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Toll-like Receptors-2 and 4 are overexpressed in an experimental model of particle-induced osteolysis

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Abstract

Aseptic loosening secondary to particle-associated periprosthetic osteolysis remains a major cause of failure of total joint replacements (TJR) in the mid- and long-term. As sentinels of the innate immune system, macrophages are central to the recognition and initiation of the inflammatory cascade which results in the activation of bone resorbing osteoclasts. Toll-like receptors (TLRs) are involved in the recognition of pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). Experimentally, polymethylmethacrylate (PMMA) and polyethylene (PE) particles have been shown to activate macrophages via the TLR pathway. The specific TLRs involved in PE particle-induced osteolysis remain largely unknown. We hypothesized that TLR-2, -4 and -9 mediated responses play a critical role in the development of PE wear particle-induced osteolysis in the murine calvarium model. To test this hypothesis, we first demonstrated that PE particles caused observable osteolysis, visible by microCT and bone histomorphometry when the particles were applied to the calvarium of C57BL/6 mice. The number of TRAP positive osteoclasts was significantly greater in the PE-treated group when compared to the control group without particles. Finally, using immunohistochemistry, TLR-2 and TLR-4 were highly expressed in PE particle-induced osteolytic lesions, whereas TLR-9 was downregulated. TLR-2 and -4 may represent novel therapeutic targets for prevention of wear particle-induced osteolysis and accompanying TJR failure.

Keywords

arthroplasty; UHMWPE; polyethylene; wear debris; Toll-like receptor; murine calvarium; osteolysis; immune response

INTRODUCTION

The demand for primary total hip arthroplasty (THA) has been projected to grow by 174% to 572,000 procedures performed per year in the U.S.A. by 2030. Furthermore, the demand for THA revision procedures has been projected to more than double during this same time period. During this time, the need for total knee arthroplasty (TKA) will grow by 673% to 3.48 million procedures performed per year in the U.S.A. The demand for revision TKA is projected to more than double in the next decade¹.

In comparison to primary surgeries, revision surgeries have higher costs, technical difficulty, and complication rates, as well as less predictable clinical outcomes¹. Despite advances in material technology and surgical technique, aseptic loosening of prosthetic implants remains one of the most common long-term complications of joint replacement surgery and results in the need for revision surgery^{2,3}. These facts are especially important given that knee and hip replacement patients are younger and more active than previous recipients of TJR, thus placing increased time and functional demands on their prosthetic implants⁴.

Millions of wear particles from the implant are generated during each gait cycle and migrate into the periprosthetic joint space where they trigger the inflammatory cascade leading to osteolysis. Both conventional and highly cross-linked ultra high molecular weight polyethylene (UHMWPE) are materials commonly used for the bearing surface⁵. Macrophages phagocytose wear particles, become activated, and subsequently release various pro-inflammatory factors⁶. This leads to the differentiation, maturation, and activation of osteoclasts, and ultimately, bone resorption^{1,7,8}. The biologic response to PE and polymethylmethacrylate (PMMA) particles from joint replacements has been experimentally and clinically characterized as a non-specific, chronic inflammatory and foreign body reaction⁹⁻¹³. However, the exact role of the innate immune system in the recognition of wear particles and the subsequent development of osteolysis has not been elucidated. This knowledge may provide important strategies for prevention and treatment of periprosthetic osteolysis, thereby decreasing the burden of revision surgery by increasing implant longevity.

Toll-like receptors (TLRs) are evolutionarily conserved transmembrane receptors that play a critical role in the innate immune system by recognizing microbes and endogenous molecules from cells subjected to stress and injury. Invading microbes are recognized via their pathogen-associated molecular patterns (PAMPS), such as lipopolysaccharide (LPS), bacterial DNA and viral RNA. Byproducts from injured cells are recognized via danger-associated molecular patterns (DAMPS) such as heat shock proteins¹⁴⁻¹⁷.

TLRs are mainly found on monocytes and macrophages, and have been previously shown to activate the inflammatory cascade by triggering the induction of various cytokines (IL-1,

IL-6, TNF-alpha), growth factors (macrophage colony-stimulating factor-1) and chemokines (MIP-1 alpha, MCP-1), and activating various downstream signaling pathways (NFkappaB, AKT, and MAPK) ¹⁵⁻²⁰.

While most TLRs are located on the plasma membrane, TLR-3, TLR-7, and TLR-9 are localized in the endosomal compartment ²¹. Each member of the Toll family (10 human and 12 murine TLRs) is activated by a different stimulus or ligand and activates a response to that specific chemical pattern ²². For example, TLR-2 recognizes various PAMPs from Gram-positive bacteria, including bacterial lipoproteins, lipomannans, and lipoteichoic acids. Of all the TLRs, TLR-2 has the greatest variety of ligands owing to its heterodimerization ²³. TLR-4 recognizes lipopolysaccharide (LPS) on the cell membrane of gram-negative bacteria ²⁴. TLR-9 detects differences between microbial and host DNA based on CpG-ODN motifs ²⁵.

TLRs signal through various adapter molecules including a common adapter known as MyD88 ^{24,26}. TLR-2 and TLR-9 signal through MyD88, though TLR-4 also signals through a MyD88-independent pathway involving the TIR domain, which contains adaptor inducing IFN-beta (TRIF) and IRF-3 transcription factor ²⁷⁻²⁹.

Signaling mechanisms that recognize DAMPS are of particular interest as these are endogenous, host-derived molecules including proteins, lipids and nucleic acids. Recent *in vitro* studies by our group have shown that phagocytosable polymer particles can activate the innate immune system independent of adherent endotoxin and that the process involves MyD88 ^{30,31}. Particles that are too large to be phagocytosed can bind to cell surfaces and also activate cells. Wear particles have been shown to come in a variety of shapes and sizes; particle size, dose and other characteristics are major determinants of particle-induced bone resorption ^{32,33}.

Extensive immunolocalization of TLR-4 and TLR-9 positive cells has been observed in the interface membrane of components revised for loosening and osteolysis compared with control synovial tissues from patients with osteoarthritis ³⁴. TLR-2, -4, -5 and -9 positive cells were also observed in peri-prosthetic tissues from cases of aseptic loosening and septic hip implants. TLR-2 and TLR-5 expression was greater than TLR-4 and TLR-9 responses. The production of TNF-alpha by macrophages exposed to hydroxyapatite particles was found to be TLR-4 dependent ³⁵. Short chain oxidized alkane polymers from the breakdown of UHMWPE was shown to activate TLR-2, resulting in endosomal damage and activation of the inflammasome ^{36,37}.

TLRs have been shown to be important in the recognition of polymethylmethacrylate (PMMA) particles using a murine calvarial model, in which particles are surgically implanted over the calvarium which is harvested and analyzed one week postoperatively; however, the expression of specific TLRs and their association with polyethylene (PE) particle-induced osteolytic lesions in an established animal model are largely unknown ³⁰. The aim of this study was to validate our hypothesis that TLR-2 and TLR-4 mediated signaling pathways play a critical role in the recognition of polyethylene wear particles in the murine calvarial model. Given that TLR modulating agents are actively being explored,

the present experiments might provide therapeutic targets to mitigate particle-induced osteolysis in humans^{18,38,39}.

MATERIALS AND METHODS

Experimental design

Twenty, 8-week old C57BL/6 (Charles River Laboratory, Wilmington, MA) were housed and fed in the Research Animal Facility on the Stanford University campus. The experimental protocol was approved by the Institutional Administration Panel for Laboratory Animal Care at our institution, and university guidelines for care and use of laboratory animals were strictly followed. Sample size calculations were carried out by a professional statistician to determine the number of animals needed for our *in vivo* study. The power analysis was based on determining a standardized effect size of 1.5, standard deviation of 1.0, with a power of 80% ($\alpha = 0.05$, $\beta = 0.20$) as we have used in our previous published work on TLR receptors.³⁰ The analysis concluded that $n = 8$ animals per group was necessary for our *in vivo* study. We used $n = 10$ animals per group in our study to accommodate for unexpected animal loss. Animals were randomly assigned to two experimental groups. The PE-treated animals ($n=10$) received PE particles in PBS solution using a surgical approach over the parietal bones, while control animals ($n=10$) only received PBS carrier solution. Animals underwent microCT of the calvarium one day before surgery (day -1) and at day 7 for comparative assessment of bone volume and bone mineral density analysis. All animals were euthanized on day 7, and the skull parietal bones were harvested for histomorphometry and immunohistochemistry.

Particles

Conventional UHMWPE particles (a gift from Dr. Timothy Wright, Hospital for Special Surgery, New York) were obtained from joint simulator tests and isolated according to an established protocol⁴⁰. The particles were isolated by density gradient centrifugation and sterilized by incubation with 95% ethanol overnight. Then, frozen aliquots of the particles containing serum were lyophilized for 4–7 days. The dried material was digested in 5M sodium hydroxide at 70 °C for 2 h. The digested particle suspension was centrifuged through a 5% sucrose gradient at 40 K rpm at 10°C for 3 h. The collected particles at the surface of the sucrose solution were ultrasonicated and centrifuged again through an isopropanol gradient (0.96 and 0.90 g/cm³) at 40 K rpm at 10°C for 1 h. The purified particles at the interface between the two layers of isopropanol were harvested and the isopropanol was evaporated from the particle mixture until dry. Particles were then re-suspended in 95% ethanol, which was evaporated completely. Ultimately, UHMWPE particles were washed in 70% ethanol and re-suspended in PBS prior to the animal surgeries. The concentration of UHMWPE was 30 mg/mL. The particles tested negative for endotoxin using a Limulus Amebocyte Lysate Kit (BioWhittaker, Walkersville, MD). The mean diameter of the particles was $1.0 \pm 0.1 \mu\text{m}$ (mean $\pm$ SE) as measured by electron microscopy. Using the density of UHMWPE particles, it was calculated that 67 μL of solution contained approximately 4.0×10^9 particles.

Surgical procedure

Surgery was performed on day 0. All animals received an injection of buprenorphine (0.1mg/kg, Ben Venue Laboratories, Bedford, OH) subcutaneously before the procedure. Animals were then anesthetized with 2-3% isoflurane in 100% oxygen at a flow rate of 1.5 L/min and were operated on a water-warmed small animal surgery station. The surgical site was first shaved using a commercial grooming shaver. After cleaning the surgical site with Betadine (Purdue Products, Stamford, CT) a 5mm incision was made over the sagittal suture starting at the level of the base of the ear and proceeding anteriorly towards the nasal bones. Two Guthrie Retractors (Fine Science Tools, Heidelberg, Germany) were then placed on either side of the incision and the scalp was retracted to create a pocket over the parietal bones. In this manner the sagittal suture, parietal bones, and anteroposterior suture could be visualized to ensure proper anatomic location for implantation of the particles. A 200 μ L pipette tip was filled with 67 μ L of saline solution with 4.0×10^9 particles for the treatment group. In the control group, animals received 67 μ L of the vehicle solution only. The pipette tip was then placed into the pocket over the parietal bones and the solution was pipetted directly on top of the periosteum overlying both parietal bones. After visual confirmation that the parietal bones were fully covered with solution, the incision was sutured with 5-0 nylon sutures (Ethicon, Somerville, NJ). The animals were checked post-operatively each day for general health and signs of infection. After 7 days, animals were euthanized using a two-port euthanasia chamber. The animals were placed in the chamber and the isoflurane port was activated until the animals were fully anesthetized. The CO₂ port was then activated to ensure complete euthanasia. Animals were then monitored for 3 minutes to ensure that they had expired. Following euthanasia, the skulls were harvested and analyzed using microCT, and then decalcified.

In vivo microCT imaging

Micro computed tomography was performed in the Small Animal Imaging Facility at Stanford University using a high-resolution MicroCAT II (Imtek Inc. Knoxville, TN) imaging system. To detect changes in bone volume (BV) and bone mineral density (BMD), microCT scan was performed one day before surgery (day -1) and at day 7 for all groups. Animals were sedated via mask inhalation of Aerrane (Isoflurane, Baxter Health Corp., Deerfield, IL). Before imaging, scout radiographs were taken to ensure that both parietal bones were entirely scanned. The following microCT settings were used: voltage of 80 kVp, anode current of 500 microA and an exposure time of 500 milliseconds for each of the 360 rotational steps. The 2D projection images were reconstructed into tomograms following a Feldkamp algorithm using a commercial software package (Cobra EXXIM, EXXIM Computing Corp., Livermore, CA). The resolution of the reconstruction was 80 micrometers. The duration of each scan was 9.5 minutes. Every scan was conducted with a phantom containing samples of hydroxyapatite, water, and air. This was used to calibrate all 40 scans to produce isosurfaces standardized at 700 Hounsfield units to allow for accurate comparisons between the various scans. Using the imaging software Microview (GE Medical Systems, Fairfield, CT), a standardized 3D volume of interest (VOI) was created (11mm $\times$ 2mm $\times$ 4mm) for BV and BMD assessment. The VOI was aligned with the skull vault landmarks of each scan (anterior lambdoid suture, sagittal suture, coronal suture, ridge

between the horizontal and vertical plate of the parietal bones. BV data was collected as mm^3 and BMD as mg/cc .

Bone histomorphometry

The left and right parietal bones were collected and freed from soft tissues. The parietal bones were fixed using paraformaldehyde (PFA) for three days, then decalcified twice for a period of seven days each using ethylenediaminetetraacetic acid (EDTA). Using a cryostat (Cambridge Instruments, Buffalo, NY), sagittally oriented frozen histological sections of 9 micron thickness were cut from the anterior lambdoid suture to the coronal suture to include the area immediately lateral to the sagittal suture where the particles were implanted. Sections were collected for histomorphometry and immunohistochemistry (IHC).

Histomorphometry was conducted on hematoxylin and eosin (H&E) stained sections (Sigma-Aldrich, Steinheim, Germany). These sections were then photographed at 20X with the use of a light microscope (Olympus BX50; Olympus Optical Co., Tokyo, Japan), camera and software package (SPOT Imaging Solutions, Sterling Heights, Michigan). The images were then imported into Photoshop (Adobe, San Jose, CA) for histomorphometric analysis by a previously established method⁴¹. The Photoshop selection tool was used to create a region of interest (ROI) that encompassed the parietal bone area between the coronal and the anterior lambdoid suture but excluded the periosteum and the marrow cavities. The pixels in the resulting ROI were used to quantify bone surface area for each section. As we found that the size of individual pixels varies depending on monitor size and resolution, we took additional steps to normalize our bone surface area results to allow for an accurate and meaningful comparison between the calvarial samples. We did this by constructing a box with a length of one millimeter and a width of one millimeter in Photoshop and used the resulting number of pixels as a conversion factor to convert the number of pixels to square millimeters.

TRAP staining

Osteoclasts were identified as large multinuclear giant cells located in the histological sections and confirmed using a leukocyte acid phosphatase kit, TRAP, (Sigma-Aldrich). Stained specimens were examined by light microscopy and evaluated by counting positive cells in the sagittally oriented histological sections from the anterior lambdoid suture to the coronal suture.

Immunohistochemistry

Immunohistochemistry with an avidin-biotin-peroxidase complex (ABC) system (Vector Laboratories, Burlingame, CA) was performed to identify osteoblasts and TLR-2, -4, and -9 positive cells. Appropriate control slides were made to ensure that there was no endogenous avidin/biotin or peroxidase activity that could obscure specific staining. Sections were treated serially with (1) Mouse (M.O.M.) Ig blocking reagent (Vector Laboratories, Burlingame, CA) for 1 hr, (2) M.O.M. protein diluent (Vector) for 5 min to reduce non-specific binding and background staining, (3) diluted primary antibody (1:100) for 30 minutes. Mouse anti-alkaline phosphatase (R&D Systems, Minneapolis, MN) was used to detect osteoblasts. Mouse anti-TLR-2 (Biolegend, San Diego, CA) was used to detect TLR-2

positive cells. Mouse anti TLR-4 (R&D Systems, Minneapolis, MN) was used to detect TLR-4 positive cells. Mouse anti-TLR-9 (eBioscience, San Diego, CA) was used to detect TLR-9 positive cells. Following primary antibody incubation, sections were treated (4) with M.O.M. Biotinylated Anti-Mouse IgG reagent (Vector) for 10 minutes, and (5) avidin-biotin peroxidase complex (Vector) for 5 minutes. The sites of peroxidase binding were visualized by incubation for 10 min in NovaRED peroxidase substrate solution (Vector). Sections were then counterstained in hematoxylin for 10 sec. Between each of the steps, the sections were washed twice in PBS. The specificity of the staining was confirmed by running appropriate control sections with and without primary antibody. All incubation steps were carried out at room temperature in a humidified chamber. Stained specimens were examined with light microscopy and TLR positive cells were visually identified as macrophages with the assistance of an experienced bone histologist and macrophage researcher^{42,43}. We then counted all positive cells in the sagittally oriented histological sections from the anterior lambdoid suture to the coronal suture.

Statistical analysis

Data are expressed as the mean $\pm$ SEM. An unpaired t-test (Prism Software, Graphpad Software Inc., San Diego, CA) was used to compare bone volume and bone mineral density between the PE-treated group and the control group. Intergroup comparisons of osteoclast, osteoblast, TLR-2 positive, TLR-4 positive, and TLR-9 positive cell numbers were made using the Mann-Whitney U Test. Statistics were conducted using Prism Software for Mac (Graphpad Software Inc., La Jolla, CA). The level of significance was set at $p < 0.05$.

RESULTS

All twenty, 8-week old C57BL/6 mice remained free of infections or any other health complications until killing on day 7.

MicroCT analysis

According to microCT analysis, the PE-treated group exhibited greater bone resorption in comparison to the control group (Figure 1, panels A and B). Analysis of volume of interest (VOI) (Figure 2, panels A-D) demonstrated that BV was significantly decreased for the PE-treated group compared to the control group (receiving PBS alone) at day 7 (Figure 1, panel C). BV at day 0 was $12.80 \pm 0.22 \text{ mm}^3$ for the control group and $13.74 \pm 0.39 \text{ mm}^3$ for the PE-treated group ($p = 0.2628$). BV at day 7 was $13.86 \pm 0.36 \text{ mm}^3$ for the control group and $12.26 \pm 0.30 \text{ mm}^3$ for the PE-treated group ($p < 0.0035$). BMD at day 0 was $49.22 \pm 1.73 \text{ mg/cm}^3$ for the control group and $51.56 \pm 1.20 \text{ mg/cm}^3$ for the PE-treated group ($p = 0.1082$). BMD at day 7 was $53.85 \pm 2.22 \text{ mg/cm}^3$ for the control group and $48.80 \pm 1.54 \text{ mg/cm}^3$ for the PE-treated group ($p < 0.0935$). Using these raw means, the change in BV was computed for each mouse (day 7 minus day 0). The intra-individual bone volume difference averaged $1.06 \pm 0.28 \text{ mm}^3$ for the control group versus $1.48 \pm 0.21 \text{ mm}^3$ for the PE-treated group ($p < 0.0001$). Analysis of the change in BMD showed similar results as the intra-individual BMD difference was $4.63 \pm 0.94 \text{ mg/cm}^3$ for the control versus $2.77 \pm 2.15 \text{ mg/cm}^3$ for the PE-treated ($p < 0.0055$).

Bone histomorphometry

Bone surface area was significantly decreased in the PE-treated group compared to the control group receiving PBS alone at day 7. Specifically, the mean bone surface area was $2376.65 \pm 70.72 \text{ mm}^2$ for the PE-treated group versus $3176.00 \pm 102.78 \text{ mm}^2$ for the control group ($p < 0.0001$).

TRAP staining

The number of TRAP positive osteoclasts was significantly greater in the PE-treated group (2.50 ± 0.40 cells per histological section) compared to the control group (0.80 ± 0.42 cells per histological section; $p < 0.0088$).

Immunohistochemistry

The number of osteoblasts in the PE-treated group was not significantly different than the number for the control group 1.10 ± 0.59 cells per histological section versus 0.70 ± 0.52 cells per histological section respectively, ($p = 0.6151$).

Immunohistochemistry showed a significantly greater number of TLR-2 and TLR-4 positive cells in the PE-treated group compared with controls (Figure 3, Figure 4). The number of TLR-2 positive cells (Figure 4) in the PE-treated group was significantly greater than the number for the control group, 45.20 ± 16.10 cells per histological section versus 5.00 ± 2.29 cells per histological section, respectively ($p < 0.0001$). Also, the number of TLR-4 positive cells in the PE-treated group was 24.10 ± 5.89 cells per histological section, which was significantly greater than the number for the control group (5.90 ± 1.39 cells per histological section, $p < 0.0001$). In contrast, there was no statistically significant difference in the number of TLR-9 positive cells in the PE-treated group (4.30 ± 1.26 cells per histological section) in comparison to the control group (3.50 ± 1.71 cells per histological section, $p = 0.3150$).

DISCUSSION

TLR signaling has been implicated in the inflammatory reaction to wear particles. TLR-2, -4, and -9 all signal through the MyD88 adaptor protein pathway. Recently, MyD88^{-/-} mice were shown to develop less particle-induced osteolysis than wild-type mice³⁰. This suggests that TLRs that signal through the MyD88 pathway are involved in particle-induced osteolysis. However the specific TLRs in the MyD88 group that are associated with particle-induced inflammation and osteolysis have not been definitively elucidated.

Therefore, our aim was to determine whether TLR-2, -4 and -9 were associated with the inflammatory response to clinically relevant polyethylene wear particles using an *in vivo* model. We showed that TLR-2 and TLR-4 positive cells were present in significantly higher numbers in PE particle-treated murine calvaria in comparison with control samples without particles. There was no difference in the number of TLR -9 positive cells in the PE particle-treated group versus the control group. As TLR positive cells were present in amounts where individual counting was feasible we were able to use a quantitative method of immunolocalization and statistical analysis.

Wear particles cause the activation of macrophages which trigger the inflammatory cascade. This inflammatory response is associated with the activation of osteoclasts which results in periprosthetic osteolysis. TLRs are found on macrophages and have been shown to trigger pro-inflammatory signaling pathways. Binding of TLRs to PAMP and DAMP ligands triggers an inflammatory response that may lead to bone resorption. Previous studies have shown that PMMA particles are an important TLR ligand using the murine calvarial model. However, the role of PE particles in the activation of TLR signaling pathways has not been elucidated. Our current study using an established wear particle-induced osteolysis model demonstrated an overexpression of TLR-2 and TLR-4, but not TLR-9.

Previous human tissue retrieval studies have highlighted the contribution of TLR-2 and TLR-4 to inflammatory bone diseases. Seibl et al showed the predominant expression of TLR-2 in rheumatoid synovium⁴⁴. Kim et al demonstrated that TLR -2 and TLR-4 activation induces RANKL expression, which in turn facilitates osteoclastogenesis from osteoclast precursor cells⁴⁵. Inflammation-dependent induction of TLR-2 and TLR-4 has been shown in intestinal macrophages⁴⁶. TLR-2, -4, and -9 were also elevated in human patients with periodontitis when compared to healthy controls, with relatively higher numbers of TLR-2 and TLR-4 positive cells than TLR-9 positive cells⁴⁷. Takagi et al found increased expression of TLR-4 and TLR-9 in interface tissues around aseptically loose total hip replacements when compared to control synovial membranes from patients with osteoarthritis³⁴.

Both the specific experimental model and particle characteristics appear to influence TLR expression. For example, *in vitro* studies carried out with ceramic and metallic particles have also shown decreased TLR-9 expression in particle-induced inflammation⁴⁸. TLR-9 mRNA expression decreased when macrophage-colony stimulating factor treated rat macrophages were stimulated with titanium particles³⁴. Interestingly, in Takagi's *in vitro* study, TLR-4 mRNA expression by macrophages did not increase when the cells were challenged with titanium particles. However Takagai's *in vitro* experiments used titanium (metallic) particles whereas our current experiments used PE particles (a polymer) in a murine calvarial model.

In the current investigation, we observed an 11% decrease in the murine calvarium bone volume over a 7-day period of particle-exposure, which is consistent with our previous observation of a 16% bone volume decrease over a 2 week-period using a similar technique⁴⁹.

Though much has been reported about the mechanisms that promote the differentiation of osteoclasts, comparatively little is known about negative regulation, especially in the setting of inflammation. Ji et al reported that TLR ligands suppressed osteoclastogenesis by inhibiting RANK expression, which in turn limited bone resorption during inflammation⁵⁰. These results support the hypothesis that TLR suppression after particle phagocytosis can protect surrounding tissues from an unregulated host inflammatory response. Thus, uncontrolled TLR stimulation leading to persistent acute and chronic inflammation must be countered by homeostatic mechanisms for survival of the organism.

Limitations of the current study include the risk of false-positive staining that would have obscured our staining due to our use of a highly sensitive avidin-biotin complex immunohistochemistry protocol. This risk was controlled with the use of control slides that left out the primary antibody to ensure specificity of our system. Control slides that left out the biotinylated secondary antibody were also used to ensure that there was no endogenous biotin activity. Control slides that left out the avidin-horseradish peroxidase complex were also used to ensure that there was no endogenous peroxidase activity.

Other limitations of our current study include the use of a short-term, single dose animal model that simulates the complex biological picture of particle disease in humans in which prosthetic implants are subjected to waves of cyclic fluid pressure and continuous delivery of particles over many years^{47,51,52}. Nevertheless, the animal model did reveal TLR activation patterns that are remarkably consistent with those that have been seen in human tissues harvested from failed joint revision surgeries.

This agreement adds validity to our conclusion that TLR-2 and TLR-4 are highly expressed in our *in vivo* model of PE-induced osteolysis. We believe that future work with specific TLR-2, -4, and -9 knockout mice would further strengthen our statement that the TLR-2 and TLR-4 but not TLR-9 signaling pathways are highly involved in PE particle-induced inflammation in the murine calvarium model. The use of TLR knockouts has proven fruitful in other clinical scenarios. For example, corneal inflammation is mitigated in TLR-2^{-/-} and TLR-9^{-/-} mice compared with wild type mice⁵³. Strategic interventions in which TLR activation could be favorably modulated might have important implications for wear particle disease associated with TJRs.

CONCLUSION

This study demonstrates that TLR-2 and TLR-4 but not TLR-9 are highly expressed when murine calvaria are exposed to PE particles for 7 days *in vivo*. Particle exposure was also associated with decreased bone volume and bone mineral density. Modulation of TLRs may provide a potential therapeutic target for the prevention of polyethylene particle-induced inflammation and periprosthetic osteolysis.

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Figure 1.

PE particle-induced osteolysis in C57BL/6 mice as assessed within the volume of interest (VOI) by longitudinal 3D micro-computed tomography (microCT). The VOI is indicated by the yellow shaded region (a), with the 2-dimensional borders indicated in the coronal plane (b), axial plane (c) and sagittal plane (d).

Figure 2.

Surgical implantation of PE particles over the calvaria of C57BL/6 mice induced bone resorption after 7 days. Representative micro-computed tomography image of the parietal bones viewed in the axial plane of a control mouse (a) and a PE particle-treated mouse (b). (c) Graphical representation of micro-computed tomography quantifying the intra-individual change (delta) in bone volume (BV) induced by injecting saline solution alone (PE-) versus PE particles in saline solution (PE+) onto the calvarium (n=10 per group, * = $P < 0.0001$).

Figure 3.

Immunohistochemistry assay. Representative sections stained with mouse anti-TLR-2, anti-TLR-4, and anti-TLR-9 primary antibodies. Large numbers of TLR-2 and TLR-4 positive cells could be observed in calvaria following PE particle exposure (arrows) as opposed to the controls without particles. No TLR-9 positive cells could be detected in either PE-particle exposed and control calvaria. Avidin-biotin complexes were conjugated with peroxidase and reacted with substrate (red-brown). Original magnification = 20X.

Figure 4.

Graphical representation of immunohistochemistry assay showing a significantly greater number of TLR-2 and TLR-4 positive cells in the PE-treated group in comparison to the control group (n=10 per group, * = $P < 0.0001$).