

# Integrin $\beta 2$ regulates titanium particle-induced inflammation in macrophages: *In vitro* aseptic loosening model

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**Abstract.** Aseptic loosening is a major complication of joint replacement surgery, characterized by periprosthetic osteolysis and chronic inflammation at the bone-implant interface. Cells release chemokines, cytokines and other pro-inflammatory substances that perpetuate inflammation reactions, while other particle-stimulated macrophages promote osteoclastic bone resorption and impair bone formation. The present study investigated integrin and inflammatory cytokine expression patterns in RAW 264.7 cells treated with titanium (Ti) particles to elucidate the role of integrins in Ti particle-mediated inflammatory osteolysis. Assessment was performed by reverse transcription-quantitative PCR, western blotting, confocal immunofluorescence, flow cytometry and enzyme-linked immunosorbent assays. Cell migration was evaluated by wound healing assay. It was found that Ti particles significantly induced integrin expression in RAW 264.7 cells, including upregulation of integrins  $\beta 2$  (CD18),  $\alpha L$  (CD11a),  $\alpha M$  (CD11b) and  $\alpha X$  (CD11c). Ti particles also enhanced the expression of Toll-like receptors (TLRs; TLR1, TLR2, TLR3

and TLR4) and triggered the release of inflammatory cytokines such as tumor necrosis factor  $\alpha$ , interleukin (IL)-1 $\beta$ , IL-8 and IL-12. Proteomics showed higher expression and activity levels of TLR2 and TLR4, along with their downstream signaling adaptors myeloid differentiation primary response protein 88 (MyD88) and Mal/TIR-domain-containing adapter protein (TIRAP), following Ti treatment. Additionally, Ti treatment significantly enhanced the migration rate of RAW 264.7 cells. The present findings indicated that Ti particles regulate the inflammatory response of RAW 264.7 cells in an *in vitro* aseptic loosening model by activating the TLR/TIRAP/MyD88 signaling pathway.

## Introduction

Total joint arthroplasty, including total hip and total knee arthroplasties, is the most effective surgical treatment for advanced symptomatic osteoarthritis. However, the inflammatory response triggered by the byproducts of joint replacement prostheses can lead to pain, bone defects and movement disorders. In severe cases, these complications may result in aseptic loosening and periprosthetic osteolysis, which are among the most common complications limiting the lifespan of prostheses (1). This poses a challenging clinical problem because significant symptoms often do not manifest until the late stage of failure, at which point the pathological changes are irreversible, necessitating further surgical intervention (2).

A previous study identified particulate wear debris from *in vivo* prosthesis degradation as a crucial factor stimulating the release of proinflammatory substances, suppressing bone formation, inducing osteoclastic bone resorption, and contributing to prosthesis-related chronic inflammation (3). Therefore, a deeper understanding of the impact of prosthesis material design and particulate wear debris on the inflammatory response after joint replacement is essential for preventing aseptic loosening and periprosthetic osteolysis (4).

Among various biomaterials, titanium (Ti)-based implants are the most widely used in the medical field, including

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dental prostheses, hearing aids, pacemakers and joint replacements (5). It is well established that oral peri-implant and periodontal bone loss, primarily caused by peri-implantitis and periodontitis, and wear debris, particularly Ti particles near the implant site, are crucial triggers of inflammation and bone resorption (6). Among the cells involved in Ti particle-induced inflammation, macrophages are of particular interest (7). Qu *et al* (8) reported that the activation of mononuclear macrophages and giant cells is the primary cause of chronic inflammation induced by Ti-wear debris. These findings suggested that macrophages play a critical role in aseptic loosening and inflammation after Ti-based joint replacement. However, the underlying mechanisms, including cytokine, inflammatory expression patterns and regulatory pathways, remain poorly understood. The present study aimed to explore the influence of Ti release on macrophage-like RAW 264.7 cells in aseptic loosening and inflammation pathogenesis.

Within the integrin family,  $\beta$ 2 integrins (CD11/CD18) are specifically expressed on the surfaces of macrophages and leukocytes and are composed of a common  $\beta$ 2 subunit (CD18) and four different  $\alpha$  subunits (CD11a-d) (9). Previous studies have shown that these  $\beta$ 2 integrins, acting as adhesion receptors, are crucial in the activation of inflammatory diseases such as rheumatoid arthritis (10) and systemic lupus erythematosus (11) by mediating inflammatory cell recruitment, cell-cell contact and downstream cellular signaling (12). Han *et al* (13) demonstrated that  $\beta$ 2 integrins expressed in macrophages contribute to cell activation, cytotoxicity, chemotaxis and phagocytosis in a mouse model of endotoxin shock. Lv *et al* (14) demonstrated that  $\beta$ 2 integrins promote the expression of inflammatory cytokines, resulting in a pro-inflammatory transformation in macrophages in multi-organ fibrosis. Among the inside-out and outside-in signaling, Toll-like receptor (TLR) signaling has been shown to regulate  $\beta$ 2 integrin-stimulated inflammatory responses, though the interaction between these pathways remains controversial (15,16). To further elucidate the signaling pathways involved and the role of integrin-mediated inflammation in Ti-induced osteolysis, the present study focused on TLR signaling.

The present study aimed to investigate the expression patterns of integrins and inflammatory cytokines in RAW 264.7 cells treated with Ti particles to understand the role of integrins in Ti particle-mediated inflammatory osteolysis and identify potential signaling pathways involved.

## Materials and methods

**Cell treatment.** Commercially pure Ti particles (mean diameter, 3.0  $\mu$ m; 93% <20  $\mu$ m) were purchased from Alfa Aesar. To sterilize the particles, they were mixed with 95% ethanol and stirred magnetically for 24 h. The particles were then resuspended in sterile phosphate-buffered saline containing 6% rat serum, penicillin (100 U/ml) and streptomycin (100 U/ml) at a concentration of  $1.2 \times 10^8$ /ml, stored at 4°C.

Murine macrophage-like RAW 264.7 cells were purchased from the Chinese Academy of Sciences and the Shanghai Institute of Cell Biology. Cells were cultured at 37°C in a humidified incubator with 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc.), supplemented with 10% heat-inactivated fetal bovine serum

and antibiotics (100 units/ml of penicillin-G and 100 pg/ml of streptomycin). Then, RAW 264.7 cells ( $3 \times 10^5$  cells/ml) were plated in 96-well plates and treated with or without Ti particles (0.1 mg/ml). Cell samples were collected at various time points (6, 12, 24, 48 and 72 h) and frozen for further analysis.

**Cell viability.** The viability of RAW 264.7 cells was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. At different time points (6, 12, 24, 48 and 72 h), cell samples (seeded at  $3 \times 10^5$  cells/ml) were incubated with MTT (0.5 mg/ml) at 37°C for 2 h. The culture medium was then replaced with an equal volume of dimethyl sulfoxide to dissolve formazan crystals. After centrifugation, the supernatant from each sample was transferred to a 96-well plate. The corresponding absorbance was measured at a wavelength of 570 nm using a microplate reader (Bio-Tek Instruments Inc.). Cell viability was presented as the mean  $\pm$  standard deviation (SD) of the optical density values. All experiments were performed in triplicates.

**Gene expression analysis: Reverse transcription-quantitative PCR (RT-qPCR).** mRNA expression levels in RAW 264.7 were quantified by RT-qPCR. RAW 264.7 samples treated with or without 0.1 mg/ml Ti particles collected at 48 and 72 h were homogenized in TRIzol<sup>®</sup> reagent (Invitrogen; Thermo Fisher Scientific, Inc.) using a glass/Teflon homogenizer. Total RNA was isolated according to the manufacturer's protocol. First-strand complementary DNA (cDNA) was synthesized using M-MLV reverse transcriptase (cDNA synthesis kit; Takara Bio, Inc.) according to the manufacturer's protocol. qPCR was performed using 1.0  $\mu$ l cDNA from each sample with a commercial kit and a Thermal Cycler Dice TP800 (Takara Bio, Inc.). Cycling conditions began with an initial 3-min denaturation step at 95°C followed by 40 cycles of 10 sec at 95°C for denaturation and 45 sec at 60°C for annealing and extension. Primer sequences for murine integrin  $\beta$ 1, integrin  $\beta$ 2 (CD18), integrin  $\beta$ 3, integrin  $\alpha$ 1, integrin  $\alpha$ 2, integrin  $\alpha$ 3, integrin  $\alpha$ 4, integrin  $\alpha$ 5, integrin  $\alpha$ 6, integrin L (CD11a), integrin M (CD11b), integrin X (CD11c), integrin D (CD11d), TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR11, interleukins (ILs; IL 1 $\beta$ , IL-6, IL-8, IL-10 and IL-12), tumor necrosis factor (TNF), interferons, matrix metalloproteinases (MMPs; MMP3, MMP8 and MMP11), *MyD88*, *TIPAR* and the housekeeping gene  *$\beta$ -actin* are listed in Table I. Relative expression values were normalized to  *$\beta$ -actin* and analyzed using the  $2^{-\Delta\Delta C_q}$  method (17). All samples were assayed in triplicates.

**Protein expression analysis by western blot assay.** RAW 264.7 macrophages were challenged with indicated concentrations of Ti particles for 6, 12, 24, 48 and 72 h, and then lysed in a buffer containing 1% Triton X-100, protease inhibitors (MilliporeSigma), and 1 mM sodium orthovanadate (MilliporeSigma). The protein concentration in the supernatant was measured using a BCA protein assay kit. Equal amounts of protein (30  $\mu$ g/lane) were separated using Tris-bis SDS-PAGE gels (8 or 10% gel concentration for different molecular weight proteins; Invitrogen; Thermo Fisher Scientific, Inc.) and transferred onto polyvinylidene fluoride membranes (MilliporeSigma). After incubation in 5%

Table I. Primer sequences used for reverse transcription-quantitative PCR analysis of gene expression.

Gene name	Primer sequence (5'-3')
Integrin $\beta$ 1 (CD29)	F: GGACGCTGCGAAAAGATGAA R: CCACAATTTGGCCCTGCTTG
Integrin $\beta$ 2 (CD18)	F: CCCTAGCTGGACTGTTCTTCC R: GTGAAGTTCAGCTTCTGGCAC
Integrin $\beta$ 3 (CD61)	F: CAGTGGCCGGGACAACCTCT R: CAGGTTACATCGGGGTGAGC
Integrin $\alpha$ 1 (CD49a)	F: ATGACGCTCTGCCAAACTCA R: TGTTGTACGCACTGTCTCCC
Integrin $\alpha$ 2 (CD49b)	F: ACCCACGGAGAAAGCAGAAG R: CGCCGATGGTTTAGCTGTTG
Integrin $\alpha$ 3 (CD49c)	F: CCGACGACTACTGAGGGGT R: AGCGGGTTCGGCTTAAAGAAG
Integrin $\alpha$ 4 (CD49d)	F: GAAGCATCCCTGGCCACTAC R: CCACTGACCAGAGTTGCACA
Integrin $\alpha$ 5 (CD49e)	F: CTTCGACAGGGAGGGACTA R: AGGGCATGTGTAAACGAGGG
Integrin $\alpha$ 6 (CD49f)	F: ACAGTGAGCGTGAGCCG R: GACAGGTAGAGCAGGCACAA
TLR1	F: CGGACGGGTAAGGTTGTCTT R: CCAACACGTGGGCTCTTAGT
TLR2	F: CAGTCTTCTAGGCTGGTG R: AAGGAAACAGTCCGCACCTC
TLR3	F: TCCTGTGTCCGAGATGTCTT R: CAGCCTGAAAGTGAACTCGC
TLR4	F: GTGGCCCTACCAAGTCTCAG R: GCTGCAGCTCTTCTAGACCC
TLR5	F: CTGCAGGGCAAAACACTGTC R: TTGTGATGTCCACCGTCCAG
TLR6	F: GGTACCGTCAGTGCTGGAAA R: TATTAAGGCCAGGGCGCAAA
TLR7	F: TGCGAGTCTCGGTTTTCTGT R: TGAGAAGGGAGCCAAGGACA
TLR8	F: TGCACATTCCCTGGAGACAC R: GGAGAGGAAGCCAGAGGGTA
TLR11	F: CTGCCAGGGACTTTGGGATT R: TTGCTAAGGCCTGTCTGTG
Integrin L CD11aLFA-1	F: TGTTCCCAGATGGAAGCCAC R: CCCTTTTGGTCCCTTGGTGT
Integrin M CD11b	F: CCACACTAGCATCAAGGGCA R: AAGAGCTTCACACTGCCACC
Integrin X CD11c	F: TCTTCTGCTGTTGGGGTTTGT R: TGCCTGTGTGATAGCCACATT
Integrin D CD11d	F: GGTAGACAGCAACAGGCTCC R: AGGAGAACCGTACCCTCTGC
IL-1 $\beta$	F: TGCCACCTTTTGACAGTGATG R: TGATGTGCTGCTGCGAGATT
IL-6	F: TCCAGTTGCCTTCTTGGGAC R: AGTCTCTCTCCGGACTTGT
IL-8	F: CTGGGATTACCTCAAGAACATC R: CAGGGTCAAGGCAAGCCTC

Table I. Continued.

Gene name	Primer sequence (5'-3')
IL-10	F: TAAGGCTGGCCACACTTGAG R: TGAGCTGCTGCAGGAATGAT
IL-12	F: TCTTCTCACCGTGCACATCC R: TGGCCAAACTGAGGTGGTTT
TNF	F: GATCGGTCCCCAAAGGGATG R: CCTCCACTTGGTGGTTTGTG
IFN	F: AGCAAGGCGAAAAAGGATGC R: TCATTGAATGCTTGGCGCTG
MMP-3	F: GGAGGCAGCAGAGAACCTAC R: AGGACCGGAAGACCCTTCAT
MMP-8	F: CTTCTGGAGACGGCATCCTC R: GCCCAGTACTGTCTGCCTTT
MMP-11	F: TCCGCTGACAACACTTTGGA R: TCCCCTGAGGAGACATAGGC
MyD88	F: GATGACTGGCCTGAGCAACT R: GCTATCCTTGGGAGCTGTCC
TIRAP	F: CTCATTTTCCCCAACCGTGC R: CTCGGGATCTGTGTTTCGGT
$\beta$ -actin	F: CCTGGCTGGCCGGGACCTGAC R: ACCGCTCGTTGCCAATAGTGATGA

F, forward; R, reverse; TLR, Toll-like receptor; TNF, tumor necrosis factor; MyD88, myeloid differentiation primary response protein 88; TIRAP, Mal/TIR-domain-containing adapter protein; IL, interleukin; MMP, matrix metalloproteinase; IFN, interferon.

skimmed milk in Tris-buffered saline with 0.05% Tween-20 (TBST) for 1 h at room temperature (to block non-specific binding), the membranes were incubated overnight at 4°C with the appropriate primary antibody (Table II). A horse-radish peroxidase-conjugated donkey anti-rabbit IgG was used as a secondary antibody (1:1,000; cat. no. NA934; Cytiva). Antibody binding was detected using the Immobilon Chemiluminescence system (MilliporeSigma). Quantitative densitometry analysis was conducted and the relative densities of the protein bands were analyzed using ImageJ software (version 1.53b; National Institutes of Health). The relative expression levels of target proteins were normalized to the corresponding intensities of tubulin.

**Confocal immunofluorescence.** RAW 264.7 cells at a density of  $1 \times 10^5$  cells/ml were seeded on coverslips using six-well plates and stained for immunofluorescence detection under confocal fluorescence microscopy. Filamentous actin was used as a housekeeping marker and stained with rhodamine-phalloidin (Molecular Probes; Thermo Fisher Scientific, Inc.) for 1 h at room temperature in the dark. Samples were stained with the following primary antibodies overnight at 4°C: CD11a (1:100; cat. no. ab186873; Abcam), CD11b (1:500; cat. no. ab184308; Abcam), CD11c (1:100; cat. no. 97585; Cell Signaling Technology, Inc.), CD18 (1:500; cat. no. 102259; SinoBiological), MyD88 (1:50; cat. no. PA5-19919; Thermo Fisher Scientific, Inc.) and TIRAP (1:100; cat. no. ab17218;

Table II. Antibody suppliers and concentrations used in western blotting.

Antibody	Species	Supplier	Cat. no.	Dilution
CD18	Rabbit mAb	Cell Signaling Technology, Inc.	72607	1:1,000
CD11a	Rabbit	Abcam	ab228964	1:1,000
CD11b	Rabbit mAb	Cell Signaling Technology, Inc.	93169	1:1,000
CD11c	Rabbit mAb	Cell Signaling Technology, Inc.	97585	1:1,000
MyD88	Rabbit mAb	Cell Signaling Technology, Inc.	4283	1:1,000
TIRAP	Rabbit mAb	Cell Signaling Technology, Inc.	13077	1:1,000
Tubulin	Rabbit	Abcam	ab6046	1:500

TIRAP, Mal/TIR-domain-containing adapter protein; MyD88, myeloid differentiation primary response protein 88.

Abcam). A secondary antibody of Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1,000; cat. no. A-11034; Invitrogen; Thermo Fisher Scientific, Inc.) was then added and incubated for 1 h at room temperature in the dark. Nuclei were visualized using Hoechst 33342 (cat. no. H1399; Thermo Fisher Scientific, Inc.) staining for 1 h at room temperature. The images were captured with a 633 or 320 objective lens using appropriate laser excitation (wavelengths of 540, 488 and 360 nm) on a laser-scanning confocal microscope (Carl Zeiss AG) or an Olympus IX81-FV1000 (Olympus Corporation) laser microscope system. Detector gain was initially optimized by sampling several regions of the coverslip and then fixed for each corresponding channel. Once set, the detector gain value was kept stable throughout the image acquisition process. Images were analyzed using Zeiss LSM Image Examiner Software and FV10-ASW 3.0 Viewer. Immunofluorescence was used to quantitatively analyze CD18, CD11b, CD11c, myeloid differentiation primary response protein 88 (MyD88) and TIR-domain-containing adapter protein (TIRAP) expression levels. Specific antibodies were used and detected using green fluorescence. Simultaneously, the cytoskeletal protein actin was labeled with red fluorescence as an internal reference standard. Relative fluorescence intensity was calculated as the ratio of the average green fluorescence intensity (target proteins) to the red fluorescence intensity (actin), yielding the fluorescence ratio. Each experiment was repeated three times to ensure reliability. Multiple group statistical analyses were performed to evaluate the significance of differences between groups. All images were captured under identical magnification and microscopy conditions.

**Flow cytometry and intracellular cytokine staining.** RAW 264.7, macrophages were treated with Ti particles for 6, 12, 24, 48 and 72 h. For the final 2 h of stimulation, macrophage Fc receptors were blocked with 2.4G2 for 10 min at 4°C, followed by surface staining with anti-F4/80 (1:100; cat. no. 14-4801-85; BM8/eBioscience; Thermo Fisher Scientific, Inc.). Peritoneal cells were surface stained with anti-Siglec F (1:100; cat. no. 564514; E50-2440/BD Biosciences) and anti-Gr1 (1:200; cat. no. MA1-10402; eBioscience; Thermo Fisher Scientific, Inc.). Macrophages were then fixed and permeabilized using the BD Cytofix/Cytoperm Fixation/Permeabilization kit (BD Biosciences) for 20 min at 4°C. Flow cytometric analysis was conducted on an LSR2

(BD Biosciences) using FACS Diva software (BD Biosciences). The gating strategy involved using Forward Scatter (FSC) and Side Scatter (SSC) to isolate viable, intact cells while excluding debris or dead cells. Fluorescence marker gating was applied to quantify integrin expression with negative controls setting baseline fluorescence and positive controls verifying accuracy. The scale bars on histograms were adjusted to ensure clear separation between marker-positive and marker-negative populations. All flow cytometry analyses and procedures were conducted using FlowJo software (version 10.8.1; Tree Star, Inc.).

**Enzyme-linked immunosorbent assay (ELISA).** RAW 264.7 cells were cultured and treated with Ti particles to induce an inflammatory response. After 6, 24, 48 and 72 h of treatment, the cells were harvested and lysed to obtain cellular extracts. Surface adhesion molecules and signaling protein levels were quantitatively assessed using specific ELISA kits according to the manufacturer's protocol. Briefly, cell lysates were added to microtiter plates pre-coated with antibodies specific to each target protein. Following incubation, the plates were washed to remove unbound proteins, and a secondary enzyme-linked antibody specific to the target protein was added. The enzyme-substrate reaction produced a colorimetric signal, measured at 450nm using a microplate reader. The concentrations of CD11a (cat. no. SEB572Mu; Cloud-Clone Corp.), CD11b (cat. no. JM-12253M2; Jiangsu Jingmei Biological Technology Co., Ltd.), CD11c (cat. no. SEB159Mu; Cloud-Clone Corp.), CD18 (cat. no. JM-13347M2; Jiangsu Jingmei Biological Technology Co., Ltd.), MyD88 (cat. no. SEB707Mu; Cloud-Clone Corp.) and TIRAP (cat. no. JM-13352M2; Jiangsu Jingmei Biological Technology Co., Ltd.) were subsequently calculated based on the appropriate standard curve.

**Wound healing assay.** Wound healing assays were performed to assess collective cell migration. After reaching 90% cell confluence, RAW 264.7 cells were scratched with a sterile 100- $\mu$ l pipette tip and then treated with serum-free medium. The distance between the wound edges was measured using an IX71 inverted light microscope (Olympus Corporation). Images were captured at 0-, 6-, 12-, 24- and 36-h time points. The experiments were performed three times for each group, and three measurements were taken for each sample using ImageJ software.

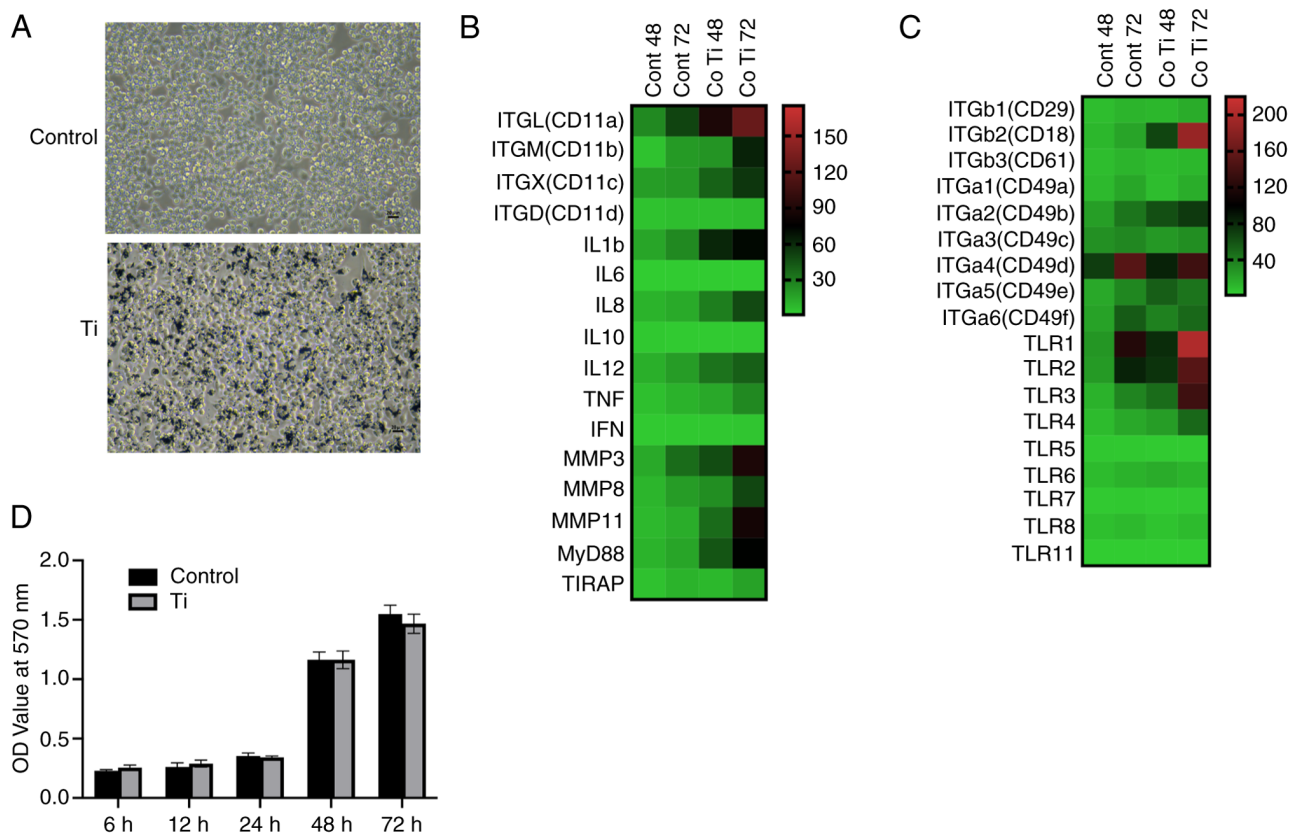


Figure 1. Effects of Ti particles on the viability of RAW 264.7 cells and expression heatmap of integrin genes and inflammatory cytokines. (A) RAW 264.7 cells co-cultured with 0.1 mg/ml Ti particles. (B and C) Heatmap of integrins and cytokines expression levels (red: high expression; green: low expression) (magnification, left: x100; right: x40). Ti, titanium; TIRAP, Mal/TIR-domain-containing adapter protein. (D) Viability of RAW 264.7 cells after co-culture with Ti particles.

**Statistical analysis.** Data are presented as the mean  $\pm$  standard deviation (SD). Statistical analyses were performed using SPSS 28 software (IBM Corp.). Differences between unpaired data groups were analyzed using the unpaired Student's two-tail t-test after normality was confirmed with the Kolmogorov-Smirnov test. A non-parametric Mann-Whitney test was conducted for data that did not follow a normal distribution.  $P < 0.05$  was considered to indicate a statistically significant difference. Data analysis was conducted by Prism 8.0 (GraphPad Software; Dotmatics) software.

## Results

**Ti particles effect on RAW 264.7 cell viability.** To evaluate the impact of Ti particles on cell viability, RAW 264.7 cells were cultured with micron-sized pure Ti particles at a concentration of 0.1 mg/ml. The viability of RAW 264.7 cells was not negatively affected by Ti particles (Fig. 1A and D). Compared with the control group, no significant decrease in cell viability was observed at any of the examined time points, including 6, 12, 24, 48 and 72 h post-treatment.

**Expression of integrin genes and inflammatory cytokines heatmap by RT-qPCR.** The expression levels of integrin family members, TLRs, and inflammation response gene classes in RAW 264.7 cells, with and without Ti particle treatment,

were detected by RT-qPCR (detailed results are presented in Fig. S1) and are presented as heat maps in Fig. 1B and C.

The expression levels of integrin subunits CD18, CD11a, CD11b and CD11c significantly increased following Ti particle treatment (Fig. 1B). Additionally, there was a notable upregulation in the gene expression of TLRs (1-4) at 48 and 72 h after Ti treatment. The relative expression folds of MyD88 and TIRAP, downstream molecules in the TLR signaling pathway, were also elevated simultaneously ( $P < 0.05$ ).

The expression patterns of inflammatory cytokines in RAW 264.7 cells treated with Ti particles are shown in Fig. 1C. There was a significant increase in the expression of TNF- $\alpha$ , IL 1 $\beta$ , IL-8 and IL-12 in cells cultured with Ti particles compared with the blank control group at 72 h ( $P < 0.05$ ). Furthermore, the matrix metalloproteinase-3 (MMP-3), MMP-8 and MMP-11 levels significantly increased following Ti treatment ( $P < 0.05$ ). Notably, the expression of IL 1 $\beta$  and IL 12 was upregulated earlier than that of the other inflammatory cytokines.

**Promoted protein expression levels with Ti stimulation by western blot assay.** Protein expression levels in the Ti co-culture group vs. the blank control group were analyzed by western blotting at the 24-, 48- and 72-h time points (Fig. 2A). Higher protein expression levels were observed in the Ti particle co-culture group, consistent with the mRNA levels of the corresponding genes, *CD18*, *CD11b*, *CD11c* and *MyD88*.

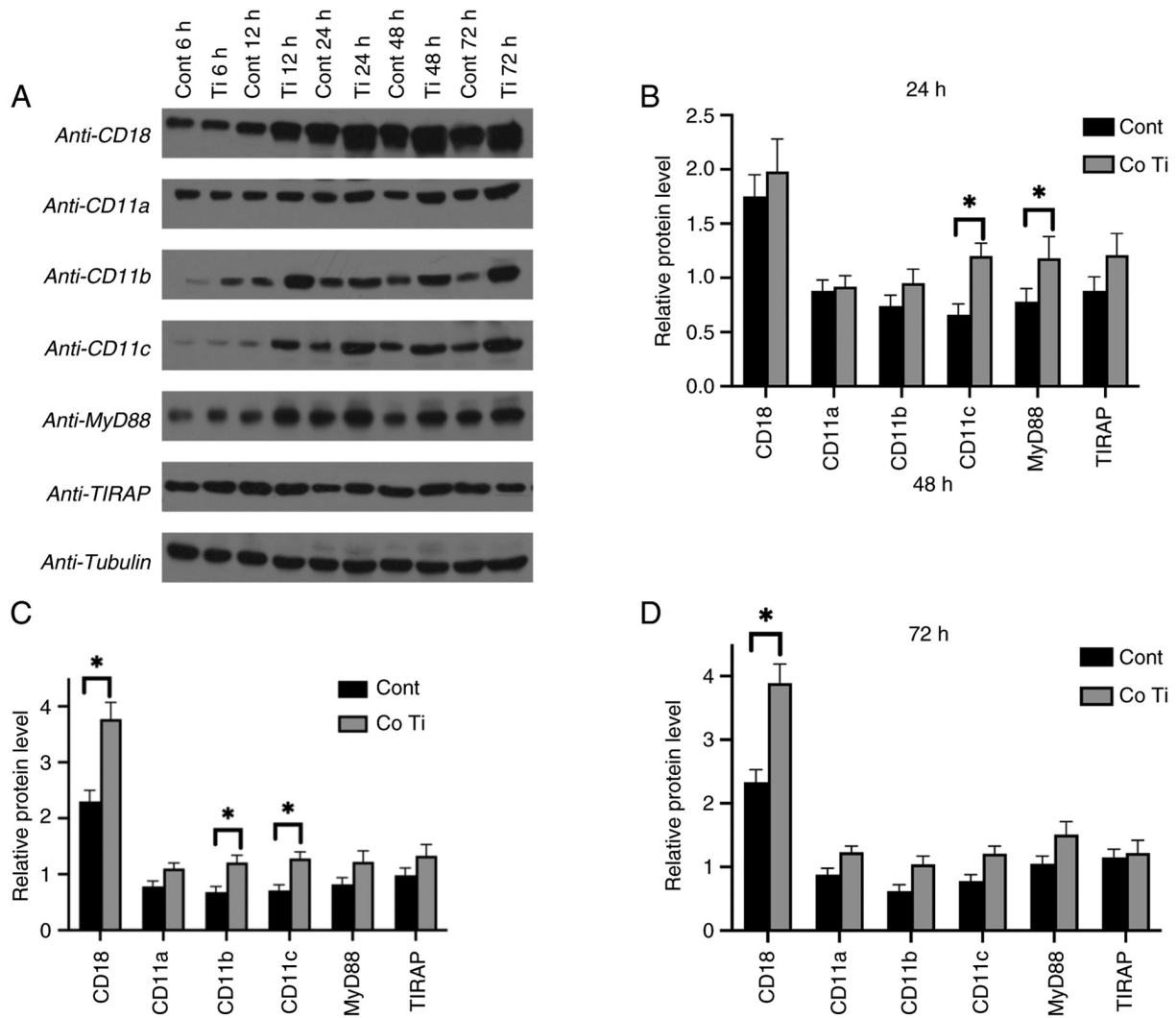


Figure 2. Western blotting and quantitative analysis of integrin levels and inflammation response proteins. (A) Western blot immunoblots results showing CD18, CD11a, CD11b, CD11c and MyD88 TIRAP. (B-D) Quantification of immunoblots using ImageJ software. \* $P < 0.05$ . Ti, titanium; TIRAP, TIR-domain-containing adapter protein.

However, discrepancies were noted where protein levels did not align with mRNA expression profiles, such as CD11a and TIRAP.

Quantitative analysis of western blot protein levels was normalized to the housekeeping protein tubulin. The results showed markedly increased protein levels of CD18 (at 48 and 72 h), CD11b (at 48h) and CD11c (at 24 and 48 h) in RAW 264.7 cells cultured with Ti particles compared with the control group ( $P < 0.05$ ) (Fig. 2B-D). The protein level of MyD88 was also significantly elevated in the Ti-treated group compared with the control group at the 24-h checking point ( $P < 0.05$ ).

**Ti-induced upregulation of integrin and inflammatory response by confocal immunofluorescence assay.** Immunofluorescent staining of RAW 264.7 cells was conducted with and without Ti treatment and observed under a confocal microscope (Fig. 3). Consistent with the immunoblotting results, the confocal immunofluorescent images demonstrated upregulation of CD18, CD11b and CD11c in response to Ti particle co-culture (Fig. 3A-C). Additionally, MyD88 and TIRAP

exhibited significant changes in immunofluorescent intensity following Ti co-culture (Fig. 3D and E). Statistical analysis was performed in term of fluorescence ratio (Fig. 3F). The observed increase in the intensity of MyD88 and TIRAP aligned with the protein and gene expression levels depicted in Figs. 1 and 2.

**Integrin characteristics by flow cytometry assessment.** Flow cytometry was used to characterize and quantify the impact of Ti particles on integrin protein expression on the surface of RAW 264.7 cells (Fig. 4A). The analysis revealed enhanced CD18, CD11b and CD11c expression in the Ti co-culture group compared with the control group. The most notable increase was observed for CD18 (from 0.2-6.2%), while CD11b and CD11c showed slight increases. However, CD11a expression decreased by over 10% (Fig. 4A).

**Ti-induced changes in RAW 264.7 cell migration features by scratch-wound healing assay.** A wound healing assay was performed to assess the migratory characteristics of RAW 264.7 cells co-cultured with Ti particles. Representative images of cell migration at different time points are revealed



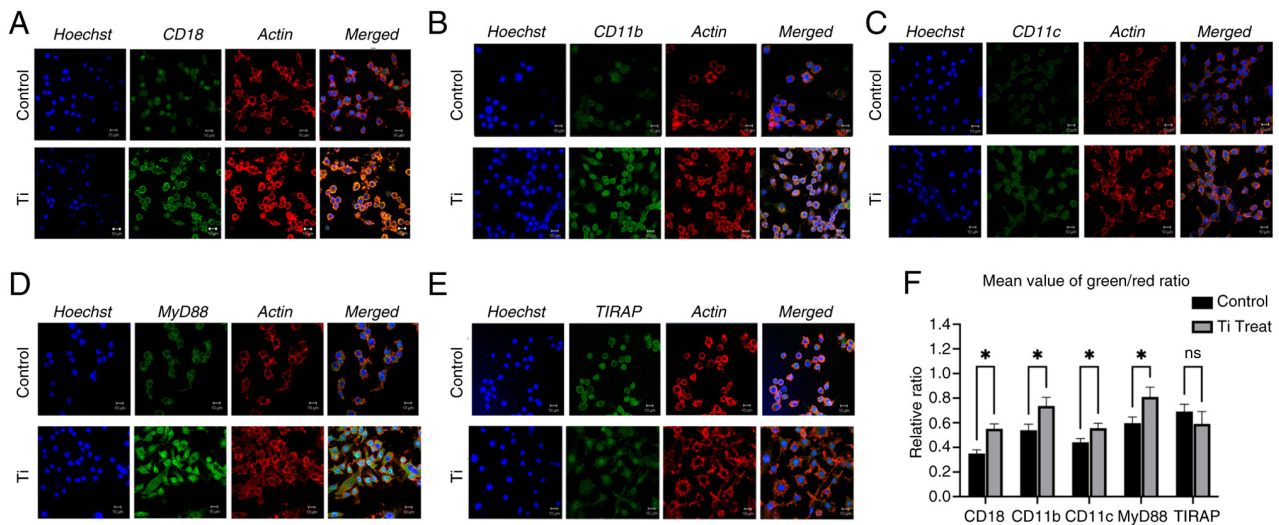


Figure 3. Confocal immunofluorescence of integrins and inflammatory response proteins with/without Ti treatment. (A-C) Localization of CD18, CD11b and CD11c after Ti co-culture. (D and E) Confocal images of Myd88 and TIRAP localizations. Non-linear adjustments. (F) Statistical analysis was performed among multiple marks comparing fluorescence ratio. Green represents anti-IgA staining. Scale bar, 10  $\mu$ m. \*P<0.05. Ti, titanium; TIRAP, TIR-domain-containing adapter protein.

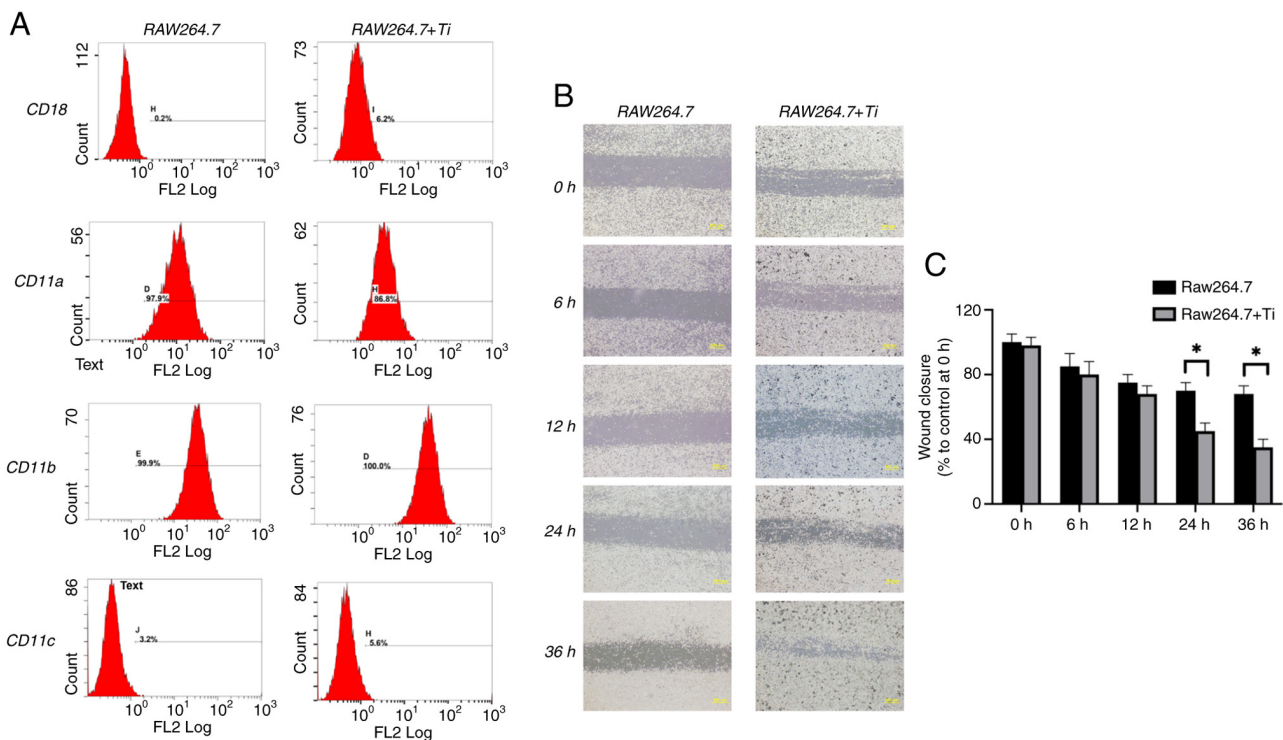


Figure 4. Flow cytometry and wound healing assay results of RAW 264.7 cells. (A) Flow cytometric analysis of integrin surface protein expression (CD18, CD11a, CD11b and CD11c) after 72 h. (B and C) Migration assessment of RAW 264.7 cells using wound healing assay. Scale bar, 500  $\mu$ m. \*P<0.05. Ti, titanium.

in Fig. 4B, with quantification presented in Fig. 4C. The results indicated that Ti particles significantly enhanced the migration of RAW 264.7 compared with the control group at 24 and 36 h (P<0.05).

**Ti-induced changes in RAW 264.7 cell protein secretion by ELISA.** The expression levels of CD18, CD11a, CD11b, CD11c, MyD88 and TIRAP were analyzed in RAW 264.7 cells following treatment with Ti particles at 6, 24, 48 and 72 h

(Fig. 5A-D). At the 6-h mark, a significant increase was observed in the expression of CD11a, CD11b and CD11c but not CD18. However, at 24 and 48 h, there was significant elevation in mostly all target molecules detected, including CD18, CD11a, CD11b, CD11c and TIRAP, indicating a pronounced response. At the 72-h time point, CD18, CD11b and TIRAP continued to show significant increases in expression. These results suggested a time-dependent activation of these markers in response to Ti particle exposure.

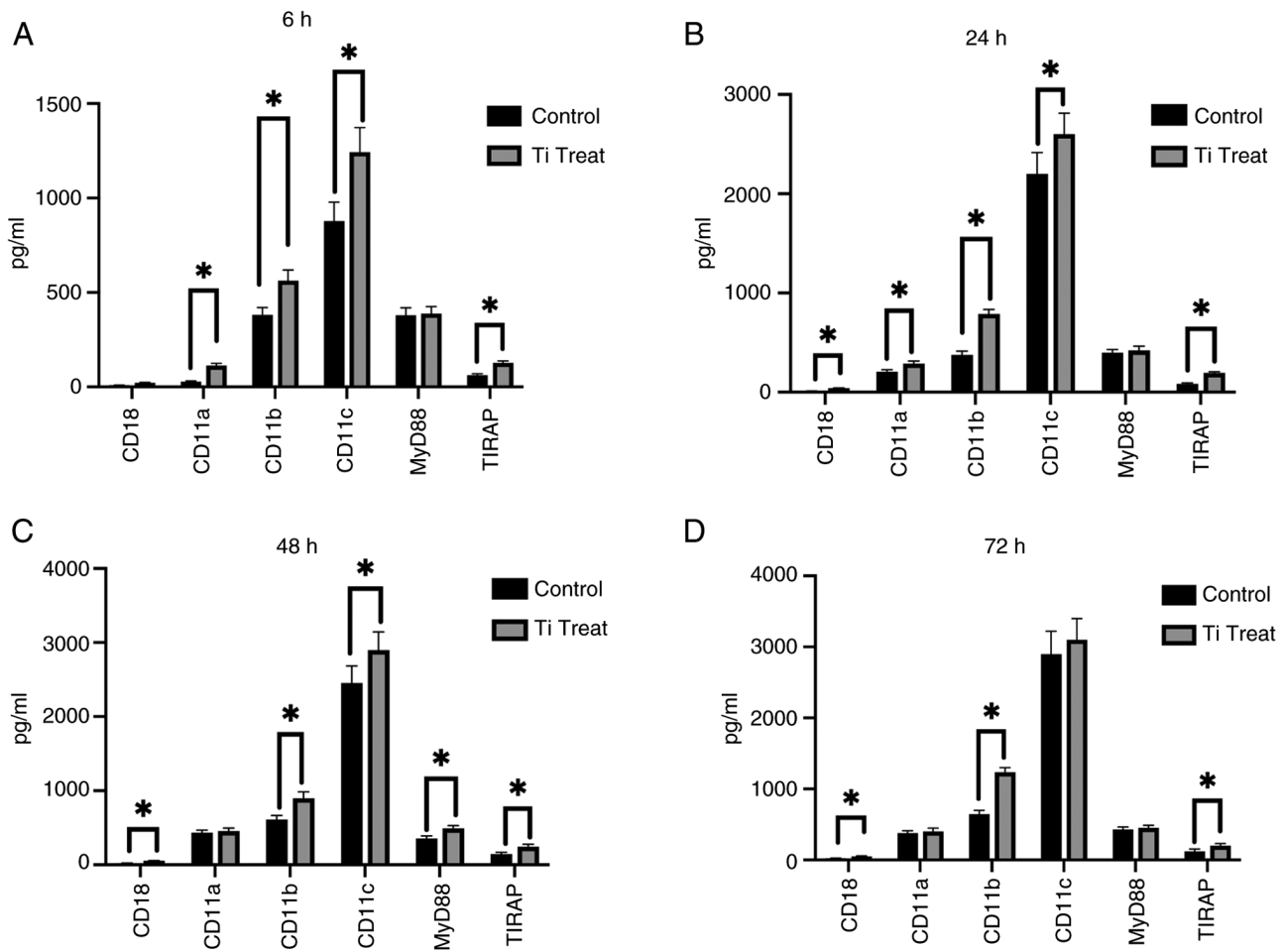


Figure 5. Time-dependent expression of CD18, CD11a, CD11b, CD11c, MyD88 and TIRAP in RAW 264.7 cells following Ti particle treatment using ELISA at (A) 6 h; (B) 24 h; (C) 48 h and (D) 72 h. Data are presented as the mean  $\pm$  SD; \* $P < 0.05$  compared with control. Ti, titanium; TIRAP, TIR-domain-containing adapter protein.

## Discussion

Previous studies have shown that macrophage-like cells are involved in the chronic inflammation induced by Ti particles. Vallés *et al* (18) found that primary macrophages released increased amounts of TNF- $\alpha$ , IL-6 and IL-1 $\beta$  after incubation with Ti particles. Pettersson *et al* (19) demonstrated that Ti particles activate lipopolysaccharide-primed macrophages and induce the release of interleukin-1 $\beta$ . Pajarinen *et al* (20) showed that Ti particles influence the local cytokine microenvironment through foreign body reactions, thereby modulating macrophage polarization. Lee *et al* (21) demonstrated that conditioned media of macrophages challenged with Ti particles (Ti-CM) suppresses early and late differentiation markers of osteoprogenitors. Bi *et al* (22) proved that conditioned media of murine marrow cells challenged with Ti particles stimulate *in vitro* bone resorption primarily by inducing osteoclast differentiation with minor effects on osteoclast activity or survival. In the present study, the impact of Ti particles on RAW 264.7 macrophage cells was investigated, focusing on cell viability, inflammatory response, migratory behavior, and particularly the underlying regulatory mechanisms of integrins.

Extensive research has focused on reducing the inflammatory response and toxicity of implant wear particles in local

tissues. Studies indicate that the cytotoxic effects of chemical components depend on factors such as dose, duration, particle size, temperature and cell type (23). For Ti-based implants, macrophage cell viability is primarily influenced by the concentration of Ti particles and the surface characteristics of the implant (24). The present results demonstrated that Ti particles did not compromise the viability of RAW 264.7 cells at any of the examined time points, suggesting that Ti particles do not exert cytotoxic effects under the conditions tested. This is consistent with previous studies reporting minimal cytotoxicity of Ti particles on macrophages at  $\sim 0.1$  mg/ml (25). Therefore, the Ti particles used in the present study were considered non-toxic and suitable for further experiments. The present study also provided insight into the safe application of Ti-based implants under well-controlled concentrations and surface modifications.

$\beta 2$  integrins are crucial in macrophage functions, including cell adhesion, migration, phagocytosis and immune responses (26). The current integrin expression analysis revealed that Ti particles significantly upregulated the expression of CD18, CD11b and CD11c at both the mRNA and protein levels, as shown by RT-qPCR, western blotting and confocal immunofluorescence assays. In macrophages, CD18, which mediates cell binding to the extracellular matrix (27),



was upregulated in response to Ti particles, indicating increased cell adhesion and proliferation, as depicted in Fig. 1. Previous studies have reported that CD11/CD18 is essential for polymorphonuclear neutrophils recruitment and activation, particularly later stages of immune cell activation through  $\beta 2$  integrin-mediated Syk activation (28). The delayed but significant increase in CD18 expression at 48 and 72 h depicted in Fig. 2 was likely due to the time required for its upregulation as part of the immune response, including cell activation, migration and adhesion, which becomes more prominent at later stages. For example, Ebnet *et al* (29) found that CD11/CD18 were involved in the regulation of leukocyte-endothelial cell interaction. Through their cytoplasmic domains, junctional adhesion molecules directly associate with various tight junction-associated proteins including ZO-1, AF-6, MUPP1 and PAR-3. CD11b and CD11c have close physical and functional relationships (30) but exhibit different expression levels and functions in various macrophages and show cell-type-specific regulation under inflammatory conditions (31). CD11c expression increased as early as 24 h after Ti treatment in RAW 264.7 cells, indicating its potential role in macrophage activation. By contrast, CD11b, critical for macrophage recruitment during the inflammatory response (32), showed significant increases at 48 h, reflecting the need for additional signaling events to mediate macrophage migration and phagocytosis. The delayed expression aligns with the role of macrophages in clearing debris and promoting tissue remodeling, processes that typically follow the initial inflammatory response. Sándor *et al* (33) reported that blocking CD11b significantly enhanced the attachment of MDDCs and MDMs to fibrinogen, demonstrating a competition between CD11b and CD11c for this ligand.

Notably, differences in cell morphology revealed by confocal immunofluorescence may be attributed to macrophage activation induced by Ti particles. Flow cytometry showed that CD18 expression increased significantly on the cell surface, while CD11b and CD11c exhibited moderate increases following Ti-particle treatment. These results align with findings from western blotting and immunofluorescence. However, while CD11a mRNA levels were elevated, its surface expression decreased, indicating potential post-transcriptional regulation or protein degradation mechanisms. Further ELISA results depicted in Fig. 5 indicated that CD11a, CD11b and CD11c are involved in early recognition with Ti particles. The delayed but significant increase in CD18 at 24 and 48 h may indicate that CD18 plays a critical role in sustaining and amplifying the immune response once upregulated.

TLRs are pivotal in mediating inflammatory responses in macrophages, primarily through the MyD88-dependent and MyD88-independent pathways (34). Previous research investigated the crosstalk between  $\beta 2$  integrins (CD11/CD18) and TLRs in macrophages and other immune cells (12), suggesting  $\beta 2$  integrin regulates TLR signaling positively or negatively depending on cell types and inflammatory status (35). Bai *et al* (36) demonstrated that CD11b enhances the TIRAP enrichment in the plasma membrane, exerting a positive regulatory effect via the MyD88-dependent pathway. Saitoh *et al* (37) also found that MyD88 is required for the interplay between TLR7 and integrin CD11a/CD18.

In the present study, increased expressions of TLR1, TLR2, TLR3 and TLR4 were observed at 48 and 72 h, along with elevated MyD88 and TIRAP expression levels following Ti particle treatment, indicating the involvement of TLRs downstream MyD88-dependent signaling in RAW 264.7 cell activation and pro-inflammatory cytokine production. MyD88 expression may peak at 24 h owing to early activation. It was reported that MyD88 can be regulated by upstream Src kinase activated by Rac1-induced F-actin formation in macrophages (38). MyD88 expression may not show significance as the signaling cascade progresses or due to negative feedback mechanisms. Avbelj *et al* (39) found a negative feedback regulation of MyD88 by inflammasome-activated caspase-1. Zhang and Ghosh (40) also found that the negative regulation of MyD88-mediated signaling through the suppression of IRAK1 and TRAF6 contributes to the attenuation of the inflammatory response over time. Additionally, the significant upregulation of pro-inflammatory cytokines, including TNF- $\alpha$ , IL-1 $\beta$ , IL-8 and IL-12, along with MMP-3, MMP-8 and MMP-11, indicated that Ti particles induce a robust inflammatory response in RAW 267.4 cells.

As a well-known pro-inflammatory factor, LPS-induced biomarker pattern in RAW 264.7 cells has been well studied (41). As early as 1-4 h period, LPS caused TLR4 and MyD88 levels to increase to more than twice those of the control group (42), while CD14 and TNF-alpha levels increased nearly 10-fold (43,44). Additionally, IL-6, MMP-9 and IL-1 $\beta$  were significantly elevated more than 3-fold compared with the untreated group (43). Among the integrin family, LPS-induced RAW 264.7 cells were characterized by an increase in CD11b/CD18, several-fold compared with the untreated group after 24 h (44). By contrast, Ti particles induced a slower and more moderate inflammatory response in RAW 264.7 cells, which was markedly different from that caused by LPS, both in terms of pattern and magnitude.

The role of  $\beta 2$  integrins in macrophage migration is complex, depending on the specific context and cellular environment (45). A previous study suggested that  $\beta 2$  integrins inhibit macrophage migration by increasing adhesion, leading to their retention at inflammation sites (46). However, other research suggested that the migration of human monocytes *in vitro* is inhibited when integrin  $\beta 2$  function is blocked (47). The activation of TLR signaling can upregulate integrin expression and other molecules, further facilitating macrophage migration toward inflammation sites (48). In the present study, Ti particles significantly promoted RAW 264.7 cell migration at all examined time points, as indicated by the wound healing assay, accompanied by increased levels of pro-inflammatory cytokines and MMPs, indicating that Ti particles enhance the inflammatory response in macrophages. This effect is likely associated with the activation of  $\beta 2$  integrins and the TLR signaling pathway.

The current study explored the role of integrin mediated TLR signaling in Ti particle-induced inflammation in RAW 264.7 macrophage cells, and provided valuable insights for developing strategies to mitigate aseptic loosening and periprosthetic osteolysis in patients with Ti-based implants. The limitations of the current study include the incomplete elucidation of the crosstalk between integrins and TLRs in the response of RAW 264.7 macrophages induced by Ti particles. Additionally, the effects of conditioned media from macrophages were not further

investigated on osteogenic and osteoclastic cells, which would clarify the indirect effect of Ti particles.

In summary, Ti particles induced an inflammatory response in RAW 264.7 macrophages, characterized by enhanced integrin expression, TLR signaling activation, pro-inflammatory cytokine production and increased cell migration, as illustrated in Fig. S2. These findings elucidated the molecular mechanisms underlying Ti particle-induced inflammation and provided valuable insights for developing strategies to mitigate aseptic loosening and periprosthetic osteolysis in patients with Ti-based implants. Future studies should identify specific regulatory pathways and potential therapeutic targets to modulate macrophage activity and improve implant longevity. Further study should also investigate the mechanisms underlying the differences in inflammatory responses to Ti particles and LPS, with the goal of identifying more targeted therapeutic strategies.

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### Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

### Authors' contributions

YS and WGW conceptualized the study. YS and HN acquired the data. HN and JFZ analyzed the data. YS wrote the original draft. HN, JFZ and GWW wrote, reviewed and edited the manuscript. All authors read and approved the final version of the manuscript. YS, JFZ and WGW confirm the authenticity of all the raw data.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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