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Role of direct estrogen receptor signaling in wear particle-induced osteolysis

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Abstract

Estrogen withdrawal following surgical ovariectomy was recently shown to mitigate particle-induced osteolysis in the murine calvarial model. Currently, we hypothesize that estrogen receptors (ERs) were involved in this paradoxical phenomenon. To test this hypothesis, we first evaluated polyethylene (PE) particle-induced osteolysis in the murine calvarial model, using wild type (WT) C57BL6J female mice, ER α deficient (ER α KO) mice, and WT mice either treated with 17 β -estradiol (E2) or with the ER pan-antagonist ICI 182,780. According to micro-CT and histomorphometry, we showed that bone resorption was consistently altered in both ER α KO and ICI 182,780 treated mice as compared to WT and E2 groups. Then, we demonstrated that ER disruption consistently decreased both PE and polymethylmethacrylate (PMMA) particle-induced production of TNF- α by murine macrophages *in vitro*. Similar results were obtained following ER blockade using ICI 182,780 in RAW 264.7 and WT macrophages. ER disruption and pre treatment with ICI 182,780 resulted in a consistent down-regulation of particle-induced TNF- α mRNA expression relative to WT macrophages or untreated RAW cells. These results indicate that the response to wear particles involves estrogen receptors in female mice, as part of macrophage activation. Estrogen receptors may be considered as a future therapeutic target for particle-induced osteolysis.

Keywords

Osteolysis; Wear debris; Estrogen receptor; Macrophages; Polyethylene

1. Introduction

Aseptic loosening secondary to periprosthetic osteolysis represents the leading cause of failure of total joint replacements in the long-term [1]. The development of periprosthetic osteolysis is driven by adverse reactions from the host toward particulate wear debris delivered from an articulating surface of a total joint replacement. Upon activation by wear particulate debris, macrophages release an array of cytokines and pro-inflammatory mediators that result in the recruitment, multiplication, differentiation, and maturation of osteoclast precursors, leading to bone resorption and eventual implant failure [2,3].

Although it is widely accepted that generation of polyethylene particles by the bearing couple correlates with the risk for revision due to aseptic loosening [4,5], a high variability in the incidence of aseptic loosening and size of the periprosthetic osteolytic lesions has been observed between individuals with similar PE wear rates [6]. Hence, the potential role for individual factors such as genetic background [7,8] and genetically determined obesity [9] to influence the development of particle-induced osteolysis has been advocated.

Recently, we evaluated the bone response to particulate wear debris in an ovariectomy (OVX) mouse model [10]. We found a significant decrease in osteolysis in this model, indicating that OVX, although known as an experimental model of menopause, could be protective against wear debris-induced osteolysis. Furthermore, we showed the biologic pathway leading to particle induced bone resorption, driven by cytokines such as IL-1 β , IL-6, and TNF- α , was consistently altered in estrogen deficient mice [11], suggesting a regulation at the cellular level.

Most biological effects of estrogens are mediated by two subtypes of nuclear receptors, described as estrogen receptor (ER) α and ER β , both being members of the nuclear steroid hormone receptor gene superfamily [12–14]. Nuclear estrogen-bound ERs act as transcription factors regulating expression of specific target genes [15–18]. Although the molecular role of bone ERs in the osteoprotection induced by estrogen remains unclear, it has been suggested that the inhibitory action of estrogens on bone resorption is mediated by the osteoclastic ER α , through the shortened lifespan of osteoclasts in female mice. In male bones, beneficial effects of estrogens could be predominantly mediated by the osteoblastic ER α [19]. Interestingly, macrophages, among other cells of the immune system, also express estrogen receptors. *In vivo*, their activation enhances their ability to produce inflammatory mediators and cytokines in conjunction with the activation of the Toll-Like Receptor (TLR) pathway [20], which has been recently reported to play a role in the recognition of wear particles [21].

Considering these observations, we hypothesized that ERs could be involved in the biologic response to wear debris in female mice. To assess the role of ERs in particle-induced osteolysis, we employed the murine calvarial model in wild-type mice treated with supra-physiological doses of estrogen or ER antagonist, and ER α knock out mice. In an attempt to shed light on the importance of macrophages, we secondly evaluated the *in vitro* inflammatory response to wear debris by macrophages subjected to either ER disruption, stimulation or blockade.

2. Materials and methods

2.1. Preparation of wear debris particles

For the *in vivo* study, we used conventional non-highly cross-linked UHMWPE (PE) particles obtained from joint simulator tests and isolated according to an established protocol [22]. Briefly, the particles were isolated by density gradient centrifugation and incubated with 95% ethanol overnight for sterilization. Then, frozen aliquots of the particles containing solution were lyophilized for 4–7 days. The dried material was digested in 5 M 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 were ultrasonicated and centrifuged again. Particles were then re-suspended in 95% ethanol, which was evaporated completely, and ultimately washed in 70% ethanol. This treatment resulted in negative testing for endotoxins using a quantitative Limulus Amebocyte Lysate (LAL) Assay (Bio-Whittaker, Walkersville, MD), at a detection level of <0.05 EU/ml. The mean diameter of the particles was $1.0 \pm 0.1 \mu\text{m}$ (mean $\pm$ SEM) measured by electron microscopy. For the purpose of the *in vivo* study, PE particles were re-suspended in phosphate buffered saline prior to implantation. The concentration of PE particle stock solution was 30 mg/ml, and using the density value of 0.94 g/ml, we calculated the appropriate volume to administer 4×10^8 particles to the calvaria of each animal.

For the purpose of the *in vitro* study, we used both PE particles, prepared as described above, and PMMA particles, since these particles have been used extensively by our group and others, and have been well documented for their ability to activate macrophages to release pro-inflammatory cytokines *in vitro* [21]. Briefly, PMMA particles (Polysciences, Warrington, PA, USA) 1–10 μm in diameter, were washed with 70% ethanol and incubated overnight with shaking at 4 °C. The particles were then washed extensively with phosphate-buffered saline (PBS) and resuspended to obtain a concentrated 5% v/v stock solution. Also, the particles were free of endotoxin according to the LAL Assay (BioWhittaker).

2.2. Animal surgery and in vivo protocol

Forty wild-type (WT) C57BL/6J female mice, aged 9–10 weeks, were used. To evaluate the consequences of estrogen receptor alpha depletion on particle-induced osteolysis, we also obtained 10 estrogen receptor alpha knockout (ER α KO) age-matched female mice (strain B6.129P2-*Esr1*^{tm1Ksk/J}). The generation of ER α KO mice has been described previously [23]. All animals were purchased from the Jackson Laboratories (Bar Harbor, ME, USA). Animals were housed in quarantine 48 h prior to experiment under local vivarium conditions (24 °C and 12 h/12 h light/dark cycle). All mice were handled according to international guidelines for care and use of laboratory animals. All experiments followed protocols approved by the Animal Facilities of Stanford University.

Surgical implantation of PE particles has been previously described in details [9]. Briefly, all mice were operated on general anesthesia via inhaled isoflurane 2–3% at 1 l/min. A $0.5 \times 0.5 \text{ cm}^2$ area of periosteum was exposed using a direct approach over the shaved and cleaned calvaria. The periosteum was preserved in all cases. In each experimental group, 5 animals received 100 μl of the PE particle suspension and another 5 animals received saline

alone (100 μ l), and were defined as “internal controls”. The incision was closed using 3–0 Ethylon sutures (Ethicon Inc., Somerville, NJ, USA).

At the time of surgery, 10 WT mice (5 saline-implanted, 5 PE-implanted) were implanted in the lateral side of the neck with subcutaneous hormone pellets (Innovative Research of America, Sarasota, FL, USA) designed for slow release of 8 μ g/day of exogenous 17 β -estradiol (E2) for a total of up to 15 days. The 8- μ g/day E2 dose was chosen to assess the impact of supra-physiological levels of E2 on particle-induced osteolysis. Ten WT mice (5 saline-implanted, 5 PE-implanted) were implanted with placebo vehicle pellets.

To test further the implication of estrogen receptors signaling in particle-induced osteolysis, the synthetic ER pan-antagonist fulvestrant (Faslodex[®]) ICI 182,780 (Sigma–Aldrich, St. Louis, MO, USA) was applied in 10 WT mice (5 saline-implanted, 5 PE-implanted). Currently, we employed ICI 182,780, an ER antagonist that, unlike other anti-estrogens such as tamoxifen, has no known estrogen agonist effect. After binding, ICI 182,780 induces a conformational change of the ER thus reducing its ability to modulate gene transcription and increasing ER degradation [24]. The ER antagonist ICI 182,780 was applied according to a protocol previously used at our Institution [25]. Starting the day of particle implantation, 10 μ l of a 100 μ M solution ICI 182,780 (in a suspension containing 0.9% normal saline, 0.01% DMSO) was injected subcutaneously in the animal’s back, 5 days a week. Vehicle only (0.9% normal saline, 0.01% DMSO) was injected in 10 littermates (5 saline-implanted, 5 PE-implanted), which were defined as “WT controls”. Table 1 shows the treatment of the different experimental groups of mice. The results are reported in four mice groups, described as “WT control”, “ER α KO”, “E2” and “ICI 182,780” groups.

After the procedure, the animals were returned to their cages where water and food were given *ad libitum*. Sacrifice was done 2 weeks after the procedure by cervical dislocation following isoflurane anesthesia. The calvariae were dissected after sacrifice. The specimen was freed of all soft tissues, and fixed in 4% neutralized paraformaldehyde prior to micro-Computed Tomography (micro-CT) and histological analyses. Also, the entire uterus was collected to assess the consequences of *in vivo* exposure to E2 or ER depletion or blockade. After the serosal fat was removed, the uterus was transected at the junction of the uterine body and cervix, and the uterine weight was recorded.

2.3. Micro-computed tomography imaging and volumetric osteolysis analysis

Calvariae were scanned using a high-resolution micro-CT system (MicroCAT II, Siemens Preclinical Solutions, Knoxville, TN, USA). The scans were individually corrected to a calibration piece placed in each scan to control for any inter-scan variability. The radiographic projections were acquired at 80 kV and 500 μ A with an exposure time of 200 ms. After scanning, 3D images were reconstructed with a voxel average size of 40 μ m using COBRA reconstruction interface software (Exxim computing corporation, Pleasanton, CA). Qualitative and quantitative data were analyzed with a global fixed threshold [26]. A cylindrical volume of interest (VOI of 6 $\times$ 6 $\times$ 2 mm) with the intersection of the sagittal suture and the coronal suture in its center was defined. For quantitative analysis of PE particle-induced osteolysis (bone volume fraction, BV/TV) within the VOI, we used eXplore MicroView V.2.0 imaging software (GE Medical, Raleigh, NC), an open source 3D volume

viewer and analysis software package. To address bone tissue mineralization following estrogen receptor stimulation, depletion or blockade, we obtained the bone mineral density (BMD, mg/cc) in saline-implanted calvariae.

2.4. Histologic evaluation of osteolysis

After scanning, calvarial bones were decalcified in 2 successive ethylene diamine tetra acetic acid (EDTA) baths, for seven days each. Calvariae were then embedded in Optimal Cutting Temperature Compound (OCT, Tissue-Tek, Sakura Finetek USA, Torrance, CA) and flash frozen in liquid nitrogen and stored at -80°C . Frozen sections ($7\text{ }\mu\text{m}$) in the coronal plane were obtained at the depth where the presence of particles was detected within the calvarial tissue using a cryostat (Cambridge Instruments, Buffalo, NY). These sections were stained with hematoxylin and eosin (H&E, Sigma–Aldrich) and a serial section stained for tartrate-specific acid phosphatase-positive (TRAP) osteoclasts (Acid Phosphatase kit, Sigma–Aldrich). Using a magnification of $20\times$, the histomorphometric analysis of each calvaria cap was performed on the most central section and on four adjacent sections. The region of interest was defined as previously recommended [9]. The sagittal suture area (SSA) was determined by tracing the area of soft tissue between the parietal bones. To determine bone thickness, sections were divided using a digital caliper in four $100\text{ }\mu\text{m}$ steps to the left and right sides of the midline suture respectively. The measurements were expressed as a percentage of the mean ratio of bone thickness to the total tissue thickness (BT/TT) of the nine regional measurements in the five adjacent sections. To quantify bone loss, results were also expressed as a percentage of the mean difference of BT/TT ((BT/TT)) between saline-implanted and particle-implanted animals in each different mice group [10]. Osteoclasts were identified as large multinucleated cells located on the bone perimeter within a resorption lacuna. Their localization was confirmed in a serial section stained for tartrate-specific acid phosphatase. The osteoclast number was measured in the region of interest of the five consecutive calvaria sections.

2.5. Murine macrophage experiments

To reproduce the *in vivo* experimental scenario, macrophages from the RAW 264.7 murine cell line and primary murine macrophages obtained from WT and from ER α KO mice were exposed to either PE or PMMA particles for 24 h.

The murine monocyte/macrophage cell line RAW 264.7 was cultured in DMEM (Invitrogen) containing 10% (v/v) fetal bovine serum (FBS), and GlutaMAX (Invitrogen), and maintained in a 5% CO_2 atmosphere at 37°C , for 7 days. Before treatment, medium was switched to phenol red free medium (white medium) since phenol red is known to activate ER α gene regulation.

Macrophages were isolated from the femurs of WT C57BL/6J mice and ER α KO mice as previously described [27]. Briefly, cells were flushed from femurs and incubated in differential medium consisting of RPMI 1640 media (Invitrogen, Carlsbad, CA), 13% FBS, 5% horse serum, 30% macrophage-colony stimulating factor and 0.5% glucose for 7 days to allow proliferation and differentiation. At confluence, cells were harvested with trypsin digestion. Cells were then seeded in culture dishes in HEPES-buffered phenol red-free

RPMI-1640 media (Cellgro, Washington, DC) supplemented with 10% charcoal dextran-stripped FBS (HyClone, Rockville, IL) and 1% penicillin/streptomycin and 1% sodium pyruvate (Invitrogen) to allow adherence. Before treatment, medium was switched to white medium as described above.

RAW 264.7 cells and primary murine macrophages from WT C57BL/6J mice were pre-incubated for 24 h in phenol red-free media containing one of the following treatments: 1) Estrogen (E2, 1 nM 17 β -estradiol (Sigma–Aldrich) in ethanol/medium 1/10⁶); 2) Ethanol/medium only as a control; 3) ER pan-antagonist (1 μ M ICI 182,780 in DMSO/medium, 1/10³); 4) DMSO/medium only as a control.

Then, all cell groups were exposed to either PE or PMMA particles for 24 h, as follows. The PE particle concentration of 3 mg/ml was achieved by suspending the calculated volume of particles in 100 μ l sterile distilled water. The particle suspension was then coated directly onto 24-well culture plates, as originally described by our group [28]. The coated plates were allowed to dry overnight to completely evaporate the water. Following the pre-incubation period in each group, cells (1 $\times$ 10⁵ cells per well, with 1 ml of medium) were seeded onto the coated plate. In parallel, cells were cultured in the presence of PMMA particles (0.1 ml per well at 0.5%) for 24 h. In addition, another set of 24-well plates seeded with the corresponding cells groups received no particles and served as internal controls.

2.6. Cytokine analysis Luminex assays

After particle exposure, cytokine production by RAW 264.7 cells and primary murine macrophages was analyzed by Luminex multiplex array (Luminex, Austin, TX) following manufacturer's recommended protocol. Specifically, we used the Mouse Cytokine Magnetic 10-Plex Panel Kit (Invitrogen) that allowed us to measure 10 analytes, including GM-CSF, IFN- γ , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 and TNF- α . Supernatants from particle-challenged cell cultures were collected at 24 h. All collected supernatants were frozen at -80 °C. All Luminex assays were analyzed in triplicate (three experiments) using manufacturer's provided buffers and protocols. Data were acquired on a Luminex 200 instrument and analyzed using the resident software (xPONENT 3.1, Luminex).

2.7. Quantitative real-time polymerase chain reaction

Following either PE or PMMA particle exposure and in the internal controls, cells were lysed with Trizol Reagent (Invitrogen) and RNA was purified using RNeasy Kit (Qiagen, Valencia, CA) per the manufacturer's protocol. The total amount of RNA was quantified using spectrophotometric OD260 readings (NanoDrop, Thermo Fisher Scientific, Waltham, MA). RNA quality was assessed by light absorbance at 260 and 280 nm. To prepare RNA for polymerase chain reaction (PCR) analysis, RNA was converted to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Real-time polymerase chain reaction (RT-PCR) was performed using probe for TNF- α (Applied Biosystems). The absolute number of gene copies was normalized using 18s ribosomal RNA and the relative quantification of the genes was calculated using the "comparative C_T (threshold cycle) method" after determining the C_T values for reference and target genes.

2.8. Data analysis

All values are presented as means $\pm$ SEM. Quantitative parameters obtained from micro-CT (BVF, BMD) were analyzed by two-tailed Student's *t*-test within each group. Comparisons across unpaired groups were made according to the Mann–Whitney *U* test. Data concerning osteoclast number, sagittal suture area, and bone thickness were initially analyzed using a one-way analysis of variance (ANOVA). *Post hoc* comparison between groups used the Fisher's protected least significant difference. Cytokine concentrations and mRNAs expression were analyzed using one-way ANOVA followed by the Bonferroni/Dunnett's test. The level of statistical significance was set at $P < 0.05$.

3. Results

3.1. In vivo consequences of estrogen receptor depletion, stimulation, or blockade

At sacrifice, profound changes in uterus weight were noted, indicating the effectiveness of ER modulation. Estrogen receptor depletion in ER α KO mice resulted in a consistent decrease in uterus weight (means, 59 ± 1 mg) compared to WT controls (mean, 135.3 ± 14 mg, $P < 0.001$). Conversely, there was a significant increase in uterus weight in E2-treated mice (mean, 250 ± 17 mg) compared to WT control animals ($P < 0.0001$). Estrogen receptor blockade using ICI 182,760 resulted in a consistent decrease in uterus weight (91 ± 0.8 mg) ($P = 0.001$) relative to WT controls (Fig. 1a).

Estrogen treatment induced no significant modification in bone mineral density (mean, 44.8 ± 3 mg/cc) as compared to WT controls (mean, 51.7 ± 1 mg/cc) ($P = 0.07$). Conversely, both ER depletion and blockade induced a significant decrease in BMD in calvariae without PE particles as compared to WT controls (Fig. 1b). Specifically, mean BMD values were 34.2 ± 6 mg/cc ($P = 0.03$) and 39.7 ± 5 mg/cc ($P = 0.04$), in ER α KO mice and in ICI 182,780 treated mice, respectively.

3.2. Micro-CT evaluation of osteolysis

As assessed by longitudinal 3D micro-CT, the presence of PE particles induced profound changes in calvarial bone microarchitecture in all groups (Fig. 2). In particle-implanted animals, the bone volume fraction (BVF) significantly decreased within the VOI as compared to saline-implanted animals in WT control group (0.07 ± 0.007 vs 0.12 ± 0.004 , $P = 0.01$) and in E2 treated group (0.06 ± 0.01 vs 0.13 ± 0.02 , $P = 0.001$) (Fig. 3). In contrast, decrease in BVF following particle implantation was consistently less marked in the ER α KO group as compared to internal controls (0.11 ± 0.01 vs 0.13 ± 0.02 , $P = 0.17$), and even less marked in the ICI 182,780 group (0.13 ± 0.008 vs 0.14 ± 0.01 , $P = 0.26$).

3.3. Histology

Histological sections showed consistent erosion and profound remodeling of the calvarial bone, mainly in normal controls and in E2-stimulated mice (Fig. 4). However, bone remodeling in response to particles appeared limited in ER α KO and ICI 182,780 groups. TRAP staining confirmed the presence of osteoclasts located in resorptive lacunae.

Histomorphometric data are presented in Table 2. According to one-way ANOVA, we found a significant difference for the sagittal suture area ($P < 0.001$). The presence of PE particles induced a significant increase in SSA in WT control group ($P = 0.02$), in E2-stimulated group ($P = 0.03$), and in ER α KO group ($P = 0.04$). No significant difference was found in SSA between saline-implanted and PE-implanted ICI 182,780 treated mice ($P = 0.13$). Two-way ANOVA revealed a significant effect of PE particles on SSA ($P < 0.001$). No independent effect of either ER α knock out or E2 treatment or ICI 182,780 treatment on SSA could be detected in the absence of particles (Two-way ANOVA, $P = 0.4$).

One-way ANOVA showed a significant difference for osteoclast number ($P < 0.05$). The presence of PE particles induced a consistent increase in osteoclast number in WT control group ($P = 0.03$), in E2-stimulated group ($P = 0.03$) and in ER α KO group ($P = 0.04$), but not in ICI 182,780 treated group ($P = 0.31$). No independent effect of either ER α knock out or E2 treatment or ICI 182,780 treatment on osteoclast number could be detected in the absence of particles (Two-way ANOVA, $P = 0.8$).

There was a significant difference for bone thickness (one-way ANOVA, $P < 0.01$). Implantation of PE particles resulted in a marked decrease in BT/TT in WT control group ($P = 0.03$), in E2-stimulated group ($P = 0.035$), and in ER α KO group ($P = 0.004$), but not in ICI 182,780 treated group ($P = 0.41$). No independent effect of either ER α knock out or E2 treatment or ICI 182,780 treatment on BT/TT could be detected in the absence of particles (Two-way ANOVA, $P = 0.3$).

3.4. Production of cytokines in RAW 264.7 cells and in primary murine macrophages

Following 24 h-exposure to PE or PMMA particles, the production of only TNF- α and IL-4 could be detected according to the Multiplex Luminex assay. The 24 h-exposure to either PE or PMMA particles induced a consistent increase in TNF- α expression in most of the cell culture groups compared to the respective internal controls (Fig. 5). In the RAW cell line, TNF- α was undetectable in the control group in the absence of particle. Following PE particle exposure, TNF- α rates reached 381.7 ± 33 pg/ml (range, 327–456 pg/ml) in the control ($P < 0.001$), and increased from 116 ± 19 pg/ml (range, 91–159 pg/ml) to 392 ± 51 pg/ml (range, 298–500 pg/ml) in the E2-treated group ($P = 0.005$). Conversely, no significant increase in TNF- α concentration could be shown in the ICI 182,780 treated group following PE particle exposure (82.7 ± 10 pg/ml vs 84.7 ± 8 pg/ml, $P = 0.45$). Relative to the control and to the E2-treated group, the blockade of ERs by ICI 182,780 significantly decreased the PE particle-induced production of TNF- α ($P < 0.01$ and $P < 0.0001$, respectively). PMMA particle stimulation resulted in a significant release of TNF- α in all RAW cells groups. TNF- α concentrations reached 1027 ± 85 pg/ml (range, 831–1148 pg/ml, $P < 0.001$ relative to the internal control), 557 ± 62 pg/ml (range, 416–655 pg/ml) ($P < 0.005$) and 201 ± 28 pg/ml (range, 159–266 pg/ml) ($P = 0.01$), in the control, the E2 and the ICI 182,780 treated groups, respectively. However, TNF- α production was significantly mitigated by E2 treatment ($P = 0.009$, as compared to the control), this effect being even more pronounced in ICI 182,780 treated cells ($P < 0.001$) (Fig. 5a).

In primary murine macrophages, TNF- α release following either PE or PMMA particle exposure was observed in all groups (Fig. 5). Following PE particle exposure, TNF- α

concentration increased from 159 ± 61 pg/ml (range, 76–300 pg/ml) to 891 ± 200 pg/ml (range, 200–1977 pg/ml) ($P = 0.03$), from 0 pg/ml to 36 ± 15 pg/ml (range, 14–58 pg/ml) ($P = 0.02$), from 151 ± 21 pg/ml (range, 121–181 pg/ml) to 329 ± 68 pg/ml (range, 183–456 pg/ml) ($P = 0.002$), and from 28 ± 9 pg/ml (range, 14–41 pg/ml) to 54 ± 14 pg/ml (range, 14–86 pg/ml) ($P = 0.165$), in the control group, the ER α KO, the E2 and the ICI 182,780 group, respectively. As compared to the WT control group, there was a significant decrease in PE particle-induced production of TNF- α in the ER α KO, the E2 and the ICI 182,780 treated groups ($P = 0.01$, $P = 0.03$, and $P = 0.001$, respectively). Following PMMA particle exposure, TNF- α concentration reached 361 ± 127 pg/ml (range, 86–673 pg/ml), 21 ± 1.2 pg/ml (range, 19–23 pg/ml), 273 ± 117 pg/ml (range, 63–526 pg/ml), and 30 ± 12 pg/ml (range, 0–45 pg/ml), in the WT control, ER α KO group, E2 treated group and ICI 182,780 treated group, respectively. Relative to WT controls, ER α KO deficiency and ER blockade were associated with a significant decrease in PMMA particle-induced production of TNF- α ($P < 0.01$ and $P = 0.02$, respectively).

ER α KO macrophages were found to express interleukin (IL)-4, both in the absence and in the presence of PE and PMMA particles. However, IL-4 expression significantly increased following PE and PMMA particles stimulation (309.1 ± 28.7 pg/ml and 669 ± 51 pg/ml, respectively) relative to internal controls (50 ± 12.4 pg/ml; $P = 0.01$ and $P < 0.001$, respectively). The expression of IL-4, a well-known anti-inflammatory cytokine, able to promote macrophage conversion from an M1 to an M2 phenotype, suggests a potential control mechanism in the mitigation of inflammatory response to PE and PMMA particles in ER α KO murine macrophages.

3.5. Quantitation of TNF- α and IL4 mRNA expressions in RAW 264.7 and primary murine macrophage cultures

Having observed that ER modulation could mitigate the particle-induced production of TNF- α and IL4 in RAW 264.7 cell and primary murine macrophage cultures, we next explored the corresponding mRNA expressions. In RAW cells, the presence of either PE or PMMA particles stimulated TNF- α mRNA expression in all groups (Fig. 5). In the controls, particle-stimulated cells expressed TNF- α mRNA 35–38 fold more than in the internal control. Similarly, E2 treatment resulted in a 15–17 fold more TNF- α mRNA expression relative to internal controls, whereas, ICI 182,780 treatment induced only a mild change in TNF- α mRNA expression in the presence of PE and PMMA particles (0.14–0.52 fold more, respectively).

In primary macrophage controls, the presence of particles consistently increased TNF- α mRNA expression (49 and 50 fold more), whereas it increased 28 and 33 fold in the E2 treated group, for PMMA and PE particles respectively. Conversely, both ER α KO and blockade by ICI 182,780 induced almost no modification in TNF- α mRNA expression (0.57 and 1.57 fold more, and 0.35 and 0.10 fold more, for PMMA and PE, respectively) (Fig. 5).

In ER α KO primary macrophages, the presence of either PE or PMMA particles consistently stimulated IL-4 mRNA expression (47 and 63 fold more, for PE and PMMA, respectively).

4. Discussion

In a recent work [10], we have shown that experimental stimulation of bone turnover by surgical ovariectomy resulted in a paradoxical decrease in PE particle-induced osteolysis in the murine calvarial model. This phenomenon could be reversed in ovariectomized mice supplemented with 17 β -estradiol, suggesting the involvement of estrogen [11]. In parallel with a limitation of particle-induced bone loss, we noted a down-regulation of local bone-resorptive cytokines TNF- α and RANKL, associated with a decrease in serum pro-inflammatory cytokine IL-6. To further explore the mechanisms by which estrogen withdrawal mitigated particle-induced osteolysis, we examined whether the modulation of estrogen receptor activity could reproduce this phenomenon both in the calvarial model and *in vitro*.

Our current experiments show that the presence of PE particles induced an osteolytic reaction in calvarial bone in WT controls and in E2-treated mice, as illustrated by an extensive bone resorption observed on micro-CT and histology. In contrast, this phenomenon was considerably attenuated in ER α KO and ICI 182,780 treated mice. In the later group, we could observe almost no modification in bone volume fraction, bone thickness, and osteoclast number, following PE implantation. Interestingly, the consequences of ER modulation appeared less marked in ER α KO mice, with only 7% decrease in bone thickness relative to internal controls, as compared to -15% in WT control and +0.6% in ICI 182,780 treated animals. This suggests that blockade of both ERs α and β with the pan-antagonist ICI 182,780 was more effective to mitigate particle-induced osteolysis than the sole disruption of ER α as occurred in the genetically modified group. These findings compare favorably with our previous observation that estrogen deficiency mitigated particle-induced osteolysis [11]. Taken together, these data support the hypothesis that ER α and β activation was involved in the development of particle-induced osteolysis in female mice. This observation appears somehow contradictory with the widely accepted paradigm that estrogen provides a protection against inflammation and that ER stimulation results in anabolic effects on bone tissue, whereas their blockade or depletion increases bone resorption [29]. However, epidemiological and immunological evidence has also suggested that female sex hormones play a role in the etiology and course of chronic inflammatory diseases such as rheumatoid arthritis, illustrating the immunomodulating action of estrogens [30–32].

Having observed that particle-induced osteolysis was consistently altered by ER disruption or blockade, we asked whether macrophages were involved in this process. Hence, the monocyte/macrophage cell lineage is widely recognized as central in wear debris recognition and phagocytosis, and initiation of inflammatory cascade that ultimately results in osteoclast hyperactivity [2]. In addition, most of the cellular components of the immune system, including macrophages, have been shown to express ERs [31], and therefore implicates the macrophage as a potential target for the estrogen-mediated inflammatory response. Currently, we found that TNF- α release following PE or PMMA particles exposure was consistently altered in ER α KO macrophages and after a 24 h ICI 182,780 treatment in both RAW cells and primary murine macrophage cultures. This was associated with a decrease in corresponding mRNA expression, suggesting a regulation at the cellular

level. We believe that TNF- α expressed by particle-stimulated macrophages was central to the regulation of subsequent bone resorption since this pro-inflammatory cytokine is reportedly crucial to both particle-induced osteolysis and estrogen deficiency-induced bone loss [33–37]. Furthermore, we noted that IL-4 expression in ER α KO macrophage cultures was stimulated by the presence of particles, in parallel with a decrease in TNF- α production. IL-4 is a key factor for the differentiation of naïve T helper (Th) cells into the Th2 type [38], and, similarly, has been shown to induce macrophage polarization from an M1 pro-inflammatory phenotype towards an M2 anti-inflammatory phenotype [39]. Thus, IL-4 appears to play an immunomodulatory role by promoting anti-inflammatory biologic response. Consistent with our observations, IL-4 has been previously shown to down-regulate TNF- α production by particle-stimulated macrophages [39]. Our finding was somehow unexpected, since the production of this potent cytokine was originally thought to be restricted to a few cell types, such as T cells, mast cells, basophils, and eosinophils [40–42]. However, it was recently shown that human alveolar macrophages may express, synthesize, and release IL-4 [43], and that IL-4 mRNA was present in rat alveolar macrophages [44]. Taken together, these data suggest that particle-stimulated IL-4 release by ER α KO macrophages may have been involved in the down-regulation of their inflammatory response, presumably through an M1/M2 polarization scheme.

We could not observe any significant differences in terms of bone volume fraction, bone thickness and osteoclast number in mice challenged with supraphysiological doses of E2 compared to WT control following PE particle implantation, indicating an absence of anti-inflammatory effect due to exogenous estrogen. Conversely, *in vitro*, E2 treatment was associated with a decrease in TNF- α expression, both at the proteomic and at the mRNA levels, in the majority of macrophage groups challenged with particles. This data sheds light on the paradox that immunomodulatory effects of E2 need to be considered in the context of both duration and level of exposure [32].

One potential mechanism by which E2 modulation influences particle induced osteolysis may be related to the modulatory effect of E2 on TLR-4 expression on macrophages [32]. TLRs are a family of different germ-line encoded transmembrane receptor proteins that enable the innate immune system to recognize numerous foreign and infectious components, such as endotoxin (lipopolysaccharides, LPS) [45–50]. TLR-4-mediated recognition of LPS leads to the activation of the mitogen activated protein kinase (MAPK) pathway via myeloid differentiation primary response protein 88 (MyD88), an adapter protein that couples the TLR to downstream signaling kinases [51]. Interestingly, high expression of TLR-4 has been detected in monocyte/macrophages of synovial-like membranes surrounding loose implants [52–54]. The implication of TLRs in wear debris recognition has been recently suggested with the finding that inhibition of MyD88 could decrease PMMA particle-induced production of TNF- α in RAW 264.7 murine macrophages. As a result, MyD88 $^{-/-}$ mice developed less particle-induced osteolysis than wild type mice, supporting the idea that the biological response to PMMA particles was dependent in part on MyD88 signaling [21]. Furthermore, removal of endogenous estrogens using surgical OVX in a murine model was shown to decrease cell surface expression of TLR-4 on macrophages, as determined by flow cytometry [32]. Not only these changes were dramatically reversed by administration of

17 β -estradiol, but OVX animals supplemented with 17 β -estradiol also exhibited an increase in endotoxin susceptibility as compared to controls or OVX animals that did not receive 17 β -estradiol. Also, a 24 h treatment with 17 β -estradiol failed to elicit significant effects on cell surface TLR-4 expression on RAW 264.7 cells and on primary murine macrophages derived from either OVX or sham-OVX animals. These observations are consistent with our finding that E2-treated macrophages challenged with particles tended to express less pro-inflammatory cytokine TNF- α compared to WT controls. Taken together, these results indicate that the response to wear particle could be consistently modulated through ERs, presumably as part of the TLR signaling pathway.

The current *in vivo* experiment has several limitations, one being that the ER α knock out model employed is known to present an incomplete gene deletion resulting in truncated receptors, still able to bind estradiol [23]. Although estrogen insensitivity has been proven in this model [23], it is not possible to confirm here that estrogen agonist effects were completely abolished. This may also explain that mitigation of particle-induced OL was only partial in ER α KO mice as compared to ER pan-antagonist treated mice. Other known limitations of the murine calvarial model may have affected our findings. Briefly, these include the differences in calvarial bone tissue relative to long bone, with respect to cells, matrix composition and mineralization. In addition, given the lack of implant, mechanical load or fluid pressure, the absence of continuous release of PE particles as opposed to the clinical scenario, the clinical relevance of our data should be considered with caution. Furthermore, since ERs are expressed in macrophages as well as osteoclasts, and although we showed that the release of TNF- α by particle-stimulated murine macrophages was consistently altered by ER modulation, it is still unclear whether decreased differentiation and survival of ER α KO or ICI 182,780 treated osteoclasts could have been involved in particle-induced mitigation. It is unknown whether ICI 182,780 treatment would result in the similar protective effects towards wear debris in male mice. In addition, it is still unclear whether our findings would be transposable to human macrophages. This question is currently the focus of ongoing studies in our laboratory.

In our hands, complete blockade of ERs was proven to dramatically mitigate particle-induced biologic response, as revealed by its protective effect on bone volume in the murine calvarial model, paralleled with a down regulation of TNF- α release in corresponding macrophage cultures. This molecule is indicated as a second line therapy for post menopausal women with advanced breast cancer who have progressed following prior endocrine therapy, and is administered intramuscularly monthly [55]. The finding that ICI 182,780 may positively influence peri-articular inflammatory response has recently been suggested in an experimental rat model of temporomandibular joint (TMJ) inflammation [56]. Using Freund's complete adjuvant to induce TMJ inflammation in female rats, authors showed that blocking the estrogen receptor partially reversed inflammatory responses, notably the transcription of NF- κ B target genes in the synovial membrane, including TNF- α mRNA. Interestingly, the supplementation of ovariectomized rats with 17 β -estradiol aggravated all of the inflammatory criteria, specifically the DNA-binding activity of NF- κ B in the synovial membrane. In line with our findings, this point underscores again the dual role of estrogens, notably in peri-articular inflammatory diseases.

5. Conclusions

Our data suggest that estrogen receptors are involved in the modulation of particle-induced osteolysis. This conclusion is supported by evidence demonstrating that blockade of estrogen receptors using a nonselective antagonist consistently altered osteolysis, whereas E α depletion only partially mitigated osteolysis. Exogenous estrogen treatment revealed dual properties, with, on one hand, the inability to modulate inflammatory biologic response to PE particles *in vivo*, and on the other hand, a global trend to decrease pro-inflammatory cytokine TNF- α expression by murine macrophages exposed to wear debris particles. Furthermore, ICI 182,780 treatment decreased macrophage TNF- α release *in vitro* following particle exposure. These results indicate that estrogen receptors may be considered as a future therapeutic target for the mitigation of wear-debris particle-induced OL.

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Fig. 1.

Estrogen receptor (ER) modulation consistently impacted mice uterus weight and calvarial bone mineral density (BMD) in the absence of PE particles, as assessed by 3D micro-CT. (a) ER α knock out or blockade using a 2 week-ICI 182,780 treatment resulted in a decrease in uterus weight relative to WT controls. Conversely, a 2 week-exogenous E2 treatment increased uterus weight, revealing the anabolic effect of 17 β -estradiol on this target organ ($n = 10$ per group). (b) Bone mineral density decreased following ER α knock out or blockade relative to WT controls, whereas it was not modified in E2-supplemented animals ($n = 5$ per group). * = $P = 0.01$, ** = $P < 0.0001$, *** = $P < 0.05$, as compared to WT control.

Fig. 2.

Both ER α knock out and blockade diminish PE particle-induced osteolysis in mice calvaria (a) PE particle-induced osteolysis in the murine calvarial model as assessed within the volume of interest (VOI) by longitudinal 3D micro-computed tomography (micro-CT). The VOI is indicated by the yellow shaded cylindrical region, with the intersection of the sagittal suture and the coronal suture in its center (red dot). (b) Representative reconstructed images of the calvarial VOI, two weeks after saline-implantation (PE (-)) or poly-ethylene particle-implantation (PE (+)) in different mice groups are shown ($n = 5$ per group). Of note is the consistent extent of bone resorption area following PE particles implantation in wild-type (WT) controls and estrogen-treated animals (E2), while bone resorption area appears limited in animals knock out for estrogen receptor α -gene (ER α KO) and mice treated with estrogen receptor antagonist (ICI 182,780). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

Effect of polyethylene particles on bone volume fraction (BV/TV), as assessed by longitudinal 3D micro-CT. Bone volume fraction consistently decreased in the wild-type (WT) controls and in the E2 treated mice, while it did not significantly differed in the estrogen receptor α knock out group (ER α KO) and the estrogen receptor antagonist treated mice (ICI 182,780), as compared to the respective saline-implanted mice ($n = 5$ per group). * = $P = 0.01$, ** $P = 0.001$, relative to internal control.

Fig. 4.

Effect of polyethylene (PE) particles on calvariae, as assessed by histology. Representative stained calvarial histology (hematoxylin and eosin, HE), and tartrate-specific acid phosphatase-positive, TRAP is presented at 100× original magnification ($n = 5$ per group). Polyethylene particles induced osteolysis, characterized by a consistent decrease in calvarial bone thickness in wild-type (WT) controls and E2 treated mice, while it was moderate in estrogen receptor α knock out mice and estrogen receptor antagonist treated mice. PE (-): saline-implanted; PE (+): PE-implanted.

Fig. 5.

The inflammatory response after a 24 h-exposure to polyethylene (PE) and PMMA particles in RAW 264.7 cells and primary murine macrophages is influenced by estrogen receptor (ER) modulation. In the RAW 264.7 cell line, (a) 24 h pre-treatment with ER pan-antagonist (ICI 182,780) resulted in a consistent decrease in TNF-α protein production (3 independent trials) relative to control. Estrogen pre treatment (E2) induced no change in PE particle-stimulated cells at the protein level relative to non treated cells, whereas it decreased TNF-α release in PMMA particle-stimulated cells. (b) Pre-treatment with ER pan-antagonist resulted in a consistent decrease in TNF-α mRNA expression (2 independent trials). TNF-α mRNA expression was downregulated in both PE and PMMA stimulated cells after E2 pre treatment. Fold change is relative to the mRNA expression level of each cell type prior to either PE or PMMA particle exposure. (c) In the primary murine macrophage cultures, both ERα knock out and ICI 182,780 pre treatment decreased TNF-α protein expression (3 independent trials, $n = 5$ per trial) relative to WT control. Estrogen pre treatment resulted in decreased expression of PE particle-induced TNF-α protein production. (d) Both ERα knock out and ICI 182,780 pre treatment decreased TNF-α mRNA expression (2 independent trials, $n = 5$ per trial). Estrogen pre treatment resulted in a decrease in both PE and PMMA particle-induced TNF-α mRNA expression. * = $P < 0.01$, ** = $P < 0.001$, *** = $P < 0.0001$, *a* = $P < 0.05$.

Table 1

Experimental mouse groups.

	WT control	ERαKO	E2	ICI 182,780	Total (n)
PE (-)	5	5	5	5	20
PE (+)	5	5	5	5	20
Total (n)	10	10	10	10	40

WT, wild-type; ERαKO, estrogen receptor α knockout; E2, 17β-estradiol; ICI 182,780, estrogen receptor antagonist; PE (-), saline-implanted; PE (+), PE-implanted;
* the WT Control group refers to the group of C57BL/6J mice that received vehicle only.

Table 2

Histomorphometric results for each mouse group.

	WT control		ERαKO		E2		ICI 182,780	
	PE(-)	PE(+)	PE(-)	PE(+)	PE(-)	PE(+)	PE(-)	PE(+)
SSA (mm ²)	0.036 ± 0.01 (0.014–0.074)	0.115 ± 0.02 (0.065–0.185)	0.048 ± 0.02 (0.01–0.097)	0.08 ± 0.015 (0.05–0.16)	0.033 ± 0.01 (0.014–0.078)	0.08 ± 0.001 (0.07–0.088)	0.05 ± 0.02 (0.01–0.135)	0.09 ± 0.06 (0.01–0.18)
BT/TT	56 ± 3.5% (48%–61%)	41 ± 6.7% (16%–73%)	63 ± 10% (12%–91%)	54 ± 11% (11%–97%)	56 ± 12% (24%–100%)	42 ± 7.5% (13%–66%)	45 ± 12% (23%–94%)	46 ± 11% (14%–98%)
(BT/TT)	-15 ± 7% (-40%–17%)		-7 ± 2.3% (-51%–38%)		-12.7 ± 4.7% (-55%–11%)		+0.6 ± 0.12% (-57%–46%)	
Oc Nb	0.33 ± 0.3 (0–2)	1.16 ± 0.55 (0–4)	0.65 ± 0.4 (0–3)	1.2 ± 0.5 (0–3)	0.42 ± 0.2 (0–1)	1.1 ± 0.5 (0–3)	0.6 ± 0.3 (0–2)	0.75 ± 0.4 (0–3)

Values are expressed as mean ± standard error of the mean (extremes). WT, wild-type; ERαKO, estrogen receptor α knockout; E2, 17β-estradiol; ICI 182,780, estrogen receptor antagonist; PE (-), saline-implanted; PE (+), PE-implanted; SSA, sagittal suture area; BT, bone thickness; TT, tissue thickness; Oc Nb, osteoclast number.