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Wear Particles Impair Antimicrobial Activity *Via* Suppression of Reactive Oxygen Species Generation and ERK1/2 Phosphorylation in Activated Macrophages

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Abstract—Implant-related infection (IRI) is closely related to the local immunity of peri-implant tissues. The generation of reactive oxygen species (ROS) in activated macrophages plays a prominent role in the innate immune response. In previous studies, we indicated that implant wear particles promote endotoxin tolerance by decreasing the release of proinflammatory cytokines. However, it is unclear whether ROS are involved in the damage of the local immunity of peri-implant tissues. In the present study, we assessed the mechanism of local immunosuppression using titanium (Ti) particles and/or lipopolysaccharide (LPS) to stimulate RAW 264.7 cells. The results indicate that the Ti particles induced the generation of a moderate amount of ROS through nicotinamide adenine dinucleotide phosphate oxidase-1, but not through catalase. Pre-exposure to Ti particles inhibited ROS generation and extracellular-regulated protein kinase activation in LPS-stimulated macrophages. These findings indicate that chronic stimulation by Ti particles may lead to a state of oxidative stress and persistent inflammation, which may result in the attenuation of the immune response of macrophages to bacterial components such as LPS. Eventually, immunosuppression develops in peri-implant tissues, which may be a risk factor for IRI.

KEY WORDS: implant-related infection; wear particles; ROS; ERK.

INTRODUCTION

Total joint replacement (TJR) is widely used to alleviate pain and to improve the quality of life. However, this operation is associated with complications such as aseptic failure or implant-related infection (IRI) [1]. IRI occurs in 1.5–2.5 % of all cases of primary hip or knee arthroplasty, despite the rigorous use of perioperative antimicrobial prophylaxis [2, 3]. Recent studies have suggested that certain cases of aseptic failure, particularly those involving aseptic loosening, may have been infected by bacteria that cannot be detected using conventional microbial cultures [4–8]. Inflammatory reactions associated with implant wear particles are believed to be the major cause of long-term failure of TJR [9, 10]. The surface of wear particles serves as a site for the adherence of bacteria, bacterial biofilms, or bacterial structural components such as lipopolysaccharide (LPS), which may promote the development of persistent infections and chronic inflammation [10–12].

The reactive oxygen species (ROS) include superoxide, hydrogen peroxide, and hydroxyl radicals, as well as a variety of their reaction products [13]. ROS play an important role in the innate immunity of macrophages. Following infection with a pathogen, macrophages begin to produce large amounts of ROS and mediate powerful microbicidal activity through nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX)—this phenomenon is known as "respiratory burst" [13–15]. However, ROS are double-edged swords in that they can also be toxic to cells and can cause tissue damage [14, 16].

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Previous studies have indicated the presence of oxidative stress in particle-stimulated cells *in vitro* [17, 18] and pre-implant tissue *in vivo* [19], which may lead to nuclear factor-κB (NF-κB) activation, proinflammatory cytokine release, and persistent inflammation [13, 20, 21]. Furthermore, ROS have been reported to activate extracellular-regulated protein kinases (ERK) 1/2 [22–24], which is a kinase of the MAPK family and primarily stimulates cell proliferation [25].

Two main families of opposing enzymes regulate the generation of ROS—oxidative enzymes such as NOX and anti-oxidative enzymes such as catalase (CAT), glutathione peroxidase, and superoxide dismutase [14, 16, 25]. NOX enzymes serve as a major source for the deliberate generation of ROS in response to inflammatory signals [14]. In particular, NOX-1 is an important enzyme of the NOX family, and has been shown to be transcriptionally induced by LPS in different cells [25, 26]. Furthermore, CAT is essential in defending cells against oxidative damage by degrading hydrogen peroxide [27].

In the present study, we examined the generation of ROS and their related enzymes in response to titanium (Ti) particles *in vitro* to elucidate the signal pathway underlying oxidative stress around implants. In addition, we tested the hypothesis that wear particles may affect respiratory burst and macrophage proliferation, which may be a risk factor for IRI.

MATERIALS AND METHODS

Preparation of Ti Particles

Commercially pure Ti particles were obtained from Alfa Aesar (catalog #00681; Ward Hill, MA, USA). Particle size was measured using scanning electron microscopy and the mean diameter of the particles was 3.2±2.7 μm (Fig. 1). The Ti particles were sterilized by baking at 180 °C for 6 h, followed by treatment with 70 % ethanol for 48 h to remove endotoxin, as described previously [28, 29]. Thereafter, the particles were reconstituted in a stock solution at a concentration of 10 mg/mL in sterile phosphate-buffed saline (PBS) and diluted to different final concentrations in experiments. Prior to the experiments, the particles were tested and confirmed to contain less than 0.1 EU/mL of endotoxin, by using a commercial Limulus assay kit (Chromogenic End-point TAL with a Diazo coupling kit; Xiamen Houshiji, Fujian, China). Previous studies have demonstrated that such commercially available Ti particles are similar to wear debris particles generated in periprosthetic tissues [29, 30].

LPS

LPS from *Escherichia coli* O111:B4, purchased from Sigma Aldrich (catalog L2630; St. Louis, MO, USA), was solubilized in sterile PBS at a concentration of 1 mg/mL at −20 °C for storage. For the experiment, LPS was diluted to 1 μg/mL in Dulbecco's modified Eagle's medium (DMEM) or PBS.

Cell Culture and Treatment

A mouse macrophage cell line RAW264.7 was obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM (Gibco, Grand Island, NY, USA), containing 10 % (*v*/*v*) fetal bovine serum (Gibco), at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air. Cells were removed from 75-cm^2 culture flasks and plated in 6-well plates or culture dishes at a density of 10^6 cells/mL. To examine the dose-dependent effects of Ti particles, the cells were incubated in serum-free DMEM containing different concentrations of Ti particles (0.001, 0.01, 0.1, and 1 mg/mL) for 12 h. Cells were incubated with LPS as a positive control. Furthermore, to assess the time-dependent effects, the RAW264.7 cells were treated with Ti particles at 0.1 mg/mL for 0.5, 1, 3, 6, 12, 24, and 48 h. To study the role of Ti particles in LPS-activated macrophages, RAW264.7 cells were pre-exposed to Ti particles for 12 h and incubated in medium containing 1 μg/mL LPS for 30 min, and were evaluated in comparison with RAW264.7 cells that were not pre-exposed to Ti particles.

Measurement of Intracellular ROS Levels in RAW 264.7 Cells by Flow Cytometry

Intracellular ROS production was measured using the fluorescence probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; Invitrogen Co., Carlsbad, CA, USA). The RAW 264.7 cells were grown in 6-well plates. Following treatment with Ti particles or LPS, the cells were collected and re-suspended in pre-warmed PBS, containing 5 μmol/L H_2DCFDA, and incubated for 20 min at 37 °C in darkness. Thereafter, the extracellular probe was removed by washing cells in PBS. We then determined the fluorescence intensity by flow cytometry (Beckman-Coulter, Fullerton, CA, USA), with an excitation wavelength of 488 nm and emission wavelength of 525 nm. In total, 50,000 cells were collected and analyzed in each sample.

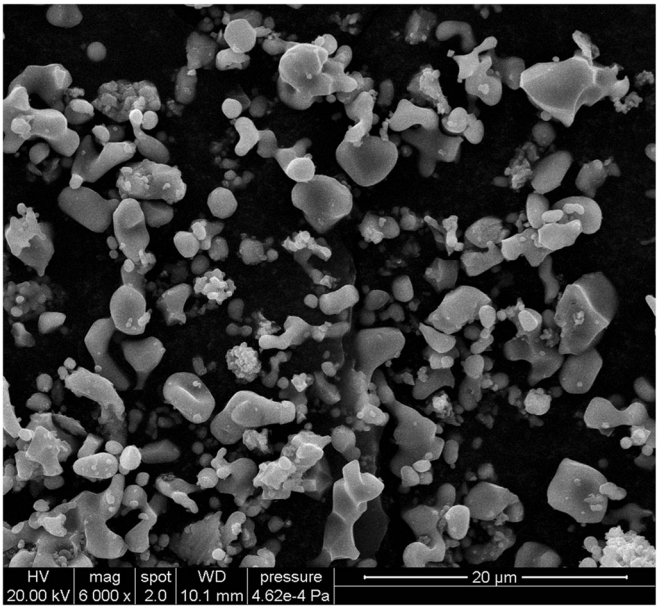

Fig. 1. Scanning electron micrograph of titanium particles used in the experiments (×6000).

The data obtained were analyzed using Kaluza software (Beckman-Coulter).

Western Blotting

Total cell extracts of RAW264.7 cells were obtained and lysed using a lysis solution containing protease inhibitors and phosphatase inhibitors. Following centrifugation at 12,000 rpm for 20 min at 4 °C, the protein in the supernatants was collected and quantified. Equal amounts of total proteins (range, 30–70 μg) were separated using 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked for 30 min in Tris-buffered saline containing 5 % skim milk at room temperature, after which probes—including anti-NOX-1(1:1000) from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-CAT (1:1000), anti-ERK1/2 (1:1000), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:5000) from Cell Signaling Technology (Beverly, MA, USA) antibodies—were added and incubated overnight at 4 °C. The anti-GAPDH antibody was used as an internal control for protein loading. The membranes were then incubated with the corresponding horseradish peroxidase-labeled secondary antibody (1:3000; Jackson Immunoresearch, West Grove, PA, USA) at room temperature for 2 h. The bands were visualized using an enhanced chemiluminescence detection system, and the band density was determined by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

Data are presented as the mean±standard deviation of at least three independent experiments. The differences between groups were assessed using one-way analysis of variance. The significance for multiple comparisons among groups was assessed using a post hoc examination with least significant difference tests. A p value of <0.05 was considered to be statistically significant. All data were processed using the SPSS 13.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

Ti Particles Stimulate a Moderate Amount of NOX-1 Expression and ROS Generation in RAW264.7 Macrophages

The protein levels of NOX-1 or CAT were determined using western blot analyses. The expression of NOX-1 in RAW264.7 macrophages increased in the presence of Ti particles in a dose- and time-dependent manner, whereas the expression of CAT remained unchanged. The addition of LPS significantly increased NOX-1 expression (Fig. 2a), in accordance with previous studies [25]. In cases receiving

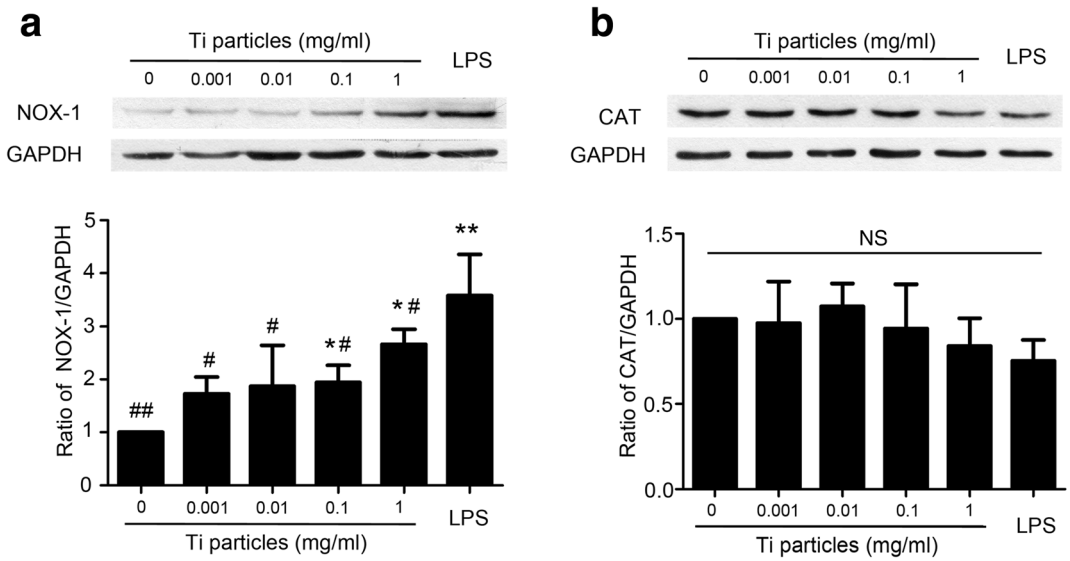

Fig. 2. Effect of Ti particles and LPS on NOX-1 and CAT expression in RAW 264.7 macrophages at different Ti particle concentrations. RAW 264.7 cells were treated with Ti particles at concentrations of 0, 0.001, 0.01, 0.1, and 1 mg/mL or LPS at 1 μg/mL for 12 h. The NOX-1 and CAT protein levels were examined using western blot assays. **a** The ratio of NOX-1/GAPDH is presented as means ± standard deviation (from three independent experiments). $*p<0.05$, $**p<0.001$ vs. negative control (NC, 0 mg/mL Ti group). $^{\#}p<0.05$, $^{\#\#}p<0.001$ vs. LPS group. **b** The ratio of CAT/GAPDH is presented as means ± standard deviation (from three independent experiments). No significant differences in the CAT/GAPDH ratio were noted among the groups. *NS* not significant.

Ti particle stimulation for 12 h, the NOX-1/GAPDH ratio increased with increasing Ti concentration (0.001–1 mg/mL) ($p<0.05$), although the induction of NOX-1 expression by Ti particles was weaker than that with LPS ($p<0.05$) (Fig. 2a); however, no significant change in CAT expression was noted ($p>0.05$) (Fig. 2b). A Ti particle concentration of 0.1 mg/mL induced NOX-1 expression at all time points, particularly at 6, 12, 24, and 48 h ($p<0.05$) (Fig. 3a, b), although it did not have any effect on CAT expression ($p>0.05$) (Fig. 3a, c). Thus, the induction of NOX-1 may contribute to ROS generation following treatment with Ti particles. Therefore, we assessed the effect of the treatment of RAW264.7 macrophages with Ti particles for 12 h on ROS generation. We noted that the Ti particles induced a moderate increase in the amount of ROS generated in a dose-dependent manner, particularly for Ti particle concentrations of 0.1 and 1 mg/mL ($p<0.05$) (Fig. 4). Similar to that noted for NOX-1 expression, Ti particles at a concentration of ≤0.1 mg/mL induced ROS generation to a lesser extent compared to that noted with LPS stimulation ($p<0.05$) (Fig. 4).

Ti Particles Suppress LPS-Induced ROS Production and ERK Phosphorylation in RAW264.7 Macrophages

Short-term LPS stimulation resulted in a large increase in ROS levels. We noted that LPS stimulation (1 μg/mL) for 30 min significantly enhanced ROS generation ($p<0.001$). To assess the role of implant wear particles on IRI *in vitro*, we used RAW264.7 macrophages that were pre-exposed to 0.1 mg/mL Ti particles (LPS-free) for 12 h before they were stimulated with LPS for 30 min. In the cultures of macrophages pre-exposed to Ti particles, in which LPS was subsequently added, we noted that the ROS generation was lower compared to the cultures of macrophages that were not pre-exposed to LPS-free Ti particles prior to LPS stimulation ($p<0.001$) (Fig. 5a, b). In addition, we assessed the expression of phosphorylated ERK1/2 (pERK1/2). We noted that phosphorylation of ERK by LPS stimulation was attenuated by Ti particles ($p<0.05$) (Fig. 5c).

DISCUSSION

Macrophages are known to play a central role in the innate immune responses to implant wear particles in peri-implant tissues [31]. Particle-stimulated macrophages have been shown to release a number of proinflammatory cytokines, including prostaglandin E2, interleukin-1 beta, interleukin-6, interleukin-17C, and tumor necrosis factor-alpha, *via* upregulation of the transcription factor NF-κB [9, 31–33]. The continuous activation of NF-κB and

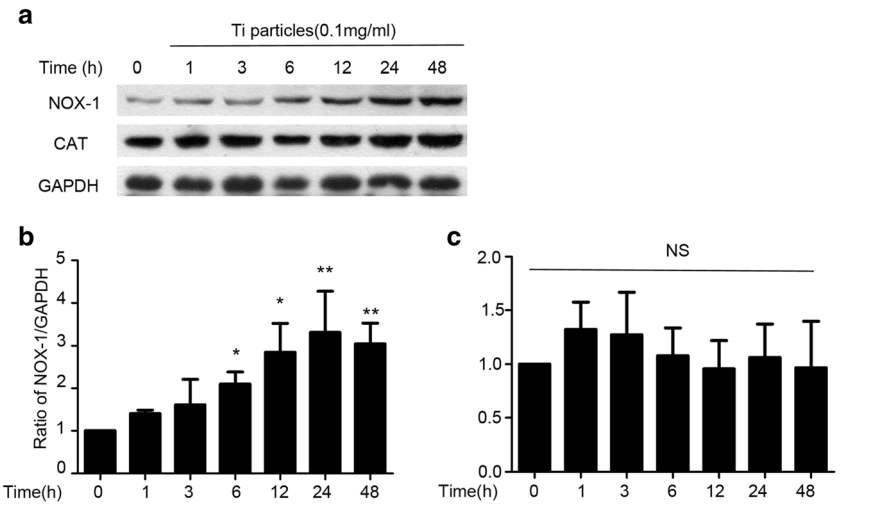

Fig. 3. Effect of Ti particles (0.1 mg/mL) on NOX-1 and CAT expression in RAW 264.7 macrophages at different time points. **a** RAW 264.7 cells were stimulated by Ti particles for 0, 1, 3, 6, 12, 24, and 48 h, respectively. The expressions of NOX-1 and CAT were assayed by western blotting. Statistical analysis of NOX-1 (**b**) and CAT (**c**) levels from three independent experiments are shown below. Data are presented as means ± standard deviation. *$p < 0.05$, **$p < 0.001$ vs. the 0 h group. *NS* not significant.

release of proinflammatory cytokines are the main causes of chronic inflammation in such cases.

During an acute infection, a sharp increase in oxygen uptake is found in macrophages, thus generating large amounts of ROS (respiratory burst) to oxidize and kill the invading pathogens [13, 15]. Moreover, the generation and accumulation of ROS is associated with chronic inflammation, while the amount is relatively low or moderate [34–36]. A moderate amount of ROS cannot eliminate detrimental stimuli thoroughly, they act as second messengers to activate NF-κB [13, 21, 36] and other proinflammatory signals [34], resulting in a low-grade, chronic inflammation [36]. In the present study, we noted that although LPS-free Ti particles stimulate ROS generation, their effect is relatively weaker compared

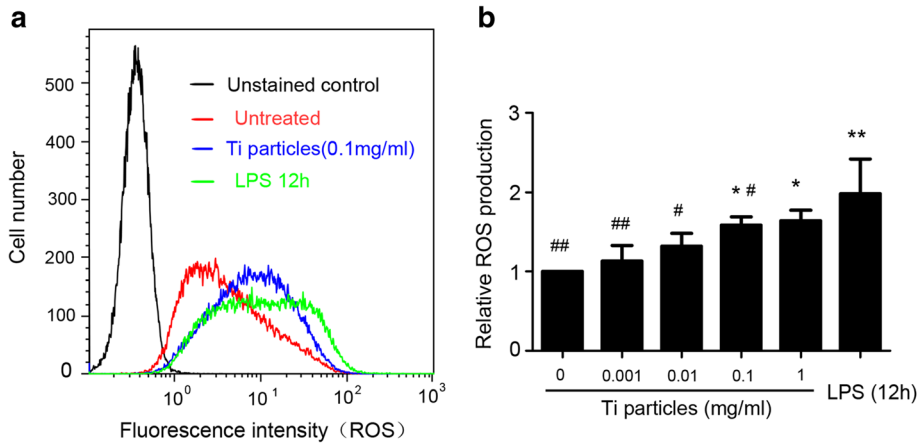

Fig. 4. Flow cytometry analysis of the effect of Ti particles and LPS on ROS production. RAW 264.7 macrophages were stimulated with different concentrations of Ti particles (range, 0–1 mg/mL) or 1 μg/mL LPS for 12 h. **a** Ti particles at a concentration of 0.1 mg/mL or LPS at a concentration of 1 μg/mL significantly increased ROS production. The data related to the stimulation with 0.001, 0.01, and 1 mg/mL Ti particles are not presented. **b** Statistical results of stimulation with different concentrations of Ti particles (range, 0–1 mg/mL) on ROS production from three independent experiments. Data are presented as means ± standard deviation (from three independent experiments). *$p < 0.05$, **$p < 0.001$ vs. negative control (NC, 0 mg/mL Ti group). $^{\#}p < 0.05$, $^{\#\#}p < 0.001$ vs. LPS (12 h) group.

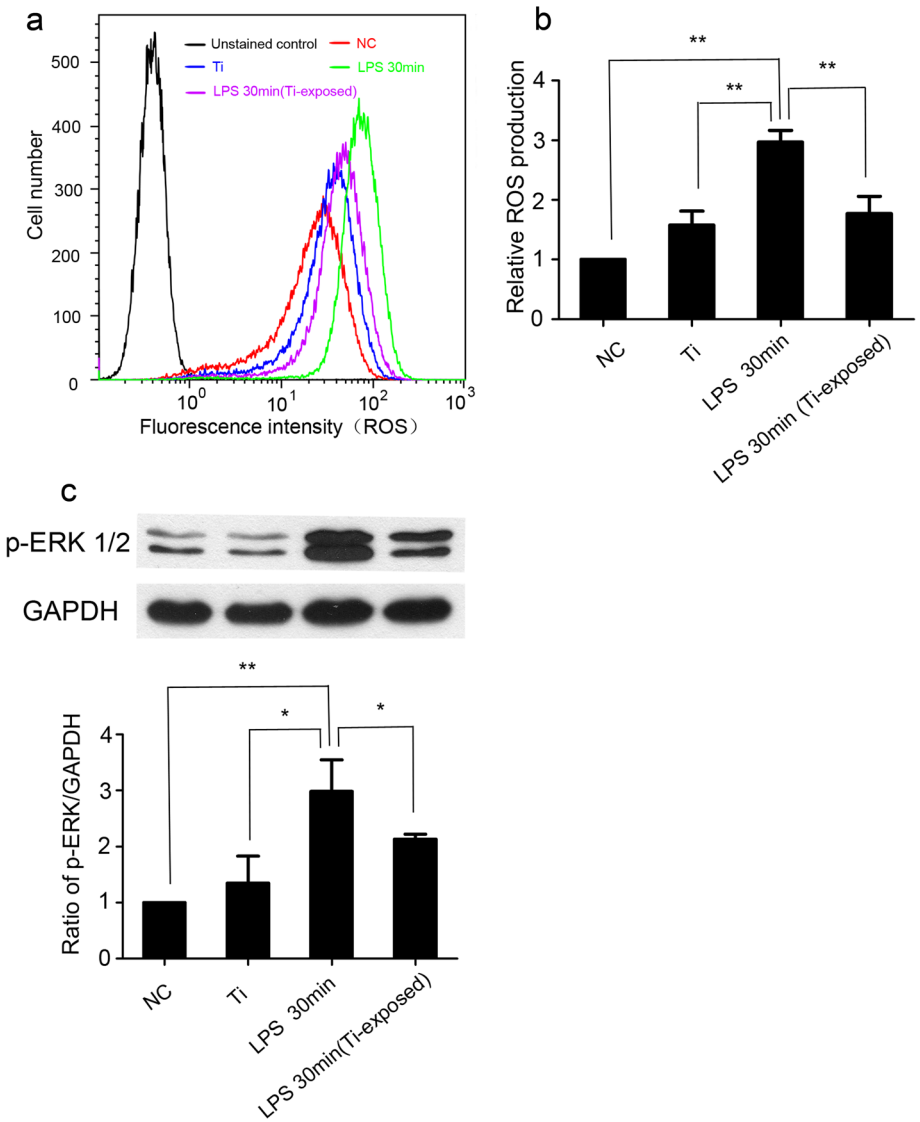

Fig. 5. Flow cytometry and western blot analysis indicating the suppressive effects of Ti particles on LPS-induced ROS generation and ERK1/2 phosphorylation in RAW264.7 cells. **a** Generation of ROS following stimulation under different conditions. NC (negative control): cells cultured without any additional stimulation; Ti: stimulation with Ti particles for 12 h; LPS 30 min: stimulation with LPS for 30 min. LPS 30 min (Ti-exposed): cells previously exposed to Ti particles for 12 h were then stimulated with LPS for 30 min. **b** Statistical results of panel **a** from three independent experiments. **c** The p-ERK/GAPDH ratio following stimulation under different conditions was also assessed. The data are presented as means±standard deviation (from three independent experiments). *$p<0.05$, **$p<0.001$ vs. negative control (NC).

to long-term (12 h) or short-term (30 min) LPS stimulation. Short-term LPS stimulation may be typically representative of an acute infection. Following short-term LPS stimulation, macrophages generate large numbers of ROS to eliminate the invasive pathogens. However, LPS-free Ti particle stimulation induces the generation of a moderate amount of ROS, which could lead to chronic inflammation. To evaluate the reasons underlying ROS accumulation in cells, we examined the expression of two ROS-related enzymes—NOX-1 and CAT—in response to stimulation by Ti particles and LPS. We noted that the upregulation of NOX-1, but not the downregulation of CAT, contributed to ROS accumulation.

The state of chronic inflammation around implant tissues due to stimulation by wear particles is a risk factor for IRI, as the wear particles could induce LPS tolerance and inhibit macrophage response to bacteria [37, 38]. Invasion of bacteria around implants is, to some extent, related to local immunosuppression. However, due to local immunosuppression, even a low level of invasive pathogens cannot be thoroughly eliminated; hence, the pathogens continue to grow and even form biofilms, which may then lead to persistent inflammation and eventually, IRI [7, 11].

In previous studies, we indicated that the Ti particles induced LPS tolerance in RAW 264.7 macrophages and had an immunosuppressive effect by inhibiting the release of proinflammatory cytokines. Furthermore, LPS stimulation can effectively activate RAW 264.7 macrophages to release a large amount of proinflammatory cytokines. However, if the macrophages have been pre-exposed to Ti particles, the stimulation of LPS causes only a moderate production of proinflammatory cytokines [38]. Therefore, although wear particles themselves have proinflammatory effects, they somewhat damage the immune function of macrophages and lead to local immunosuppression.

To further confirm the immunosuppressive effect of Ti particles, we examined ROS generation using methods similar to those used in our previous studies [38]. The present study indicated that pre-exposure to Ti particles can suppress ROS production and the respiratory burst mechanism in macrophages, thus indicating that local immunosuppression, or LPS tolerance, is induced. In addition, we noted that phosphorylation of ERK by LPS was inhibited by pre-exposure to Ti particles, indicating that the ability of macrophages to proliferate was damaged. The damage to both the respiratory burst mechanism and macrophage proliferation could contribute to local immunosuppression in peri-implant tissues. That is, macrophages cannot response rapidly and potently to bacterial components to clear invasive pathogens, which may be a risk factor for IRI.

In conclusion, the results of the present study support the hypothesis that the presence of wear particles can lead to chronic inflammation and LPS tolerance around implants through the suppression of ROS generation and macrophage proliferation. In such cases, immune function is eventually impaired. These tissues would thus be more susceptible to infections. Therefore, appropriate regulation of ROS and ERK may serve as a potential new strategy in the treatment of IRI.

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