

Pitavastatin attenuates monocyte activation in response to orthopedic implant-derived wear particles by suppressing the NF- κ B signaling pathway

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Abstract. Aseptic loosening secondary to particle-induced periprosthetic osteolysis is considered to be the primary cause of long-term implant failure in orthopedic surgery. Implant-derived wear particles activate and recruit macrophages and osteoclasts, which cause a persistent inflammatory response with bone destruction that is followed by a loosening of the implant. Thus, strategies for inhibiting macrophage and osteoclast function may provide a therapeutic benefit for preventing aseptic loosening. The aim of the present study was to determine the effects of pitavastatin on the activation and cytokine response of polymethyl methacrylate (PMMA) particle-induced monocytes. Peripheral blood monocytes were obtained and treated with PMMA and pitavastatin. ELISA demonstrated that pitavastatin inhibited mRNA and protein expression of interleukin (IL)-1 β , IL-6 and tumor necrosis factor- α . Western blot analysis and immunofluorescence staining demonstrated that pitavastatin downregulated inhibitor of κ B phosphorylation and degradation, and nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) p65 translocation. Together, these results indicate that pitavastatin may attenuate monocyte activation in response to orthopedic implant wear particles by suppression of the NF- κ B signaling pathway.

Introduction

Total joint replacement (TJR), by the implantation of permanent prosthetic components, is currently considered to be a particularly successful clinical procedure in orthopedic

surgery (1). However, wear particle-induced periprosthetic osteolysis and subsequent aseptic loosening continue to be major causes of arthroplasty failure (2). Implant-derived wear particles activate and recruit macrophages and osteoclasts around and at the implant-host interface (3). These cells secrete high levels of inflammatory cytokines, such as interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α that mediate and accelerate the inflammatory and osteolytic responses, which result in the loosening and subsequent failure of bone implants (4). Thus, the application of pharmacological agents for the prevention of this osteolytic response to wear debris has attracted significant interest, with particular emphasis on anti-inflammatory agents.

Pitavastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitor, effectively modifies atherogenic lipid profiles and thereby reduces cardiovascular risk in individuals presenting with dyslipidemia and cardiometabolic diseases (5). Unlike other statins, the characteristic structure of pitavastatin provides improved pharmacokinetics and significant low-density lipoprotein cholesterol-lowering efficacy at low doses (6). In addition, increasing evidence indicates that pitavastatin exhibits numerous pleiotropic effects beyond its lipid-lowering potencies. In a previous study, pitavastatin at a low dose (1 μ M) inhibited nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) activation and decreased IL-6 production, which was induced by TNF- α in human breast cancer cells (7). Takano *et al* (8) demonstrated that pitavastatin prevented acute lung injury development in septic mice by increasing glucocorticoid receptor expression and downregulating NF- κ B activation in alveolar macrophages. Katsuki *et al* (9) showed that pitavastatin treatment inhibited atherosclerotic plaque destabilization and rupture in a mouse model of diet-induced lipodystrophy by regulating monocyte chemoattractant protein-1/C-C chemokine receptor type 2-dependent monocyte recruitment. Furthermore, various clinical studies identified that pitavastatin therapy significantly decreased in-hospital or 28-day mortality in patients exhibiting infection and sepsis (10). However, pitavastatin has not been considered as an effective tool with therapeutic potential in the prevention of osteolysis in response to orthopedic wear particles.

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The present study investigated whether treatment with pitavastatin (a new synthetic statin drug) was able to alleviate the activation of the monocyte inflammatory response induced by polymethyl methacrylate (PMMA) particles. The production of pro-inflammatory cytokines and the intracellular NF- κ B signaling pathway were also examined to identify the role of pitavastatin in monocyte activation..

Materials and methods

Preparation of PMMA particles and pitavastatin. Spherical PMMA particles (Polysciences, Inc., Warrington, PA, USA) 1–10 μ m in diameter (mean diameter, 6.0 μ m; 95% <10 μ m) were used for all experiments as previously reported (11–13). The particles were rinsed four times in 70% ethanol, sterilized in 70% ethanol (Sigma-Aldrich, St. Louis, MO, USA) overnight, then washed four times in sterile phosphate-buffered saline (PBS; Wuhan Boster Biological Technology, Ltd., Wuhan, Hubei, China). Particles were resuspended in serum-free Minimum Essential Medium (MEM; Life Technologies, Grand Island, NY, USA) and stored at -20°C. A Limulus Amebocyte Lysate kit (BioWhittaker, Walkersville, MD, USA) was used to detect bacterial endotoxins. The optimal PMMA particle concentration for cell culture experiments was identified to be 2 mg/ml. The pitavastatin (Sigma-Aldrich) was stored at -20°C until it was diluted in serum-free MEM immediately prior to use.

Monocyte isolation. Peripheral whole blood was obtained from healthy volunteers (n=12) into heparinized bottles (Shandong Weigao Group Medical Polymer Co., Ltd., Weihai, China). The twelve subjects recruited for the study were healthy male volunteers aged between 23–45 years. All volunteers enrolled in the present study provided written informed consent. Blood collection was conducted between January and June 2014 at the Southwest Hospital of the Third Military Medical University (Chongqing, China). The heparinized blood was diluted in equal volumes of 10 ml Dulbecco's modified Eagle's medium (Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco Life Technologies) and maintained at 37°C. The 20 ml of diluted blood samples were then layered onto 10 ml of Ficoll-Paque PLUS (GE Healthcare Bio-Sciences, Uppsala, Sweden) and centrifuged at 500 x g for 30 min. The buffer mononuclear layer was removed and washed three times with PBS, and then plated in 12-well plates (Corning Incorporated, Corning, NY, USA) in 5x10⁵/ml Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. All steps were carried out at room temperature. The monocyte population was allowed to adhere for 120 min at 37°C in an atmosphere of 5% CO₂. Cells (lymphocytes) that had not adhered were subsequently washed off using PBS and the remaining monocyte population was used.

Measurement of cytokines. Monocytes were treated with various concentrations of PIT (0–100 μ M) together with 2 mg/ml PMMA for 12 h. The culture medium was subsequently collected and the concentrations of TNF- α , IL-1 β and IL-6 were measured using a commercially available ELISA kit (Wuhan Boster Biological Technology, Ltd.) according to the manufacturer's instructions. The absorbance was measured at

450 nm using a microplate reader (Bio-Tek ELX800; Bio-Tek Instruments, Inc., Winooski, VT, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay. Total cellular RNA was isolated using TRIzol (Invitrogen Life Technologies, Camarillo, CA, USA) and cDNA was synthesized using SuperScript[®] Reverse Transcriptase (Invitrogen Life Technologies) according to the manufacturer's instructions. The primer sequences (Shanghai Shenggong Co., Ltd., Shanghai, China) used in the present study were as follows: Forward, 5'-CGAGTCTGGGCAGGTCTA-3' and reverse, 5'-CGAAGTGGTGGTCTTGTTG-3' for TNF- α ; forward, 5'-CTTCAGGCAGGCAGTATCACTC-3' and reverse, 5'-TGCAGTTGTCTAATGGGAACGT-3' for IL-1 β ; forward, 5'-TCAATGAGGAGACTTGCCTG-3' and reverse, 5'-GATGAGTTGTCTATGTCCTGC-3' for IL-6; and forward, 5'-TCACCACCATGGAGAAGGC-3' and reverse, 5'-GCTAAGCAGTTGGTGGTGCA-3' for GAPDH. RT-qPCR was performed using a LightCyclerH 480 Instrument (Roche Diagnostics GmbH, Mannheim, Germany) and SYBR[®] Green SuperMix (Sigma-Aldrich). The amplification program included an initial denaturation step at 95°C for 5 min and 45 cycles (each consisting of denaturation at 95°C for 10 sec), annealing at 60°C for 10 sec and extension at 72°C for 10 sec. The comparative cycle threshold (Ct) method (2^{- $\Delta\Delta$ Ct}) was used to calculate relative gene expression. The results were expressed as the fold change over the control values.

Western blotting. Cells were collected and lysed in radioimmunoprecipitation assay buffer (Sigma-Aldrich) supplemented with a Broad Spectrum Protease Inhibitor Cocktail (BD Pharmingen, San Diego, CA, USA). Following centrifugation for 10 min at 10,000 x g, the protein content of the supernatant was determined using a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology, Shanghai, China). The protein lysates were separated by 10% SDS-PAGE (Beyotime Institute of Biotechnology) and subsequently electrotransferred onto a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). The membrane was blocked with 5% bovine serum albumin (Sigma-Aldrich) for 1 h at room temperature. The blocked membranes were incubated overnight at 4°C with the following primary antibodies: Rabbit anti-NF- κ B monoclonal antibody (1:600; cat. no. sc-372), rabbit anti-p-inhibitor of κ B (I κ B) monoclonal antibody (1:600; cat. no. sc-101713), rabbit anti-I κ B monoclonal antibody (1:600; cat. no. sc-371), rabbit anti-GADPH monoclonal antibody (1:1,000; cat. no. sc-25778) and rabbit anti-TATA box binding protein monoclonal antibody (1:1,000; cat. no. sc-33736), which were all purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The membranes were then incubated with horseradish peroxidase-linked goat anti-rabbit immunoglobulin G (1:10,000; Cell Signaling Technology, Inc., Danvers, MA, USA) for 1 h at room temperature. Protein bands were visualized using a western blotting detection system (ChemiDoc[™] XRS+; Bio-Rad Laboratories, Hercules, CA, USA) and analyzed by a densitometry system (Quantity One v4.6.2; Bio-Rad Laboratories) according to the manufacturer's instructions.

For extraction of the nucleoprotein, cells were collected and lysed in the lysis buffer [10 mM Hepes (pH 7.9), 1.5 mM

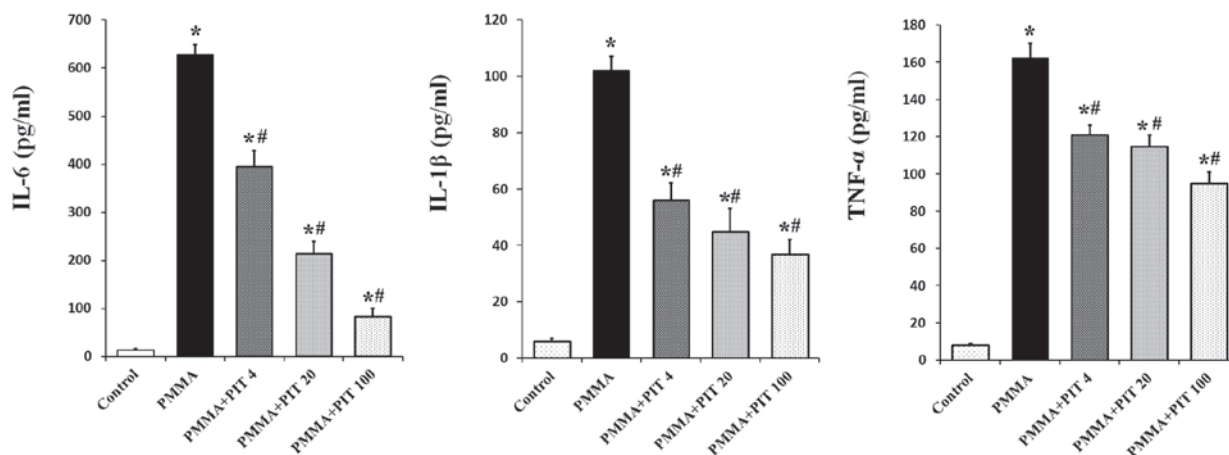


Figure 1. Effects of PIT on PMMA-induced production of pro-inflammatory cytokines. Monocytes were treated with different concentrations of PIT (4, 20 and 100 μ M) together with 2 mg/ml PMMA for 12 h. TNF- α , IL-1 β and IL-6 in supernatants were measured by ELISA. Results are presented as means \pm standard deviation for three independent experiments. *P<0.05 vs. the control group. #P<0.05 vs. the PMMA group. PIT, pitavastatin; PMMA, polymethylmethacrylate; TNF, tumor necrosis factor; IL, interleukin.

MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 2% NP-40, 1 mM phenylmethylsulfonyl fluoride] (Beyotime Institute of Biotechnology) for 20 min, and the lysis buffer was centrifuged at 1,000 \times g for 10 min. The protein content of the supernatant was collected as the cytoplasmic protein. The precipitate was washed twice and lysed in lysis buffer containing Triton X-100 (Sigma-Aldrich) and collected as the nucleoprotein.

Immunofluorescence. Monocytes were seeded onto 12-well plates, grown in chamber slides and treated with 2 mg/ml PMMA and/or 100 mM pitavastatin for 1 h. Cells were fixed with 4% paraformaldehyde (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and permeabilized with 0.5% Triton X-100. Immunostaining of p65 was performed using rabbit anti-p65 monoclonal antibody (cat. no. sc-372; Santa Cruz Biotechnology, Inc.) at a dilution of 1:200, then with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G secondary antibody (Abcam, Cambridge, MA, USA) at a dilution of 1:5,000. Finally, the cells were stained with DAPI (Beyotime Institute of Biotechnology) for 10 min to localize the nuclei, which served as a reference point. For the negative controls, the primary antibodies were excluded from the staining procedure described above. The activation of NF- κ B was defined by its translocation into the nucleus, which was visualized by fluorescence microscopy (magnification, \times 20) using a Nikon Eclipse E1000 (Nikon Corporation, Tokyo, Japan).

Statistical analysis. All results are expressed as the mean \pm standard deviation. The results were analyzed by analysis of variance, followed by Student's t-test to determine the significance. P<0.05 was considered to indicate a statistically significant difference. All the statistical analyses were performed using the SPSS 12.0 statistical software (SPSS, Inc., Chicago, IL, USA).

Results

Pitavastatin inhibits PMMA-induced pro-inflammatory cytokine production. PMMA particles are widely derived from

implants used in TJR, as they result from wear of the material in the prosthetic. Thus, in the present study, PMMA particles served as a stimulant to trigger inflammatory monocytes. Varying concentrations of PMMA (1, 2 and 4 mg/ml) were added into the monocyte culture medium 6, 12 or 24 h prior to analysis. It was found that in all of the PMMA concentration groups, the pro-inflammatory cytokines were increased in a time-dependent manner and there was no significant differences noted between the 2 and 4 mg/ml PMMA groups (data not shown). Therefore, a PMMA particle concentration of 2 mg/ml was selected and administered 24 h prior to the cell culture experiments.

To study the effect of pitavastatin, 2 mg/ml PMMA was applied to stimulate the monocytes, and 0–100 μ M pitavastatin was simultaneously administrated. After 24 h, as seen in Fig. 1, 2 mg/ml PMMA induced a significant production of TNF- α , IL-1 β and IL-6 in the monocytes (P<0.05). However, pitavastatin treatment significantly inhibited the production of pro-inflammatory cytokines in a dose-dependent manner (P<0.05).

Pitavastatin inhibits PMMA-induced TNF- α , IL-1 β and IL-6 mRNA expression. RT-qPCR was conducted to assess whether pitavastatin inhibits TNF- α , IL-1 β and IL-6 mRNA expression. As demonstrated in Fig. 2, PMMA stimulation of monocytes resulted in a marked increase in TNF- α , IL-1 β and IL-6 at the transcriptional level. However, treatment with varying concentrations of pitavastatin (4, 20 and 100 μ M) significantly downregulated PMMA-induced transcription of TNF- α , IL-1 β and IL-6 mRNA in a concentration-dependent manner (P<0.05).

Pitavastatin inhibits PMMA-induced I κ B phosphorylation and degradation. NF- κ B is considered to be a master switch in the regulation of inflammation and immunity (14). As a transcription factor, NF- κ B controls an array of pro-inflammatory genes involved in the inflammatory signaling cascade, such as TNF- α , IL-1 β , and IL-6 (15). Therefore, the effect of pitavastatin on PMMA-induced NF- κ B activation in monocytes

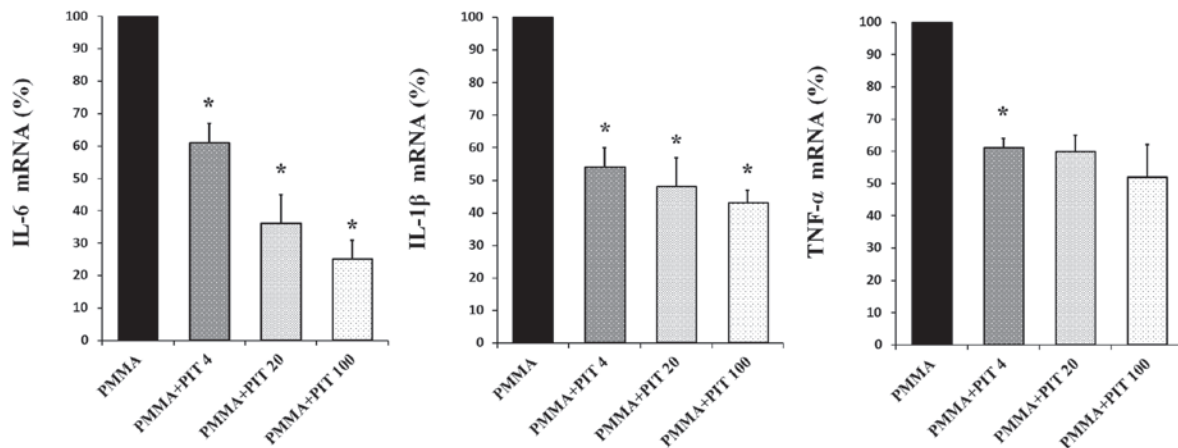


Figure 2. Effects of PIT on PMMA-induced mRNA expression of TNF- α , IL-1 β and IL-6. Monocytes were treated with different concentrations of PIT (4, 20 and 100 μ M) together with 2 mg/ml PMMA for 5 h. The mRNA expression levels of TNF- α , IL-1 β and IL-6 were detected by reverse transcription-quantitative polymerase chain reaction. Results are presented as means \pm standard deviation for three independent experiments. *P<0.05 vs. the PMMA group. PIT, pitavastatin; PMMA, polymethylmethacrylate; TNF, tumor necrosis factor; IL, interleukin.

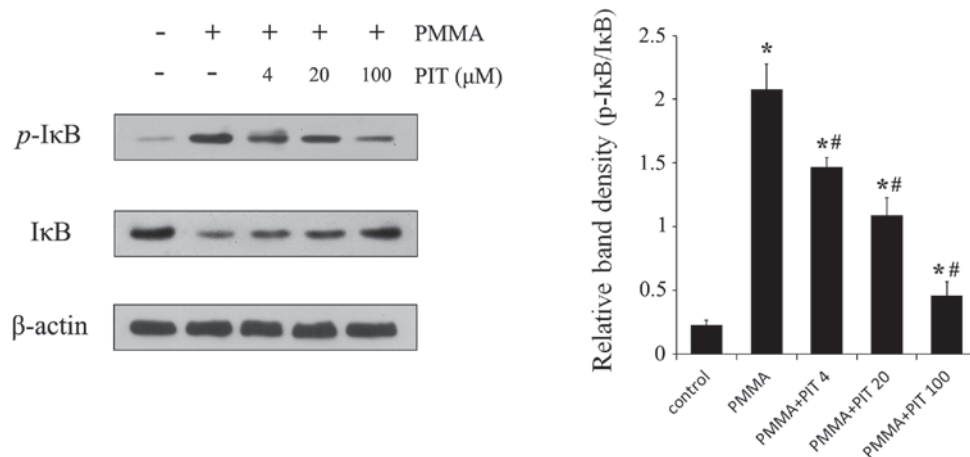


Figure 3. Effects of PIT on PMMA-induced I κ B phosphorylation and degradation. Monocytes were treated with different concentrations of PIT (4, 20 and 100 μ M) together with 2 mg/ml PMMA for 30 min. Cells were collected and lysed. I κ B and p-I κ B were determined by western blotting. The expression ratio of p-I κ B/I κ B was quantitatively determined by densitometry. *P<0.05 vs. the control group. #P<0.05 vs. the PMMA group. PIT, pitavastatin; PMMA, polymethylmethacrylate; p, phosphorylated.

were investigated. As shown in Fig. 3, monocytes treated with PMMA exhibited significant phosphorylation of I κ B, whereas the various treatment concentrations of pitavastatin (4, 20 and 100 μ M) prevented I κ B phosphorylation. In addition, pitavastatin inhibited degradation of I κ B, which had been induced by PMMA, in a concentration-dependent manner. These results indicate that pitavastatin significantly blocks the NF- κ B signaling pathway in PMMA-stimulated monocytes by suppressing I κ B phosphorylation and degradation.

Pitavastatin inhibits PMMA-induced NF- κ B p65 translocation. Having established that pitavastatin inhibits I κ B phosphorylation and degradation, further investigations regarding its effects on NF- κ B signaling were performed. The p65 protein levels in the cytoplasmic and nuclear fractions of PMMA-induced monocytes were analyzed. As shown in Fig. 4, PMMA stimulation resulted in significant nuclear translocation of p65 in the monocytes, whereas pitavastatin treatment prevented the accumulation of PMMA-induced nuclear p65 in

a concentration-dependent manner. Furthermore, immunocytochemical analysis demonstrated that pitavastatin treatment exhibited reduced nuclear translocation of endogenous p65 following PMMA stimulation (Fig. 5). These results indicate that pitavastatin significantly inhibits NF- κ B p65 translocation in PMMA-stimulated monocytes.

Discussion

TJR is considered to be the most effective treatment strategy for end-stage joint diseases, such as osteoarthritis and rheumatoid arthritis (16). However, despite the clinical effectiveness of TJR, aseptic loosening of prostheses continues to present a major problem, particularly for the long-term success and survival of a prosthesis (17). There are various causes of aseptic loosening, however, an inflammatory reaction induced by excessive production of wear particles from the implant components and consequent peri-implant osteolysis is regarded as the primary cause (18).

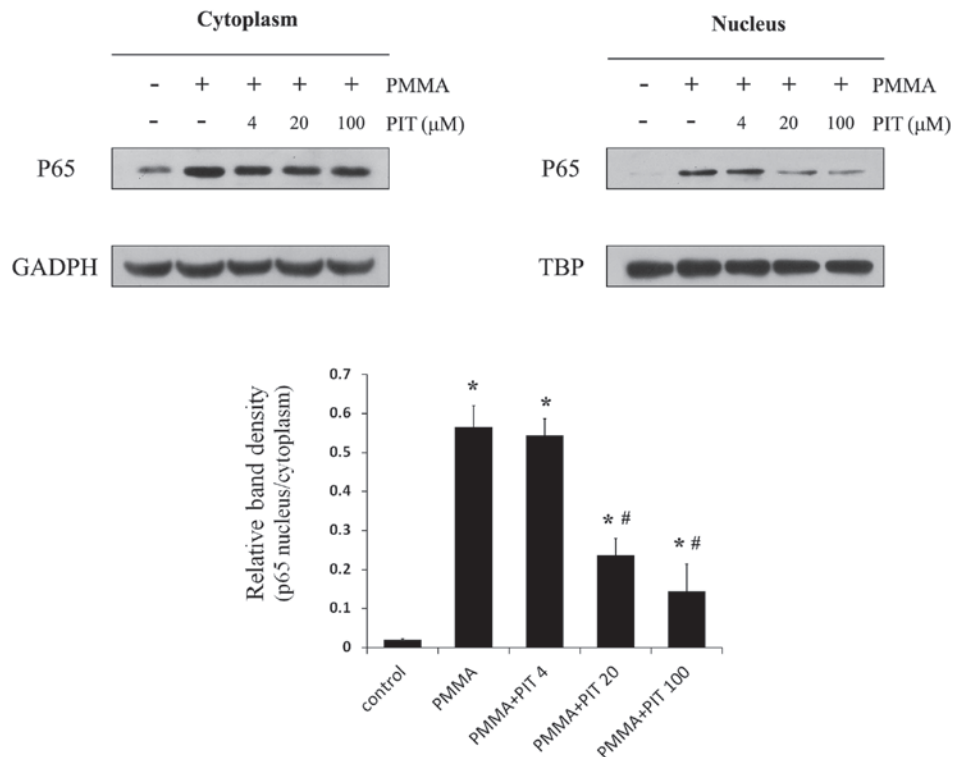


Figure 4. Effects of PIT on PMMA-induced NF- κ B p65 translocation. Monocytes were treated with different concentrations of PIT (4, 20 and 100 μ M) together with 2 mg/ml PMMA for 30 min. Cells were collected and lysed, and the levels of NF- κ B p65 in the cytoplasm and nucleus were determined by western blotting. The ratio of nuclear p65 to cytoplasmic p65 was quantitatively determined by densitometry. * $P < 0.05$ vs. the control group. # $P < 0.05$ vs. the PMMA group. PIT, pitavastatin; PMMA, polymethylmethacrylate; TBP, TATA box binding protein; NF- κ B, nuclear factor κ -light-chain-enhancer of activated B cells.

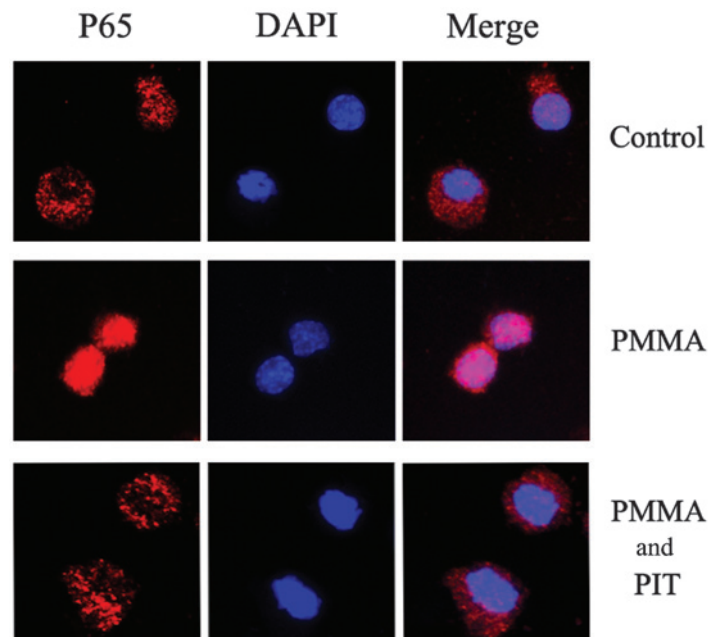


Figure 5. Effects of PIT on NF- κ B p65 translocation induced by PMMA. Monocytes were seeded onto 12-well plates, grown in chamber slides and treated with 2 mg/ml PMMA and/or 100 mM PIT for 1 h. NF- κ B p65 translocation was measured by immunofluorescence microscopy. Magnification, x600. PIT, pitavastatin; PMMA, polymethylmethacrylate; NF- κ B, nuclear factor κ -light-chain-enhancer of activated B cells.

PMMA particles, a material widely derived from orthopedic implants, elicit a marked inflammatory response due to macrophage and enhanced osteoclast formation and activity (19-21). Upon exposure to PMMA, osteoblasts

contribute to periprosthetic osteolysis by secreting mediators that drive the inflammatory process (22). These mediators, which include TNF- α , IL-1 β and IL-6, interact with one another contributing significantly to the inflammatory cascade.

TNF- α is considered to be a key cytokine, which mediates particle-driven osteoclastogenesis and osteolysis (23). TNF- α increases osteoclast differentiation by promoting the expression of receptor activation of NF- κ B ligand and macrophage-colony stimulating factor, which are essential factors involved in the expansion, commitment and differentiation of osteoclast precursors into mature osteoclasts (24,25). Fuller *et al* (26) demonstrated that a low level concentration of TNF- α stimulation activates osteoclasts and indicated that this effect cannot be inhibited by osteoprotegerin. Furthermore, an adenovirus-mediated small interfering RNA targeting TNF- α was observed to significantly inhibit titanium wear particle-induced osteoclastogenesis and bone resorption in macrophages (27). IL-1 β is also a well-established pro-inflammatory cytokine that contributes to aseptic loosening. As an important downstream molecule in TNF- α -induced osteoclast differentiation, IL-1 β promotes multinucleation of osteoclast progenitor cells and enhances mature osteoclast-associated bone resorption (28-30). Therefore, controlling the synthesis of inflammatory cytokines in the periprosthetic environment may be a potential target for the prevention or reduction of wear particle-induced osteolysis. In addition, the anti-inflammatory effects of statins are well recognized. Uekawa *et al* (31) indicated that statin pretreatment ameliorated early brain injury, following a subarachnoid hemorrhage, via the attenuation of oxidative stress and NF- κ B-mediated inflammation. Moon *et al* (32) reported that short-term administration of statins to patients who had suffered an atherosclerotic stroke exerts antioxidant effects against lipid peroxidation via lipid-lowering-dependent and -independent mechanisms. McGuire *et al* (33) demonstrated that healthy male subjects who were administered with statins for three weeks showed a decline in TNF- α plasma concentrations and toll-like receptor-4 expression in blood monocytes. A meta-analysis of observational studies demonstrated that, although the use of statins did not significantly decrease the in-hospital or 28-day mortality, it did present a survival advantage in patients with infection and sepsis (10). Thus, it is hypothesized that statins may inhibit wear particle-induced inflammatory reactions from implant components.

The ability of pitavastatin to abrogate PMMA-mediated monocyte activation was examined in the present study. The peripheral blood monocyte/macrophage model is a well-recognized *in vitro* model for particle stimulation. Therefore, this was considered to be the most appropriate model for the established primary instigators of foreign-body inflammatory response and subsequent osteolysis, as monocytes represent the circulatory precursors of tissue macrophages. In the current study, stimulation of monocytes with 2 mg/ml PMMA resulted in a marked increase in TNF- α , IL-1 β and IL-6 at the transcriptional and translational levels. However, pitavastatin treatment significantly downregulated the PMMA-induced TNF- α , IL-1 β and IL-6 expression at the transcriptional and translational levels in a concentration-dependent manner (Figs. 1 and 2).

PMMA particles are potent inducers of the NF- κ B signaling pathway, which is considered to be an important mediator of inflammatory responses, and essential for osteoclast differentiation and function (34). In its inactivated state, NF- κ B is located in the cytoplasm as an inactive NF- κ B/I κ B

complex, and its activity is tightly controlled by the inhibitory protein, I κ B (35). Upon I κ B phosphorylation and subsequent degradation, NF- κ B p65 is released and enters the nucleus to activate specific target gene expression. Therefore, the activation of NF- κ B was assessed in monocytes in the present study by measuring the quantity of I κ B protein expression. Incubation of monocytes with PMMA caused marked phosphorylation and degradation of cytosolic I κ B, and NF- κ B p65 translocation into the nucleus, whereas pitavastatin treatment significantly inhibited the phosphorylation and degradation of I κ B, as well as NF- κ B p65 nuclear translocation in a dose-dependent manner (Figs. 3-5). This suggests that pitavastatin may suppress PMMA-induced activation of the NF- κ B signaling pathway, indicating that the NF- κ B pathway may be involved in the anti-inflammatory effects of pitavastatin.

In conclusion, pitavastatin has been demonstrated to inhibit PMMA-induced monocyte activation and inflammatory cytokine release by inhibiting phosphorylation and degradation of I κ B, and subsequent NF- κ B p65 translocation. Considering these findings, pitavastatin may be an efficacious candidate for administration as a therapeutic agent for periprosthetic osteolysis and aseptic loosening, which occur following TJR.

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