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# Endotoxin Contributes to Artificial Loosening of Prostheses Induced by Titanium Particles

Authors' Contribution:  
Study Design A  
Data Collection B  
Statistical Analysis C  
Data Interpretation D  
Manuscript Preparation E  
Literature Search F  
Funds Collection G

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**Background:** Aseptic loosening of orthopedic implants caused by wear particles is a major cause of joint replacement failure. However, the mechanism of aseptic loosening has not yet been defined. The present study explored whether endotoxin adherent on the titanium (Ti) particles contributes to aseptic loosening.





**Material/Methods:** Limulus amoebocyte lysate detection was conducted to detect the levels of endotoxin adhered to the Ti particles. A mouse air pouches model was established and mice were divided into 4 groups and injected with phosphate-buffered saline (PBS) or Ti particles suspensions (0.1, 1, 10 mg/mL), following detection of the number of macrophages and the level of endotoxin. Scanning electron microscopy (SEM) was used to characterize the microstructures of Ti particles adhered with endotoxin.

**Results:** *In vitro* experiments showed that the level of endotoxin adhered to the Ti particles was significantly increased after adding LPS back to these "endotoxin-free" particles. *In vivo* experiments showed that Ti particles injection significantly increased the number of macrophages and the level of endotoxin.

**Conclusions:** In conclusion, these results suggest that adherent endotoxin may play an important role in aseptic loosening induced by Ti particles.

**MeSH Keywords:** **Endotoxins • Macrophages • Titanium**

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## Background

Total joint replacement (TJR) is one of the most successful procedures for the treatment of rheumatoid arthritis, osteoarthritis, femoral neck fracture, and other end-stage joint diseases [1,2]. TJR can effectively relieve joint pain, improve function, and elevate the health-related quality of life of patients [3]. Aseptic loosening is a major complication of total joint replacement, affecting the long-term use of the artificial joint, and is a major cause of arthroplasty failure in humans [3]. However, the mechanism of aseptic loosening has not yet been defined.

An extensive study has reported that wear debris cause aseptic loosening of prostheses [4]. Prosthesis wear particles stimulate the macrophages and other inflammatory cells around the prosthesis to produce a variety of pro-inflammatory cytokines and enhanced bone absorption, affect osteoblast differentiation (OD), and inhibit bone formation, resulting in aseptic loosening [5]. Titanium (Ti) particles are the most common wear debris particles in periprosthetic tissue [6]. Ti particles stimulate monocytes and macrophages around periprosthetic tissue to secrete many proinflammatory cytokines and chemokines, including interleukin-1 $\beta$  (IL-1 $\beta$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-6 (IL-6), transforming growth factor- $\beta$  (TGF- $\beta$ ), colony stimulating factor-1 (CSF-1), metalloproteinases (MMPs), and macrophage-CSF (M-CSF) [7,8]. These proinflammatory cytokines then result in bone absorption and osteolysis, thus contributing to aseptic loosening of the replaced joint [9,10]. However, the mechanisms by which wear particles induce cytokine production and aseptic loosening are still unclear.

Because of the many similarities between endotoxin and abrasive particles, we speculated that endotoxins participate in the biological response caused by wear particles [11]. For example, endotoxin and wear particles activate similar signal transduction pathways, increase production of prostaglandins and proinflammatory cytokines, and stimulate bone resorption. Bacterial endotoxins can adhere to wear particles and accelerate inflammatory host responses [12]. There are at least 3 potential sources of endotoxin in patients with aseptic loosening. First, the implants themselves may contain substantial amounts of adherent endotoxin [13]. Second, the high affinity of wear particles for endotoxin may lead to accumulation of circulating endotoxin [14]. Third, it is likely that the quantitatively most important source of endotoxin is the bacterial biofilms that exist on many implants from patients with aseptic loosening [15]. These 3 sources of endotoxin make it likely that a biologically significant amount of endotoxin adheres to wear particles in patients with aseptic loosening.

Lipopolysaccharide (LPS) is the classical endotoxin and is the main component of the outer cell wall of Gram-negative

bacteria [16]. Accumulating evidence indicates that particulate biomaterial debris generated from the mechanical wear of prosthetic components plays a critical role in aseptic loosening. The purpose of this study was to determine whether endotoxin adherent to Ti particles participates in induction of the biological responses that lead to aseptic loosening.

## Material and Methods

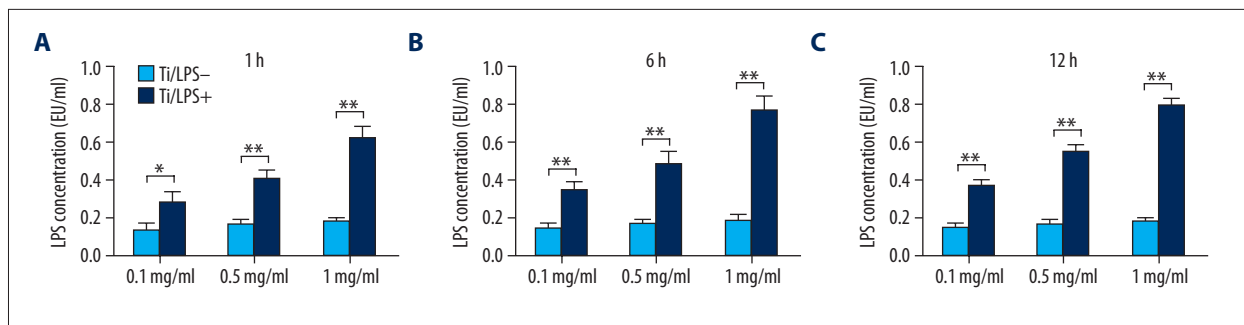
### Ti particle preparation

Commercial pure Ti particles with an average diameter of 5.6  $\mu\text{m}$  were obtained from Johnson Matthey Company (Ward Hill, MA). As previously described, Ti particles were sterilized by baking at 180°C for 6 h, then incubated in 70% ethanol for 48 h with shaking and washed 3 times with phosphate-buffered saline (PBS, pH 7.4) (Boster Biological Technology Co. Ltd., AR0030). The concentration of endotoxins was less than 0.1 EU/ml on particles. "Endotoxin-free" Ti particles (Ti/LPS<sup>-</sup>) were prepared by 5 cycles of alternating treatment with 25% nitric acid for 18–20 h at room temperature and a mixture of 0.1 N NaOH and 95% ethanol for 18–20 h at 30°C, as previously described [17]. LPS-coated Ti particles (Ti/LPS<sup>+</sup>) were produced by incubating 10 ng/ml LPS (*Escherichia coli*O55: B5; Sigma, St. Louis, MO) back to Ti/LPS<sup>-</sup> for 12 h at 37°C. Shumei KQ218 (100 w) Ultrasonic Cleaning equipment (Kunshan Ultrasonic Instruments Co., Ltd., Jiangsu, China) was used to suspended the Ti/LPS<sup>-</sup> and Ti/LPS<sup>+</sup> particles in PBS at a concentration of 50 mg/ml as stock solutions and were autoclaved. The concentration of particles used for incubation were 0.1, 0.5, and 1 mg/ml. It has been demonstrated that such particles are similar to the wear particles retrieved from periprosthetic tissues [18]. Endotoxins of the particles were detected by a limulus amoebocyte lysate detection kit (QCL-1000; BioWhittaker, Walkersville, MD, USA) at 1, 6, and 12 h after incubation.

### Murine air pouches

Twenty-four healthy C57BL/6 male mice aged 8 weeks and weighing 18–20 g were housed in a 12-h light/dark cycle and received water and food ad libitum. Air pouches were generated based on method previously reported by Sedgewick et al. [19]. Twenty-four mice were randomly assigned into 4 experimental groups (6 mice in each group): a control group (PBS), a Ti particle with PBS vehicle (0.1 mg/ml) group, a Ti particle with PBS vehicle (1 mg/ml) group, and a Ti particle with PBS vehicle (10 mg/ml) group. The dose of titanium particles was adjusted according to previous reports [20,21].

Air pouch cavities were produced according to a previous report [22]. Briefly, a 2-cm<sup>2</sup> area of the dorsal skin was sterilized using alcohol and shaved to provide the pouch site.



**Figure 1.** LPS added back increased the level of LPS adhering to Ti particles. (A) The concentrations of LPS at 1 h after “endotoxin-free” Ti particles incubation with or without LPS. (B) The concentrations of LPS at 6 h after “endotoxin-free” Ti particles incubation with or without LPS. (C) The concentrations of LPS at 12 h after “endotoxin-free” Ti particles incubation with or without LPS. \*  $p < 0.05$ , \*\*  $p < 0.01$  vs. Ti/LPS<sup>-</sup>. Ti – Titanium; LPS – lipopolysaccharide.

An air pouch was established by injecting 3 ml sterile air. Three days later, 2 ml sterile air was injected again to maintain the pouch. After 7 days, in mice with established air pouches, we injected 1 ml of each of the 4 different concentrations of Ti particles (0, 0.1, 1, 10 mg/ml) into the air pouch cavity on both sides of the body, and 1 week later the same dose of Ti particles were injected again. All animal procedures were approved by the Institutional Animal Care Committee.

#### Collection of exudates

Two week later, the mice were anesthetized with ether and killed by cervical dislocation. We injected 4 ml PBS with 5 U heparin/ml into the air pouch cavity to harvest the cells. Then, cavities were briefly massaged and an incision was made in each mouse to allow collection of the resulting cell suspension of the inflammatory exudate.

#### Air pouch macrophage population in mouse pretreatment with Ti particles

Ti particles (0, 0.1, 1, 10 mg/ml) were injected into the murine air pouch cavity, and 2 week later the macrophages were collected and counted using an Automatic Hematology Analyzer (Celltac E MEK-7222, Nihon Kohden, Japan), and then were compared to the number of the identical cells obtained from a group of untreated animals (control).

#### Endotoxins detection

Endotoxins in murine air pouch cavities were detected by a limulus amoebocyte lysate detection kit (QCL-1000; BioWhittaker, Walkersville, MD, USA) after Ti particles (0, 0.1, 1, 10 mg/ml) were added.

#### Morphological and structural evaluations

The morphology of the Ti particles and adherent endotoxins was assessed using scanning electron microscopy (SEM, Quanta-200, FEI, The Netherlands). Specimens were prepared for SEM viewing by double fixation, dehydration, and gold coating.

#### Statistics

Statistical analyses were performed with the SPSS version 17.0 software package (SPSS, Inc., Chicago, IL, USA). All values are expressed as mean  $\pm$  standard error of the mean (SEM). Analysis of variance (ANOVA) was used to analyze differences between groups. A value of  $p < 0.05$  was considered statistically significant.

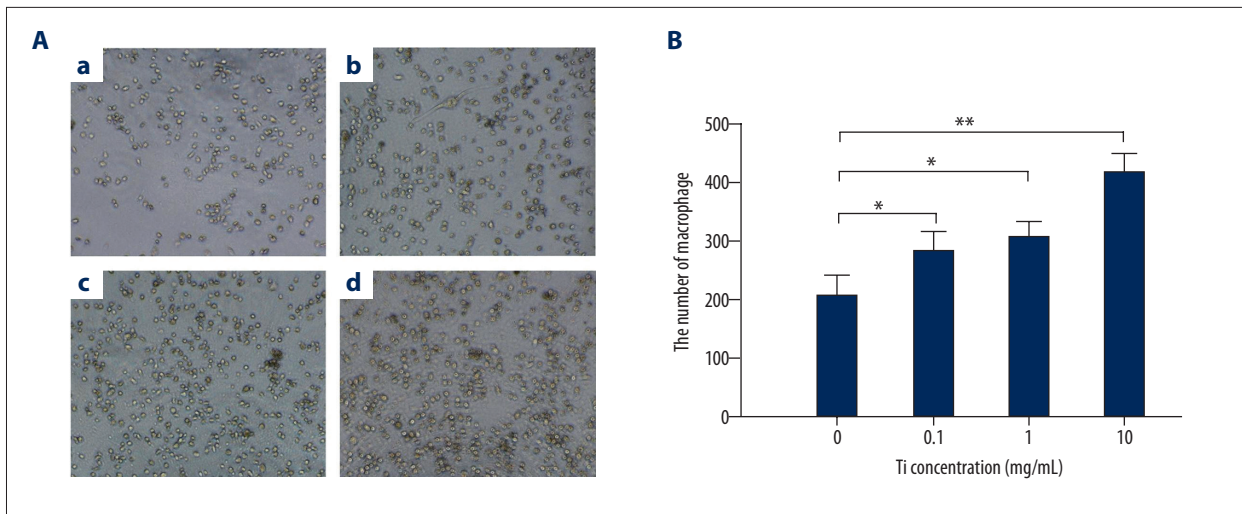
## Results

#### LPS added back increased the level of LPS adhering to Ti particles

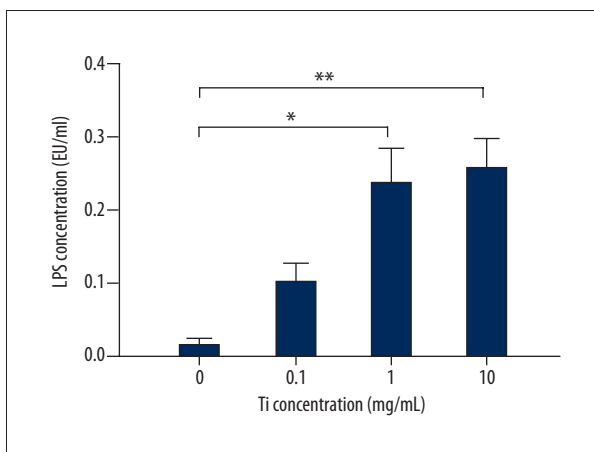
In order to detect the adhesion of Ti particles to endotoxin, we added 10 ng/ml LPS back to “Endotoxin-free” Ti particles. As shown in Figure 1, *in vitro* experiments indicated that after incubation with 0.1, 0.5, and 1 mg/ml Ti/LPS<sup>-</sup> and Ti/LPS<sup>+</sup> for 1 h, 6 h, 12 h, the level of LPS adhered to the Ti particles was significantly increased in Ti/LPS<sup>+</sup> compare to Ti/LPS<sup>-</sup>.

#### Ti particles increased macrophage population *in vivo*

As shown in Figure 2, injection of Ti particles (0.1, 1, 10 mg/ml) into the air pouch cavity of mice significantly increased the number of macrophages compared with the control group.



**Figure 2.** Ti particles increased macrophage population *in vivo*. (A) Ti particles (0.1, 1, 10 mg/ml) were injected into the air pouch cavity and 2 week later the macrophages were collected and counted. (a) Ti particles nontreated group, (b) Ti particles (0.1 mg/ml) group, (c) Ti particles (1 mg/ml) group, (d) Ti particles (10 mg/ml) group. (B) Quantitative analysis of the number of macrophage in Ti particles (0, 0.1, 1, 10 mg/ml) groups. \*  $p < 0.05$ , \*\*  $p < 0.01$  vs. nontreated group. Ti – Titanium.



**Figure 3.** Ti particle increased the level of LPS *in vivo*. The concentration of LPS in Ti particles (0, 0.1, 1, 10 mg/ml) groups. \*\*  $p < 0.01$  vs. nontreated group. Ti – titanium; LPS – lipopolysaccharide.

### Ti particle increased the level of LPS *in vivo*

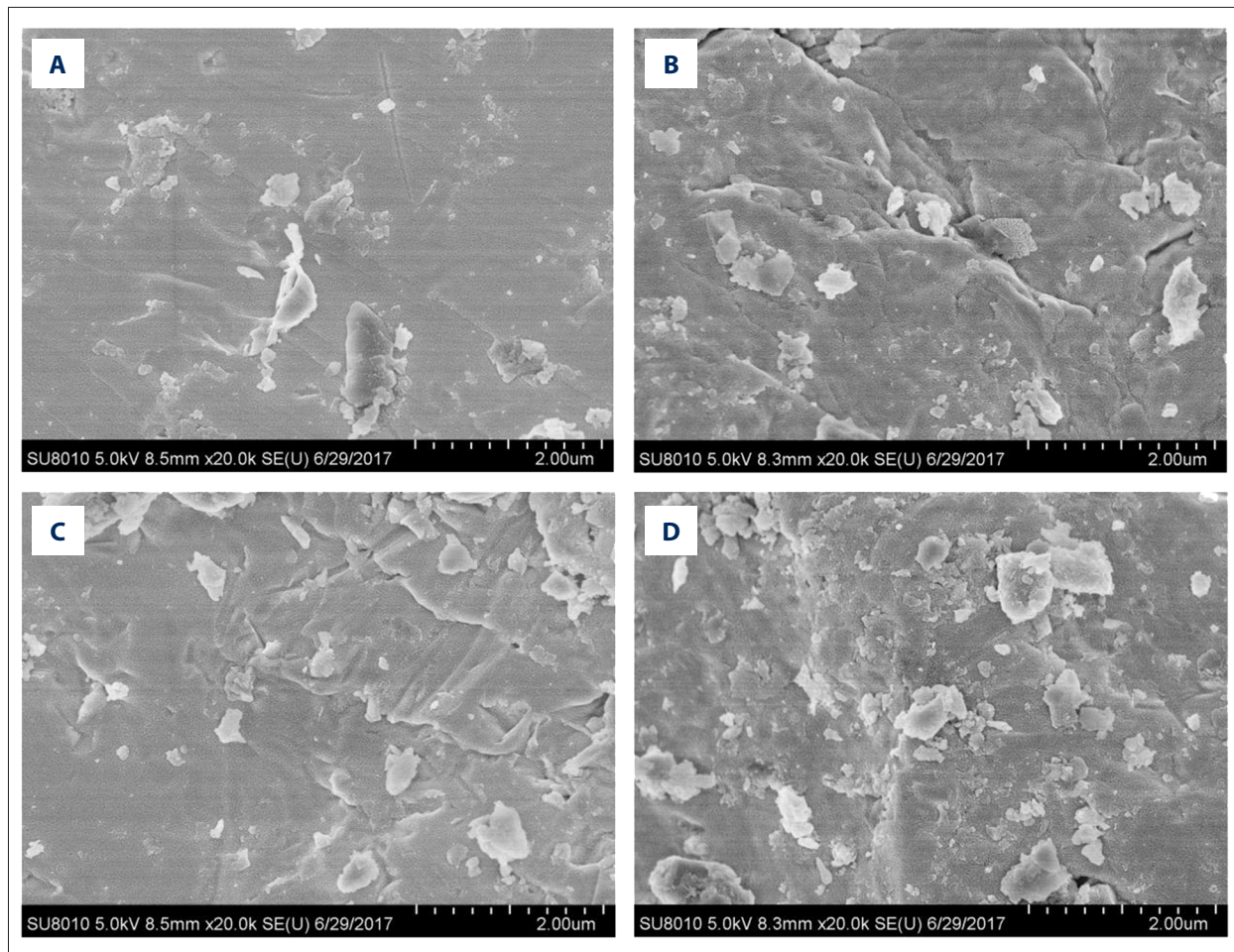
As shown in Figure 3, the concentration of LPS was markedly increased in Ti particle (0.1, 1, 10 mg/ml) groups compared with the control group. However, there was no significant difference between the Ti particle (1 mg/ml) group and the Ti particle (10 mg/ml) group. In addition, we found the ultrastructure of Ti particles adhered to endotoxin. As shown in Figure 4, the level of LPS adhesion to Ti particles was significantly increased in Ti particle (0.1, 1, 10 mg/ml) groups compared with the control group.

## Discussion

TJR is frequently used to treat severe joint disease [1]. However, the premature failure of TJR remains a significant problem. The most frequent cause of TJR failure is aseptic loosening of the implant and bone resorption. Our results demonstrate that the level of endotoxin adhering to the Ti particles was significantly increased after adding endotoxin back to these “endotoxin-free” particles, and Ti particles injection significantly increased the number of macrophages and the level of endotoxin. Therefore, this study provides evidence that endotoxin may be involved in the process of aseptic loosening.

In this study, Ti particles, an integral component of the implant wear debris, and the murine air pouch model were used according to methods described in a previous study [19]. The histiocytic infiltration and inflammation mediators in this model are similar to those of human joint inflammation in rheumatoid arthritis. A previous study reported that endotoxin accumulates on the endotoxin-free particles and/or in the surrounding tissue during the experiments [23]. Endotoxin also similarly accumulates after implantation of endotoxin-free polyethylene particles [24]. Our results indicate that the level of LPS adhering to the Ti particles was significantly increased after adding “endotoxin-free” Ti particles with LPS. A previous study reported that LPS was detected in periprosthetic tissues from patients with aseptically loosened implants, even in the absence of any clinical or microbiologic signs of infection [25]. Studies with a more specific LPS assay indicate that LPS exists in periprosthetic tissue from a subset of patients with aseptic loosening [26]. Our results showed that Ti particles injection significantly increased the level of LPS *in vivo*.





**Figure 4.** Scanning electron microscopy showed Ti particles adhered to endotoxin. A scanning electron microscope (JSM-5900LV) was used to characterize the microstructures and a laser diffraction particle size analyzer (Mastersizer 2000) was used to determine the size of various particles. (A) Ti particles nontreated group, (B) Ti particles (0.1 mg/ml) group, (C) Ti particles (1 mg/ml) group, (D) Ti particles (10 mg/ml) group.

A previous study reported that wear debris promote periprosthetic inflammation and subsequent bone resorption, and that this is an important cause of aseptic loosening of implants and bone resorption [27]. Ti particles stimulate the gathering of macrophages cells to produce a variety of proinflammatory cytokines, such as TNF- $\alpha$  and IL-6, in interfacial membranes [28–30]. In addition, the fibrous interface membrane around a loosening periprosthetic shows a predominance of macrophages, which represent 60–80% of the entire cellular population [31]. In the present study, our results also indicated that Ti particles increased the macrophage population, which is consistent with previous reports. However, the potential mechanism of wear debris-induced osteolysis and aseptic loosening is unclear.

Adherent endotoxin participates in aseptic loosening in patients and has important clinical implications [32]. There are at least 3 potential sources of endotoxin in patients with aseptic

loosening. However, studies with a more specific LPS assay indicate that LPS exists in periprosthetic tissue from a subset of patients with aseptic loosening [33].

## Conclusions

In the present study, our results indicated that Ti particles injection significantly increased the number of macrophages and the level of endotoxin. Therefore, this study may provide evidence that endotoxin is involved in the process of aseptic loosening. Reducing the level of adherent endotoxin without inhibiting osseointegration of orthopedic implants might also reduce aseptic loosening. However, how endotoxin, through the regulation of Ti particles, affects the mechanism of joint loosening needs further study.

## Acknowledges

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## References:

- Rubak TS, Svendsen SW, Soballe K, Frost P: Total hip replacement due to primary osteoarthritis in relation to cumulative occupational exposures and lifestyle factors: A nationwide nested case-control study. *Arthritis Care Res (Hoboken)*, 2014; 66: 1496–505
- Ollivere B, Wimbush JA, Clark IM, Donell ST: Current concepts in osteolysis. *J Bone Joint Surg Br*, 2012; 94: 10–15
- Gallo J, Vaculova J, Goodman SB et al: Contributions of human tissue analysis to understanding the mechanisms of loosening and osteolysis in total hip replacement. *Acta Biomater*, 2014; 10: 2354–66
- Amirhosseini M, Andersson G, Aspenberg P, Fahlgren A: Mechanical instability and titanium particles induce similar transcriptomic changes in a rat model for periprosthetic osteolysis and aseptic loosening. *Bone Rep*, 2017; 7: 17–25
- Bi Y, Seabold JM, Kaar SG et al: Adherent endotoxin on orthopedic wear particles stimulates cytokine production and osteoclast differentiation. *J Bone Miner Res*, 2001; 16: 2082–91
- Holt G, Murnaghan C, Reilly J, Meek RM: The biology of aseptic osteolysis. *Clin Orthop Relat Res*, 2007; 460: 240–52
- Zhao YP, Wei JL, Tian QY et al: Progranulin suppresses titanium particle induced inflammatory osteolysis by targeting TNFalpha signaling. *Sci Rep*, 2016; 6: 20909
- Lin TH, Tamaki Y, Pajarinen J et al: Chronic inflammation in biomaterial-induced periprosthetic osteolysis: NF-kappaB as a therapeutic target. *Acta Biomater*, 2014; 10: 1–10
- Wang Z, Liu N, Shi T et al: ER stress mediates TiAl6V4 particle-induced peri-implant osteolysis by promoting RANKL expression in fibroblasts. *PLoS One*, 2015; 10: e137774
- O'Neill SC, Queally JM, Devitt BM et al: The role of osteoblasts in peri-prosthetic osteolysis. *Bone Joint J*, 2013; 95-B: 1022–26
- Samelko L, Landgraber S, McAllister K et al: TLR4 (not TLR2) dominate cognate TLR activity associated with CoCrMo implant particles. *J Orthop Res*, 2017; 35: 1007–17
- Xing Z, Pabst MJ, Hastly KA, Smith RA: Accumulation of LPS by polyethylene particles decreases bone attachment to implants. *J Orthop Res*, 2006; 24: 959–66
- Ragab AA, Van De Motter R, Lavish SA et al: Measurement and removal of adherent endotoxin from titanium particles and implant surfaces. *J Orthop Res*, 1999; 17: 803–9
- Inada K, Endo S, Takahashi K et al: Establishment of a new perchloric acid treatment method to allow determination of the total endotoxin content in human plasma by the limulus test and clinical application. *Microbiol Immunol*, 1991; 35: 303–14
- Neut D, van Horn JR, van Kooten TG et al: Detection of biomaterial-associated infections in orthopaedic joint implants. *Clin Orthop Relat Res*, 2003; 413: 261–68
- Henderson B, Poole S, Wilson M: Bacterial modulins: A novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. *Microbiol Rev*, 1996; 60: 316–41
- Ragab AA, Van De Motter R, Lavish SA et al: Measurement and removal of adherent endotoxin from titanium particles and implant surfaces. *J Orthop Res*, 1999; 17: 803–9
- Kong L, Yang C, Yu L et al: Pyrroloquinoline quinone inhibits RANKL-mediated expression of NFATc1 in part via suppression of c-Fos in mouse bone marrow cells and inhibits wear particle-induced osteolysis in mice. *PLoS One*, 2013; 8: e61013
- Edwards JC, Sedgwick AD, Willoughby DA: The formation of a structure with the features of synovial lining by subcutaneous injection of air: An *in vivo* tissue culture system. *J Pathol*, 1981; 134: 147–56
- Geng DC, Xu YZ, Yang HL et al: Inhibition of titanium particle-induced inflammatory osteolysis through inactivation of cannabinoid receptor 2 by AM630. *J Biomed Mater Res A*, 2010; 95: 321–26
- Geng DC, Zhu XS, Mao HQ et al: Protection against titanium particle-induced osteoclastogenesis by cyclooxygenase-2 selective inhibitor. *J Biomed Mater Res A*, 2011; 99: 516–22
- Martins HB, Selis ND, Souza CL et al: Anti-inflammatory activity of the essential oil citral in experimental infection with staphylococcus aureus in a model air pouch. *Evid Based Complement Alternat Med*, 2017; 2017: 2505610
- Bi Y, Seabold JM, Kaar SG et al: Adherent endotoxin on orthopedic wear particles stimulates cytokine production and osteoclast differentiation. *J Bone Miner Res*, 2001; 16: 2082–91
- Tatro JM, Taki N, Islam AS et al: The balance between endotoxin accumulation and clearance during particle-induced osteolysis in murine calvaria. *J Orthop Res*, 2007; 25: 361–69
- Nalepka JL, Lee MJ, Kraay MJ et al: Lipopolysaccharide found in aseptic loosening of patients with inflammatory arthritis. *Clin Orthop Relat Res*, 2006; 451: 229–35
- Greenfield EM, Beidelschies MA, Tatro JM et al: Bacterial pathogen-associated molecular patterns stimulate biological activity of orthopaedic wear particles by activating cognate Toll-like receptors. *J Biol Chem*, 2010; 285: 32378–84
- Goodman SB, Gibon E, Pajarinen J et al: Novel biological strategies for treatment of wear particle-induced periprosthetic osteolysis of orthopaedic implants for joint replacement. *J R Soc Interface*, 2014; 11: 20130962
- Hirayama T, Tamaki Y, Takakubo Y et al: Toll-like receptors and their adaptors are regulated in macrophages after phagocytosis of lipopolysaccharide-coated titanium particles. *J Orthop Res*, 2011; 29: 984–92
- Peng L, Wang H, Song K et al: Lentivirus-mediated TNF-alpha gene silencing and overexpression of osteoprotegerin inhibit titanium particle-induced inflammatory response and osteoclastogenesis *in vitro*. *Mol Med Rep*, 2016; 13: 1010–18
- Antonios JK, Yao Z, Li C et al: Macrophage polarization in response to wear particles *in vitro*. *Cell Mol Immunol*, 2013; 10: 471–82
- Liu G, Guo T, Zhang Y et al: Apoptotic pathways of macrophages within osteolytic interface membrane in periprosthetic osteolysis after total hip replacement. *APMIS*, 2017; 125: 565–78
- Bi Y, Seabold JM, Kaar SG et al: Adherent endotoxin on orthopedic wear particles stimulates cytokine production and osteoclast differentiation. *J Bone Miner Res*, 2001; 16: 2082–91
- Nalepka JL, Lee MJ, Kraay MJ et al: Lipopolysaccharide found in aseptic loosening of patients with inflammatory arthritis. *Clin Orthop Relat Res*, 2006; 451: 229–35

## Conflict of interest

None.