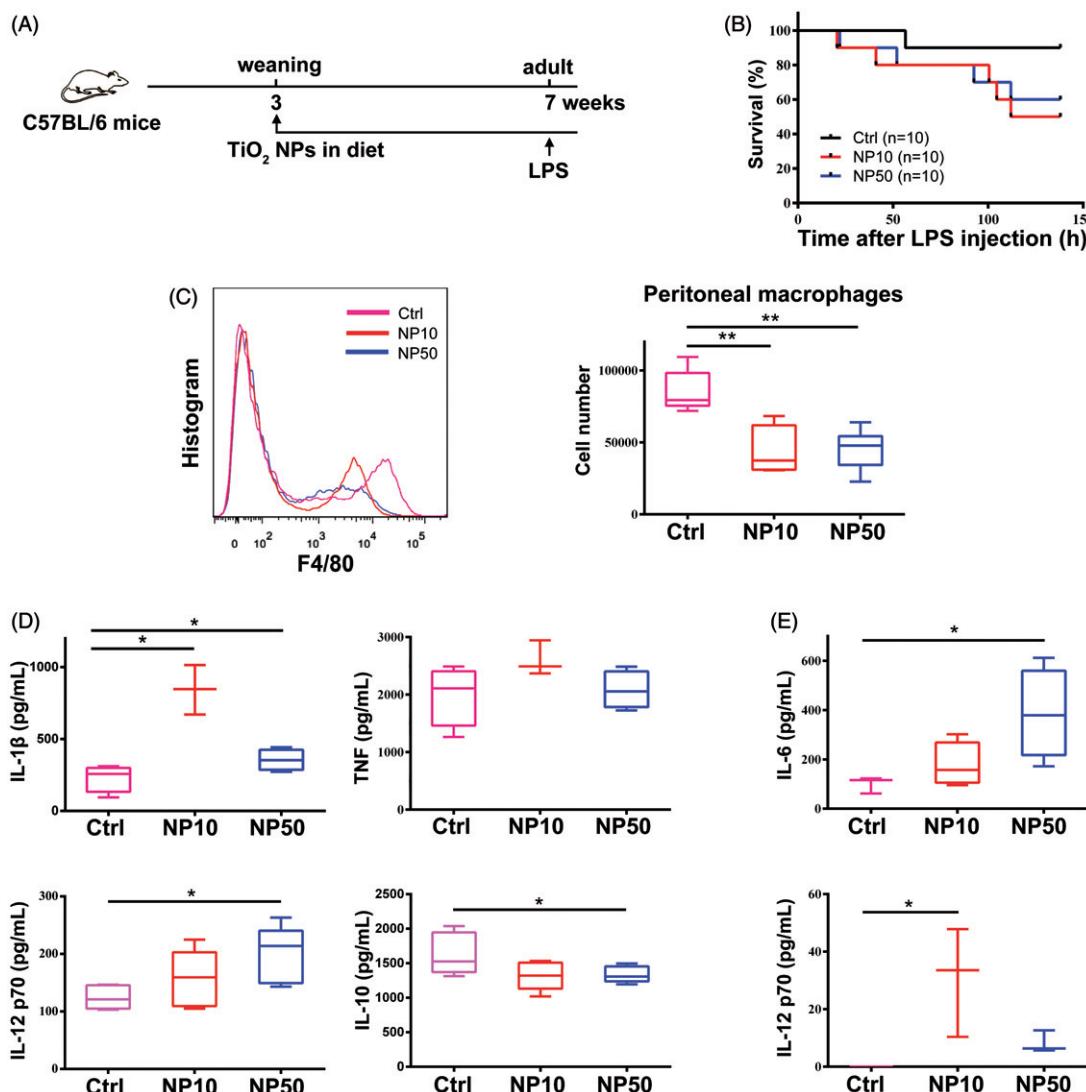


**Figure 4.** TiO<sub>2</sub> NPs decrease the chemotactic, phagocytic, and bactericidal activity of macrophages. (A) J774a.1 and Raw264.7 cells were exposed to TiO<sub>2</sub> NPs (0, 0.1, 1, or 10 µg/mL) for 48 h, then the chemotaxis of macrophages towards *E. coli* was examined with Transwell assays. (B,C) BMDMs were primed by TiO<sub>2</sub> NPs (0, 0.1, 1, or 10 µg/mL) for 48 h. Then TiO<sub>2</sub> NPs were removed and macrophages were infected with GFP-*E. coli* (MOI = 100) for 45 min. The GFP intensities of macrophages were analyzed by flow cytometry (B), and the numbers of GFP-positive cells were normalized to that of the control group (C). (D) After priming with TiO<sub>2</sub> NPs, BMDMs were incubated with *E. coli* (MOI = 10), lysed, and coated on ampicillin plates to measure the CFUs. (E) TiO<sub>2</sub> NPs-primed BMDMs were infected with *E. coli* (MOI = 10), and the bactericidal rate was measured as described in 'Material and Methods' section. (F) TiO<sub>2</sub> NPs-primed BMDMs were infected with heat-activated *E. coli* (MOI = 50), and the production of NO in the culture medium was measured after 24 h. The results represent at least three independent experiments. \**p* < .05; \*\**p* < .01.

macrophages in *TLR4*<sup>−/−</sup> mice fed diets containing TiO<sub>2</sub> NPs showed similar inflammatory gene expressions and phagocytic activity compared with the control (Figure 7(C,D)). Taken together, these results demonstrate that, in macrophages, the effects of TiO<sub>2</sub> NPs on immunity are dependent on TLR4.

## Discussion

In the present study, we identified, in macrophages primed by TiO<sub>2</sub> NPs, a specific activation state that is distinguished from M1 and M2 and characterized by elevated inflammatory and depressed innate immune responses. This type of macrophage



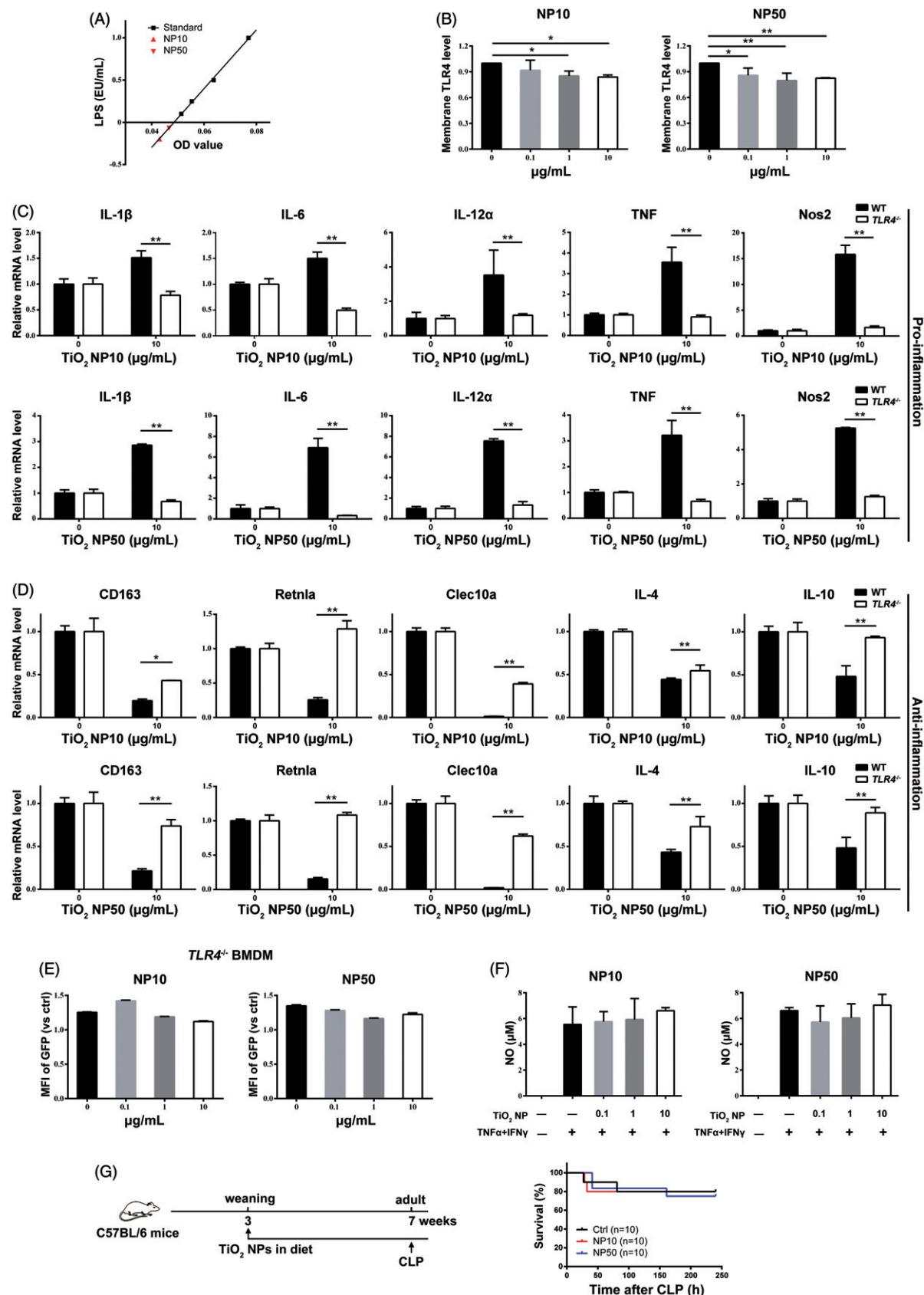
**Figure 5.**  $\text{TiO}_2$  NPs exposure exacerbated the response of mice to LPS shock. (A) Experimental design. (B) After injection i.p. with LPS, the survival rate was then monitored ( $n=10$ ). (C) At 48 h after LPS injection, peritoneal cells were collected, and  $\text{F}4/80^+$  macrophages were detected and quantified by flow cytometry ( $n=4-6$ ). (D) Serum concentrations of IL-1 $\beta$ , TNF, IL12 p70, and IL10 after 4 h of LPS injection were determined ( $n=3-5$ ). (E) Serum concentrations of IL-6 and IL-12 p70 at 48 h after injection were determined ( $n=3-5$ ). \* $p < .05$ , \*\* $p < .01$ .

could disturb the balance of the immune system and enhance the inflammation and mortality of mice in response to LPS septic shock. We further demonstrated that TLR4 is involved in the activation of macrophages primed by  $\text{TiO}_2$  NPs. These findings indicate that  $\text{TiO}_2$  NPs reduce the protective effects of macrophages against bacterial infections and thus are likely to be a risk for human health.

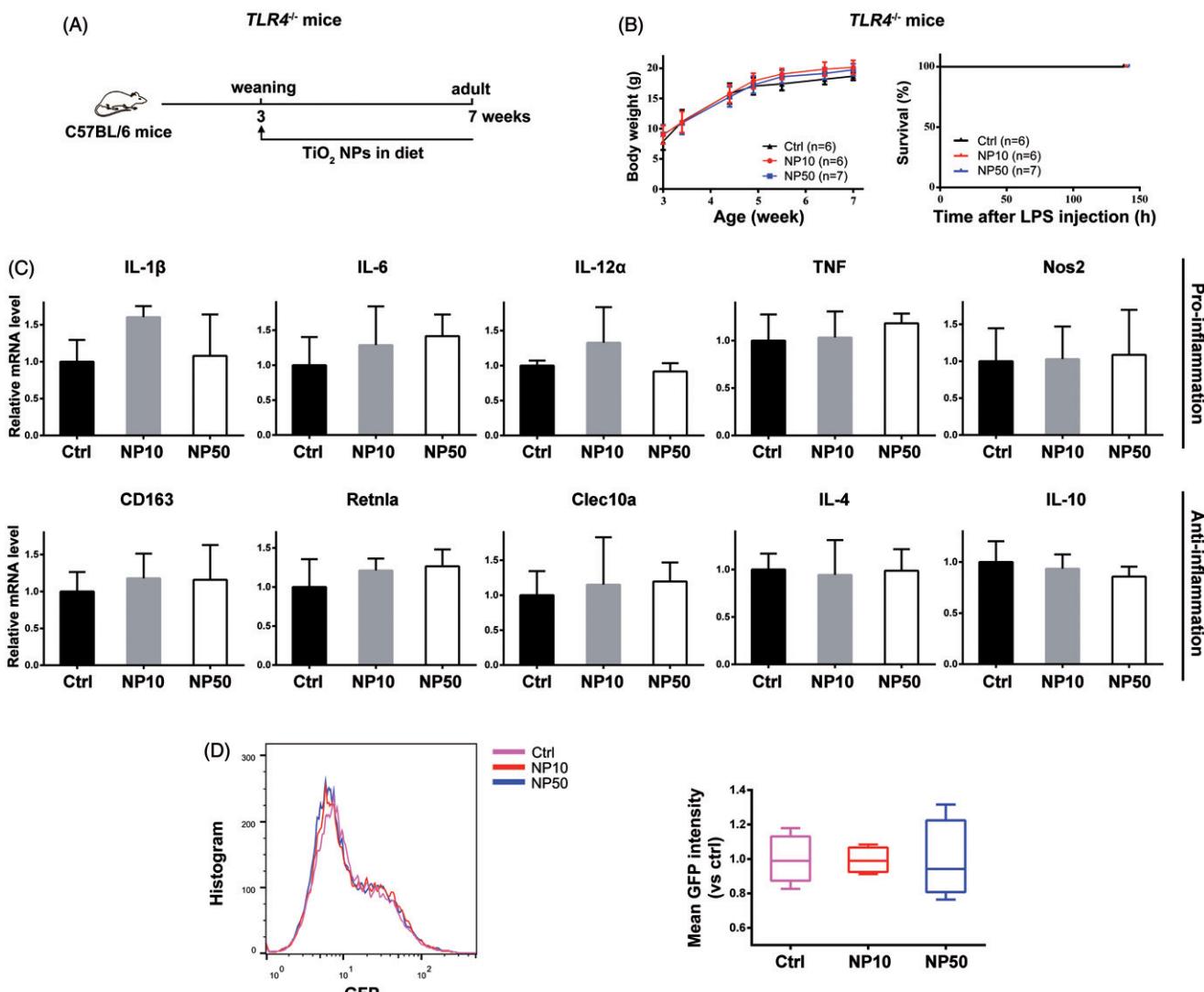
Macrophages, which are essential for immunity, have various roles in host defense. For macrophages to execute their diverse functions, they are primed to different activation states in response to various stimuli. Among the activated forms of macrophages, two typical groups are designated M1 and M2 (Sica & Mantovani, 2012). Macrophages that recognize LPS in bacteria undergo classical M1 activation, which is characterized by secretion of high levels of pro-inflammatory cytokines and strong bactericidal activity (Murray et al., 2014; Zanoni, et al., 2011). M2, alternatively activated macrophages, turn off the inflammation by producing anti-inflammatory cytokines. In recent years, more types of macrophage activation that differ from M1 and M2 have been found (Jantsch et al., 2015; Murray et al., 2014; Zhang et al., 2015). In our study, although  $\text{TiO}_2$  NPs could induce M1-like polarization,

$\text{TiO}_2$  NPs primed a specific activation state of macrophages that differs from M1 and M2. On one hand, despite promoting inflammation, M1 macrophages show concomitant increases of expression of anti-inflammatory genes to prevent prolonged and uncontrolled inflammation (Kinjyo et al., 2002; Medzhitov & Horng, 2009; Ramirez-Carrozzi et al., 2009; Wynn et al., 2013). Whereas, macrophages primed by  $\text{TiO}_2$  NPs boost the pro-inflammatory cytokines, IL-1 $\beta$ , IL-6, IL-12 $\alpha$ , and TNF, but repress the secretion of the anti-inflammatory factors, CD163, Retnla, Clec10a, IL-4, and IL-10. On the other hand, unlike other activated forms, macrophages primed by  $\text{TiO}_2$  NPs exhibit suppressed phagocytic and bactericidal activities, thus representing an inefficient response to bacterial infection and weak functions in innate immunity.

Based on previous report and our observations, we have proposed a model for the effects of  $\text{TiO}_2$  NPs on macrophage activation and function (Figure 8). In the normal state, macrophages produce basal levels of cytokines and NO. *Escherichia coli* are recognized by TLR4, which subsequently induces classical macrophage activation (M1), represented by high levels of pro-inflammatory cytokines (TNF, IL-1 $\beta$ , IL-6, and IL-12 $\alpha$ ) and NO, to exert



**Figure 6.** TiO<sub>2</sub> NPs had little effect on TLR4<sup>-/-</sup> macrophages and TLR4-independent stimuli. (A) The LPS content in TiO<sub>2</sub> NPs stock solutions was measured. (B) BMDMs were exposed to TiO<sub>2</sub> NPs at different concentrations (0, 0.1, 1, or 10  $\mu\text{g/mL}$ ) for 48 h, and the membrane levels of TLR4 were measured by flow cytometry. (C,D) Wild-type and TLR4<sup>-/-</sup> BMDMs were exposed to TiO<sub>2</sub> NPs (10  $\mu\text{g/mL}$ ) for 48 h, and the expressions of pro-inflammatory (C) and anti-inflammatory genes (D) was determined by quantitative RT-PCR. (E) After exposure to TiO<sub>2</sub> NPs, TLR4<sup>-/-</sup> BMDMs were infected with GFP-*E. coli* (MOI = 100). GFP-positive macrophages were analyzed by flow cytometry. (F) After exposure of wildtype BMDMs to TiO<sub>2</sub> NPs, the cells were treated with TNF $\alpha$  (50 ng/mL) + IFN $\gamma$  (50 ng/mL). The production of NO in the culture medium was measured after 24 h. (G) Wild-type mice received normal diet or diets containing 0.1% TiO<sub>2</sub> NPs for 1 month and subjected to CLP. The survival rate was then monitored ( $n = 10-12$ ). The above results are representative of three different experiments. \* $p < .05$ ; \*\* $p < .01$ .



**Figure 7.**  $\text{TiO}_2$  NPs had little effect on  $\text{TLR4}^{-/-}$  mice. (A) Experimental design. (B)  $\text{TLR4}^{-/-}$  mice ( $n=6-7$ ) were received normal diet or diets containing 0.1%  $\text{TiO}_2$  NPs for 1 month and injected i.p. with LPS. The body weight during feeding period and the survival rate after injection were monitored. (C)  $\text{TLR4}^{-/-}$  mice ( $n=4-6$ ) fed with normal or  $\text{TiO}_2$  NPs-containing diets were injected i.p. with sterile TG medium, and peritoneal macrophages were collected. The expression of genes in peritoneal macrophages isolated from mice with different diets were determined by quantitative RT-PCR ( $n=4-6$ ). (D) Peritoneal macrophages isolated from  $\text{TLR4}^{-/-}$  mice with different diets were infected with GFP-*E. coli* (MOI = 100) for 45 min, the GFP-positive cells were analyzed by flow cytometry and normalized to the control group ( $n=4-6$ ).

bactericidal activity (Wynn et al., 2013).  $\text{TiO}_2$  NPs modulate the macrophages toward a specific pro-inflammatory state. When challenged by *E. coli* infection, the specifically activated macrophages induced by  $\text{TiO}_2$  NPs exhibit suppressed innate immune functions (chemotaxis, phagocytosis, and bactericidal activity) but enhanced inflammatory responses.

The suppression of innate immunity may be due to reduced amounts of cell-surface TLR4. As a pattern recognition receptor, internalized TLR4 is likely to trigger the expression of inflammatory genes (Medzhitov & Horng, 2009). However, low levels of membrane-associated TLR4, which was induced by  $\text{TiO}_2$  NPs, may block TLR4-mediated recognition and the innate immune response of macrophages to external *E. coli*.  $\text{TiO}_2$  NPs-primed activation was not evident in  $\text{TLR4}^{-/-}$  macrophages or  $\text{TLR4}^{-/-}$  mice, suggesting that TLR4 is essential for this process. Although it is reported that overexpressed TLR4 is involved in the uptake of  $\text{TiO}_2$  NPs in HepG2 hepatoma cells (Chen et al., 2013), we found no difference in the internalization of  $\text{TiO}_2$  NPs between wild-type and  $\text{TLR4}^{-/-}$  macrophages (Supplementary Figure S7), which may be due to the compensatory effect in macrophages. In addition to

TLR4, macrophages express a variety of membrane receptors (such as scavenger receptors) on the cell surface, which could work together in mediating internalization of NPs (Kodali et al., 2013; Wang et al., 2014). Notably, SR-AI/II-deficient mice showed increased pro-inflammatory cytokine levels in lung lavage fluid after challenge of the airways with  $\text{TiO}_2$  NPs (Arredouani et al., 2006); thus, it can be speculated that TLR4 might play important roles in these mice.

Another possible explanation for suppressed immunity is that internalized  $\text{TiO}_2$  NPs inhibit the aggressive reactions of macrophages to bacteria. After  $\text{TiO}_2$  NPs treatment, the production of NO, a free radical that damages microbes, was reduced in response to *E. coli* infection. In addition, endocytosed  $\text{TiO}_2$  NPs triggered an abnormal inflammatory response, which may be caused by the indigestibility of  $\text{TiO}_2$  NPs. Different from *E. coli*,  $\text{TiO}_2$  NPs are resistant to breakdown by macrophages, thereby sustaining a pro-inflammatory stimulus and disturbing immune homeostasis. In terms of distribution, most internalized  $\text{TiO}_2$  NPs were wrapped by membranes, suggesting a probable localization in the endo/lysosomal compartment. It has been reported that

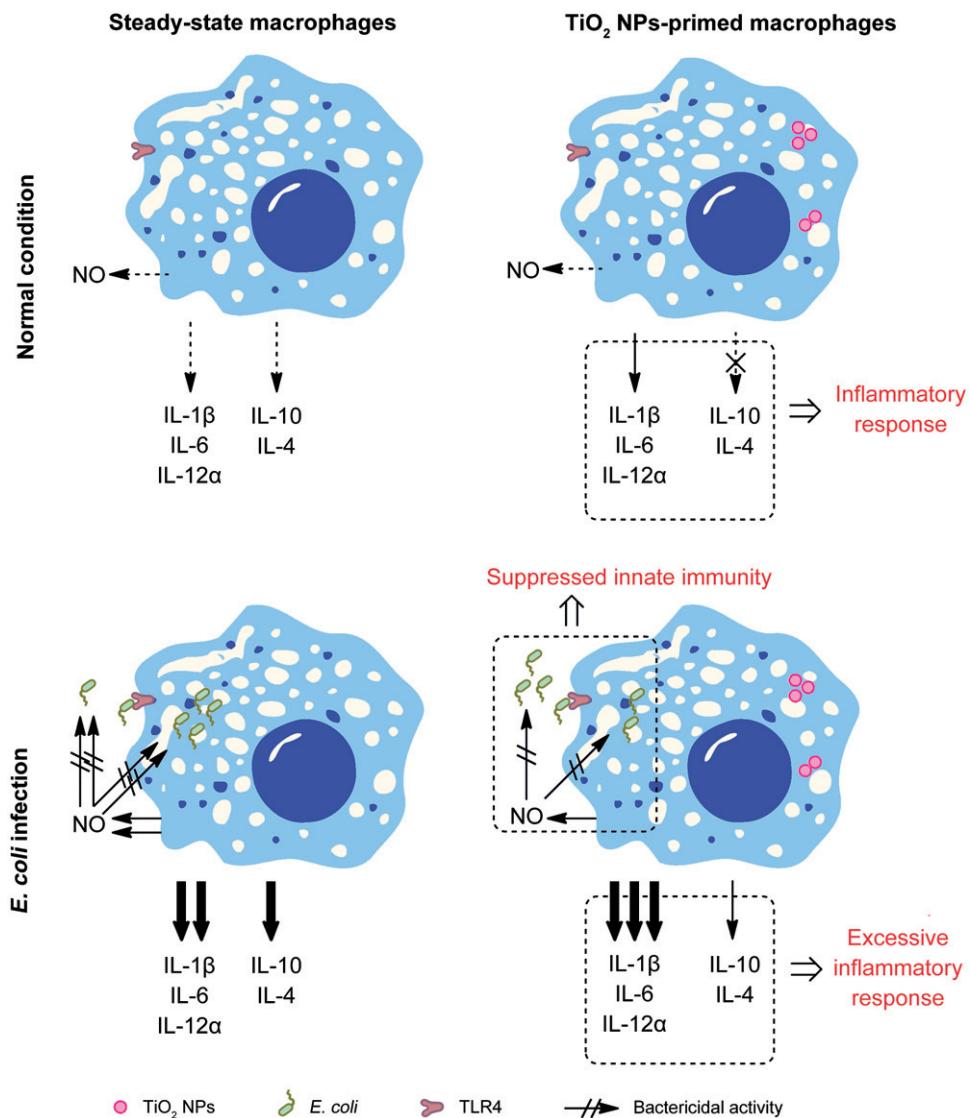


Figure 8. A schematic representation of the activating effects of  $\text{TiO}_2$  NPs on macrophages.

$\text{TiO}_2$  NPs could induce lysosomal destabilization (Hussain et al., 2010), which contributes to necrosis if complete lysosomal rupture occurs or drives apoptosis by the release of cathepsins, which are lysosomal hydrolases (Droga-Mazovec et al., 2008). However, no abnormalities for lysosomal destabilization-related toxicity pathway were observed, possibly due to the dose of  $\text{TiO}_2$  NPs, which caused no appreciable cytotoxicity.

The possible health outcomes of macrophages primed by  $\text{TiO}_2$  NPs were also investigated. Repressed innate immunity and sustained inflammation of peritoneal macrophages were confirmed in mice with dietary exposure of  $\text{TiO}_2$  NPs, which may result in detrimental effects, especially in the presence of second hits, such as bacterial infections (Kinjyo et al., 2002; Medzhitov & Horng, 2009; Ramirez-Carrozzi et al., 2009; Wynn et al., 2013). As determined with mice, our results revealed that LPS induced aggravated septic shock in animals exposed to  $\text{TiO}_2$  NPs. After such exposure of mice, serum pro-inflammatory cytokines were elevated, anti-inflammatory cytokines were lowered, and the overall survival of mice was reduced. Consistent with our observations, inhalation of some other NPs, including carbon nanotubes or copper NPs, decreased lung elimination of bacterial in mice (Kim et al., 2011; Shvedova et al., 2008). Thus, exposure to  $\text{TiO}_2$  NPs, even at low amounts, represents a potential risk for human health.

Most of the previous  $\text{TiO}_2$  NPs studies used relative high-dose inhalation or intratracheal exposure and focused on the pulmonary inflammation, which is valuable for assessing risk in occupational exposure population. However, the dietary exposure to low-dose  $\text{TiO}_2$  NPs may exert different biological effects and has been less evaluated. In this study, we determined the biological effects of dietary  $\text{TiO}_2$  NPs at low concentrations. According to the food intake, body weight, and body surface area in mice, the dietary exposure in our experimental setting is roughly comparable to 10–20 mg  $\text{TiO}_2$ /kg/day in human. The realistic exposure to  $\text{TiO}_2$  is 2–3 mg  $\text{TiO}_2$ /kg/day for children in UK (Weir et al., 2012). Thus, the dose in this study can simulate the potential human exposure. The effect of dietary  $\text{TiO}_2$  NPs on macrophage activation was supported by the results in primary cells. Although the results were most remarkable at 10  $\mu\text{g}/\text{mL}$ ,  $\text{TiO}_2$  NPs caused the same trend at 0.1 and 1  $\mu\text{g}/\text{mL}$ , thus suggesting a potent macrophage-priming capacity. In addition, two types of particles with different primary sizes, NP10 and NP50, were investigated. However, few obvious phenotypic changes related to particle size were evident. One possible reason is that NP10 is more likely to aggregate to particles with sizes close to NP50. Nevertheless, the possibility that the effects of  $\text{TiO}_2$  NPs on macrophages are irrelevant to particle size cannot be excluded.

## Conclusions

In summary,  $\text{TiO}_2$  NPs could prime macrophages into a specific state, with excessive pro-inflammatory reactions and reduced innate immune functions, in a TLR4-dependent manner. In mice, these activated macrophages disturb the immune balance and enhance the susceptibility to bacterial infection. These findings reveal a comprehensive influence of  $\text{TiO}_2$  NPs on macrophages, which represents the health significance in a broader population.

## Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (81573161, 81630086, 91529305, and 81427805), the Chinese Academy of Sciences (ZDRW-ZS-2017-1, XDA12020319), and the Science and Technology Commission of Shanghai Municipality (16391903700).

## Disclosure statement

No potential conflict of interest was reported by the authors.

## Funding

This work was supported by grants from the National Natural Science Foundation of China (81573161, 81630086, 91529305, and 81427805), the Chinese Academy of Sciences (ZDRW-ZS-2017-1, XDA12020319), and the Science and Technology Commission of Shanghai Municipality (16391903700).

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