Orthopaedic implant materials drive M1 macrophage polarization in a spleen tyrosine kinase- and mitogen-activated protein kinase-dependent manner

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Abstract

Total joint replacements (TJR) are costly procedures required to relieve pain and restore function in patients suffering from end-stage arthritis. Despite great progress in the development and durability of TJRs, the generation of prosthesis-associated wear particles over time leads to an inflammatory cascade which culminates in periprosthetic osteolysis. Studies suggest that wear particles drive the polarization/differentiation of immature macrophages towards a pro-inflammatory M1 phenotype rather than an anti-inflammatory M2 phenotype associated with normal bone and wound healing. This, in turn, contributes to the initiation of peri-implant inflammation. As a result, modulating M1 macrophage cytokine production has been recognized as a viable therapeutic option. The aim of this study was to examine the impact of hydroxyapatite (HA) and poly(methyl methacrylate) (PMMA) particles on human macrophage polarization by comparing their effect on M1/M2-associated gene expression using real-time PCR. Furthermore, using immunoblotting to assess kinase activation, we sought to identify the intracellular signalling molecules activated by PMMA/HA particles and to determine whether pharmacological blockade of these molecules impacts on macrophage phenotype and cytokine production as measured by ELISA. We report that wear particles preferentially polarize macrophages towards an inflammatory phenotype, an effect that is dependent on activation of the membrane proximal kinase, Syk and members of the mitogen-activated protein kinase (MAPK) family of signalling molecules. Pre-treatment of macrophages with Syk inhibitors (R788/piceatannol) or MAPK inhibitors (SB203580 and PD98059), not only prevents M1 polarization, but also attenuates production of key pro-inflammatory mediators that have been specifically implicated in periprosthetic osteolysis and osteoclast differentiation.

Statement of Significance

It is now well established that wear-debris particles from implanted materials drive deleterious inflammatory responses which can eventually lead to implant loosening. In this study, we provide further insight into the specific cellular pathways activated by wear particles in primary human immune cells. We demonstrate that PMMA bone cement and hydroxyapatite, a commonly used biomaterial, drive the polarization of macrophages towards an inflammatory phenotype and identify the specific signalling molecules that are activated in this process. Pre-treatment of macrophages with pharmacological inhibitors of these molecules in turn prevents macrophage polarization and dampens inflammatory cytokine production. Hence these signalling molecules represent potential therapeutic targets to treat or possibly prevent particulate induced osteolysis.

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1. Introduction

Total joint replacements (TJR) are costly procedures required to alleviate pain and improve ambulation in patients with end stage osteoarthritis (OA) and rheumatoid arthritis. While many advances have been made in implant technology, revision surgeries are often required due to the limited lifespan of orthopaedic devices. The predominant factor contributing to implant failure is aseptic loosening which arises due to progressive inflammation at the bone-implant interface. Several studies have shown that synthetic materials currently marketed for orthopaedic applications drive potent host immune responses during the early post-implantation period and contribute significantly to local bone loss or osteolysis [1]. Specifically, ‘wear debris’ from ceramic and metal based prostheses can drive macrophage-mediated inflammatory responses leading to chronic synovitis, osteoclast activation and eventual implant loosening. In fact, periprosthetic osteolysis accounts for 75% of implant failures, and as a result, individuals must undergo revision surgery which, in itself, is associated with shorter duration of implant survival and higher patient risk [2].

A number of studies have reported that macrophages play an integral role in the initiation of periprosthetic osteolysis [3–5]. These cells can undergo phenotypic polarization to form pro-inflammatory M1 macrophages, which promote pathogen killing and are associated with chronic inflammation, or anti-inflammatory M2 macrophages which are associated with immunoregulation, tissue repair and remodelling. In the case of TJR, classically activated M1 macrophages are required to initiate the foreign body response necessary for early post-implantation wound healing, while their eventual transition to alternatively activated M2 macrophages is required to prevent fibrous capsule formation and chronic inflammation [6]. However, it has been reported that synovial tissues from patients undergoing revision surgery exhibit higher ratios of M1 versus M2 macrophages compared to tissues from patients undergoing primary TJR [7]. Furthermore, studies have shown that wear particles directly activate M1 macrophages leading to a sustained inflammatory response characterised by the production of chemokines and pro-inflammatory cytokines such as tumor-necrosis factor (TNFα), interleukin (IL)-1 and IL-6 [8–12]. As well as causing tissue damage, this increased production of pro-inflammatory mediators contributes to an imbalance in the levels of the key osteoclastogenesis regulators, RANKL and OPG, and facilitates the formation and activation of bone-resorbing osteoclasts [13–16]. As a result, efforts are underway to develop strategies to modulate periprosthetic inflammatory responses using selective small molecule inhibitors or anti-cytokine therapies. For example, studies have shown that the COX-2 inhibitor, celecoxib, suppresses wear debris-induced osteolysis in both the murine calvaria model and rabbit prosthesis model [17,18]. Furthermore, etanercept, the decoy receptor for TNFα, has been shown to reduce bone resorption and osteoclastogenesis in mice [19], as has gene delivery of the anti-inflammatory cytokine, IL-10 [20]. In addition to specific targeting, efforts are also being made to manipulate the interplay between the implant material itself and the host immune system, and recent evidence suggests that promoting specific interactions between the two can boost immune tolerance and positive healing outcomes [21].

Despite these efforts, many aspects of macrophage-mediated responses to particulate wear debris remain poorly characterised. We and others have previously reported that osteoarthritis-associated basic calcium phosphate (BCP) crystals which are composed mainly of hydroxyapatite (HA), a biomaterial commonly used to coat orthopaedic implants, drive deleterious pro-inflammatory cytokine production and the up-regulation of damage associated molecules that can further exacerbate inflammation in the joint [22–25]. PMMA bone cement, the fixative used to hold implants in place, is also known to generate wear debris particles and has been shown to induce inflammatory responses in murine macrophages and ex vivo patient samples [7,26,27]. We have previously reported that BCP and cholesterol crystals activate the membrane proximal kinase, Syk, in a receptor-independent manner and by a process known as membrane affinity-triggered signalling (MATS) which involves lipid raft formation and culminates in the expression of pro-inflammatory genes [22,28]. Syk inhibition has also been reported to inhibit HA-induced IL-6 production in murine chondrocytes [29] and PMMA induced IL-1β production and cell binding in murine macrophages [27]. Furthermore, it is known that MAPKs are activated downstream of Syk activation following Fc receptor engagement [30,31]. Therefore the aim of this study was to examine the effect of PMMA and HA particles on M1- and M2-associated gene expression in primary human macrophages, and to determine if Syk and MAPKs are involved in wear particle induced polarization and inflammatory responses. We also sought to determine whether pharmacological blockade of specific signalling pathways can impact PMMA- and HA-induced macrophage polarization and inflammation.

2. Materials and methods

2.1. Reagents

Ultrapure lipopolysaccharide (LPS), p38 (SB203580) and MEK/ERK (PD98059) inhibitors were from Invivogen (Toulouse, France). Syk inhibitor, R788, was from AdooQ BioScience (Irvine, CA). Recombinant human M–CSF was from PeproTech (Rocky Hill, NJ). Lymphoprep was from Stemcell Technologies (Grenoble, France). PMMA particles were obtained from Polysciences, Inc. (Warrington, PA). Primary antibodies were obtained from Cell Signalling Technology (Beverly, MA). The Syk inhibitor, piceatannol, hydroxyapatite (HA) particles methyl-β-cyclodextrin (MβCD), secondary antibodies, cell culture reagents and all other chemicals were from Sigma Aldrich (St. Louis, MO).

2.2. Scanning electron microscopy (SEM) and particle sizing

The size and morphology of the HA particles was characterized using scanning electron microscopy (SEM). The HA and PMMA samples were deposited onto aluminium SEM mounts (Ted Pella, USA) and sputter coated with Au-Pd (Agar Scientific, USA) prior to imaging with a Carl Zeiss Ultra scanning electron microscope, aligned at 5 kV (Carl Zeiss, Germany). Size distribution and zeta potential of HA particles were measured using dynamic light scattering (DLS) (ZetaSizer and DTS software 3000 HS, Malvern instruments, UK). Measurements were carried out under monochromatic, coherent He-Ne laser light of fixed wavelength (633 nm) at room temperature with each size determination yielding an average particle size expressed as the mean diameter (Zave) together with a graph of the size range.

2.3. Monocyte isolation and macrophage differentiation

Monocytes were isolated through density gradient centrifugation from leukocyte-enriched buffy coats, obtained with permission from the Irish Blood Transfusion Board, St. James’s Hospital, Dublin. CD14+ cells were subsequently acquired using the MagniSort Human CD14 Positive Selection Kit (ebioscience, San Diego, CA) and determined, by flow cytometry, to be >90% pure. To drive macrophage differentiation, CD14+ cells were cultured for 6 days in RPMI 1640 medium containing 1% penicillin-streptomycin and 10%...
Foetal Bovine Serum and treated with M-CSF (50 ng/ml) on days 0 and 3, as adapted from [32]. CD14 and CD11b were used as macrophage markers and cells were shown, by flow cytometry, to be >95% pure (Fig. S2A). These cells are used throughout the study and are referred to as primary human macrophages; any additional treatments (e.g., wear particles, inhibitors) are performed after this 6-day differentiation protocol.

2.4. Kinase activation

HA and PMMA particles were first tested for LPS contamination using the HEK-Blue™ hTLR4 assay system (Invivogen). HEK-blue cells expressing TLR4 were treated with the positive control, LPS (10–100 ng/ml), HA particles (250 µg/ml) or PMMA particles (500 µg/ml) for 24 h. The expression of SEAP which is under the control of NF-κB and AP-1 was tested by incubating cell supernatants with HEK-blue detection medium for 30 min at 37 °C and absorbance was read at 650 nm. Primary macrophages (2 x 106/ml 1 ml/well) were stimulated with PMMA (500 µg/ml) or HA (250 µg/ml) particles over the course of 30 min. Cells were lysed by addition of RIPA buffer (Tris 50 mM; NaCl 150 mM; SDS 0.1%; sodium deoxycholate 0.5%; Triton X 100) containing phosphatase inhibitor cocktail 3 (Sigma-Aldrich). Samples were subjected to SDS-PAGE (12% gel) prior to transfer to PVDF membranes and detection with anti-phospho-Syk, anti-phospho-p38 or anti-phospho-ERK antibodies.

2.5. Cytokine measurements

For IL-1β measurement in vitro, 2 signals are required; the first ‘priming’ signal upregulates the intracellular expression components of the NLRP3 inflammasome, as well as pro-IL-1β, the inactive precursor form of the cytokine while a second signal activates NLRP3 and drives the processing of pro-IL-1β into its mature, active form that is secreted from the cell. In vitro, LPS drives signal 1 while the particles act as signal 2. [24,27]. For IL-1β detection, primary macrophages (1 x 106/ml; 0.5 ml/well) were primed with the LPS (100 ng/ml) for 2 h to drive pro-IL-1β production prior to treatment with piceatannol (20 µM), R788 (25 µM), MCC950 (1 µM, 5 µM) or MyD88 (10 mM) for 40 min and stimulation with PMMA (500 µg/ml) or HA (250 µg/ml) particles for 24 h. For TNFα, IL-6 and IL-8 detection, macrophages were pre-treated with piceatannol (20 µM), R788 (25 µM), PD98059 (20 µM) or SB203580 (20 µM) for 40 min prior to stimulation with PMMA (500 µg/ml) or HA (250 µg/ml) particles for 24 h. Cytokine concentrations in the cell supernatants were quantified by ELISA (eBioscience, San Diego, CA).

2.6. Real-time polymerase chain reaction (PCR)

Human macrophages (1 x 106/ml; 0.5 ml/well) were treated with PMMA (250 µg/ml) or HA (500 µg/ml) particles for 24 h or were pre-treated with piceatannol (20 µM), R788 (25 µM), PD98059 (20 µM) or SB203580 (20 µM) for 40 min, prior to particle treatment for 24 h. RNA was extracted using High Pure RNA Isolation Kits (Roche), and equalised following determination of concentration and purity using the NanoDrop 2000c UV–Vis spectrophotometer. RNA from triplicate samples was reverse transcribed using the Applied Biosystems High-Capacity cDNA reverse transcription kit, prior to real-time PCR carried out on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, California). Reactions included cDNA, TaqMan fast universal PCR Master Mix and pre-designed TaqMan gene expression probes (Applied Biosystems) for CXC9, CXCL10, CXCL11, MRC1, CCL13 and the housekeeping gene, 18S ribosomal RNA. The 2–ΔΔCT method was used to analyse relative gene expression.

2.7. Statistical analysis

For real-time PCR and ELISA, three technical replicates, per donor, were obtained and the mean for each donor ± SEM was then plotted for n ≥ 3 healthy donors. Statistical analysis was performed by one way analysis of variance (ANOVA) with Tukey post-test where applicable or student’s t test when comparing only two observations. All experiments were run at least three times and analysed on GraphPad Prism 6 software. P value ≤ 0.05 was considered to be statistically significant.

3. Results

3.1. PMMA and HA particles directly promote M1 macrophage polarization

Prior to analysis, SEM was performed to assess the size and morphology of the individual particles. As expected, PMMA particles are microparticles ranging in diameter from 1 µm to 10 µm, while HA particles are irregular in shape (Fig. S1A & S1B). HA particles are prone to aggregation therefore we also performed particle sizing using dynamic light scattering. Using this method, the mean particle size was found to be approximately 1.3 µm. The zeta potential of the HA particles was also assessed using electrophoretic light scattering confirming their tendency to aggregate (Fig. S1C & S1D). Particles were also tested for LPS contamination using the HEK-Blue™ LPS Detection Kit and endotoxin levels were found to be below the threshold required for cell activation (Fig. S1E).

In order to examine the direct impact of HA and PMMA particles on macrophage polarization, primary human macrophages were treated with previously published doses of PMMA particles (500 µg/ml) or HA (250 µg/ml) for 24 h and expression of established M1/M2-associated genes was assessed by real-time PCR. PMMA significantly enhanced the mRNA expression of the M1 markers, CXCL9 (50–500 fold; p = .0079), CXCL10 (20–80-fold; p = .0097) and CXCL11 (20–120 fold; p = .0079) compared to control samples (Fig. 1A–C). HA also significantly enhanced the expression of CXCL9 (50–500 fold; p = .0119), CXCL10 (20–500 fold; p = .0118) and CXCL11 (20–700 fold; p = .0171) (Fig. 1F–H). In contrast, treatment of macrophages with either particulate led to a significant decrease in basal levels of the M2 macrophage markers, MRC1 and CCL13 (PMMA: Fig. 1D & E; HA: Fig. 1 & J). Due to donor–donor variability, we also carried out a direct comparison of the two particles using macrophages from two individual donors. The magnitude of response to PMMA and HA was similar in this side by side comparison in both cases (Fig. S3A and S3B). Surface marker expression of CD86 (a marker of activated macrophages) and the M2-associated surface marker, CD163, was assessed by flow cytometry. Both HA and PMMA were found to enhance the expression of CD86 above the level of the positive control, IFNγ, while CD163 surface expression was reduced upon HA or PMMA treatment (Fig. S2B and S2C).

3.2. PMMA and HA particles drive pro-inflammatory cytokine production in a Syk dependent manner in human macrophages

Having established that PMMA and HA particles preferentially polarize macrophages towards an M1 phenotype, we next sought to examine the signalling events involved in this transition. Given that Syk is involved in particulate induced cell signalling, we first sought to determine if PMMA and HA particles can directly activate this kinase in primary human macrophages. To test this, cells were stimulated with PMMA (500 µg/ml) or HA particles (250 µg/ml) over the course of 30 min and activation of Syk, as indicated by
Fig. 1. PMMA and HA particles promote M1 macrophage polarization. Primary human macrophages (0.5 × 10^6 cells/ml; n = 5 healthy donors) were stimulated with PMMA (500 µg/ml) or HA (250 µg/ml) particles for 24 h. mRNA levels of CXCL9, CXCL10, CXCL11, MRC1 and CCL13 were analysed by real-time PCR. Results shown are means (± SEM) of five independent experiments. (*) P ≤ .05, (**) P ≤ .01 and (***) P ≤ .001.

Fig. 2. PMMA and HA particles drive pro-inflammatory cytokine production in a Syk-dependent manner in primary human macrophages. Human macrophages (1 × 10^6 cells/ml; n = 3 healthy donors) were stimulated with (A) PMMA particles (500 µg/ml) or (B) HA particles (250 µg/ml) for the indicated time points. Phosphorylation of Syk was detected by immunoblotting using phospho-specific antibodies. Representative blots of three independent experiments are shown. (C & D) Densitometric analysis of western blots from three individual donors was performed using ImageJ software. Bar graphs illustrate the mean (±SEM) increase in phosphorylation of Syk relative to the untreated sample (0) and normalised to total Syk protein. Primary human macrophages (0.5 × 10^6 cells/ml) were treated with two Syk inhibitors, Piceatannol (20 µM) or R788 (25 µM) for 45 min prior to stimulation with PMMA (500 µg/ml) or HA (250 µg/ml) particles for 24 h. Cell supernatants were assessed for (E & F) IL-6, (G & H) TNF-α and (I & J) IL-8 by ELISA. Results shown are means (±SEM) of three independent experiments. (*) P ≤ .05, (**) P ≤ .01 and (***) P ≤ .001.
phosphorylation, was examined by immunoblotting. Phosphorylation of Syk was detected within 10–15 min of PMMA particle treatment (Fig. 2A) and within 5 min of HA treatment (Fig. 2B). Densitometric analysis of western blots from three individual donors revealed that maximal phosphorylation occurs at 30 min post-stimulation with both particle types, with approximate 15–30-fold increase in Syk phosphorylation compared to control cells (Fig. 2C & D).

In order to investigate if Syk is involved in PMMA- and HA-induced pro-inflammatory cytokine production in primary human macrophages, cells were pre-treated with previously published doses of either piceatannol (25 μM), or the orally available Syk inhibitor, R788 (25 μM), for 1 h prior to stimulation with PMMA or HA particles for 24 h. In the case of PMMA, pre-treatment with both inhibitors resulted in a significant reduction (piceatannol: p = .0049; R788: p = .0028) in IL-6 production (Fig. 2E) with R788 also significantly reducing TNFα production (Fig. 2G, p = .0176). HA-induced IL-6 and TNFα was significantly reduced upon Syk inhibition with piceatannol (p = .0025 and p = .0178, respectively) while the alternative Syk inhibitor, R788, also abrogated HA-induced IL-6 (p = .0011) and TNFα (p = .018) (Fig. 2F & H). It has been reported that wear particles can drive expression of the chemokine, IL-8 [33], and while we did observe enhanced IL-8 production in PMMA and HA treated cells, pre-treatment with either piceatannol or R788 prior to particle stimulation and both inhibitors were shown to attenuate IL-1β production confirming that, in addition to IL-6 and TNFα, Syk is also involved in PMMA- and HA-induced secretion of IL-1β (Fig. 2C & D). In order to ascertain whether lipid raft formation and MATS is involved in wear particle-induced IL-1β production in primary human macrophages, LPS-primed cells were depleted of membrane cholesterol with MβCD (10 mM) to prevent lipid sorting, prior to stimulation with PMMA or HA. Treatment with MβCD significantly reduced PMMA-induced IL-1β production (p = .008; Fig. 3E) and there was a trend towards reduced IL-1β in HA-treated cells (p = .0889; Fig. 3F).

3.3. PMMA and HA particles activate ERK and p38 MAPKs in primary human macrophages

In order to examine whether wear particles can activate MAPKs, primary human macrophages were stimulated with PMMA and HA over the course of 30 min and ERK and p38 activation, as indicated by phosphorylation, was assessed by immunoblotting. Robust phosphorylation of ERK and p38 was evident from the 15 min time point for both particle types. Densitometric analysis of western blots from three individual donors revealed that phosphorylation of ERK was significantly increased by PMMA (maximal phosphorylation approx. 80-fold) while p38 phosphorylation was increased...
by approximately 30-fold. In the case of HA, an approximate 200-fold increase in ERK phosphorylation was observed while there was an approximate 80-fold increase in p38 phosphorylation at the 30 min time point (Fig. 4A–D).

Having observed that PMMA and HA activate MAPKs, we next sought to establish whether particle-induced cytokine production is dependent on ERK and p38 MAPK activation. Primary human macrophages were pre-treated with PD98059 or SB203580 in order to inhibit ERK and p38, respectively, prior to stimulation with PMMA or HA particles for 24 h. Inhibition of p38 resulted in a significant decrease in PMMA induced IL-6 (∼0.15) and TNFα (∼0.024) production while inhibition of ERK also significantly reduced PMMA induced TNFα (∼0.113) (Fig. 5A & C). In the case of HA, both IL-6 and TNFα were significant reduced upon p38 inhibition (∼0.031 and ∼0.0175, respectively) while inhibition of ERK also abrogated HA-induced IL-6 (∼0.049) and TNFα (∼0.0178) (Fig. 5B & D), suggesting that MAPK activation is directly coupled to PMMA- and HA-induced pro-inflammatory cytokine expression. As was the case for Syk inhibition, MAPK inhibition had no effect on particle-induced IL-8 production, again suggesting an alternative activation pathway (Fig. 5E & F).

3.4. PMMA and HA particles induce M1 macrophage marker expression in a Syk- and MAPK-dependent manner

Having observed that PMMA and HA particles drive M1-associated gene expression, and that both Syk and ERK/p38 MAPKs are coupled to HA- and PMMA-induced pro-inflammatory cytokine production, we next sought to establish whether this M1 macrophage phenotype is dependent on Syk and MAPK activation. To test this, primary human macrophages were pre-treated with the MAPK inhibitors, PD98059 (ERK), SB203580 (p38), or the Syk inhibitor, Piceatannol and R788, prior to stimulation with PMMA or HA particles and mRNA expression levels of M1 markers were measured. Inhibition of p38 resulted in a significant decrease in mRNA expression of CXCL9 (∼0.0008) and CXCL10 (∼0.001) in PMMA particle-treated macrophages, while ERK inhibition also significantly reduced expression of both genes (CXCL9: ∼0.01; CXCL10: ∼0.001) (Fig. 6A & B). Inhibition of p38 significantly reduced HA-induced CXCL9 (∼0.014) and CXCL10 (∼0.0068) mRNA expression and, as with PMMA, this was also the case upon ERK inhibition (CXCL9: ∼0.0152; CXCL10: ∼0.0085) (Fig. 6C & D). Similarly, blockade of Syk prior to particle treatment resulted in a significant inhibition of these M1-associated genes (Fig. 6A–D).

4. Discussion

Due to increased life expectancy and the current obesity crisis, the number of individuals requiring joint replacement surgery is predicted to rise to 4 million annually by 2030 [36,37]. Several studies have reported that wear particles generated from orthopaedic implants can activate innate immune cells to drive deleterious inflammatory responses and osteolysis leading to aseptic loosening and implant failure. However, the precise mechanism through which this occurs has not been fully elucidated. We report that PMMA and HA particles activate the membrane-proximal kinase, Syk, and downstream MAP kinases in primary human macrophages and show that pharmacological inhibition of these enzymes abrogates particle-induced pro-inflammatory cytokine production and M1 macrophage polarization. SEM confirmed that the PMMA particles used in this study are microspheres ranging in diameter from 1 µm to 10 µm, while the HA particles are irregular in shape and have a similar morphology to those imaged in a recent study by Lebre et al. [38]. These particle sizes are clinically relevant as HA particles ranging in size from 1 to 5 µm as well as larger particles of 20 µm have been identified in histological samples from soft tissue biopsies from hip arthroplasty [39] while PMMA particles of widely variable size (0.2–10 µm) are produced as a result of fragmentation of polymerized PMMA and are also known to elicit inflammatory responses [40].

As well as inducing cytokines and genes associated with classically activated macrophages, both particle types were found to induce the expression of the M1-associated cell surface marker, CD86, while simultaneously downregulating the expression of the M2 surface marker, CD163. Our results complement ex vivo histological studies from patients undergoing revision TJR, which demonstrated that the specific presence of PMMA and/or HA particles in soft tissue biopsies was associated with increased numbers of activated macrophages. Specifically, higher numbers of macrophages expressing the M1 macrophage associated marker, HLA-DR, were found, while fewer macrophages expressing the M2 marker CD163 were observed (1). We have expanded on these studies and confirmed that PMMA and HA also impact on the expression of additional M1 associated genes including CXCL9, CXCL10, CXCL11 and, in a side by side comparison, the magnitude of the response was similar for both particle types.

In most circumstances, Syk is activated following Fc receptor engagement on immune cells and upon phagocytosis [41]. However, recent studies have reported that Syk is activated by particulates via MATS, a receptor-independent process that involves direct binding of particulates to the cell membrane. This results in lipid raft formation and aggregation of ITAM-containing molecules which mediate the recruitment of Syk, to the plasma membrane and facilitate its subsequent activation [42]. Syk has been implicated in both the internalisation of gout-associated MSU crystals and subsequent MSU-induced signalling in neutrophils and dendritic cells [42,43]. We have also previously reported that...
Atherosclerosis-associated cholesterol crystals and osteoarthritis-associated BCP crystals activate Syk via MATS leading to IL-1β processing and secretion [22,28]. In this study, treatment of macrophages with MblogCD, which is used to prevent lipid raft formation, effectively reduced PMMA- and HA-induced IL-1β production which is in agreement with a previous study showing similar effects in PMMA-activated murine macrophages [27]. We also show that pharmacological inhibition of the NLRP3 inflammasome, which is activated downstream of Syk [35], prevents wear particle induced IL-1β production. This is in agreement with in vitro studies using NLRP3 deficient murine macrophages [24,27], however there is evidence to suggest that this complex may be dispensable for the in vivo production of IL-1β [44,45] and further study is required to determine if MCC950 (or derivatives of) represents a viable therapeutic for the treatment of periprosthetic inflammation. Atomic force microscopy should also be performed to confirm that Syk activation is required for high affinity interactions between wear particles and the cell membrane of human macrophages.

Fig. 5. PMMA and HA particles drive TNFα and IL-6 production in a MAPK-dependent manner. Primary human macrophages (1 x 10⁶ cells/ml; n = 3 healthy donors) were treated with the p38 inhibitor, SB203580 (SB; 20 μM) or the ERK inhibitor, PD98059 (PD; 20 μM) for 45 min prior to stimulation with PMMA (500 μg/ml) or HA (250 μg/ml) particles for 24 h. Cell supernatants were assessed for (A & B) TNFα, (C & D) IL-6 and (E & F) IL-8 by ELISA. Results shown are means (±SEM) of 3 independent experiments. ( * P ≤ .05, ** P ≤ .01 and *** P ≤ .001).
We also provide evidence that PMMA and HA particles activate the downstream MAPKs, ERK and p38, in primary human macrophages and, as with Syk inhibition, pharmacological blockade of these molecules prevents M1 macrophage polarization and inflammatory gene expression. It has previously been reported that PMMA particles activate MAPKs in murine osteoclast precursor cells, contributing to osteoclast differentiation [46] hence, MAPK targeting has the potential to modulate the two key processes leading to aseptic implant loosening - periosteal inflammation and osteoclast formation. Interestingly, while we observed inhibition of IL-1β, TNFα and IL-6, wear particle-induced IL-8 production was not affected by Syk or MAPK inhibitors suggesting that this chemokine is regulated in a different manner. Our findings that PMMA particles drive M1 macrophage polarization are consistent with previous in vitro/ex vivo studies and it will now be of interest to determine if Syk and/or MAPK inhibitors can alter macrophage phenotype and prevent bone loss in mouse calvaria models.

There are currently no drugs specifically approved to prevent or inhibit peri-implant inflammation and subsequent periprosthetic osteolysis. Inhibitors of osteoclastogenesis and osteoclast function are being considered. For example, bisphosphonates are known to have anti-osteoclast activity through the induction of apoptosis, with riserdronate and zoledronic acid proving to be particularly potent and, in addition to alendronate, have been shown to reduce wear debris-induced osteolysis in animal models [47]. Statins and inhibitors of RANKL and recombinant parathyroid hormone are also reported to show efficacy in animal models, however it remains to be determined if any of these agents are efficacious in osteolysis patients [19,48–50]. Given the pathological similarities between periprosthetic osteolysis and arthritis, it has also been suggested that treatments that have been developed for the latter may be applicable for the prevention of aseptic implant loosening [1]. As mentioned previously, specific targeting of TNFα with etanercept (a decoy receptor for TNFα), has been shown to have anti-resorptive effects in a murine calvaria model. However, in a pilot study of 20 patients with established periprosthetic osteolysis, no significant difference in osteolysis progression was observed between the etanercept-treated group and placebo controls (although the small sample size may have been a factor here) [51]. While anti-cytokine therapies are indeed promising candidates for periprosthetic osteolysis, preventing cytokine expression rather than inhibiting their activity may prove more efficient. The orally available Syk inhibitor used in this study (R788) has previously shown efficacy in clinical trials for rheumatoid arthritis, however, reports of side effects during phase III trials led to trial termination [52]. Nevertheless, a modification on the current drug or indeed, an alternate method of administration, such as local delivery into the joint, is worthy of consideration given our...
in vitro results showing dual inhibition of IL-1β and TNFα, both of which are known to promote osteoclastogenesis even when RANKL levels are low [53].

Of note, HA and PMMA particles are not the only wear materials generated at the implant site. Metallic and polyethylene particles, which were not assessed in this study, are even more problematic and macrophages laden with these particles have been shown to have a direct relationship to the degree of bone resorption seen in periprosthetic tissue [54,55]. In vivo studies using the murine air pouch model of inflammation and periprosthetic osteolysis, have demonstrated that UHMWPE and Ti-6Al-4V particles increase IL-1β production and macrophage numbers at the site of injection. Furthermore, it has been recently been demonstrated that titanium particles can drive IL-1β production via the NLRP3 inflammasome [56]. Hence, until the development of more inert and durable biomaterials, an improved understanding of the signalling cascades produced by wear debris activated by wear particles may pave the way for new treatments to either prevent or modulate inflammation and periprosthetic osteolysis. Furthermore, the development of early detection methods for osteolysis would aid the prevention of aseptic implant loosening, which continues to represent a major complication of total joint replacement.

Disclosure
The authors declare no conflicts of interest.

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Appendix A. Supplementary data
Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.actbio.2017.10.041.

References


