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# Fluid Pressure Induces Osteoclast Differentiation Comparably to Titanium Particles but Through a Molecular Pathway Only Partly Involving TNFα

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## ABSTRACT

In contrast to the well-understood inflammatory pathway driven by TNFα, by which implant-derived particles induce bone resorption, little is known about the process in which loosening is generated as a result of force-induced mechanical stimulus at the bone–implant interface. Specifically, there is no knowledge as to what cells or signaling pathways couple mechanical stimuli to bone resorption in context of loosening. We hypothesized that different stimuli, i.e., fluid flow versus wear particles, act through different cytokine networks for activation and localization of osteoclasts. By using an animal model in which osteoclasts and bone resorption were induced by fluid pressure or particles, we were able to detect distinct differences in osteoclast localization and inflammatory gene expression between fluid pressure and titanium particles. Fluid pressure recruits and activates osteoclasts with bone marrow contact away from the fluid pressure exposure zone, whereas titanium particles recruit and activate osteoclasts in areas in direct contact to particles. Fluid pressure induced weaker expression of the selected inflammatory related genes, although the eventual degree of osteoclast induction was similar in both models. Using TNFαRa (4 mg/kg) (Enbrel) and dexamethasone (2 mg/kg) as specific and more general suppressors of inflammation we showed that the TNFαRa failed to generate statistically impaired osteoclast generation while dexamethasone was much more potent. These results demonstrate that fluid pressure induces osteoclasts at a different localization than titanium particles by a molecular pathway less associated with TNFα and the innate system, which open up for other pathways controlling pressure induced osteoclastogenesis. J. Cell. Biochem. 113: 1224–1234, 2012. 

**KEY WORDS:** PRESSURE; FLOW; OSTEOCLASTOGENESIS; CYTOKINES; OSTEOLYSIS

The current paradigm suggests a single major cause for implant loosening, namely particle-induced stimulation of a local inflammatory response [Revell, 2008]. One less explored causative factor for implant loosening is the mechanical stimuli around implants or at the bone–implant interface. Clinical observations show that hydrostatic pressure alone can stimulate osteoclastogenesis and osteolysis. For instance, micromotion of an implant in itself substantially results in significant local bone loss as shown by resorption around fibulo-tibial screws inserted at surgery for ankle fractures [Perren, 2002]. The same phenomenon occurs around screw holes of acetabular components during implant loosening [Walter et al., 2005], suggesting involvement of fluid pressure and flow as an additional component in aseptic loosening. Thus, it is of vital importance to identify other mechanisms that mediate prosthetic loosening with a view to developing additional therapeutic approaches.

Osteoclasts originate from haematopoetic stem cells in the bone marrow and are the main target cells to suppress osteolysis in which the RANKL/OPG system is of crucial importance. The former cytokine is the key osteoclastogenic factor, while the latter acts to a secreted decoy molecule. The growth factor macrophage colony stimulating factor (M-CSF) binds to the receptor c-fms on osteoclast precursors and activates a signaling cascade, which will together with RANK-ligand (RANKL) commit the cell further into the

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osteoclastic linage [Bar-Shavit, 2007]. Several signaling pathways impact production of RANKL and/or OPG. TNFα together with IL-1 is the most potent osteoclastic cytokines produced in inflammation acting by activating NF-γB [Kitaura et al., 2004; Wei et al., 2005]. Through these signals, the precursors become committed to the osteoclast linage, and start to express osteoclast specific genes such as tartrate-resistant acid phosphatase (TRAP) and Cathepsin K.

Wear debris released from joint surfaces is phagocytosed by macrophages, releasing pro-inflammatory cytokines such as IL-1β, IL-6, and TNFα, all of which are associated with progressive bone resorption and focal bone loss [Ingham and Fisher, 2005; Rubin et al., 2006]. Numerous deposits of wear particles are found in peri-implant tissue together with increased expression of TNFα [Holding et al., 2006], Cathepsin K [Shen et al., 2006], RANK, and RANKL [Mandelin et al., 2003]. The involvement of specific cytokines was re-inforced by the association of aseptic loosening with genetic variants of TNFα and IL-6 in patients with severe osteolysis [Gallo et al., 2009].

Expression of specific chemoattractants such as CX3CL1 and CCL2 have been identified recently at sites of particle deposition [Goodman and Ma, 2010]. During osteoclastogenesis, RANKL induces the expression of CX3CL1, which then enhances adhesion and migration of bone marrow macrophages [Koizumi et al., 2009]. CCL2, a CC-chemokine, is expressed at high levels during osteoclastogenesis in orthodontic movement [Garlet et al., 2008] induced by a specific mechanical stimulus.

Nitric oxide (NO) is another important regulator of mechanical loading but also for osteoclastogenesis. NO generated by inducible nitric oxide synthase (iNOS) mediates osteoclast activity while at the same time protects against osteocyte apoptosis in vitro [Bonewald, 2011]. Other important regulators are TGFβ and prostaglandins in the lacuno-canalicular system, activating osteoblasts and osteoclasts when subjected to fluid flow [Bonewald, 2011].

The importance of optimizing initial fixation to avoid mechanical induced osteolysis is well known, but the mechanisms underlying bone loss are unknown. Our recent data show clearly that fluid pressure per se can induce osteoclast formation, the key process preceding aseptic loosening [Fahlgren et al., 2010]. We show further that bone resorption in response to pressure was completely inhibited by OPG-Fc treatment, confirming the key role for the RANKL/OPG ratio activity [Aspenberg et al., 2011]. Moreover, a previous study in vitro showed that prolonged cyclic hydrostatic pressure on osteocytes stimulates up-regulation of RANKL and down-regulation of OPG, in contrast to a shorter stimulus that results in reciprocal effects [You et al., 2008; Bonewald, 2011]. In summary, there is growing evidence for a direct effect of pressure leading to osteoclastogenesis. Further analysis of the signaling pathways that initiate bone resorption following fluid pressure is, therefore, of importance to understand and potentially inhibit this catabolic process.

We hypothesized that fluid pressure and wear particles, respectively, act through different cytokine networks to induce differentiation and activation of osteoclasts. Titanium particles alone, previously shown to induce osteoclast activation in vivo [Taki et al., 2005], were used as a known stimulus for inflammatory cytokine-induced osteoclast activation. We show now, using our established animal model in which bone resorption can be induced independently by fluid pressure and particles, that fluid pressure per se is associated with lower levels of inflammatory cytokines compared to particle-induced osteolysis. Nevertheless, we found the same number of osteoclasts per unit area in both models. While systemic administration of the TNFα receptor antagonist Enbrel failed to generate statistically less numbers of osteoclasts, dexamethasone as an overall inhibitor of the innate inflammatory system was more potent, suggesting targeting other mediators than TNFα for osteoclast differentiation might be crucial to inhibit pressure induced osteolysis.

## MATERIALS AND METHODS

### ANIMAL MODEL

Twelve-week-old Sprague Dawley rats were used in an animal model for pressure-induced osteolysis previously described elsewhere [Skripitz and Aspenberg, 2000; Fahlgren et al., 2010]. The rats were anesthetized with 5% isoflurane gas and received a preoperative subcutaneous injection of 25 mg/kg Tetracycline and 5 mg/kg Carprofen. A titanium plate with a center plug was fastened under aseptic conditions at the medial aspect of the rat proximal tibia after the periosteum was reflected (Fig. 1a). The center plug protruded 0.5 mm into a milled depression in the bone cortex. New

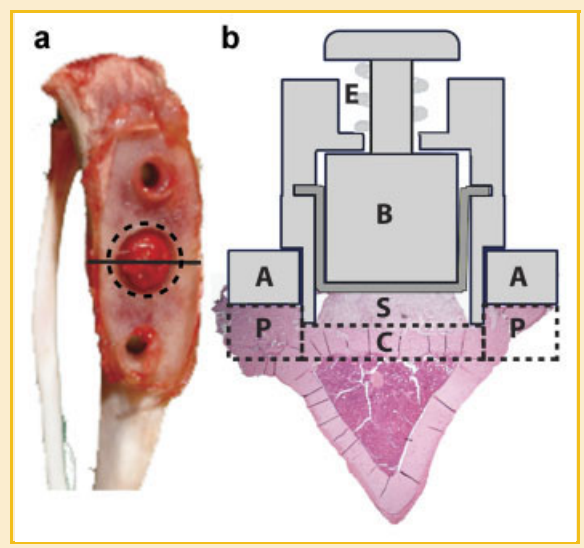


Fig. 1. (a) Proximal tibia showing the bone surface with the soft tissue under the pressure piston and the central localization for histological analysis. The dashed circle is showing the area were the tissue core for qPCR was taken. (b) Transverse section of proximal tibia showing titanium plate (A) with pressure piston (B) sealed with a silicon detail with an internal spring force (E). By applying force on the piston, load is transmitted to the soft tissue (S) without propagating down to the underlying cortical bone. Instead of pressure piston a hollow plug filled with titanium particles were inserted into similar titanium plate (not shown). Osteoclast numbers was counted at the central zone (C) and the peripheral zone (P) of the cortical bone. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

bone was allowed to grow up to the titanium surface of the central plug to form a flat osseointegrated bone surface. In a second operation the plug was replaced by a pressure piston or a hollow screw with titanium particles (Fig. 1b). With the piston in place, a 0.5 mm wide area was left for soft tissue to form between the piston and the flat bone surface. The soft tissue was compressed by the piston, creating fluid pressure propagated down onto the underlying bone. Each episode of pressure comprised 20 pressure cycles at 0.17 Hz, applied twice a day.

All experiments were carried out within the context of institutional guidelines for care and treatment of experimental animals after approval from Linköping Ethical Committee.

### TITANIUM PARTICLE PREPARATION

Pure titanium particles (Alfa Aesar, Karlsruhe, Germany) with 93% of the particles $<20\,\mu m$ and 90% of particles $<3.6\,\mu m$ were used in this study [Kaar et al., 2001; Taki et al., 2005]. To remove lipopolysaccharide (LPS), the particles were first suspended for 30 min in 95% ethanol and then heated in an oven for 3 h at 200°C. Particles were all found to have LPS amounts $<0.1$ pmol/mg, using gas chromatography-tandem mass spectrometry (GC-MS-MS) [Skoglund et al., 2002]. LPS-free particles were saturated with sterile NaCl solution and then poured onto a sterilized filter paper. The particle paste (20 mg) was transferred to the interior of the hollow central screw, corresponding to the amounts used in earlier studies [Kaar et al., 2001].

### HISTOLOGICAL ANALYSES

To identify and quantify osteoclasts by different histological and immunohistochemical parameters, the rats were randomized into fluid pressure, titanium particles or controls and followed for 0, 1, 3, and 5 days of treatment (Table I). Controls were sacrificed before the first stimuli after 5 days of soft tissue formation, whereas samples were obtained from the experimental groups after the last pressure episode or at the same time point as the particle group. The tibiae was disarticulated from the knee joint and dissected free from soft tissue. The bone specimens were fixed in 4% paraformaldehyde solution for 48 h and then decalcified in 10% formic acid for 28 days. After decalcification, specimens were embedded in paraffin, transverse sections of $5\,\mu m$ were cut and stained with hematoxylin and eosin or used for immunohistochemistry.

For immunohistochemistry the primary antibody was rabbit-anti rat Cathepsin K (1:600) [Zenger et al., 2007]. The sections were deparaffinated, rehydrated, and blocked for endogenous peroxidase with 3% $H_2O_2$ for 5 min, followed by blocking with 0.5% bovine serum albumin (BSA) (Sigma, MO) for 20 min. Primary antibodies diluted in Dako real™ antibody diluent (S2022, DakoCytomation, Denmark) were applied for 1 h and 20 min, followed by incubation of biotinylated secondary antibodies (Dakopatts AB) for 40 min. Vectastain Elite ABC kit (Vector Laboratories, CA) was applied for 30 min followed by 5 min of 3,3′diaminobenzidine (DAB) (Sigma). All antibody-reactions were performed at room temperature. The sections were then counter-stained with Mayer's hematoxylin, dehydrated and mounted.

TABLE I. Study Design Histological and Gene Expression Analysis

| Treatment | Analysis | 0 day (n) | 1 day (n) | 3 days (n) | 5 days (n) |
|---|---|---|---|---|---|
| Pressure | IHC | 8 | 7 | 6 | 7 |
| Pressure | qPCR | – | 7 | 7 | – |
| Titanium | IHC | 7 | 9 | 7 | 7 |
| Titanium | qPCR | – | 7 | 6 | – |
| Control | qPCR | – | 6 | 6 | – |

For histomorphometry, a blinded single investigator (A.N) analyzed one representative section from two levels of the cortical bone with a distance of 450 μm (Fig. 1a). Osteoclasts were defined as large Cathepsin K positive cells within a distance of 0.1 mm from bone surfaces and counted at 10× magnification using an Olympus microscope and the software Cell-D (Olympus Europe GmbH, Germany). Osteoclast numbers were evaluated at two different surfaces: (i) attached to the cortical bone below the former piston or hollow plug (central region), or (ii) at the sides (peripheral region) (Fig. 1b).

To assess the localization of osteoclasts, the ratio between the central zone and the peripheral zone for each individual was compared between pressurized and particle exposed specimens.

### GENE EXPRESSION ANALYSES

To monitor gene expression after fluid pressure and titanium particles, the rats were randomized into fluid pressure, titanium particles or controls and followed for 1 or 3 days of treatment (Table I). The area under the piston or hollow plug were harvested by a trephine with internal diameter of 4.2 mm before being snap frozen in liquid nitrogen and stored at −80°C before RNA isolation, cDNA synthesis and quantitative real-time PCR.

Extraction of total RNA was performed using the TRIspin method described in detail in [Eliasson et al., 2008]. Frozen bone specimens were placed one by one in a liquid-nitrogen-cooled vessel and pulverized using a Retsch Mixer Mill MM 200 (Retsch GmbH, Germany). RNA extraction was preformed using the RNeasy Total RNA kit (Qiagen GmbH, Germany). Samples were then treated with DNase I (MERCK Eurolab, Sweden). 0.1 μg total RNA was reverse-transcribed from each sample using a high-capacity cDNA reverse transcription kit (Applied Biosystems, UK). Primers for TNFα (Rn00563754_m1), IL-1β (Rn01410545_m1), IL-6 (Rn01410330_m1), CCL2 (Rn00580555_m1), CX3CL1 (Rn00593186_m1), TGF-β1 (Rn00572010_m1), PGES (Rn00572047_m1), iNOS (Rn00561646_m1), RANKL (Rn00589289_m1), OPG (Rn00563499_m1), RANK (Rn01426423_m1), Cathepsin K (Rn00580723_m1), MMP-9 (Rn00579162_m1), TRAP (Rn00569608_m1), CD68 (Rn01495634_g1) and CD163 (Rn01498101_m1) were purchased from Applied Biosystems. Amplification was performed in 15 μl reactions using TaqMan Fast PCR MasterMix (Applied Biosystems).

Each sample was analyzed in duplicate. Real-time PCR reactions were conducted using a standard curve methodology to quantify the specific gene targets of interest. The standard curve was made with rat spleen RNA (Labinova AB, Sweden) for the inflammation-related genes (TNFα, IL-1β, IL-6, CCL2, CX3CL1, TGF-β1, PGES, iNOS, RANKL, OPG, RANK, CD68, and CD163) and embryonic rat RNA (Labinova AB) for the osteoclast specific genes (Cathepsin K, TRAP, and MMP-9). Each sample was normalized to 18S rRNA, which was constantly expressed over time of the experiments. Reactions with

no reversed transcriptase enzyme or no template were included as negative controls.

### SUPPRESSION OF THE INNATE INFLAMMATORY SYSTEM AND TNFα

To determine the role of the innate inflammatory system, and more specifically TNFα, a major cytokine controlling wear debris-induced osteoclast differentiation [Schwarz et al., 2000; Childs et al., 2001], we used two approaches; the specific TNFα receptor antagonist Enbrel or the anti-inflammatory steroid dexamethasone. Twenty-seven rats were randomized into three groups, Enbrel 4 mg/kg (n = 9), saline (n = 8), or dexamathasone 2 mg/kg (n = 10) every third day, starting 3 days prior to initiation of the pressure stimuli. Dexamethasone was used as a positive control to suppress activation of the innate immune system. After 5 days of pressure animal were sacrificed and tissue were processed as earlier described. Our primary readout was the number of Cathepsin K positive osteoclasts counted as described above.

### STATISTICS

Osteoclast numbers were analyzed by non-parametric statistics. With significant Kruskal–Wallis ANOVA, group differences were analyzed by Mann–Whitney U-test. For gene expression data, animals with fluid pressure and titanium particles were analyzed separately. In both cases the hypothesis that a difference between controls and treated animals would be present was tested. With significant Kruskal–Wallis ANOVA, effects of titanium particle exposure or fluid pressure were tested against controls separately at specific time points with separate Mann–Whitney U-tests. Non-parametric Spearman's rank correlation test was used to determine the correlation between RANKL and Cathepsin K against inflammatory genes in experimental animals. $P$-value $<0.05$ was considered to be statistically significant for all measurements.

## RESULTS

### DESCRIPTIVE HISTOLOGY OF OSTEOCLAST ACTIVATION INDUCED BY FLUID PRESSURE OR TITANIUM PARTICLES

To assess osteoclast development after fluid pressure or particle treatment, 1, 3, and 5 days after initial application of stimulus were selected, representing different stages of osteoclast recruitment, differentiation and activation.

The first osteoclasts recruited to the area were seen after 3 days with distinctive difference in osteoclast localization between the two treatment groups (Fig. 2). With fluid pressure, multinucleated osteoclasts and resorption lacunas were abundant within pre-formed cavities at the periphery. With titanium particle exposure, osteoclasts were accentuated and restricted to just beneath the exposure zone at the interface between the soft connective tissue and bone. After 5 days of fluid pressure, osteoclasts were detected on the bone marrow side or intracortically of the central cortical bone in addition to the periphery (Fig. 2). Areas of the central region of the cortical bone were completely resorbed as well as within the cavities at the periphery. After titanium particle exposure, in cases where the bone was resorbed completely with access to the marrow cavity after titanium particle exposure, particles were also found in the bone marrow (data not shown).

### SEMI-QUANTIFICATION OF OSTEOCLAST NUMBERS

To determine the number and localization of osteoclasts activated by fluid pressure or titanium particles, Cathepsin K-positive cells were counted in the central and peripheral exposure zone of the lesion (Fig. 1b).

There was a clear difference in number of osteoclasts in the different locations between the treatment groups (Fig. 3). After fluid pressure, osteoclasts were increased at periphery by time. This effect was already evident after 1 day although not significant ($P = 0.06$), but became more evident after 3 ($P < 0.05$) and 5 days ($P < 0.01$). At the central exposure zone there were no effect seen over time by fluid pressure. In contrast, titanium particles stimulated osteoclast number at the central exposure area but not at the peripheral sites. At day five, there was a trend ($P = 0.07$) of higher number of Cathepsin K positive osteoclasts within the whole area after fluid pressure exposure ($147 \pm 71$) compared to the group exposed to titanium particles ($83 \pm 18$). In the controls, the few osteoclasts detected were spread randomly.

### GENE EXPRESSION OF INFLAMMATORY MARKERS

To characterize the expression pattern of inflammation-related genes after fluid pressure or titanium particle exposure: IL-6, TNFα, IL-1, Prostaglandin E Synthase (PGES), TGFβ, inducible Nitric Oxide (iNOS) as well as selected genes involved in migration of inflammatory cells (CCL2, CX3CL1). The tissue in the experimental area was harvested at 1 and 3 days in order to detect early changes compared to controls.

After 1 day of fluid pressure, three of eight genes related to an inflammatory response were up regulated compared to the controls (Fig. 4), namely PGES (3-fold), iNOS (6-fold) and IL-6 (6-fold). After an additional 2 days, none of these genes were differently expressed. After exposure to titanium particles for 1 day, six of eight genes were up-regulated compared to controls, such as PGES (8-fold), iNOS (4-fold), TNFα (2-fold), IL-β (3-fold), CCL2 (3-fold), and IL-6 (67-fold). After two more days of titanium particle exposure, the expression of CCL2 was up-regulated 5-fold, TGFβ 2-fold, and IL-6 declined to a 7-fold up-regulation.

### GENE EXPRESSION OF MACROPHAGE AND OSTEOCLAST MARKERS

Differentiation and activation of osteoclasts involves a complex interplay of gene products. Therefore, several genes were selected specific to different stages of the macrophage lineage (CD68, CD163), migrating precursor cells (TRAP, MMP9) as well as the RANKL-OPG system (RANKL, RANK, OPG) and functional osteoclasts (Cathepsin K).

After 1 day of fluid pressure or particle exposure, there was a slight down-regulation of CD68 mRNA expression compared to controls (1.5-fold) (Fig. 5). A 8- or 15-fold up-regulation of Cathepsin K mRNA expression was detected after two more days of fluid pressure or titanium particles, respectively. In the particle exposed group, there was a down-regulation of MMP-9 (2-fold), and TRAP (3-fold) after 1 day, with no significant changes at day 3. After 3 days of particle treatment, RANKL (5-fold), OPG (6-fold), and RANK (9-fold) were up regulated compared to the controls. No

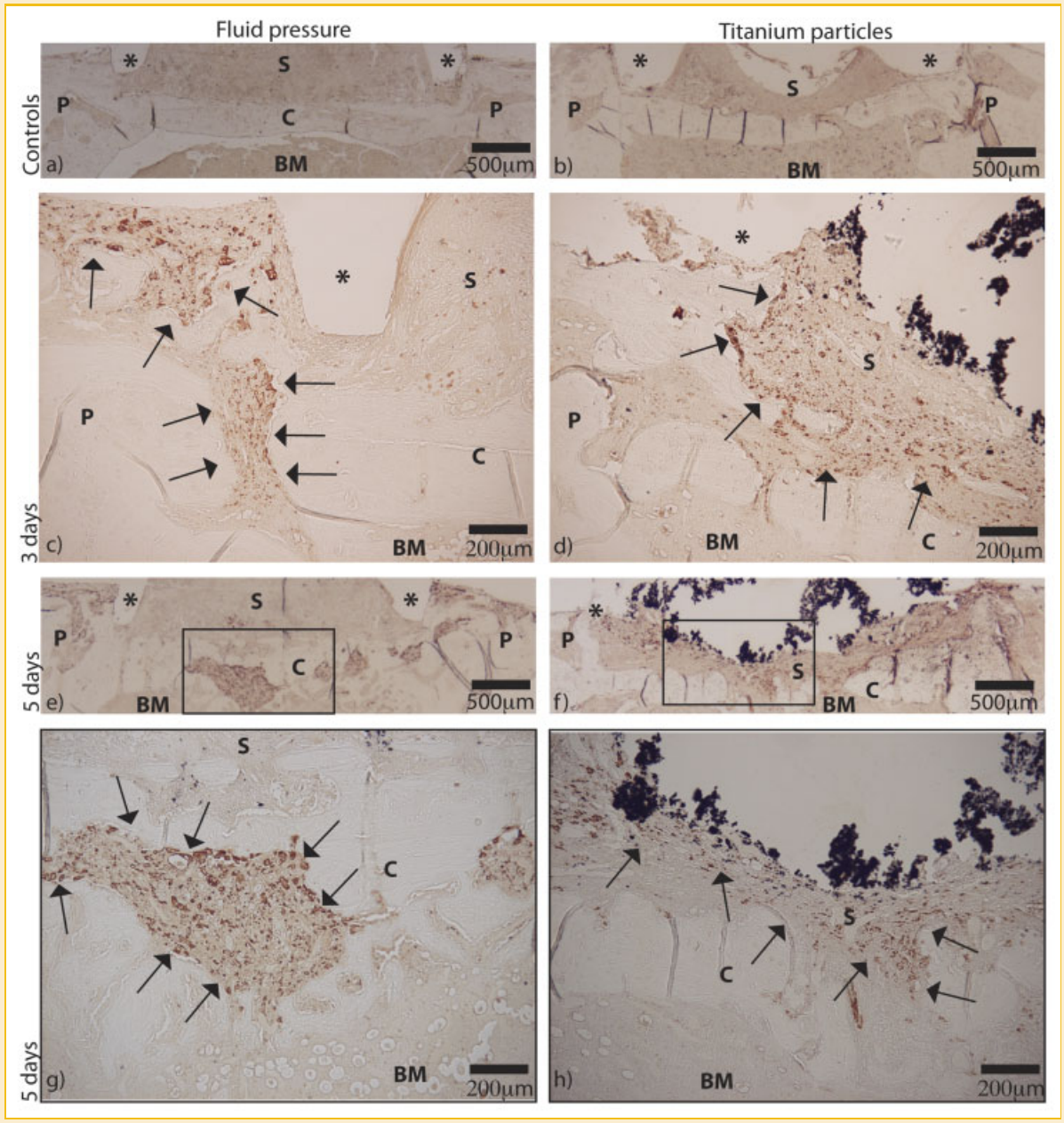


Fig. 2. Histological sections showing the distribution of Cathepsin K positive osteoclasts in the central (C) and peripheral (P) localization of the bone under and beside the soft tissue (S) and bone marrow (BM) underneath the pressure piston (a, c, e, g), or the hollow screw confusing delete in samples exposed to titanium particles (b, d, f, h). Controls are showing areas without any positive osteoclasts after both fluid pressure (a) and titanium particle exposure (b). After 3 days are osteoclasts were recruited mainly to the peripheral location after fluid pressure (b) and the central location after titanium particle exposure (c). Boxes in (e) and (f) after 5 days of exposure indicate location of higher magnification of osteoclasts induced by fluid pressure (g) or Titanium particles (h). Arrow indicates numerous Cathepsin K positive osteoclasts. Asterisk indicates the placement of the edge of the former piston or the hollow screw. BM, bone marrow; C, central location; P, peripheral location, S, soft tissue. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

significant change in RANKL, RANK or OPG mRNA expression was observed at any time point in the fluid pressure group.

### CORRELATION BETWEEN EXPRESSION OF INFLAMMATORY MARKERS AND RANKL

The correlation between RANKL and the inflammatory markers was compared after fluid pressure or titanium particle exposure (Table II).

RANKL was positively correlated with CCL2, PGES and iNOS after both fluid pressure or titanium particle exposure. RANKL was highly or moderately correlated with TGF-β, TNFα, and CX3CL1 after titanium particle exposure, while there was lack of correlation between these genes after fluid pressure. After fluid pressure RANKL was correlated with IL-6, but this link was not observed after titanium particle exposure.

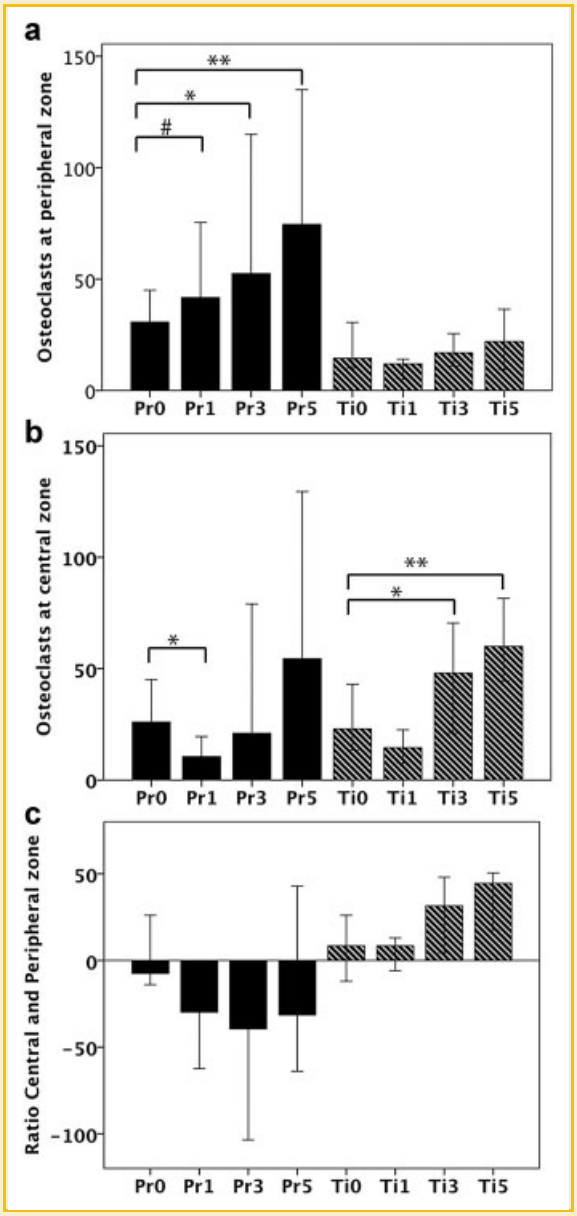


Fig. 3. Osteoclast number at the periphery (a) and central zone (b) of the cortical bone under the piston at 0, 1, 3, and 5 days of fluid pressure (Pr0, Pr1, Pr3, Pr5) or titanium particle exposure (Ti0, Ti1, Ti3, Ti5). The ratio between the central and peripheral zone (c) illustrate the different location of osteoclasts induced by fluid pressure or titanium particles. Black bars indicate pressure (Pr) and dashed bars indicate titanium particles (Ti). $^{\#}P < 0.05$–$0.02$, $^{**}P < 0.01$, $^{***}P < 0.001$, represents the $P$-value compared to controls.

### CORRELATION BETWEEN EXPRESSION OF INFLAMMATORY MARKERS AND CATHEPSIN K

The correlation between Cathepsin K and the inflammatory markers was compared after fluid pressure or titanium particle exposure (Table III).

Cathepsin K was positively correlated with CX3CL1, CCL2, IL-1β, and iNOS after fluid pressure or titanium particle exposure. Cathepsin K was highly or moderately correlated to TGF-β, TNFα and PGES after titanium particle exposure while there was lack of significant correlation between these genes after fluid pressure.

### OSTEOCLAST NUMBER AFTER TREATMENT WITH DEXAMETHASONE AND ENBREL

TNFα has been shown to be critical for osteoclastogenesis induced by wear particles in several in vivo [Schwarz et al., 2000; Childs et al., 2001] and in vitro models [Merkel et al., 1999]. To determine if the innate immune system by itself or more specifically if TNFα would be able to control fluid pressure induced osteolysis we treated animals with dexamethasone or a TNFα receptor antagonist (Enbrel), and additionally saline treated pressurized controls.

Dexamethasone clearly suppressed the osteoclast number after 5 days of pressure compared to controls ($P = 0.00$). With Enbrel treatment, a trend of suppression was seen, but this was not significant ($P = 0.13$) (Fig. 6).

## DISCUSSION

Present and previous studies demonstrate that forces generated around the implant contribute to bone loss [Fahlgren et al., 2010; Aspenberg et al., 2011]. We were able to show that fluid pressure-induced osteoclastogenesis is of the same magnitude as that generated by the presence of titanium particles. One important difference, however, is that the former outcome is accompanied by only a moderate expression of important regulators of the innate inflammatory pathway, most notably TNFα. Surprisingly the presence of the well-documented TNFα inhibitor Enbrel, failed to generate statistically less osteoclast generation and bone loss while dexamethasone, a known inhibitor of the innate inflammatory system, was more potent in this regard. This observation suggests strongly that targeting other mediators of osteoclast activation may be a reasonable approach to inhibit pressure-induced osteoclastogenesis.

Compared to titanium particle exposure, fluid pressure resulted in an overall lower level of expression of innate inflammatory response genes but also showed a different pattern of osteoclast locations. Osteoclasts stimulated by fluid pressure appeared at localizations inside the bone compared to those stimulated by particles, which were seen at the surface. Osteoclasts were also found mainly in the peripheral area beside the piston [Fahlgren et al., 2010] while osteoclast stimulated by titanium particles was present just below the pressure piston. This might suggests that fluid pressure recruited cells mainly from the bone marrow meanwhile titanium particles seemed to recruit cells from the soft tissue beneath the piston.

We corroborated that titanium particle-induced osteolysis displays an up-regulation of a pro-inflammatory response and subsequent osteoclast activation as previously described in both animal models [Taki et al., 2005] and in vitro studies [Gordon et al., 2010]. TNFα, IL-1, and IL-6 were all up regulated at the mRNA level after 24 h, which might be related to osteoblastic and/or macrophage-driven stimulation of osteoclastogenesis. Up-regulation of several cytokines and chemokines, as described in the present study, has been demonstrated to correlate with periprosthetic osteolysis [Wang et al., 2010] and increased risk for revision [Gallo et al., 2009]. Other factors upregulated by pressure were prostaglandin E

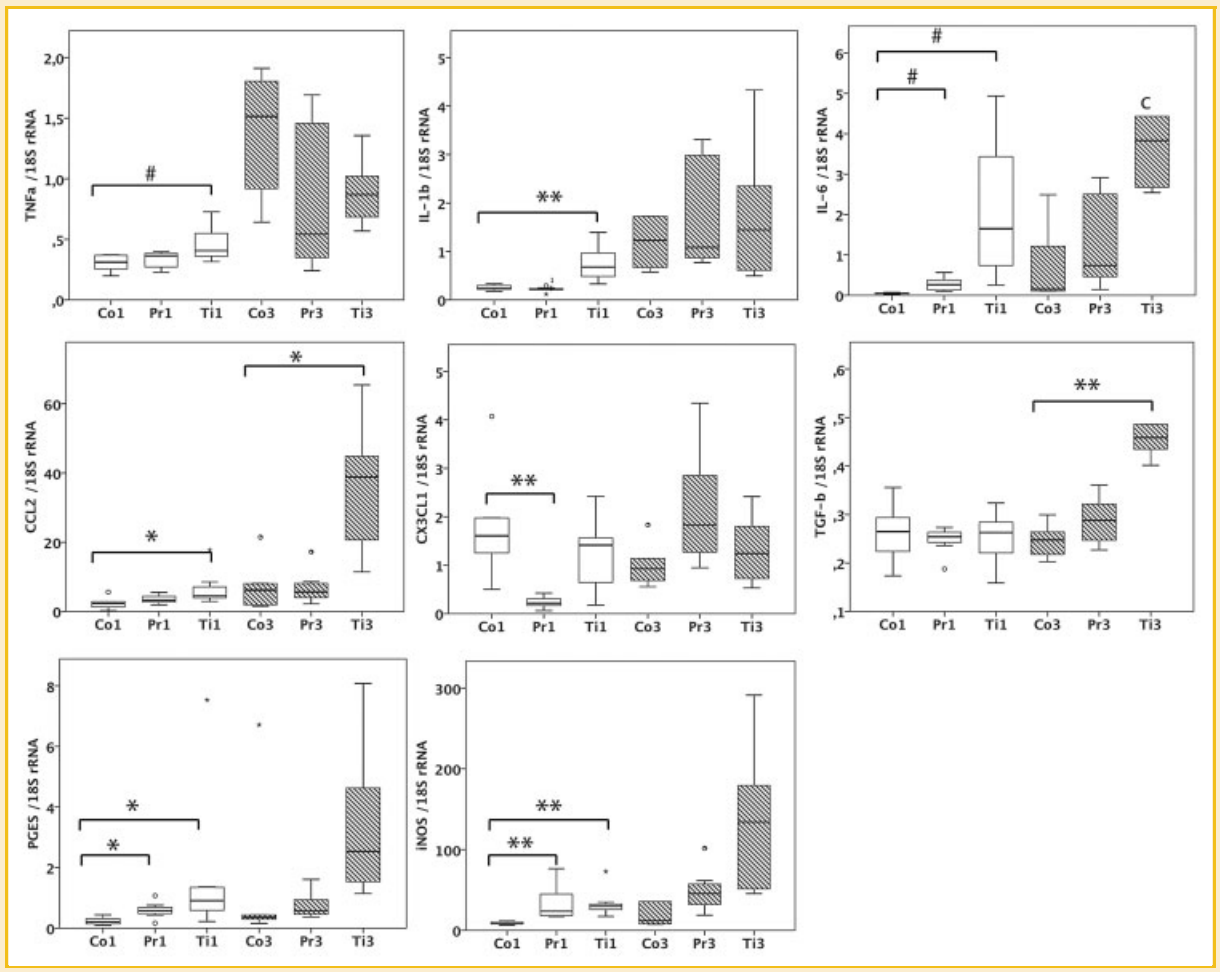


Fig. 4. Box plots show the expression of selected inflammatory genes, which showed significant differences within groups. The vertical axis shows the ratio to a housekeeping gene (18s rRNA). The horizontal axis shows time of exposure (1 or 3 days) after fluid pressure (Pr), titanium particles (Ti) or untreated controls (Co). Pressure (Pr) has white boxes and titanium (Ti) has hatched boxes. $^{\#}P < 0.051$, $^{*}P < 0.02$, $^{**}P < 0.009$, represents the *P*-value compared to controls.

synthase (PGES) and iNOS [Sanchez et al., 2009]. The products of these enzymes have been suggested to be involved in the response of osteocytes to mechanical stimulation [Klein-Nulend et al., 1998; Lau et al., 2010]. $PGE_2$, synthesized by PGES is of particular interest as a direct osteoclast regulator, through modulation of β-catenin and OPG-levels [Kitase et al., 2010]. Low β-catenin level in osteocytes results in low secretion of OPG and hence increased osteoclast activation. Consistent with these findings, there is a marked increase in the number of empty osteocyte lacuna in response to fluid pressure [Van der Vis et al., 1999; De Man et al., 2005]. Our observations are also in line with previous in vivo studies in which mechanical regulation and bone trauma led to osteocyte apoptosis preceding osteoclast activation, seen after osteotomy [Clark et al., 2005], tooth movement [Sakai et al., 2009], and in response to bone fatigue [Cardoso et al., 2009]. Moreover, iNOS and $PGE_2$ are as well expressed in the prosthesis interface membrane in patients with aseptic loosening [Hukkanen et al., 1997].

CCL2 is expressed both in the membrane around loose prosthesis and at the pressure side during periodontal movement [Garlet et al., 2008; Wang et al., 2010]. CX3CL1, on the other hand, is induced by RANKL, stimulates osteoclast differentiation, and is a novel target for bone destruction diseases such as rheumatoid arthritis and bone metastasis [Ha et al., 2010]. CX3CL1 and CCL2 were highly correlated with RANKL and Cathepsin K in response to fluid pressure or titanium particles, suggesting that these chemoattractants are important for homing of precursors in response to both stimuli. Moreover pressure- and wear particle-stimulated osteolysis is induced rapidly, re-enforcing the important role of progenitor recruitment. Bone marrow precursor cells can be differentiated rapidly depending of the degree of commitment [de Souza Faloni et al., 2010], as demonstrated by identification of cycle-arrested quiescent osteoclast precursors in vivo. These cells were localized at bone surfaces in the vicinity of osteoblasts and were shown to survive for several weeks [Mizoguchi et al., 2009].

We have previously found that bone resorption induced by fluid pressure was completely inhibited by OPG-Fc [Aspenberg et al., 2011]. Thus, we were surprised to find that despite massive and rapid increases in osteoclastogenesis and increased Cathepsin K levels

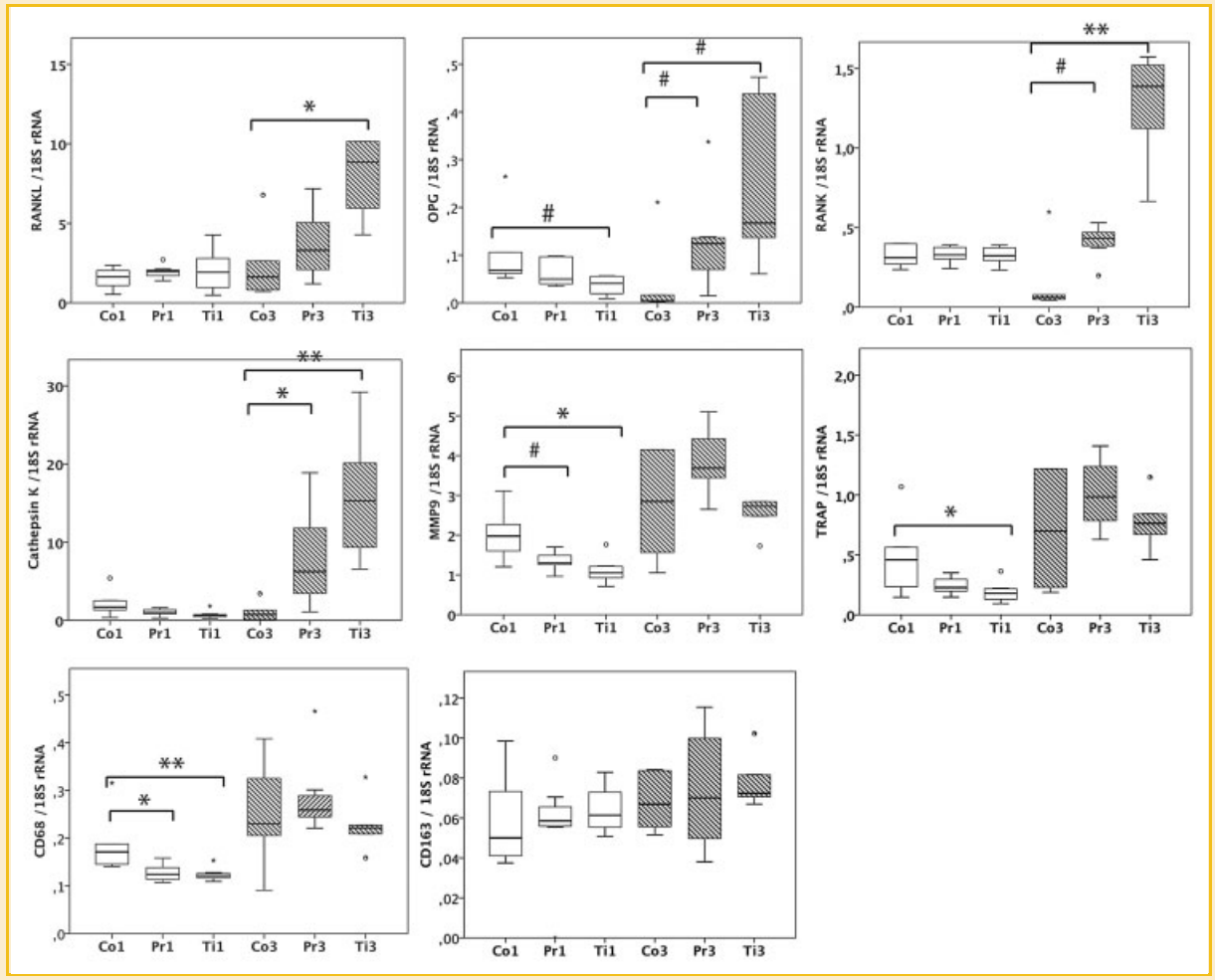


Fig. 5. Box plots show the expression of osteoclast and macrophage related genes, which was shown significant differences within groups. The vertical axis shows the ratio to a housekeeping gene (18s rRNA). The horizontal axis shows time of exposure (1 or 3 days) after fluid pressure (Pr), titanium particles (Ti) or untreated controls (Co). Pressure (Pr) has white boxes and titanium (Ti) has hatched boxes. $^{\#}P < 0.051$, $^{*}P < 0.02$, $^{**}P < 0.009$, represents the p-value compared to controls.

compared to controls there was no change in genes defining the RANKL/OPG pathway. One possible explanation is based on the fact that osteoclastogenesis can be induced by the actions of inflammatory cytokines, working synergistically with the RANKL/RANK pathway [Masui et al., 2005]. In particular, TNFα in the presence of M-CSF can stimulate osteoclast differentiation in vitro, but need IL-1 for full potency [Sabokbar et al., 2003]. IL-1 can mediate the osteoclastogenic effect of TNFα by enhancing RANKL expression in bone stromal cells [Wei et al., 2005]. It was sufficient to use less than one percent of RANKL for priming of osteoclast

TABLE II. Correlation Between mRNA Expressions of Inflammatory Related Genes and RANKL

| | Titanium particles (n = 12) | | Fluid pressure (n = 14) | |
|---|---|---|---|---|
| | Correlation to RANKL ($R^2$) | *P*-value | Correlation to RANKL ($R^2$) | *P*-value |
| CX3CL1 | 0.44 | 0.05 | 0.15 | 0.16 |
| CCL2 | 0.64 | 0.01 | 0.94 | 0.01 |
| TGFβ | 0.74 | 0.01 | −0.06 | 0.42 |
| PGES | 0.34 | 0.05 | 0.45 | 0.01 |
| iNOS | 0.58 | 0.01 | 0.31 | 0.05 |
| TNFα | 0.69 | 0.01 | 0.00 | 0.83 |
| IL1β | 0.00 | 0.96 | 0.10 | 0.26 |
| IL6 | 0.02 | 0.66 | 0.34 | 0.01 |

TABLE III. Correlation Between mRNA Expressions of Inflammatory Related Genes and Cathepsin K

| | Titanium particles (n = 12) | | Fluid pressure (n = 14) | |
|---|---|---|---|---|
| | Correlation to Cathepsin K ($R^2$) | *P*-value | Correlation to Cathepsin K ($R^2$) | *P*-value |
| CX3CL1 | 0.48 | 0.05 | 0.49 | 0.01 |
| CCL2 | 0.78 | 0.01 | 0.40 | 0.05 |
| TGFβ | 0.72 | 0.01 | 0.58 | 0.09 |
| PGES | 0.41 | 0.05 | 0.26 | 0.06 |
| iNOS | 0.74 | 0.01 | 0.29 | 0.05 |
| TNFα | 0.76 | 0.01 | 0.14 | 0.19 |
| IL1β | 0.32 | 0.05 | 0.40 | 0.05 |
| IL6 | 0.00 | 0.81 | 0.22 | 0.09 |

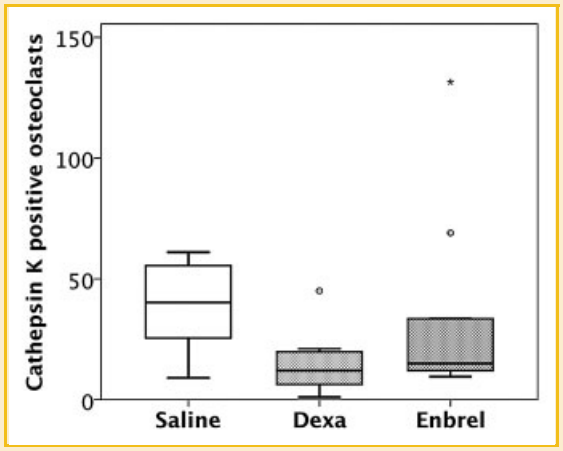


Fig. 6. Box plot shows suppression of osteoclasts by dexamethasone ($P = 0.002$) and Enbrel ($P = 0.13$) after 5 days of fluid pressure exposure.

precursor cells to synergize with TNFα to promote osteoclastogenesis [Lam et al., 2000]. With priming, also a lower concentration of the cytokine is needed to activate stromal cells for induction of osteoclast formation [Kitaura et al., 2004]. In vitro bone stromal cells [Kim et al., 2009] and osteocytes [You et al., 2008] respond to mechanical loading such as fluid flow and pressure by release of RANKL and OPG within 2 h. Thus, we propose that in the current study, the expression of RANKL, if occurring at an earlier time point than 24 h, might be sufficient to synergize with even a low concentration of other cytokines, including but not restricted to TNFα.

We examined the role of both TNFα and other inflammatory cytokines in augmenting fluid-pressure-induced osteoclastogenesis using well-documented antagonists. Nevertheless, even with a low mRNA expression of TNFα and other important regulators of the innate inflammatory system we found that dexamethasone inhibited osteoclastogenesis. Although dexmethasone has a dual role in osteoclastogenesis we used dexamethasone as a positive control to suppress the majority of the T-cell- and macrophage-mediated inflammatory system. However, since dexamethasone also has been shown to affect the osteoclasts both indirectly by influencing osteoclastogenesis through osteoblast differentiation [Rauch et al., 2010], and directly by influencing the osteoclast bone degrading capacity mainly through disruption of organization of the cytoskeleton [Kim et al., 2006], we cannot unequivocally conclude that the suppression of osteoclast number was solely due to inhibition of the inflammatory cascade. On the contrary, we were able to see a strong upregulation of osteoclast stimulating factors such as IL-6, PGES, and iNOS by fluid pressure, which was most likely suppressed by dexamethasone.

Unexpectedly the TNFα receptor antagonist Enbrel, failed to provide a statistically significant decrease in the process, but merely a trend. Alternatively, it could also open up possibilities that presence of other inflammatory factors such as IL-6, which was highly expressed in the current study may have an important role in pressure induced osteoclastogenesis.

In summary, additional mechanisms leading to the fluid pressure-induced osteoclastogenesis are unidentified, but may include

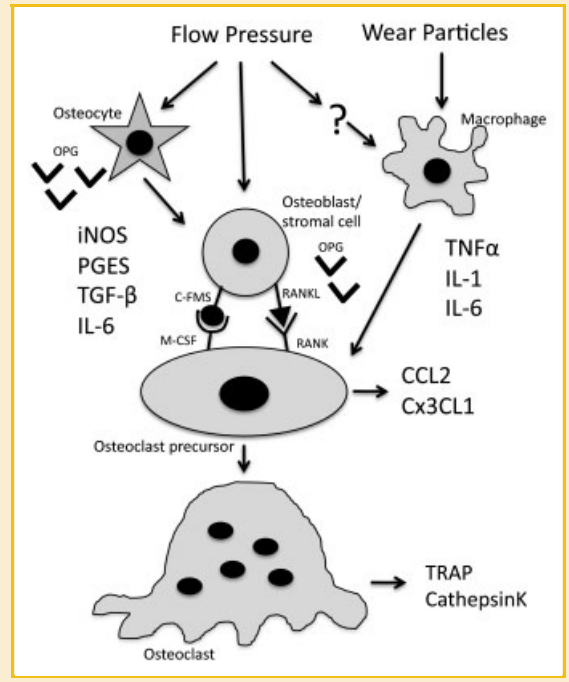


Fig. 7. Flow chart proposing additional mechanisms for pressure induced osteolysis comparing to wear-particle induced osteolysis. Fluid pressure may include an osteoblast and/or osteocyte driven osteoclastogenesis compared to the predominantly macrophage driven process induced by wear particles.

osteocyte apoptosis driven osteoclastogenesis (Fig. 7). There is support for the osteocyte hypothesis by increased RANKL/OPG ratio in osteocytes in response to increased pressure in vitro or following bone fatigue in vivo [Lau et al., 2010; Cheung et al., 2011], which was further shown to be diminished by specific inhibitors of apoptosis [Cardoso et al., 2009]. However, further studies are required to target this pathway, but also other mediators that might be crucial for pressure induced osteolysis.

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## REFERENCES

Aspenberg P, Agholme F, Magnusson P, Fahlgren A. 2011. Targeting RANKL for reduction of bone loss around unstable implants: OPG-Fc compared to alendronate in a model for mechanically induced loosening. Bone 48:225–230.

Bar-Shavit Z. 2007. The osteoclast: A multinucleated, hematopoietic-origin, bone-resorbing osteoimmune cell. J Cell Biochem 102:1130–1139.

Bonewald LF. 2011. The amazing osteocyte. J Bone Miner Res 26:229–238.

Cardoso L, Herman BC, Verborgt O, Laudier D, Majeska RJ, Schaffler MB. 2009. Osteocyte apoptosis controls activation of intracortical resorption in response to bone fatigue. J Bone Miner Res 24:597–605.

Cheung WY, Liu C, Tonelli-Zasarsky RM, Simmons CA, You L. 2011. Osteocyte apoptosis is mechanically regulated and induces angiogenesis in vitro. J Orthop Res 29:523–530.

Childs LM, Goater JJ, O'Keefe RJ, Schwarz EM. 2001. Efficacy of etanercept for wear debris-induced osteolysis. J Bone Miner Res 16:338–347.

Clark WD, Smith EL, Linn KA, Paul-Murphy JR, Muir P, Cook ME. 2005. Osteocyte apoptosis and osteoclast presence in chicken radii 0–4 days following osteotomy. Calcif Tissue Int 77:327–336.

De Man FH, Tigchelaar W, Marti RK, Van Noorden CJ, Van der Vis HM. 2005. Effects of mechanical compression of a fibrous tissue interface on bone with or without high-density polyethylene particles in a rabbit model of prosthetic loosening. J Bone Joint Surg Am 87:1522–1533.

de Souza Faloni AP, Schoenmaker T, Azari A, Katchburian E, Cerri PS, de Vries TJ, Everts V. 2011. Jaw and long bone marrows have a different osteoclastogenic potential. Calcif Tissue Int 88(1):63–74.

Eliasson P, Fahlgren A, Aspenberg P. 2008. Mechanical load and BMP signaling during tendon repair: A role for follistatin? Clin Orthop Relat Res 466:1592–1597.

Fahlgren A, Bostrom MP, Yang X, Johansson L, Edlund U, Agholme F, Aspenberg P. 2010. Fluid pressure and flow as a cause of bone resorption. Acta Orthop 81:508–516.

Gallo J, Mrazek F, Petrek M. 2009. Variation in cytokine genes can contribute to severity of acetabular osteolysis and risk for revision in patients with ABG 1 total hip arthroplasty: A genetic association study. BMC Med Genet 10:109.

Garlet TP, Coelho U, Repeke CE, Silva JS, Cunha Fde Q, Garlet GP. 2008. Differential expression of osteoblast and osteoclast chemmoatractants in compression and tension sides during orthodontic movement. Cytokine 42:330–335.

Goodman SB, Ma T. 2010. Cellular chemotaxis induced by wear particles from joint replacements. Biomaterials 31:5045–5050.

Gordon A, Greenfield EM, Eastell R, Kiss-Toth E, Wilkinson JM. 2010. Individual susceptibility to periprosthetic osteolysis is associated with altered patterns of innate immune gene expression in response to pro-inflammatory stimuli. J Orthop Res 28:1127–1135.

Ha J, Choi HS, Lee Y, Kwon HJ, Song YW, Kim HH. 2010. CXC chemokine ligand 2 induced by receptor activator of NF-kappa B ligand enhances osteoclastogenesis. J Immunol 184:4717–4724.

Holding CA, Findlay DM, Stamenkov R, Neale SD, Lucas H, Dharmapatni AS, Callary SA, Shrestha KR, Atkins GJ, Howie DW, Haynes DR. 2006. The correlation of RANK, RANKL and TNFalpha expression with bone loss volume and polyethylene wear debris around hip implants. Biomaterials 27:5212–5219.

Hukkanen M, Corbett SA, Batten J, Konttinen YT, McCarthy ID, Maclouf J, Santavirta S, Hughes SP, Polak JM. 1997. Aseptic loosening of total hip replacement. Macrophage expression of inducible nitric oxide synthase and cyclo-oxygenase-2, together with peroxynitrite formation, as a possible mechanism for early prosthesis failure. J Bone Joint Surg Br 79: 467–474.

Ingham E, Fisher J. 2005. The role of macrophages in osteolysis of total joint replacement. Biomaterials 26:1271–1286.

Kaar SG, Ragab AA, Kaye SJ, Kilic BA, Jinno T, Goldberg VM, Bi Y, Stewart MC, Carter JR, Greenfield EM. 2001. Rapid repair of titanium particle-induced osteolysis is dramatically reduced in aged mice. J Orthop Res 19:171–178.

Kim HJ, Zhao H, Kitaura H, Bhattacharyya S, Brewer JA, Muglia LJ, Ross FP, Teitelbaum SL. 2006. Glucocorticoids suppress bone formation via the osteoclast. J Clin Invest 116:2152–2160.

Kim JH, Jin HM, Kim K, Song I, Youn BU, Matsuo K, Kim N. 2009. The mechanism of osteoclast differentiation induced by IL-1. J Immunol 183: 1862–1870.

Kitase Y, Barragan L, Qing H, Kondoh S, Jiang JX, Johnson ML, Bonewald LF. 2010. Mechanical induction of PGE2 in osteocytes blocks glucocorticoid-induced apoptosis through both the beta-catenin and PKA pathways. J Bone Miner Res 25:2381–2392.

Kitaura H, Sands MS, Aya K, Zhou P, Hirayama T, Uthgenannt B, Wei S, Takeshita S, Novack DV, Silva MJ, Abu-Amer Y, Ross FP, Teitelbaum SL. 2004. Marrow stromal cells and osteoclast precursors differentially contribute to TNF-alpha-induced osteoclastogenesis in vivo. J Immunol 173:4838–4846.

Klein-Nulend J, Helfrich MH, Sterck JG, MacPherson H, Joldersma M, Ralston SH, Semeins CM, Burger EH. 1998. Nitric oxide response to shear stress by human bone cell cultures is endothelial nitric oxide synthase dependent. Biochem Biophys Res Commun 250:108–114.

Koizumi K, Saitoh Y, Minami T, Takeno N, Tsuneyama K, Miyahara T, Nakayama T, Sakurai H, Takano Y, Nishimura M, Imai T, Yoshie O, Saiki I. 2009. Role of CX3CL1/fractalkine in osteoclast differentiation and bone resorption. J Immunol 183:7825–7831.

Lam J, Takeshita S, Barker JE, Kanagawa O, Ross FP, Teitelbaum SL. 2000. TNF-alpha induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. J Clin Invest 106:1481–1488.

Lau E, Al-Dujaili S, Guenther A, Liu D, Wang L, You L. 2010. Effect of low-magnitude, high-frequency vibration on osteocytes in the regulation of osteoclasts. Bone 46:1508–1515.

Mandelin J, Li TF, Liljestrom M, Kroon ME, Hanemaaijer R, Santavirta S, Konttinen YT. 2003. Imbalance of RANKL/RANK/OPG system in interface tissue in loosening of total hip replacement. J Bone Joint Surg Br 85:1196–1201.

Masui T, Sakano S, Hasegawa Y, Warashina H, Ishiguro N. 2005. Expression of inflammatory cytokines, RANKL and OPG induced by titanium, cobalt-chromium and polyethylene particles. Biomaterials 26:1695–1702.

Merkel KD, Erdmann JM, McHugh KP, Abu-Amer Y, Ross FP, Teitelbaum SL. 1999. Tumor necrosis factor-alpha mediates orthopedic implant osteolysis. Am J Pathol 154:203–210.

Mizoguchi T, Muto A, Udagawa N, Arai A, Yamashita T, Hosoya A, Ninomiya T, Nakamura H, Yamamoto Y, Kinugawa S, Nakamura M, Nakamichi Y, Kobayashi Y, Nagasawa S, Oda K, Tanaka H, Tagaya M, Penninger JM, Ito M, Takahashi N. 2009. Identification of cell cycle-arrested quiescent osteoclast precursors in vivo. J Cell Biol 184:541–554.

Perren SM. 2002. Evolution of the internal fixation of long bone fractures. The scientific basis of biological internal fixation: Choosing a new balance between stability and biology. J Bone Joint Surg Br 84:1093–1110.

Rauch A, Seitz S, Baschant U, Schilling AF, Illing A, Stride B, Kirilov M, Mandic V, Takacz A, Schmidt-Ullrich R, Ostermay S, Schinke T, Spanbroek R, Zaiss MM, Angel PE, Lerner UH, David JP, Reichardt HM, Amling M, Schutz G, Tuckermann JP. 2010. Glucocorticoids suppress bone formation by attenuating osteoblast differentiation via the monomeric glucocorticoid receptor. Cell Metab 11:517–531.

Revell PA. 2008. The combined role of wear particles, macrophages and lymphocytes in the loosening of total joint prostheses. J R Soc Interface 5:1263–1278.

Rubin J, Rubin C, Jacobs CR. 2006. Molecular pathways mediating mechanical signaling in bone. Gene 367:1–16.

Sabokbar A, Kudo O, Athanasou NA. 2003. Two distinct cellular mechanisms of osteoclast formation and bone resorption in periprosthetic osteolysis. J Orthop Res 21:73–80.

Sakai Y, Balam TA, Kuroda S, Tamamura N, Fukunaga T, Takigawa M, Takano-Yamamoto T. 2009. CTGF and apoptosis in mouse osteocytes induced by tooth movement. J Dent Res 88:345–350.

Sanchez C, Gabay O, Salvat C, Henrotin YE, Berenbaum F. 2009. Mechanical loading highly increases IL-6 production and decreases OPG expression by osteoblasts. Osteoarthritis Cartilage 17:473–481.

Schwarz EM, Lu AP, Goater JJ, Benz EB, Kollias G, Rosier RN, Puzas JE, O'Keefe RJ. 2000. Tumor necrosis factor-alpha/nuclear transcription factor-kappaB signaling in periprosthetic osteolysis. J Orthop Res 18:472–480.

Shen Z, Crotti TN, McHugh KP, Matsuzaki K, Gravallese EM, Bierbaum BE, Goldring SR. 2006. The role played by cell-substrate interactions in the pathogenesis of osteoclast-mediated peri-implant osteolysis. Arthritis Res Ther 8:R70.

Skoglund B, Larsson L, Aspenberg PA. 2002. Bone-resorptive effects of endotoxin-contaminated high-density polyethylene particles spontaneously eliminated in vivo. J Bone Joint Surg Br 84:767–773.

Skripitz R, Aspenberg P. 2000. Pressure-induced periprosthetic osteolysis: A rat model. J Orthop Res 18:481–484.

Taki N, Tatro JM, Nalepka JL, Togawa D, Goldberg VM, Rimnac CM, Greenfield EM. 2005. Polyethylene and titanium particles induce osteolysis by similar, lymphocyte-independent, mechanisms. J Orthop Res 23:376–383.

Van der Vis HM, Aspenberg P, Tigchelaar W, Van Noorden CJ. 1999. Mechanical compression of a fibrous membrane surrounding bone causes bone resorption. Acta Histochem 101:203–212.

Walter WL, Clabeaux J, Wright TM, Walsh W, Walter WK, Sculco TP. 2005. Mechanisms for pumping fluid through cementless acetabular components with holes. J Arthroplasty 20:1042–1048.

Wang CT, Lin YT, Chiang BL, Lee SS, Hou SM. 2010. Over-expression of receptor activator of nuclear factor-kappaB ligand (RANKL), inflammatory cytokines, and chemokines in periprosthetic osteolysis of loosened total hip arthroplasty. Biomaterials 31:77–82.

Wei S, Kitaura H, Zhou P, Ross FP, Teitelbaum SL. 2005. IL-1 mediates TNF-induced osteoclastogenesis. J Clin Invest 115:282–290.

You L, Temiyasathit S, Lee P, Kim CH, Tummala P, Yao W, Kingery W, Malone AM, Kwon RY, Jacobs CR. 2008. Osteocytes as mechanosensors in the inhibition of bone resorption due to mechanical loading. Bone 42:172–179.

Zenger S, Hollberg K, Ljusberg J, Norgard M, Ek-Rylander B, Kiviranta R, Andersson G. 2007. Proteolytic processing and polarized secretion of tartrate-resistant acid phosphatase is altered in a subpopulation of metaphyseal osteoclasts in cathepsin K-deficient mice. Bone 41:820–832.