Chapter 2
A Bone Remodeling Lab-on-a-Chip to Study Periprosthetic Osteolysis: Early Evidence that Osteocyte Signaling Contributes to Increased Osteoclast Resorption

M. M. Saunders

Abstract Total joint replacement surgeries are highly prevalent throughout the United States. However, it is estimated that within 10 years of surgery, 20% to 30% of patients will present with a complication known as periprosthetic osteolysis (PPOL), or a degradation of the bone surrounding the implant [1]. Patients experiencing PPOL often require revision surgeries, which are associated with higher rates of morbidity and mortality [2]. Thus, there is a great need for therapeutic or pharmaceutical approaches that could delay the need for revision arthroplasties. Currently, the mechanisms that drive this osteolytic process are not fully understood. However, evidence suggests that particulate debris generated from repetitive wear of implant surfaces leads to inflammation within the joint, which ultimately disrupts bone remodeling. Remodeling is a lifelong metabolic process involving three bone cell types (osteocytes, osteoclasts, and osteoblasts) that maintains bone homeostasis by tightly coordinating bone resorption and formation in a dynamic loading environment. Furthermore, mechanical load has also been shown to play a crucial role in bone remodeling and long-term implant stability. The goal of this project was to develop a lab-on-a-chip (LOC) platform that recapitulates the bone dynamic microenvironment and allows for analysis of multicellular signaling processes that lead to PPOL. This *in vitro* system will allow us to study complex signaling between osteocytes, osteoclasts and osteoblasts while eliminating confounding factors present in *in vivo* studies. Following development, the LOC platform was utilized to preliminarily investigate the effects of particulate debris in a dynamic loading environment on osteocyte signaling and the subsequent effect on osteoclast resorption.

Keywords Lab-on-a-Chip · Periprosthetic Osteolysis · Bone Remodeling · Mechanobiology

Introduction

One of the leading causes of late-stage joint failure is aseptic loosening, a condition in which the bond between the bone and the implant fails in the absence of infection. This loosening is often associated with PPOL, or degradation of the bone tissue surrounding the implant. While the mechanisms that drive these processes are not fully understood, many studies have shown that particulate debris generated from wear of the implant surfaces triggers an inflammatory response within the joint, which ultimately leads to bone loss [3–4]. Additionally, long-term implant survival is dependent on the physical activity of the patient. This highlights the crucial role of mechanical load on bone metabolism and long-term implant stability. A more thorough and comprehensive understanding of the underlying mechanisms that regulate load-induced bone remodeling and implant debris-induced osteolysis may lead to novel interventions that mitigate late-stage joint failure.

Background

An investigation of the mechanisms responsible for PPOL requires a deeper understanding of the complex multicellular signaling cascades that regulate bone regeneration. This lifelong metabolic process, known as bone remodeling, is necessary

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for maintaining the structural integrity of the skeletal system. During this complex multicellular process, old or damaged bone is removed (resorbed) by osteoclasts and new bone is deposited by osteoblasts. A tight coordination between these two processes is required for maintaining overall bone mass. Osteocytes, which account for more than 90% of the total bone cells, play a critical role in this coordination effort. Embedded within the bone matrix, osteocytes exhibit a high degree of interconnectivity and are responsive to external stimuli, such as mechanical load. Once stimulated, osteocytes have been shown to regulate the activity of both osteoclasts and osteoblasts. Furthermore, crosstalk between osteoclasts and osteoblasts has proven essential for coupling the resorption and formation stages of remodeling. While multicellular signaling is crucial to the tight regulation of bone remodeling, many *in vitro* studies are limited to investigating a single, isolated cell type.

In recent years, our lab along with others, has emphasized the need for more complex bone remodeling platforms that more fully recapitulate the intricate bone microenvironment. Our lab has emphasized the benefits of developing a LOC platform for bone remodeling that incorporates osteoclasts, osteoblasts and osteocytes [5]. Here we modified our current platform (Fig. 1) which utilizes fluid shear forces to induce mechanical stimulation to model the periprosthetic microenvironment and investigate cell-cell signaling modalities that lead to PPOL. Per our focus on PPOL and bone resorption, our initial efforts focused upon analyzing the interactions between osteocytes and osteoclasts. The design of our LOC allowed osteocytes to be stimulated via particulate debris under dynamic load while exposing osteoclasts to real-time osteocyte soluble signaling. However, as is typical of novel models, significant effort was required to establish the fidelity of the system. These efforts, which are presented below, included optimizing cell feeding, loading profile and particulate debris dosing. In addition, combined dynamic particulate studies incorporating osteocyte and osteoclast activity were completed with preliminary results provided.

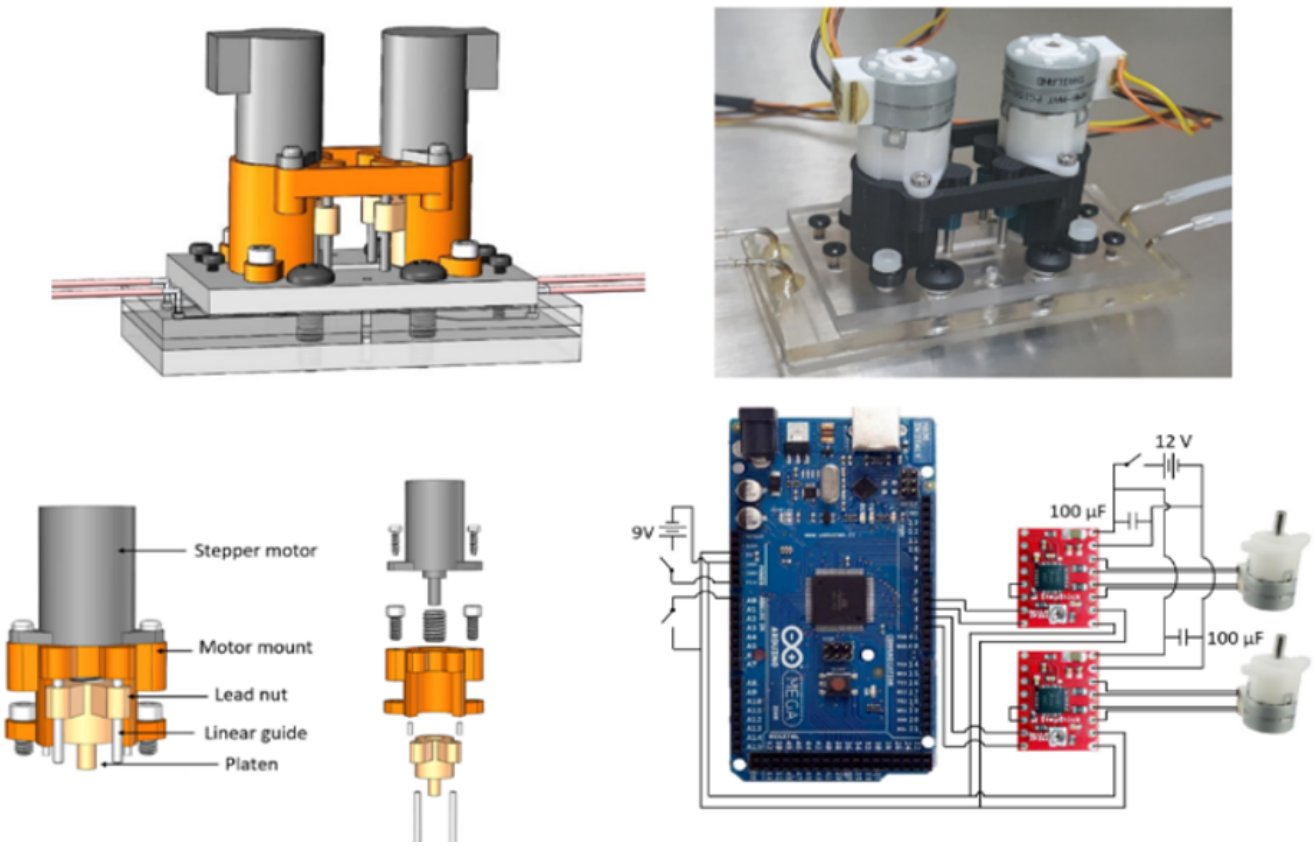

Fig. 1 (top) Schematic of multicellular LOC with pumping platform applied exposing cells to fluid shear forces and photograph of LOC. (bottom) Design of 3D printed linear actuator that used to control fluid flow within the osteocyte channel with exploded view of the linear actuator and design of circuitry used to control movement of stepper motors within the linear actuators.

Analysis

Our previously developed dynamic LOC design incorporating fluid shear was modified to fabricate a multicellular platform that recapitulated the bone microenvironment to serve as a model for PPOL [6]. The modified platform is shown in (Fig. 2). This multilayer system contains two culturing chambers separated by a permeable membrane. In the top culturing chamber, osteocytes are selectively stimulated with particulate debris and mechanical shear stress via oscillating fluid flow. This layer consists of a main cell culturing chamber with a medium reservoir on each end and two sets of access channels. The central access channel is used for filling the medium reservoirs, and the outer access channel is used for seeding cells. The lower culturing chamber contains osteoclasts cultured on bone wafers with a single set of access channels. The two chambers are separated by a thin polyethylene terephthalate (PETE) porous membrane (12 μm thick, 0.4 μm pore size). The design allows osteoclasts to be shielded from the mechanical stimulation and particulate debris present in the osteocyte chamber, while still permitting the real time diffusion of osteocyte soluble signals. Fluid flow within the osteocyte chamber is driven by custom-designed 3D printed linear actuators controlled by an Arduino microcontroller with custom-written software.

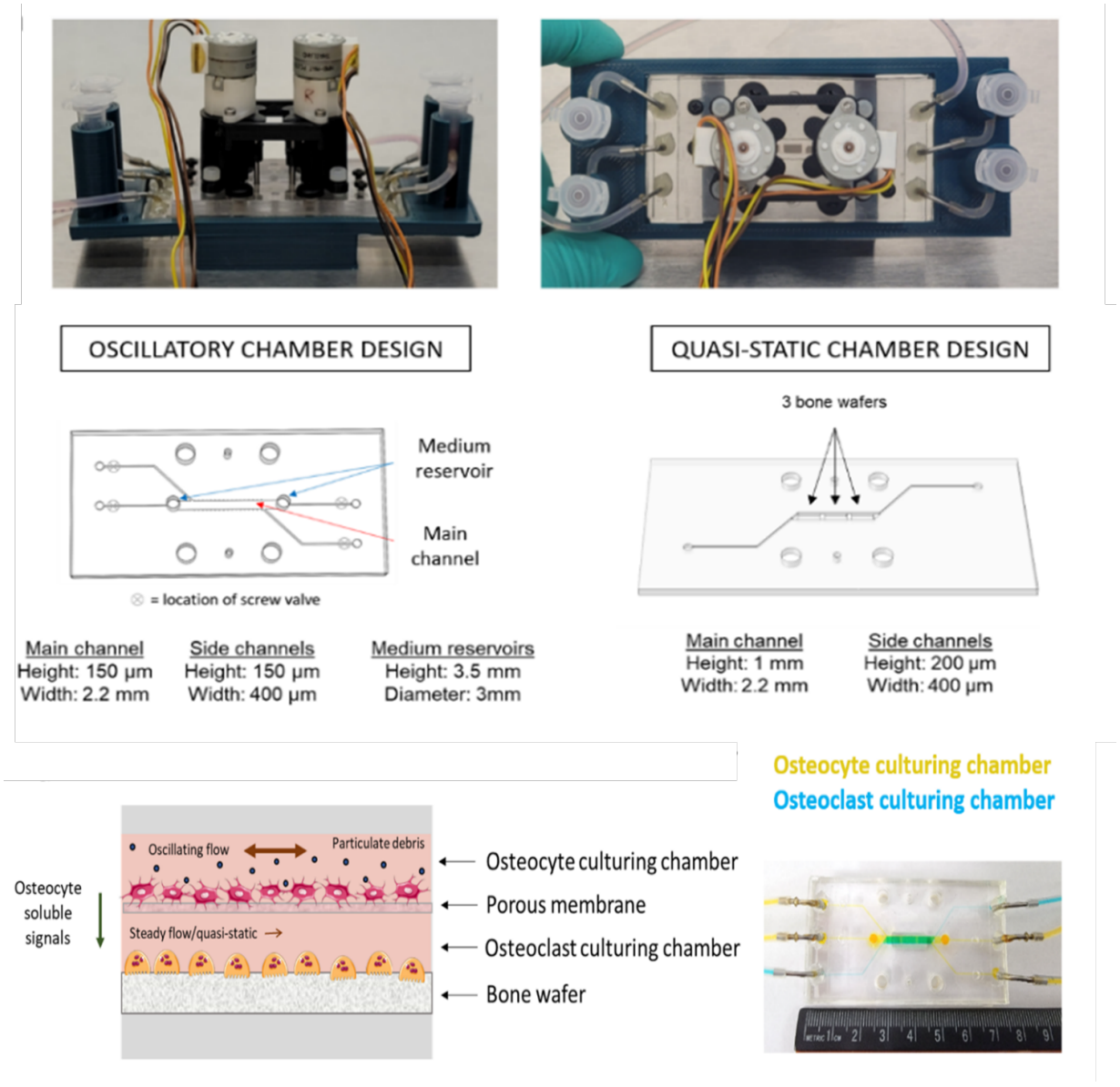

Fig. 2 The assembly utilized for co-culture loading studies quantifying the effects of polyethylene particles in bone loss, modeling PPOL.

To first analyze the effect of mechanical stimulation and particulate debris on osteocytes, only the osteocyte layer of the LOC was used. The fully cured PDMS osteocyte layer was sealed with a PDMS membrane on top and a slab of PDMS (2 mm thick) on the bottom. The main channel of the LOC was coated with collagen type I by flowing the solution through the outside access channels. After 1 h the channel was rinsed with Dulbecco's phosphate-buffered saline. The central access channels were then used to fill the entire chip with Minimum Essential Medium (MEM) α supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

Optimizing osteocyte feeding in the LOC: MLO-Y4 osteocytes were seeded within the main channel of the chip at a density of 10^4 cells/cm^2 and were given 4 h to adhere to the surface. Assuming a similar culture medium consumption rate as osteocytes grown within a T-25 flask, it was determined that the medium within the main channel should subsequently be replaced with medium from the reservoirs every 3 h. The feeding protocol was fully automated using the linear actuators to drive fluid movement. Chips with a reservoir depth of 3.5 mm contained sufficient culture medium to maintain the cells for 24 h. Every 24 h the medium in the reservoirs was manually replaced. To address the concern of excessive evaporation of culture medium through the PDMS that would result in the formation of air bubbles in the main channel, a humidity chamber was designed and 3D printed to house the chip within the laboratory incubator. This chamber fit on top of a standard 6-well culture plate that was filled with sterile dH_2O. Volumetric flow rates used during the automated portion of the feeding protocol were analyzed to balance osteocyte viability with minimal stimulatory effects. A flow rate of 14.9 µl/h (lower feed rate) was initially tested, which generated a wall shear stress two orders of magnitude less than the lowest value reported in literature that is known to stimulate osteocytes. At this flow rate, osteocyte proliferation rates were substantially less than that of cells grown in a 96-well plate. By increasing the flow rate to 29.8 µl/h (higher feed rate), proliferation rates were comparable to that of cells grown in the 96-well plate (Fig. 3). Additionally, cells displayed typical dendritic morphology as shown for 4 and 72 h (scale bars represent 100 µm); rhodamine phalloidin and DAPI stains at 72 h revealed the presence of an organized cytoskeleton (scale bar represents 50 µm). Based upon osteocytic iNOS production, the higher feed rate was utilized for subsequent studies.

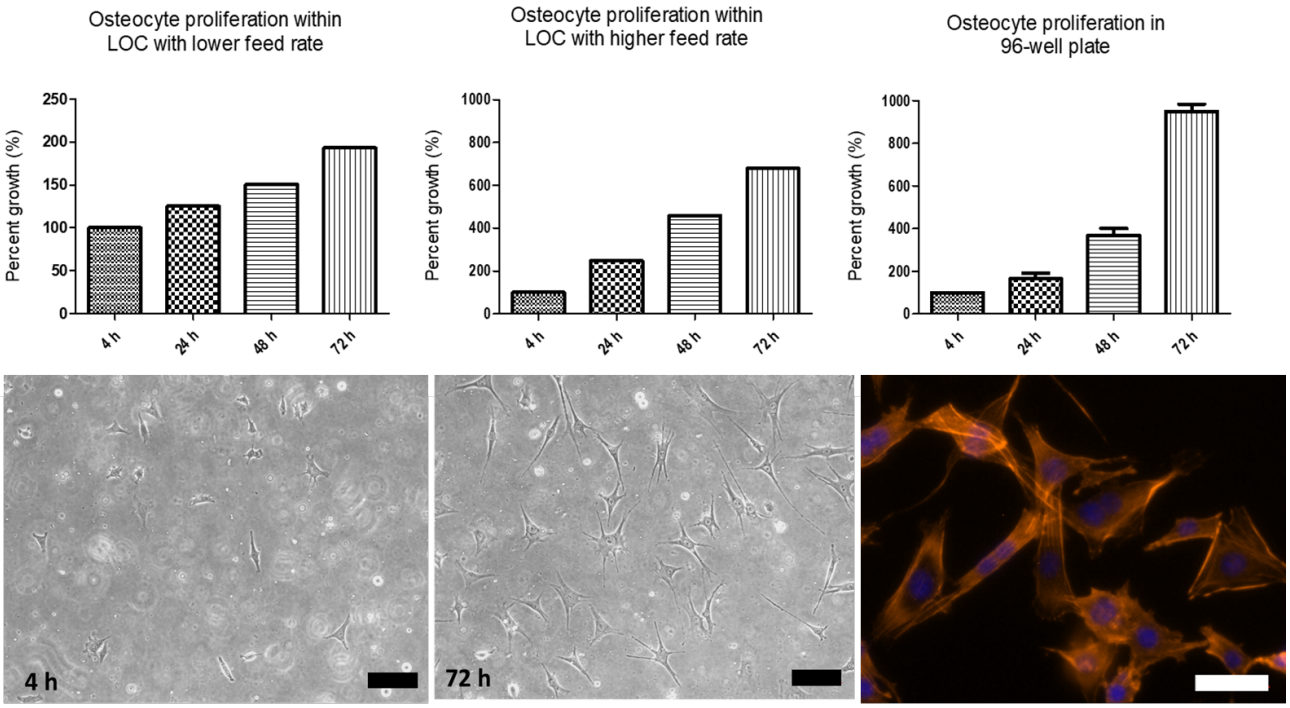

Fig. 3 Osteocytes in the LOC channels maintained viability with cellular proliferation more responsive to a higher feeding rate (29.8 µl/h). At the higher feeding rate, proliferation was comparable to that observed under identical feeding conditions in the 96-well plate.

Validating experimental flow rates: Micro-particle image velocimetry (µPIV) was used to validate fluid flow rates within the osteocyte culture chamber (Fig. 4). A chip was filled with 0.02% solution of fluorescent polystyrene tracer particles

with a diameter of 1 μm in minimum essential alpha medium (MEMα) supplemented with 5% fetal bovine serum (FBS), 5% calf serum (CS) and 1% penicillin/streptomycin. The linear actuators were turned on to induce oscillating fluid flow with a sinusoidal wave (1 Hz) and maximum velocity corresponding to a maximum wall shear stress of 1 Pa. Images were obtained using a Zeiss Axio Observer A1 inverted microscope. Burst mode was used to collect 100 images at a rate of 47.6 frames per second. Images were analyzed pairwise using PIVLab, an open-source add-on for MATLAB, and three passes with a decreasing interrogation window (128, 64, and 32 pixels) to generate a velocity vector field. The average particle velocity was determined for each image and then plotted versus time. A one-term sum of sine approximation for the data was obtained using the MATLAB curve fitting application. The amplitude of the model was compared to the theoretical amplitude (previously described), and a calibration coefficient was determined. The calibration coefficient was incorporated into the model, and the experiment was repeated. Osteocytic iNOS production showed that mechanical load continued to have a cellular effect at 24 h. This waveform was used for subsequent loading studies.

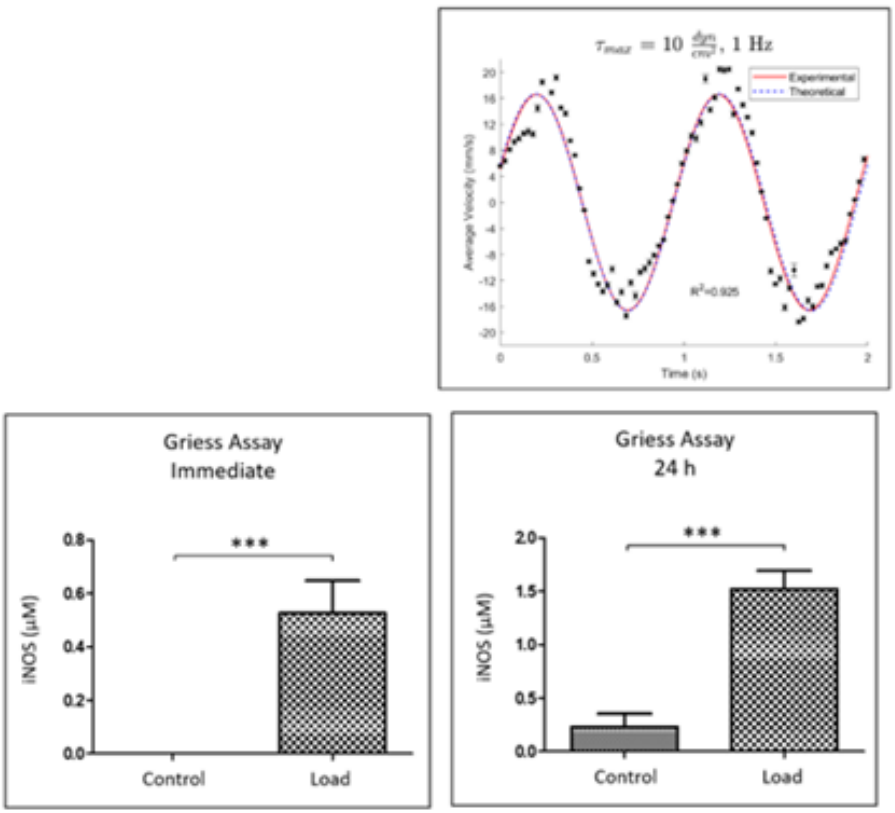

Fig. 4 μPIV analysis to validate fluid flow rate.

Validating experimental particulate debris dosing (particulate preparation): Wear debris was modeled using commercially available polyethylene nanospheres (Cospheric LLC) with a diameter range of 0.2 to 9.9 μm. Evidence suggests that the biological response to debris depends on particle size, and particles with a diameter between 0.1 and 10 μm have been reported to be the most biologically active. A solution of polyethylene particles in culture medium was generated using an established protocol. Briefly, particles were suspended in 70% ethanol then sonicated for 1 h to disperse any clumps. To ensure particles remained in the upper osteocyte chamber, the solution was filtered to remove particles smaller than 0.4 μm. The remaining particles were then resuspended in 70% ethanol, sonicated for 1 h and left at RT for 48 h to eliminate endotoxin contamination. The particles were then washed three times with endotoxin-free PBS, resuspended at 1 mg/ml in MEMα supplemented with 5% FBS, 5% CS and 1% penicillin/streptomycin and sonicated for 30 min. The solution was streaked on nutrient agar plates and incubated for 48 h at 37 °C to confirm the absence of any bacterial or fungal contamination. Prepared PDMS chips were rinsed two times with sterile dH_2O using a 5 ml syringe and Tygon tubing. Using the outside access channels, the osteocyte culture chamber was coated with collagen type I in 0.2 M acetic acid for 1 h then rinsed two times with Dulbecco's phosphate-buffered saline (with calcium and magnesium). The osteocyte chamber and medium reservoirs were then completely filled with MEMα supplemented with 5% FBS, 5% CS and 1% penicillin/streptomycin, taking care to remove

all air bubbles from the system. MLO-Y4 osteocytes were seeded in the chips at 20,000 cells/cm^2 using the outside access channels and a syringe pump set to a speed of 5 ml/h and a volume of 40 µl per chip. The cells were allowed to adhere to the chips for 4 h prior to motor attachment. The linear actuator pump was then used to automatically feed the cells every 3 h with medium from the reservoirs using a flow rate of 29.8 µl/h. Medium in the reservoirs was manually replaced every 48 h. LDH staining and Griess assays were employed to quantify the dose response ranging from 0-1 mg/mL after 48 h. iNOS production was determined using a Griess assay. First, 50 µl of each sample was added to 50 µl of Griess reagent (a solution containing 0.5% sulfanilamide, 0.05% N-(1-Naphthyl)ethylenediamine dihydrochloride and 2.5% phosphoric acid) within a 96-well plate. The absorbance at a wavelength of 540 nm was determined for each sample and converted to a concentration of iNOS. Cells were stained with LDH reaction solution—a mixture containing HBSS, Polypep, diglycine, lactic acid, β-Nicotinamide adenine dinucleotide hydrate and nitroblue 34 tetrazolium. Cells were incubated for 90 min at 5% CO_2 and 37 °C. They were washed with HBSS and fixed in 4% paraformaldehyde for 15 min at RT. They were washed again and stored in HBSS for imaging. Based upon analysis (Fig. 5), particles were resuspended in 1 mg/ml for subsequent experimental studies.

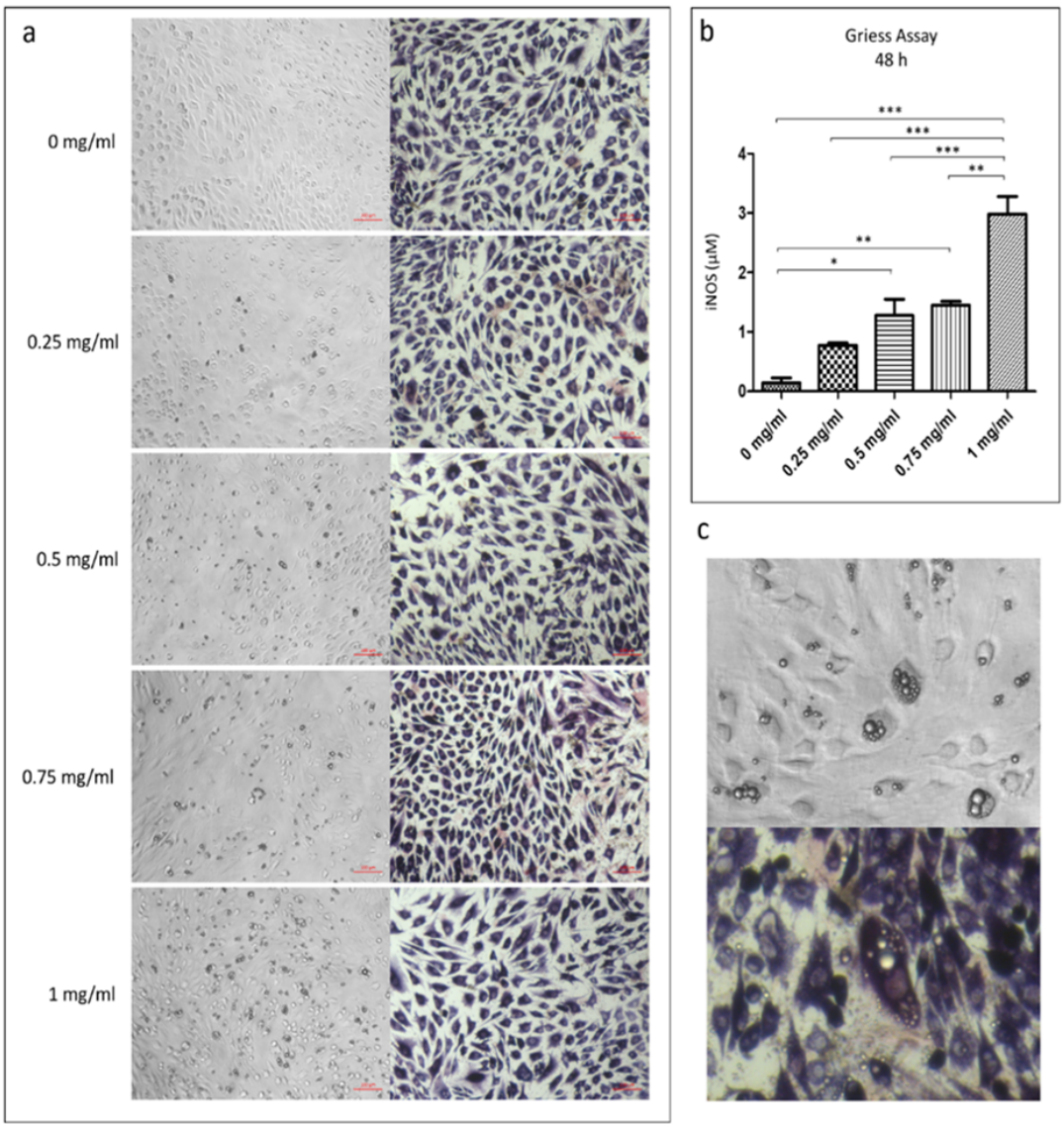

Fig. 5 The results of LDH staining and the Griess assays in quantifying iNOS production as a function of polyethylene particle dose response. Of particular interest are the magnified phase contrast and stained images clearly demonstrating the successful cellular uptake of the polyethylene particles.

Fabricating chips for multicellular studies: Chip fabrication has been previously described. Briefly, a custom-designed mask was designed in SolidWorks 2018 (Dassault Systèmes) and fabricated from Accura SL 5530 using high-resolution stereolithography (Protolabs). Chips were cast from PDMS with a 10:1 ratio of elastomer base to curing agent. The polymer was mixed vigorously, poured over the mask, degassed and cured for 18 h at 45 °C. A similar process was used to fabricate the osteoclast culturing chamber and a thin (0.5 mm thick) PDMS membrane. This membrane served as a diaphragm that, when deflected downward, drove fluid from the medium reservoirs and across the osteocyte culturing chamber. A biopsy punch was used to create holes for the access channels. The PDMS membrane was attached to the top of the osteocyte chamber layer by plasma oxidation for 30 s using a medium RF setting (200 mTorr, 10W). The PETE membrane was attached to the bottom of the osteocyte layer using aminosilanization of the membrane and plasma oxidation. To ensure the PETE membrane was taut, the membrane was initially oversized, and the edges were taped to filter paper that was in turn taped to a flat metal tray. After attaching the membrane to the PDMS layer the excess PETE membrane was trimmed off with a scalpel. The osteoclast culture chamber was then plasma oxidized and pressed to the underside of the PETE membrane following careful alignment with the osteocyte chamber. The chips were then baked at 65 °C for 30 min with weight (∼9 N) placed on top. Angled dispensing tips (18-Gauge, 12.7 mm, 90 °) were inserted into the access holes and secured with epoxy.

Analyzing the effects of particulate debris and mechanical stimulation on osteocyte signaling and subsequent osteoclast resorption: The LOC was fabricated with both the osteocyte and osteoclast culturing chambers; experiments were conducted as shown in the experimental design chart (Fig. 6).

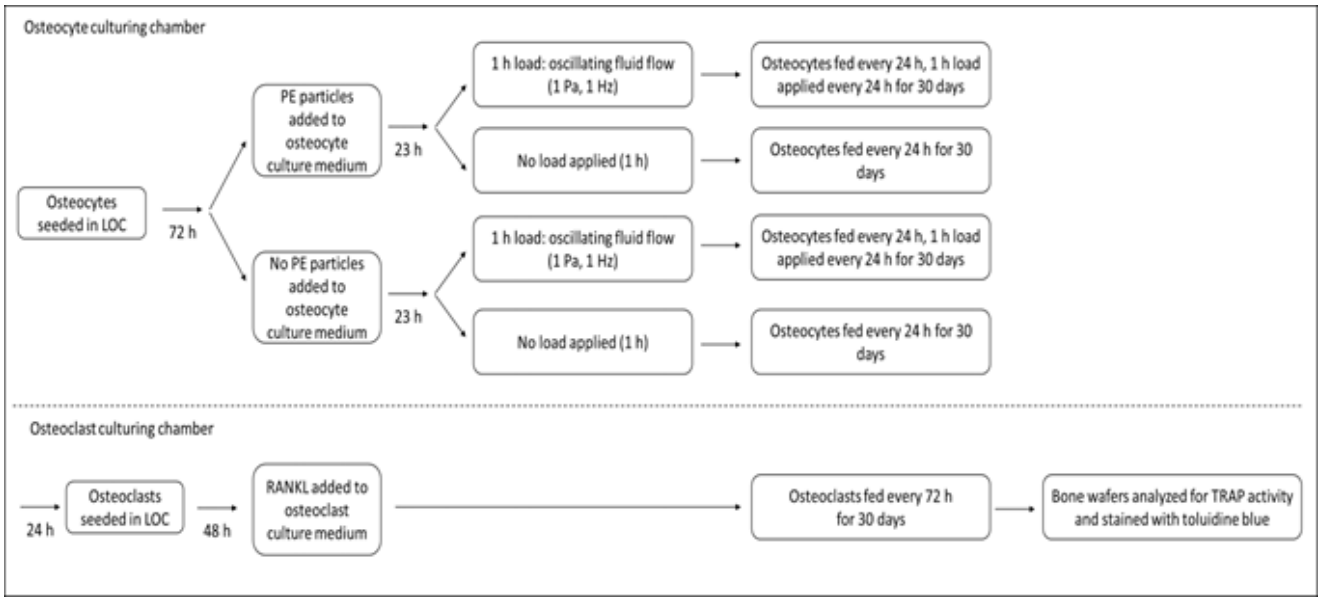

Fig. 6 Experimental design for initial investigation of osteoclast resorption as a function of exposure to osteocytes subjected to mechanical stimulation in the presence of particulate debris simulating PPOL in a dynamic loading environment.

The osteocyte and osteoclast chambers were separated by a porous PETE membrane to allow for diffusion of soluble signals between the culturing chambers. The device was fabricated following the procedures described above. The osteoclast culturing chamber contained three bovine bone wafers (2 mm x 5 mm) secured to the bottom of the channel. Following experimentation, bone wafers were removed from the chip and each wafer was analyzed separately. MLO-Y4 osteocytes were seeded in the upper chamber at a density of 10^4 cells/cm^2 using a flow rate of 5 ml/h. At 48 h RAW264.7 pre-osteoclasts were seeded in the lower chamber at a density of 5,000 cells/cm^2 in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS and 1% penicillin/streptomycin. After 24 h, osteoclasts were induced to undergo osteoclastogenesis with the addition of 120 ng/ml RANKL to the medium. Osteoclasts were fed with induction medium every 72 h using 100% medium replacement. Osteocytes were stimulated with polyethylene particles at 72 h and with oscillating fluid flow (1Pa, 1Hz) for 1 h every 24 h for 30 days. Conditioned medium was collected for analysis via ELISAs. The soluble factors, interleukin (IL)- 1β, and IL-6 were quantified. IL-1β enhances osteoclast differentiation while IL-6 indirectly promotes osteoclast differentiation by enhancing osteocyte-secreted RANKL (Fig. 7). At 30 days, bone wafers were removed from the LOC and assessed for TRAP activity and stained with toluidine blue for quantification of bone resorption (Fig. 8).

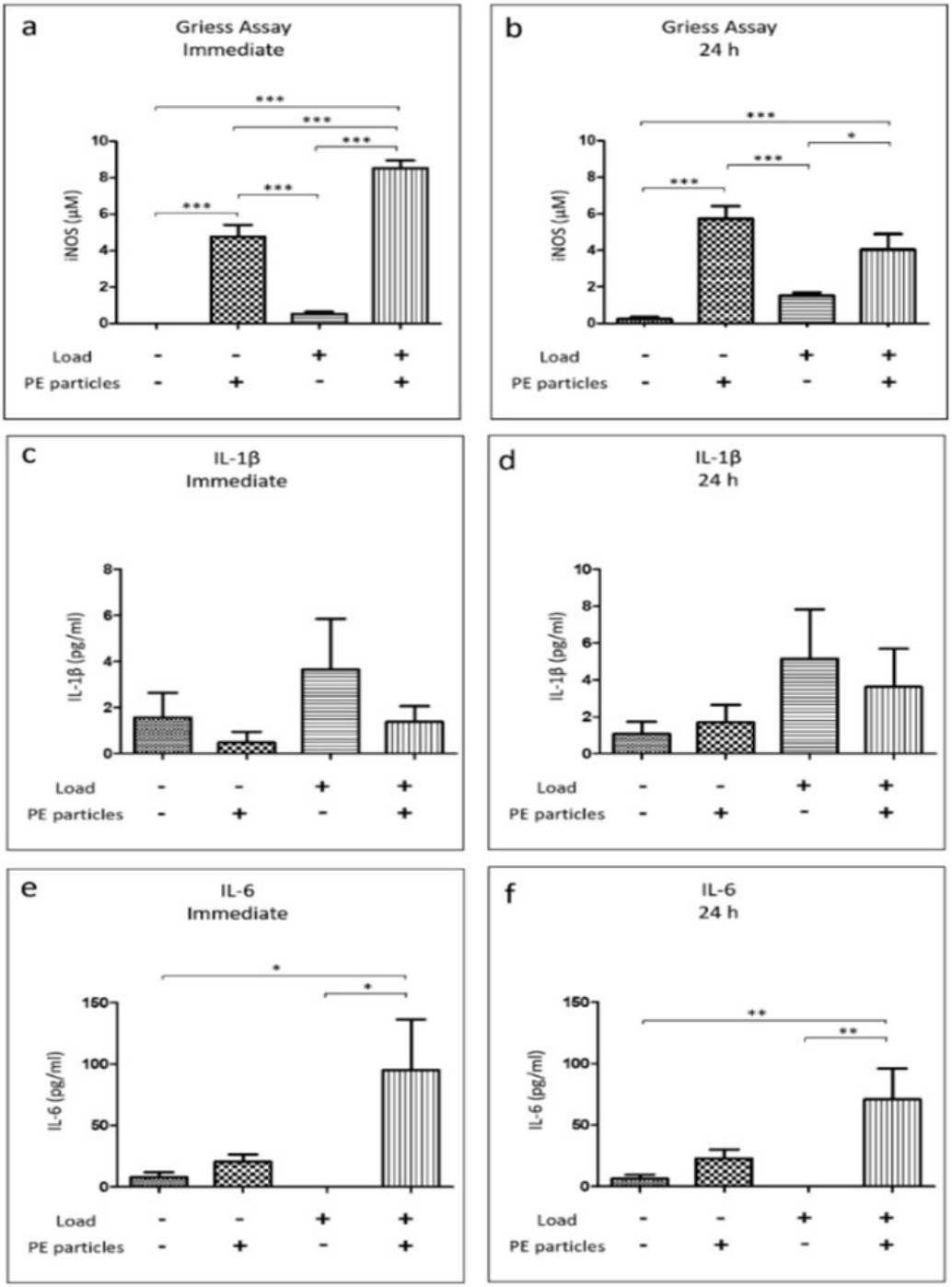

Fig. 7 Analysis results of osteocyte conditioned medium.

Results

To date, osteocyte conditioned medium assays showed the effects of combined loading and debris upon immediate collection or following a 24 h incubation period. Initial results suggested that in the LOC the combined effects on inflammation (osteocytic iNOS production) were largest immediately following collection. These effects decreased after 24 h but were not significantly different from the effects of particulate debris alone. Results from IL-1β quantification suggested that this cytokine production was not significantly different under the various combinations of load and particulate debris at the

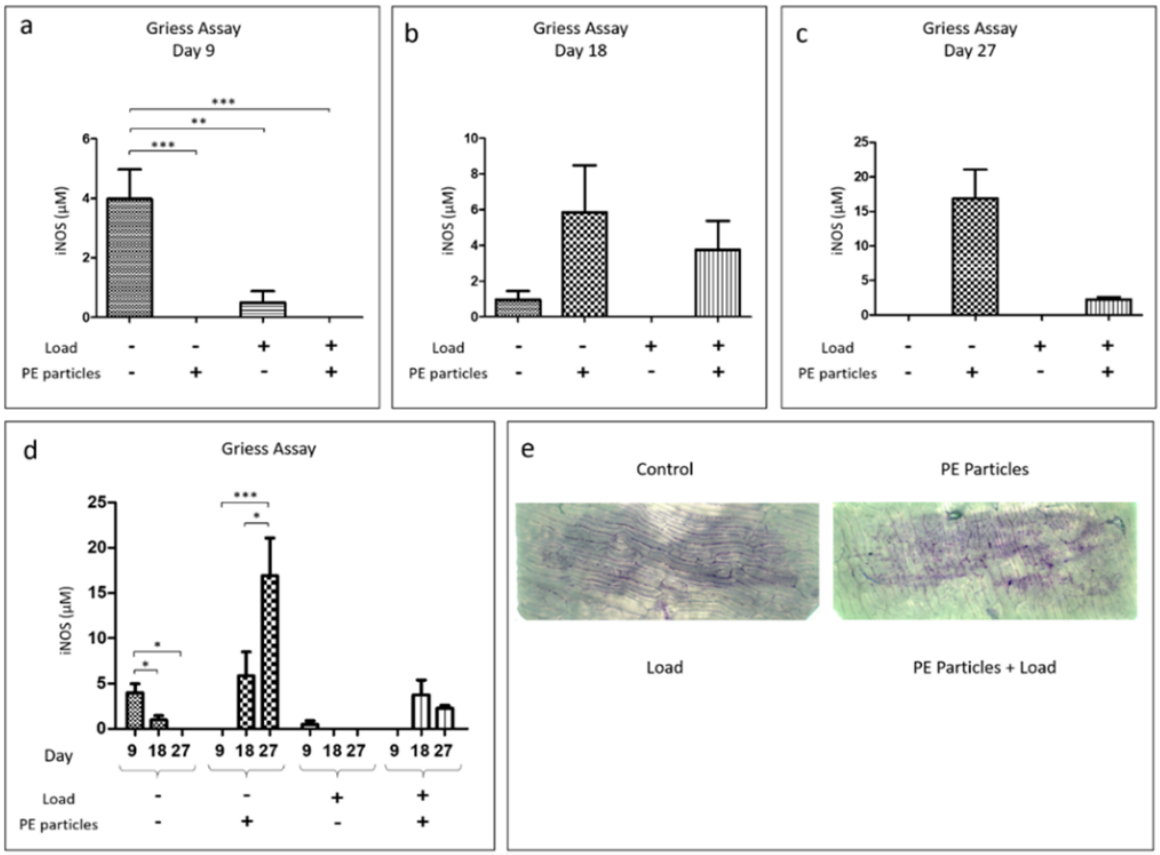

Fig. 8 Osteoclast iNOS production and bone resorption.

various collection times, while IL-6, which more indirectly promotes osteoclast activity by acting directly on RANKL, was significantly increased in combined cultures over baseline. To date, osteoclast conditioned medium assays showed that when osteocytes were not exposed to polyethylene particles, iNOS levels in the osteoclast chamber peaked at day 9 and then dropped significantly. But when osteocytes were exposed to polyethylene particles, OS levels in the osteoclast chamber were not detectable at day 9 but then increased significantly by day 27. Wafer resorption analysis has not been completed. While we are still working to define these results in the context of current knowledge, similar trends are emerging for the collection periods. However, the biomarker activity will help us to better understand the added benefit of the indirect measurements in the absence of the resorption data. This is also being completed to better vet the LOC and loading system for a wide range of research uses where it will be necessary to first establish true fidelity of the system and approach.

Conclusion

Although osteocytes make up the vast majority of bone cells and are known to play an essential role in regulating osteoclast and osteoblast activity, surprisingly little is known regarding their role in PPOL. In contrast, the biological response of macrophages to implant debris has been well established. Significant clinical evidence has demonstrated that wear debris from implants stimulate macrophages and trigger the release of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6 [7–8]. This inflamed environment ultimately leads to enhanced osteoclast activity and subsequent bone loss. While it is widely accepted that implant debris is a major cause of PPOL, some studies have suggested that mechanical stimulation may provide an additional mechanism leading to osteolysis that is independent of wear debris [9–10]. Researchers have hypothesized that micromotion at the bone-implant interface may result in a cyclic pressure-induced flow of joint fluid into the surrounding cancellous bone [11–12]. Further, some studies have reported that exposure to both wear particles and mechanical stimulation has a synergistic effect on pro-inflammatory cytokine expression in macrophages [13–14].

Recent studies suggest that osteocytes may also contribute to the pro-inflammatory response and subsequent increase in osteoclast resorption [15–16]. Ormsby et al demonstrated that exposure of human primary osteocyte-like cells to either polyethylene or metal particles upregulated expression of pro-inflammatory cytokines TNF-α and IL-6 [16]. Exposure of MLO-Y4 osteocytes to polyethylene particles has been shown to induce an upregulation of receptor activator of nuclear factor kappa-B ligand (RANKL), which promotes osteoclast differentiation [15–16]. And TNFα and IL-6 were shown to be upregulated in RAW264.7 macrophages exposed to titanium particles. However, this upregulation was suppressed by two inhibitors of SPHKs (FTY720 and ABC294640) suggesting that release of TNFα and IL-6 depends on SPHK-2 activity [17]. In osteocytes, it remains to be seen if a similar mechanism regulates particle-induced expression of these cytokines.

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