IRAK-4 in macrophages contributes to inflammatory osteolysis of wear particles around loosened hip implants

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Abstract
TLRs recognizing PAMPs play a role in local immunity and participate in implant-associated loosening. TLR-mediated signaling is primarily regulated by IL-1 receptor associated kinase-M (IRAK-M) negatively and IRAK-4 positively. Our previous studies have proved that wear particles promote endotoxin tolerance in macrophages by inducing IRAK-M. However, whether IRAK-4 is involved in inflammatory osteolysis of wear particles basically, and the specific mechanism of IRAK-4 around loosened hip implants, is still unclear. IRAK-4 was studied in the interface membranes from patients in vivo and in particle-stimulated macrophages to clarify its role. Also, IL-1β and TNF-α levels were measured after particle and LPS stimulation in macrophages with or without IRAK-4 silenced by siRNA. Our results showed that the interface membranes around aseptic and septic loosened prosthesis expressed more IRAK-4 compared with membranes from osteoarthritic patients. IRAK-4 in macrophages increased upon particle and LPS stimulation. In the former, IL-1β and TNF-α levels were lower compared with those of LPS stimulation, and IRAK-4 siRNA could suppress production of pro-inflammatory cytokines. These findings suggest that besides IRAK-M, IRAK-4 also plays an important role in the local inflammatory reaction and contributes to prosthesis loosening.

Keywords
Total joint arthroplasty, septic loosening, aseptic loosening, TLR, IRAK-4

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Introduction
Implant loosening is the most common complication in the long-term follow-up of joint arthroplasty, which is mediated by acute and chronic inflammation produced chiefly by macrophages stimulated by prosthesis-derived wear particles.¹⁻⁵ The causes of loosening are complicated, and the implant loosening is mainly classified into two different categories, namely aseptic and septic implant loosening. Septic implant loosening is deemed to be caused by deep prosthesis-associated infections, while the aseptic is thought to be due to inflammatory cytokines, mostly from particle-stimulated macrophages. Recent studies suggested that some pathogenic microorganisms and their structural components (PAMP), for instance, LPS, decomposed into the blood during skin injuries or mucous membrane contusion, and gingivitis.⁶,⁷ They could be discovered in apparently aseptic peri-prosthetic tissues, germ cultures of which were negative.⁵,⁸⁻¹¹ It was also demonstrated that, from in vivo and in vitro studies, pure wear particles cannot lead to substantial and effective inflammatory osteolysis causing implant loosening, which merely accounted for a small percentage of cases.⁴,¹²⁻¹⁵ Osteoclastogenesis, as has been found in...
suppurative arthritis and prosthesis-related pyogenic infections, is seemingly more efficaciously provoked by pathogenic microorganisms, bacterial components and PAMP, all of which adhere to the superﬁcicies of implants and eventually wear particles. These stimuli activate a series of inﬂammatory cytokines and cause prosthetic loosening. Hence, adhered microorganisms and their components are afﬁrmatively participating in loosening.

Recent studies demonstrated that the tissues surrounding loosened total hip arthroplasty (THA) prostheses were equipped with various TLRs, and these TLRs expressed in macrophages of interface membranes played a major role in the local inﬂammatory and immunological reactions. In the signal pathway of TLRs, the TLRs’ ligand-mediated signaling procedures are regulated by various negatively and positively adjusting components, particularly the IL-1 receptor associated kinases (IRAKs). IRAKs are the important signal pathway leaguers of the TLR/IL-1R family. Up to now, four categories of IRAK-like molecules have been identiﬁed, characterized by the expression of a serine/threonine kinase domain and an N-terminal death domain, i.e. IRAK-1, IRAK-2, IRAK-M, and IRAK-4. IRAK-1 and 4 are active kinases, while IRAK-2 and M are inactive kinases. Except for the role of IRAK-2, those of the others have been elucidated clearly. Although IRAK-1 and IRAK-4 up-regulate innate immunological reactions positively by the activation of downstream kinases like MAPKs, IRAK-4 plays a key role in both innate and adaptive immunological reactions and is a crucial component in TLR/IL-1R signaling.

In our study, immunohistochemical techniques were used to analyze IRAK-4 expression in peri-prosthetic interface membranes from patients and its levels in vitro regulated by the extent of concentrations of titanium (Ti) particles and simplex P polymethylmethacrylate bone cement (BC) particles. The design was employed to clarify the expression of IRAK-4 protein and topology in the interface membranes of loosening, but also to elucidate whether wear particles, without any PAMP, could up-regulate IRAK-4 of macrophages and make sure the role of IRAK-4 in the inﬂammatory osteolysis of wear particles around loosened hip implants.

**Material and methods**

**Patients and samples**

Twenty samples of interface membrane formed between prosthesis and bone were acquired at THA revision, and these samples came from two places on each hip; one was from the acetabular side and the other was from the femoral side. Using classical standards, ﬁve cases were revised because of infection (one woman and four men, aged 56–78 yr, the average age was 63 yr) (Table 1) and 15 cases for implant loosening (10 women and ﬁve men, aged 56–85 yr, the average age was 68 yr) (Table 2). The average period from the ﬁrst operation to the prosthetic revision was 4.6 yr for infected prosthesis and 14.2 yr for implant loosening. All the seemingly loosening and non-infective cases were negative in germ cultures. For the infected peri-prosthetic interface membrane samples, both frozen sections and germ cultures conﬁrmed the septic implant loosening, with one case positive for *Staphylococcus epidermis* and four for *Staphylococcus aureus*. As controls, ﬁve synovialis samples were acquired from the ﬁrst total hip replacement operated for primary osteoarthritis of the hip (two women and three men, aged 49–72 yr, the average age was 62 yr). Inflammatory arthritis such as rheumatoid arthritis, uarthritis, spondylitis ankylpoietica, etc., were excluded by medical histories, clinical manifestations, imaging diagnosis, physical examinations, and laboratory tests. The samples were treated by formalin ﬁxation for 24–48 h, then dehydrated in absolute ethyl alcohol and clariﬁed in xylene, before ﬁnally being embedded in parafﬁn. The thickness of the parafﬁn sections was 3 µm and they were treated immuno- histochemically by deparafﬁnization, rehydration, and dye, as described below. The research agreement was consented by the ethical committee of the medical college of Sun Yat-sen University, and the ethics approval permit number is 201508918.

**Immunohistochemical analysis**

Ag repair was processed on 3 µm sections of interface membrane tissue as described before. Intrinsic peroxidase was suppressed with 3% H2O2 for about 10 min. Then Ags were retrieved for 5 min in a microwave oven. The specimens were incubated with (I) 10% blocking normal goat serum at 22°C for 30 min, (II) 10 µg/ml rabbit anti-human polyclonal IRAK-4 IgG (Abcam, Cambridge, UK) in humidified chambers at 4°C overnight, (III) biotinylated goat-anti-rabbit IgG at 37°C for 20 min, and (IV) avidinbiotinperoxidase complex at 37°C for 20 min. The peroxidase-conjugated sites were incubated visually in 3,3’-diaminobenzidine tetrahydrochloride (DAKO A/S, Glostrup, Denmark) and 0.03% H2O2 for 5 min, followed by counterstaining in hematoxylin for 10 s. In between, sections were washed with PBS (Boster, China) at least three times. Staining was afﬁrmed by using normal rabbit IgG at the identical concentration and substitution of the primary Ab.
Stained sections were analyzed and photographed by a Leica DM2500b microscope and semiautomatic Leica Application Suite V4 imaging analysis and procedures. Semi-quantitative evaluation of immunohistochemical dye was performed as follows: negative dyeing was defined as (–); the fraction of occasional positive cells (less than 5% of the section area) was defined as (×/C6); some positive cells (5–25%) were defined as (þ); a medium number of positive cells (25–50%) were defined as (þþ); and a high number of positive cells (more than 50% of the tissue) were defined as (þþþ).

All the immune-stained tissues were blindly graded and checked through optical microscopy by two different researchers. The evaluations were combined with a histological grading of wear particles. Metal particles were examined microscopically like black stain. Ultrahigh molecular mass-polylethylene particles (UHMMP) were examined by a polarized optical microscope. Polymethylmethacrylate (PMMA) particles were observed as “ghosts” combined with residually small particles, unsolvable 0.5–1 µm barium sulfate residues.22 The extent of the particles were evaluated as follows: (–) absent; (×) occasionally found in the tissues covering under 5% of the area examined; (þ) often found in 5–25% of the area; (þþ) moderately

| Case | Diagnosis | Age/sex | Yr from THA to revision | Type of alloy | Type of Liner | Cement | Immunoreactivity* | Wear particles |
|------|-----------|---------|-------------------------|---------------|--------------|--------|------------------|----------------|
| 1    | SOA       | 56M     | 8                       | CoCrMo        | UHMMPE      | no     | + + +            | –              |
| 2    | SOA       | 69M     | 10                      | TiAlV         | UHMMPE      | no     | + + +            | –              |
| 3    | POA       | 74M     | 22                      | TiAlV         | Ceramic     | yes    | + + +            | –              |
| 4    | SOA       | 76F     | 18                      | TiAlV         | UHMMPE      | yes    | + + +            | –              |
| 5    | SOA       | 64F     | 15                      | TiAlV         | UHMMPE      | no     | + + +            | –              |
| 6    | POA       | 57F     | 8                       | TiAlV         | UHMMPE      | yes    | + + +            | –              |
| 7    | SOA       | 59M     | 11                      | CoCrMo        | UHMMPE      | yes    | + + +            | –              |
| 8    | POA       | 76F     | 22                      | TiAlV         | UHMMPE      | yes    | + + +            | –              |
| 9    | POA       | 56F     | 8                       | CoCrMo        | UHMMPE      | no     | + + +            | –              |
| 10   | POA       | 64F     | 10                      | TiAlV         | UHMMPE      | no     | + + +            | –              |
| 11   | SOA       | 77M     | 20                      | CoCrMo        | UHMMPE      | yes    | + + +            | –              |
| 12   | SOA       | 80F     | 17                      | TiAlV         | Ceramic     | yes    | + + +            | –              |
| 13   | SOA       | 85F     | 21                      | TiAlV         | UHMMPE      | yes    | + + +            | –              |
| 14   | POA       | 60F     | 13                      | CoCrMo        | UHMMPE      | no     | + + +            | –              |
| Ave- rage |        | 68.2    | 14.2                    |              |             |        |                   |                |

THA: total hip arthroplasty; CoCrMo, cobalt-chromium-molybdenum; TiAlV, titanium-alumina-vanadium alloy; UHMMPE, ultrahigh molecular mass polyethylene; PMMA, polymethylmethacrylate; SA: S. aureus; SE: S. epidermidis; *Immunoreactivity for IRAK-4. Semi-quantitative evaluation of immunohistochemical dye and wear particles were performed as follows: negative dyeing or absent particles defined as (–); the fraction of coverage of occasional positive cells or particles area less than 5% of the section area defined as (+×); some positive cells or particles area, 5–25% of the coverage defined as (+); a medium number of positive cells or particles area, 25–50% of the coverage defined as (+×); and a large number of positive cells or particles area, covered more than 50% of the tissue defined as (+++×).

Microscopy and grading

Stained sections were analyzed and photographed by a Leica DM2500b microscope and semiautomatic Leica Application Suite V4 imaging analysis and procedures. Semi-quantitative evaluation of immunohistochemical dye was performed as follows: negative dyeing was defined as (–); the fraction of occasional positive cells (less than 5% of the section area) was defined as (×); some positive cells (5–25%) were defined as (+×); a medium number of positive cells (25–50%) were defined as (+××); and a high number of positive cells (more than 50% of the tissue) were defined as (++++×). All the immune-stained tissues were blindly graded and checked through optical microscopy by two different researchers. The evaluations were combined with a histological grading of wear particles. Metal particles were examined microscopically like black stain. Ultrahigh molecular mass-polyethylene particles (UHMMP) were examined by a polarized optical microscope. Polymethylmethacrylate (PMMA) particles were observed as “ghosts” combined with residually small particles, unsolvable 0.5–1 µm barium sulfate residues.22 The extent of the particles were evaluated as follows: (–) absent; (×) occasionally found in the tissues covering under 5% of the area examined; (+×) often found in 5–25% of the area; (+××) moderately
found in 25–50%; (+++), abundantly more than 50%.23

**Titanium particles and BC particles**

Commercial titanium particles (Ti) used in cellular experiments were bought from Pfizer (New York, NY). The size of Ti particle was defined by an electronic scanning microscope and NIH imaging software, and the average diameter was observed to be 4.74 ± 1.82 μm. The particles were rinsed for no endotoxic conglutination, according to the protocol by Ragab et al.24 The Ti particles were disposed in 25% nitric acid for 20 h, followed by incubation in a mixture of 95% ethanol and 0.1 N NaOH at 30°C for 20 h. The procedure was performed twice. Between each step, the Ti was rinsed 3–5 times with PBS until the used PBS pH was not more than 1 U of the fresh PBS. Then the stock solution was concentrated to 1 mg/ml in DMEM (Boster, China) and 1% antibiotics. Simplex P BC powder (Howmedica Osteonics, Allendale, NJ) was constituted of 10% barium sulfate, 15% PMMA, and 75% methylmethacrylate styrene copolymer. According to the manufacturer the BC particle diameters ranged from < 1 μm to approximately 100 μm. One mg/ml BC mixture was made in 1% antibiotic DMEM.

**Endotoxin detection**

Before experimentation, particles prepared in in vitro tests were ensured by Limulus Ameobocyte Lysate tests (Fitzgerald Industries International, Acton, MA) to contain < 0.125 EU/ml of endotoxin.

**LPS**

LPS from *Escherichia coli* 0111:B4 (Sigma, USA) was dissolved in PBS in a concentration of 1 mg/ml and stored at -20°C. Before experiment, LPS was diluted to a 100μg/l solution in 1% antibiotic DMEM.

**Cell culture and particle stimulation**

RAW264.7 macrophages were resuscitated from a deep freezer at −80°C. The macrophages were cultured at 37°C in high-Glc-DMEM (HG-DMEM) with 1% antibiotics and 10% FCS (Jetway, China). Cells were moved to 6 cm culture flasks from 75 cm² dishes for further tests, 2 × 10⁶ cells per dish in 2 ml medium. Macrophages were incubated for 12 h in 1% antibiotic HG-DMEM. Then the cultural medium was transformed to 2 ml solution with added 100 μg/l LPS, 0.1mg/ml Ti particles, 0.1mg/ml BC particles, and normal medium alone, all of which were cultured in eight dishes. Here, macrophages were isolated by scraping at different times (0, 0.5, 1, 3, 6, 9, 12, and 24 h). Meanwhile, supernatants were collected and stored at −80°C until used for cytokine determination.

**Total RNA isolation and RT-PCR**

After the above-mentioned treatments, RAW264.7 macrophages were rinsed three times with PBS and incubated with Trizoll reagent (Invitrogen, Carlsbad, CA). Then the extracted total RNA was stored at −80°C. cDNA was prepared by reverse transcription (ThermoscriptRT; Invitrogen) and amplified by real-time PCR (ABI PRISM 7000 Real-Time PCR, Applied Biosystems, Carlsbad, CA). The primers were planned using known mouse IRAK-4 and β-actin sequences, obtained online using software Primer 3. The following primer pairs were used: IRAK-4 (104bp), 5’-CCA TCG TGG CGG TGA AG-3’ (forward primer) and 5’-GTG CGT CAT TAC T-3’ (reverse primer); for β-actin (121bp), 5’- ATG GTG GGA ATG GGT CAG AA-3’ (forward primer) and 5’-TCT CCA TGT CGT CCC AGT TG-3’ (reverse primer). Mouse β-actin was designed as a house-keeping gene in RT-PCR analysis.

A total of 1 μg cDNA was used and combined with the primers described above in a 25 μl reactive admixture, applying SYBR Green as a marker for DNA content, supplied in the SYBR Green PCR Master Mix (Stratagene Europe, Amsterdam, NL). The following amplification proceeded for a maximum of 45 cycles as: 45 s at 95°C, 45 s at 59°C, and 45 s at 72°C. Nonbyproducts were obtained, as indicated by the dissociation pattern at the last phase of the reaction and by agarose gel electrophoresis (data not shown). The amplified effect of the mouse IRAK-4 was the same as that of the β-actin, as indicated by the normal curves of amplification. Quantitative analyses were performed by LightCycler Software to examine the real-time fluorescent signal related directly to the concentration of the target cDNA.

**Western blotting**

The cells and the tissue samples (the samples came from the subgroups, which were the same as those in immunohistochemistry) were thawed from −80°C in an ice box and lysed using lysis buffer, which contained (in mM) 50 Tris-HCl, 40 NaF, 150 NaCl, 5 ethylene diamine tetraacetic acid (EDTA), 1 Na-orthovanadate, 5 ethylene glycol tetraacetic acid
(EGTA), 1% (v/v) triton×-100, 0.5 phenylmethanesulfonyl fluoride (PMSF), 0.1% (w/v) SDS, and protease inhibitor mixture, pH 7.4. Each sample was homogenized and insoluble material or cell debris was removed by centrifugation at 12,000 g for 10 min at 4°C. The supernatant was removed and the protein concentration in the supernatant was determined using a Pierce protein assay kit (Pierce Bio-technology, USA) according to the manufacturer’s instructions.

Thirty μg of each sample were denatured by boiling in 5 × Laemmli gel loading buffer at 100°C for 5 min, solubilized by SDS sample buffer, electrophoresed by a 12% SDS-PAGE in Tris/glycine/SDS buffer (25 mM Tris, 250 mM glycine, and 0.1% SDS), and transferred to Immobilon-P PVDF Membranes (Bio-Rad, CA) at 300 mA for 1.5 h at 4°C. After blocking for 1 h in 20 mM Tris-HCl, 150 mM NaCl, and 0.2% Tween 20 (TBS-T) containing 5% non-fat milk, the membranes were washed three times in TBS-T alone and incubated for 20 h at 4°C with gentle agitation with a rabbit polyclonal IgG Ab anti-mouse IRAK-4 (1:1000 dilutions) (Abcam, Cambridge, UK) diluted in TBS-T. Following washing in TBS-T, the membranes were incubated with secondary HRP-conjugated goat anti-rabbit IgG (1:2000 dilutions) for 2 h at room temperature and washed three times in TBS-T. The bound Abs were detected using ECL Plus reagents according to the manufacturer’s instructions and then exposed to X-ray film (Fuji xRX-U, Tokyo, Japan). To re-probe for β-actin, the membranes were washed three times with wash buffer (TBS containing 0.1% Tween-20 and 0.5% skimmed milk) and blotted with anti-β-actin Ab (Cell Signaling Technology, Boston, USA) according to the method described above. The membrane was exposed to X-ray film, processed, and photographed. A PC-based Image Analysis program was used to quantify the intensity of each band (Image Analysis).

ELISA

Cellular supernatant was recovered from -80°C to 25°C and inflammatory cytokines were detected using ELISA kits (Shanghai Excell Biology, Shanghai, China). IL-1β and TNF-α levels were detected, based on the instructions of the manufacturer. The OD was measured at a wavelength of 450 nm on a Tecan Sunrise Absorbance Reader and each specimen was examined three times.

Silencing of IRAK-4 by siRNA

Small interfering RNAs (siRNAs) for IRAK-4 and scrambled control siRNA were purchased from Invitrogen company (Invitrogen, USA). The target sequences of murine IRAK-4 were 5’-GCU CAU CAU CUU GCA UGU CAA GAU UTT-3’ (sense) and 5’-AAU GAC AUG CAA UTT-3’ (antisense). The constructs were approved by sequencing. RNA interference expression vectors or control vectors (containing a scrambled sequence that did not show significant sequence homology to rat, mouse, or human gene sequences) were transfected into RAW264.7 macrophages using Lipofectamine reagent (Invitrogen, USA) according to the illustrations. Macrophages were incubated in 10% FBS for 24 h post-transfection and then treated with LPS, Ti particles and BC powder for 12 h.

Statistical analysis

All the experiments above were performed five times. Results are shown as the mean and the standard deviation and the normal distribution of this data was checked by the Shapiro-Wilk test. Differentiation between the groups was analyzed by ANOVA, the Student’s t test was used for analysis of normally distributed data, and the Mann-Whitney U test was used for analysis of skewed data. The relevance between IRAK-4 and particles was analyzed by Pearson’s correlation. A value of P < 0.05 was considered as statistically significant. Pearson’s correlation coefficient (r) was calculated for all the standard curves to confirm the proper PCR performance. Microsoft Excel and SPSS14.0 software were used to analyze all the data.

Results

Interface membranes and synovialis detection

More macrophages were detected in the interface membranes from loosened hip joints and infected prostheses. In OA synovial specimens, diffused and fewer distribution of macrophages were detected in the lining and sub-lining layers of synovialis and perivascular synovialis. In implant loosening (Figure 1a) and infection (Figure 1b), lots of macrophages in the interface membranes expressed IRAK-4 obviously.

In aseptic-like implant loosening, IRAK-4 staining was not related to the present wear particles, such as UH-MWEP, metallic particles, and/or PMMA (Table 2) (P > 0.05). The relativity in the infection was still not clear (P > 0.05). As for OA synovialis, IRAK-4 staining (Figure 1c) was only observed occasionally in stromal macrophages and the extent of the immunoreactivity was quite scarce when compared with interface membranes from loosened hip prostheses and infected prostheses. Some feeble staining of IRAK-4 was also observed in other cells such as synovial lining cells and vascular endothelial cells. The controls with negative staining by non-immune rabbit IgG...
proved the specificity (Figure 1d). To further confirm the expression of IRAK-4 in these tissues, we used the same batch of samples in immunohistochemical analysis and examined the interface membranes of loosened hip joints and infected prostheses and the osteoarthritis synovialis by Western blot. It was found that IRAK-4 in the interface membranes around infected and loosened total hip replacement implants was increased compared with osteoarthritis synovialis ($P < 0.05$), while there was no statistical difference between infected and loosened interface membranes ($P > 0.05$) (Figure 2).

**Expression of IRAK-4 mRNA in macrophages**

Melting curve analyses revealed exact PCR representation with a lone peak without non-specific byproducts. Pearson’s correlation coefficients ($r$) of all curves were $<-0.994$, affirming the PCR amplification exactly. Expression of IRAK-4 mRNA in macrophages

| Group          | $n$ | ++++ | ++ | + | ±/− | $\chi^2$ | $P$   |
|----------------|-----|------|----|---|-----|----------|-------|
| Infection      | 5   | 5 (100%) | 0 (0) | 0 (0) | 0 (0) | 27.083 | <0.001 |
| Loosening      | 15  | 11 (73.33%) | 4 (26.67%) | 0 (0) | 0 (0) |
| Synovialis     | 5   | 0 (0) | 0 (0) | 1 (20%) | 4 (80%) |

*Figure 1.* Immunostaining of IRAK-4 in the interface membranes around infected (a) and loosened (b) total hip replacement implants is increased compared with the osteoarthritis synovial membrane (c). The negative control staining using non-immune rabbit IgG at the same concentration as, and instead of, the primary specific Abs confirmed the specificity of the staining (d). The quantitative analysis of IRAK-4 in interface membranes and OA synovialis.
increased after BC and Ti particle stimulation. Particles activated IRAK-4 in a dose-dependent and time-dependent way. For BC particle stimulation and Ti particle stimulation for 6 h, the IRAK-4/β-actin ratio (%) in both at 0, 0.0001, 0.001, 0.01, and 0.1 mg/ml increased (P < 0.05), while at 1 mg/ml it decreased, especially for BC particle stimulation (P < 0.05) (Figure 3).

In the 0.1 mg/ml BC particle stimulation, with 0.1 mg/ml Ti particle stimulation, and 100 μg/l LPS stimulation, all IRAK-4/β-actin ratios (%) at 0, 0.5, 1, 3, 6, and 9 h increased (P < 0.05), while at 12 and 24 h it decreased, especially in LPS stimulation (P < 0.05) (Figure 4).

Expression of IRAK-4 protein in macrophages

Expression of IRAK-4 protein levels in macrophages increased after BC and Ti particle stimulation. As with IRAK-4 mRNA described above, particles activated IRAK-4 protein in a dose-dependent and time-dependent way. For BC particle stimulation and Ti particle stimulation for 6 h, the IRAK-4/β-actin ratio (%) in both at 0, 0.0001, 0.001, 0.01, and 0.1 mg/ml increased (P < 0.05), while at 1 mg/ml, the trend of continued increase was not obvious, compared with the former 0.1 mg/ml (P > 0.05) (Figure 5).

In the 0.1 mg/ml BC particle stimulation, 0.1 mg/ml Ti particle stimulation, and 100 μg/l LPS stimulation, all IRAK-4/β-actin ratios (%) at 0, 0.5, 1, 3, 6, and 9 h increased (P < 0.05), while they were stable at 12 and 24 h (Figure 6), which was somewhat different to that of IRAK-4 mRNA described above.

IL-1β and TNF-α detection after stimulation

Two typical inflammatory cytokines, IL-1β and TNF-α, were examined. The results indicated that after 0.1 mg/ml BC particle, 0.1 mg/ml Ti particle, and 100 μg/l LPS stimulations, for 0, 0.5, 1, 3, 6, 9, 12, and 24 h, the IL-1β (Figure 7) and TNF-α (Figure 8) levels (pg/ml, mean ± SD) presented some interesting tendencies over time.

Compared with macrophages cultured in either BC particles or Ti particles, the levels of IL-1β and TNF-α were lower than in the group of LPS stimulation, especially after 3 h (P < 0.0001). Meanwhile, the levels of IL-1β and TNF-α in Ti particle stimulation were lower than those of BC particles at 6, 9, 12, and 24 h (P < 0.05), however, there were no distinct differences.
at 0, 0.5, 1, and 3 h ($P > 0.05$) between the groups stimulated with Ti particles and BC particles. Compared with the above-mentioned three groups, the levels of IL-1β and TNF-α were much lower in the group with macrophages cultured in the absence of any stimulus.

**Inflammatory cytokines after silencing of IRAK-4 by siRNA**

In order to further demonstrate the role of IRAK-4 in macrophage immunoreactions and loosening, we silenced the expression of IRAK-4 genes in RAW264.7 cells by siRNA. After the RAW264.7 cells were transfected with IRAK-4 siRNA, they were pre-exposed to LPS for 12 h, and then the cell culture medium samples were collected for cytokine measurements. IL-1β concentration in the media of the IRAK-4 silenced samples was 32 ± 15 pg/ml, and the control RNA and non-treated control values were 190 ± 16 pg/ml and 185 ± 20 pg/ml, respectively. Therefore, silencing of IRAK-4 significantly interfered with IL-1β production ($P < 0.001$). A similar interference as a result of silencing of IRAK-4 was seen in samples stimulated with BC particles and Ti particles ($P < 0.05$). For Ti particles, the IL-1β concentrations in the siRNA silenced samples, control RNA samples, and non-treated control samples were 20 ± 5 pg/ml, 62 ± 7 pg/ml, and 65 ± 8 pg/ml, respectively. As for BC particles, the IL-1β concentrations were 32 ± 15 pg/ml, 190 ± 16 pg/ml, and 185 ± 20 pg/ml, respectively.

**Figure 5.** Effect of bone cement (BC) particles and titanium (Ti) particles on IRAK-4 protein level. After BC and Ti particle stimulation, the ratios of IRAK-4 to β-actin increased in a concentration-dependent manner.

**Figure 6.** Effect of bone cement (BC) particles, titanium (Ti) particles, and LPS on IRAK-4 protein levels. In time course studies, BC particles, Ti particles, and LPS increased IRAK-4 protein expression in relation to β-actin in a time-dependent manner over 24 h.
As for BC particles, they were $144 \pm 25$ pg/ml, $767 \pm 162$ pg/ml, and $754 \pm 109$ pg/ml. In the Ti particle-stimulated cells and cells stimulated with BC particles and LPS, the TNF-α levels were lower in IRAK-4 siRNA macrophages than in the control RNA group and the non-treated controls ($P < 0.001$). The TNF-α concentration in media of the blank group was only $98 \pm 5$ pg/ml (Figure 9).

### Discussion

It is well known that macrophages play an important role in implant loosening, especially in THA. Around the prosthesis, wear particles originating from the metallic femoral stem, BC, and polymeric acetabular cup represent a primary stimulus. In vivo and in vitro research has proven previously that “aseptic implant loosening” results from inflammatory osteolysis, nevertheless, it may be mainly provoked by pathogenic microorganisms and/or at least their structural components, namely PAMP, adhering to wear particles and implants. It has been claimed that pure wear particles could not cause enough cytokine production to contribute to inflammatory osteolysis, while PAMP, adhering to the prosthesis as well as to the surface of wear particles, could effectively stimulate cascades of pro-inflammatory cytokines and lead to prosthesis loosening.

According to this hypothesis, it has been manifested that the interface membranes surrounding the prosthesis express plentiful and various genres of TLRs, mostly in macrophages. Indeed, lower levels of PAMP ligands around implants could activate macrophages through TLRs and generate chronic inflammation, which at last accelerates to osteoclast formation and proliferation, peri-prosthesis osteolysis, and implant loosening.

IRAK-4 is a serine/threonine-specific protein kinase that is essential for innate and adaptive immune responses. It plays a key mediator role in IL-1 and TLR signaling pathways and protection from bacterial infections. Upon IL-1 stimulation, IRAK-4 and IRAK-1 co-localize at IL-1R and both kinases bind to the adaptor protein MyD88. IRAK-1 is phosphorylated by IRAK-4 and subsequently by autophosphorylation. Phosphorylated IRAK-1 dissociates from the receptor and associates with TNFR-associated factor 6 to initiate downstream signaling cascades, resulting in activation of NF-κB, p38, and JNK MAPK. IRAK-4-deficient mice $(-/-)$ revealed that IRAK-4 is essential for mediating the majority of innate immune responses, and the IRAK-4 kinase-inactive knock-in mice were completely resistant to LPS-induced shock, due to impaired TLR-mediated...
induction of pro-inflammatory cytokines and chemokines.32,36 Macrophages and mouse embryonic fibroblasts derived from IRAK-4-/-mice exhibit severe defects in cellular signaling in response to IL-1 and TLR ligands. IRAK-4-deficient-mice in humans has been described as resulting in several defects in the immune responses to pathogens, such as a lack of induction of IFN-α and -β production in response to viral stimulation of TLR7, 8, and 9, and a defect in generating a sustained Ab response during childhood.37,38 Given the central role of IRAK-4 in Toll-like/IL-1R signaling and immunological inflammation, we clarify the essentiality of IRAK-4 in the prosthesis loosening.

Here we demonstrate that IRAK-4 is mainly found in macrophages and strongly expressed in the implant loosening and infected peri-prosthetic interface membranes. However, the expression levels of IRAK-4 seem to be irrelevant to the extent of aggradation of prosthesis-derived wear particles in peri-prosthetic tissues, both in aseptic implant loosening and peri-prosthetic infection. Wear particles per se may not play a role in this process in vivo in quite the way it has been reported for TLRs in peri-prosthetic tissues.23 The potential role of the pathogenic microorganism and its components, like biofilms, LPS, and so on, and indolent infections in the apparently aseptic loosening has raised much attention lately.13,31 Hence, in vivo, whether IRAK-4 would be directly or indirectly induced by wear particles or affected by pathogenic microorganism and its components, like PAMPs. All of which were unclear, because of the complex internal environment. In order to clarify the effect of wear particles on IRAK-4 in macrophages, we used Ti particles and BC particles, which are the most common used particles in particle-related loosening, to observe procedures and trends, and illustrate that wear particles could bring about the up-regulation of IRAK-4 in macrophages, although Ti or BC particles cannot provide all the information about the role of prosthesis-derived wear particles like UHMMP and metal particles in regulating IRAK-4 expression. The reaction of macrophage IRAK-4 responses to wear particles can be regulated, especially in vivo, by related components of the wear particles as well. From procedures and trends displayed in vitro, we deduced that wear particles could increase the expression of IRAK-4 in macrophages. According to the cell culture stimulating experimentation, we consider that, compared with other different concentrations of particle stimulations, 0.1 mg/ml is more sensitive for the expression of IRAK-4 mRNA in the density of 1×10⁶/ml RAW264.7 macrophages. Despite the expression levels of IRAK-4 in peri-prosthetic interface membranes not being associated with the presence of wear particles, probably because of lower number of patients and/or the extremely complex internal environment, we found that the relationship between IRAK-4 expression and the concentration of wear particles was dose-dependent in vitro to some extent. It is deduced that, after THA operation, under a single factor such as wear particles, the more wear particles are performed, the higher the expression of IRAK-4 in the local macrophages of the peri-prosthetic interface membrane. Although in vitro the highest amplitude of the reaction was shown at 9 h, it should not be ignored that the higher IRAK-4 level continued for at least 24 h in vitro. The detected down-regulation of IRAK-4 might have been caused by autocrine inflammatory cytokine signaling, revealing that mRNA expression of IRAK-4 was modulated

![Figure 9](https://example.com/figure9.png)

**Figure 9.** The expression of IRAK-4 was effectively inhibited by siRNA, not by control or normal RNA. The effect of knock-down of IRAK-4 using siRNA on the production of pro-inflammatory cytokine protein IL-1β (left) and TNF-α (right) in RAW264.7 cells compared with controls without siRNA interference. After bone cement particles, titanium particles and LPS stimulations for 12 h, supernatants were collected for ELISA. Mean values ± SD are represented.
by a degenerative feedback system. The up-regulation of IRAK-M induced by particles that we found previously might inhibit its expression, yet could also have other explanations.\textsuperscript{4,5} Further, in in vitro experimentation, the arranged LPS was added at different time points, which showed that LPS enhances IRAK-4 expression in macrophages as others have already reported, and that it was higher than that of particle stimulation. It suggested that wear particles could not induce enough pro-inflammatory cytokines by TLR. So we still think that wear particles lead to production of pro-inflammatory cytokines, but adherence of PAMP, like LPS, to wear particle surfaces significantly increases macrophage-mediated production of pro-inflammatory cytokines as previously proved.

It is well known that TNF-$\alpha$ is one of the important pro-inflammatory cytokines emerging in response to wear particles.\textsuperscript{39–41} TNF-$\alpha$ activates macrophages to release other inflammatory cytokines such as IL-6 and IL-1$\beta$.\textsuperscript{31,40} In this study, we also showed that IRAK-4 up-regulates the levels of TNF-$\alpha$ and IL-1$\beta$ upon different stimulations. Pure wear particles represent a relatively weak excitant of inflammatory cytokine release, and LPS stimulation strongly stimulated the macrophages to produce a considerable series of inflammatory cytokines, with or without wear particles. Comparatively, BC particles stimulated inflammation in vitro more easily than Ti particles did. In order to further confirm the role of IRAK-4 in macrophages and implant loosening, we silenced IRAK-4 genes using siRNA. IRAK-4-deficient macrophages stimulated with LPS, Ti particles and BC particles did not effectively produce TNF-$\alpha$ and IL-1$\beta$. In fact, cytokine levels were significantly lower than in the control RNA and non-treated control groups. This showed that silencing of IRAK-4 in macrophages decreased their ability to respond to stimulation.

In conclusion, both human peri-implant pathology and the procedure of implant loosening are much more complex in vivo, thus we understand that it would be perfect if we would present in vivo experiments in an animal model. However, current prosthetic animal models still cannot imitate well the effect of mechanical stress and inflammatory osteolysis on humans and the actual pathological process of clinical loosening.\textsuperscript{42–45} Nevertheless, combined with our early studies, besides IRAK-M previously proved, this study indicates that IRAK-4 kinase activity also plays a critical and positive role in the local inflammatory reaction and prosthesis loosening. The therapeutic strategies which interfere with the synthesis and activity of IRAK-4 and IRAK-M may be more efficacious in reduction of the PAMP-accelerated prosthesis loosening.

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