Molecular Mechanisms Leading to Interleukin-1β Release by Macrophages in Response to Wear and Corrosion Products from Metal Implants

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List of Abbreviations

AAOS	American Academy of Orthopaedic Surgeons
AIM2	Absent in melanoma 2
ALVAL	Aseptic lymphocyte-dominated vasculitis-associated lesions
ARMD	Adverse reactions to metal debris
ASC	Apoptosis-associated speck-like protein containing a caspase recruitment domain
ATP	Adenosine triphosphate
Al_2O_3	Aluminum(III) oxide
β-ME	β-Mercaptoethanol
BMDM	Bone marrow-derived macrophages
BRCA1	Breast cancer 1 DNA repair associated (gene)
BRCA2	Breast cancer 2 DNA repair associated (gene)
BRCC3	BRCA1/BRCA2-containing complex subunit 3
CARD	Caspase recruitment domain
CGM	Complete growth medium
CJRR	Canadian Joint Replacement Registry
CoC	Ceramic-on-ceramic
CoCrMo	Cobalt-chromium-molybdenum (alloy)
CoP	Ceramic-on-polyethylene
Cr_2O_3	Chromium(III) oxide
DAMP	Damage-associated molecular pattern
DEP	Diesel exhaust particles
DMEM	Dulbecco’s modified Eagle medium
DPBS	Dulbecco’s phosphate buffered saline
ds	Double-stranded
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases
EU	Endotoxin units

FBS	Fetal bovine serum
HR	Hip resurfacing
HIN	Hematopoietic interferon-inducible nuclear (domain)
HXLPE	Highly-crosslinked polyethylene
ICE	IL-1β-converting enzyme
IL-1β	Interleukin-1β
IL-6	Interleukin-6
kDa	kiloDalton
L-AA	L-Ascorbic acid
LAL	Limulus amebocyte lysate
LPS	Lipopolysaccharide
LRR	Leucine-rich-repeat
M-CSF	Macrophage-colony stimulating factor
MAVS	Mitochondrial antiviral signalling protein
MEM	Mitochondrial-associated membrane
MC	Million cycles
MMP	Matrix metalloproteinase
MoM	Metal-on-metal
MoP	Metal-on-polyethylene
mtDNA	Mitochondrial DNA
M2/9I	MMP-2/MMP-9 Inhibitor I
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κβ	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	NOD-like receptor
NLRP3	NOD-, leucine-rich-repeat-, pyrin domain-containing protein 3
NLRC4	NOD-, leucine-rich-repeat-, caspase recruitment domain-containing protein 4
NOD	Nucleotide binding and oligomerization domain
NOX	NADPH oxidases
OA	Osteoarthritis
PA	Palmitic acid
PAMP	Pathogen-associated molecular pattern
PE	Polyethylene

PMMA	Polymethyl methacrylate
ppb	Parts per billion
ppm	Parts per million
PRR	Pathogen recognition receptor
PTFE	Polytetrafluoroethylene
PYD	Pyrin domain
PYHIN	Pyrin and HIN domain-containing proteins
RNAi	RNA interference
ROS	Reactive oxygen species
RT	Room temperature
SD	Standard deviation
SEM	Standard error of the mean
SM	Smooth muscle cells
Ti6Al4V	Titanium-6Aluminum-4Vanadium
TiO_2	Titanium dioxide
THA	Total hip arthroplasty
THR	Total hip replacement
TJA	Total joint arthroplasty
TJR	Total joint replacement
TKA	Total knee arthroplasty
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
UHMWPE	Ultra-high molecular weight polyethylene
WT	Wild type
ZrO_2	Zirconium dioxide

Abstract

Wear particles and ions from cobalt-chromium-molybdenum (CoCrMo)-based implants have been shown to cause adverse immune responses, including periprosthetic osteolysis leading to aseptic loosening, the main cause of implant failure. Previous studies have shown that these wear and corrosion products can lead to the release of inflammatory cytokines, including interleukin-1β (IL-1β), suggesting the involvement of the NLRP3 inflammasome. However, the mechanisms leading to IL-1β release have not been fully elucidated. The primary objectives of this thesis were to determine if, in murine macrophages, IL-1β release induced by micrometre-size CoCrMo particles and nanometre-size chromium oxide (Cr_2O_3) particles is: 1. Caspase-1-dependent; 2. Reduction-oxidation (redox)-dependent; and 3. NLRP3 inflammasome-dependent. Additionally, the effects of metal ions (Co^{2+}, Cr^{3+}, and Ni^{2+}) on NLRP3 inflammasome activation and the effects of matrix metalloproteinase (MMP) inhibition on IL-1β release induced by CoCrMo particles were analyzed. Results showed that IL-1β release induced by CoCrMo particles was partly caspase-1-, redox-, and MMP-dependent, but NLRP3 inflammasome-independent. On the other hand, IL-1β release induced by Cr_2O_3 particles appeared to be NLRP3 inflammasome-dependent. Finally, IL-1β release induced by Cr^{3+}, but not Co^{2+}, appeared to be NLRP3 inflammasome-dependent, while Ni^{2+}-induced IL-1β release appeared to be only partially NLRP3 inflammasome-dependent, suggesting that other pathways may also be involved. These findings, which provide additional insights into the mechanisms leading to IL-1β release induced by wear particles and ions from CoCrMo-based implants, may help the future development of therapeutic treatments to modulate wear product-induced inflammation and increase implant longevity.

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“Remember to look up at the stars and not down at your feet. Try to make sense of what you see and wonder about what makes the universe exist. Be curious. And however difficult life may seem, there is always something you can do and succeed at. It matters that you don't just give up.”

– Stephen Hawking

Thesis Organization

This thesis is divided into six main chapters.

Chapter 1 is the literature review.

Chapter 2 describes the objectives and hypotheses of this thesis.

Chapter 3 covers the materials and methods employed in the present research project.

Chapter 4 presents the results of this thesis.

Chapter 5 is the overall thesis discussion, which includes the conclusions from the work and future studies.

Chapter 6 lists the references.

1 Literature Review

1.1 Biomaterials and Orthopaedic Implants

The inception and development of biomaterials has led to numerous advances in the medical field that have enhanced our healthcare. Within the last century, rapid progression in the realm of biomaterials has given rise to numerous applications that are integral to the treatment of many medical conditions [1]. In orthopaedics, one of the most prominent and successful contributions stemming from biomaterials was the invention of implants. The development of suitable biomaterials for orthopaedic applications allowed for the innovation of said implants, enabling the repair or substitution of damaged tissues and joints, and providing enhanced solutions for clinical orthopaedic problems [2].

Total joint replacement or arthroplasty (TJR/TJA) revolutionized the treatment of damaged and arthritic joints. As defined by the American Academy of Orthopaedic Surgeons (AAOS), TJR is a "surgical procedure in which parts of an arthritic or damaged joint are removed and replaced with a metal, plastic or ceramic device called a prosthesis" [3]. Hip and knee replacements are the most commonly performed joint replacement surgeries, although replacement of other joints, such as the shoulder, elbow, ankle and wrists also occur. The success of joint replacements, however, would not have been feasible without the development of suitable biomaterials that are the building blocks of the implants themselves. Of note, the context of this thesis focuses on hip implants, however, the content discussed is not exclusive to hip implants and can be applied to other orthopaedic implants.

1.2 Synovial Joints

Synovial joints allow articulation and movement characterized by low rates of wear and friction, owed mainly to the presence of articular cartilage and a joint cavity filled with synovial fluid [4]. Articular cartilage covers the articulating ends of each bone in the joint cavity, acting as a tough, yet smooth surface promoting joint mobility without damaging underlying bony structures, while also acting as a shock absorber [5]. Synovial fluid, which is secreted by synoviocytes localized within the synovial membrane, provides further lubrication and reduced friction in the joint space, while also supplying nourishment to the articular cartilage [5]. Both the hip and knee are synovial joints, the former a ball-and-socket

joint, and the latter a modified hinge joint. The hip joint is formed by the articulation of the femoral head (ball) and the acetabulum (socket), which allows for multi-axial rotation and movement in a wide range of motion. The hip is responsible for affording stability to the upper body while also withstanding the accompanying body weight during stance and gait [6]. The load exerted on the hip joint can exceed the total body weight multiple times, as is the case when performing a single-leg stance (a load three times the body weight), or during activities such as running and jumping [7]. As such, it is imperative to maintain stability and strength in the hip joint.

The knee joint is comprised of two articulations: one between the femur and tibia, and another between the patella and femur, with the former bearing the majority of body weight [8]. Although not as free-moving as the hip, the knee joint still plays an integral role to movement, allowing the flexion and extension of the lower leg, with a limited degree of rotation [9].

Both joints can become compromised for a variety of reasons, although this is mainly due to osteoarthritis (OA), a degenerative joint disorder that is indicative of the aging process. OA is characterized by the degradation of articular cartilage and other joint tissues, with age being one of the most common risk factors, although joint injury, obesity, and heredity play a role in OA etiology [10]. Damage can also occur through trauma injuries such as fractures, osteonecrosis and rheumatoid arthritis [9], [11]. If all non-surgical treatments have been exhausted and failed to remedy joint pain, surgical intervention is performed.

1.3 Joint Arthroplasty

1.3.1 Prevalence of Joint Arthroplasty

The Canadian Joint Replacement Registry (CJRR), a Canada-wide database on hip and knee replacements launched in 2001, is comprised of information on clinical results and outcomes regarding primary and revision hip and knee replacements. The most recent report released by the CJRR, which details the 2017-2018 period, reported that 58,492 hip replacements and 70,502 knee replacements were performed in Canada [12]. Comparing these numbers to those reported five years earlier indicates a 17.4% and 17.0% increase, respectively, which is mostly attributed to both the aging and growth of the population. Of these reported findings, hip and knee revision surgeries (a procedure carried out to replace all or some of the components of the original implant) accounted for 8.2% and 6.9%, respectively, of the joint replacements performed [12]. CJRR coverage for

2017-2018 represented 72% of the estimated national total. A noted caveat, however, is that submission of primary and revision joint surgeries to the CJRR in 2017-2018 was mandatory only for Ontario, Manitoba, British Columbia, and two regions in Saskatchewan, with submission from all other provinces being voluntary.

In the United States, over 1 million total hip and knee arthroplasties are performed every year and in 2010, it was estimated that 2% (approximately 7 million people) of the population were living with either a total hip or knee replacement, or both [13]. A proportion of these surgeries are due to revisions, with Ong et al. [14] stating that 18% of hip arthroplasties and 8% of knee arthroplasties were revisions. With an aging population and an increasing number of TJA occurring in younger patients (in part because of the introduction of hard-on-hard bearings aimed at increasing implant longevity in more active patients [15]), the prevalence of TJA is expected to increase in the coming years. In the next 10 years, primary arthroplasties for the hip and knee are expected to grow by 71% and 85%, respectively, in the United States [16].

TJA is regarded as one of the most successful innovations in orthopaedic surgery [17] and generally has good clinical outcomes. Nevertheless, implant longevity remains a concern, as no implant will last indefinitely. Although infection, instability, implant malpositioning, and fracture can contribute to implant failure, the majority of failures are attributed to aseptic loosening as a result of periprosthetic osteolysis [11], [18], [19]. Failure of the primary arthroplasty requires revision surgery, which is more complex, resulting in increased healthcare costs and patient recovery [14]. As such, the need to improve the longevity of implants and avoid revision is apparent.

1.3.2 Hip Arthroplasty

The type of implant device used in a hip arthroplasty is dependent on the procedure being performed. A total hip arthroplasty or replacement (THA/THR) replaces both the femoral head and acetabulum, effectively restoring the hip joint. THR utilizes a stem-like device, which includes a femoral and acetabular component, realizing the articulation of the two components through the femoral head portion of the implant [7]. THR involves excising the natural femoral neck and head and inserting a metal stem-like implant into the femur. The acetabulum is typically fitted with a metal cup containing an inserted liner made from a material of choice that allows for rotation between the two implant components. In the context of this thesis, THR will refer to the stem-like

implant to differentiate from the other forms of arthroplasty. Of note, a similar procedure is hemiarthroplasty, where the acetabulum is kept intact and only the femoral head is replaced. However, this procedure is largely used to treat femoral neck fractures, typically in older, low-mobility patients, although it may sometimes be used in cases of arthritis [20].

Hip resurfacing (HR) is another type of arthroplasty that is very similar to a THR as defined above, but differs in that the femoral head and neck are not excised. Instead, the natural femoral head is reshaped to be fitted with a cap (typically metallic), and as such, HR is seen as a bone preserving procedure, mainly performed in younger patients with good bone stock [21]. HR is also advantageous in that it reduces stress-shielding and lowers the risk of dislocation compared to THR [22]. The arthroplasty procedure and implant device used is patient-specific, and depends on both the personal preference of the surgeon and the overall health, age, activity level and bone quality of the patient.

1.3.3 Knee Arthroplasty

In knee arthroplasty, up to three bone surfaces may be replaced. Total knee arthroplasty (TKA) replaces the articulating surfaces of the femur and tibia and if necessary, the back surface of the patella (kneecap) [23]. Both joint surfaces are reshaped to be fitted with corresponding implant components. A curved femoral component is fitted on the distal end of the femur and a flat tibial component replaces the tibial surface, stabilized through a small stem that inserts into the centre of the tibia [9]. A spacer component or insert is placed between the femoral and tibial components to facilitate smooth articulation and reduce wear, and if the patella is also damaged, the posterior or underside is fitted with a patellar component [23]. Typically, the femoral and tibial components are metallic, whereas the insert and patellar components are made of polyethylene (PE) [9]. Although TKA is the most common procedure to remedy a deteriorated knee joint, a less invasive and tissue preserving procedure is a partial or unicompartmental replacement. This procedure sees only the damaged portions (typically one side) of the joint replaced [9], [23].

1.4 Implant Design

1.4.1 Introduction to Implant Design

Selecting an implant for arthroplasty is highly patient-specific; accordingly, different implants with varying characteristics such as composition, fixation method, and component design are

available for TJA. Gard et al. [24] stated that in the Orthopaedic Device Reference, there are 55 different femoral components that are suitable for primary THR. A study by Murray et al. [25] found that in the United Kingdom alone, there are 62 different THRs manufactured by 19 companies, showing there is no shortage of selection for orthopaedic implants.

1.4.2 Composition and Materials

In orthopaedics, the success of a biomaterial relies largely on its ability to reproduce natural mechanical properties and functionality of the tissue it is repairing or replacing, and on its biocompatibility. The biomaterials used in TJR are selected for their strength, fatigue and wear resistance, minimal deterioration (i.e., corrosion and degradation) and biocompatibility with existing tissue [26], [27]. Currently, the major types of biomaterials used in the manufacture of orthopaedic implants are metal alloys, ceramics, and polymers [28]. Although the advent and development of these materials has been largely successful in providing a treatment for joint degeneration, they are not exempt from complications. Because these materials are used to replicate articulating surfaces, they are subject to the natural phenomena of wear and its sequelae, the primary culprit in limiting to implant longevity [29]. Wear products from implants have been shown to cause adverse immune responses, including periprosthetic osteolysis leading to aseptic loosening, hypersensitivity reactions (mostly with metal implants) and pseudotumors [30], [31], indicating a need to reduce the wear of implant materials and mediate the immune response.

1.4.2.1 Polyethylene

The pioneer of the modern THR is Sir John Charnley, who in the 1960’s introduced the concept of low friction arthroplasty, by pairing a metal head with a polytetrafluoroethylene (PTFE) cup and using a stainless steel stem [32]. This initial design proved to be unsuccessful however, due to the poor wear resistance of PTFE and excessive production of wear particles. This led to the introduction of ultra-high molecular weight polyethylene (UHMWPE) to replace PTFE due to its improved mechanical properties such as better wear resistance, lower friction and greater toughness [2]. Despite these advantages, the use of UHMWPE in hip implants still produced clinically significant levels of wear particles, resulting in continued research and development into improving wear resistance. Initial attempts at improving UHMWPE involved sterilization by gamma irradiation, however this process caused the formation of free radicals that led to oxidative degradation of the material, leaving it brittle and prone to fatigue failure [32], [33]. By the late

1990's, improvements to the gamma irradiation process and addition of thermal treatments allowed for the production of highly-crosslinked polyethylene (HXLPE), which displayed increased oxidation resistance and reduced wear rates [34]. Performance of PE was further improved through the addition of Vitamin E to act as a free radical scavenger, which has been shown to prevent fatigue cracks in UHWMPE [35], [36]. Metal-on-polyethylene (MoP) is the most common bearing couple used in THR.

1.4.2.2 Ceramics

The high wear rates of UHMWPE in arthroplasty led to the development of bearing surfaces that had reduced volumetric wear, and as such, ceramics were introduced in THR design. Ceramics are bioinert, convey hardness, which allows for wear and scratch resistance, and they have high wettability for low friction [28]. The two most commonly employed ceramics in orthopaedics are aluminum oxide (alumina; Al_2O_3) and zirconium dioxide (zirconia; ZrO_2). Ceramics were first used as a bearing in THR by Pierre Boutin in 1972 [37], where he introduced a ceramic-on-ceramic (CoC) hip implant made of Al_2O_3. The reduced volumetric wear of ceramic bearings in joint arthroplasty has been studied extensively [38]–[41], providing an improved alternate choice to the use of PE. However, ceramic materials are not without drawbacks; CoC articulations can be prone to squeaking and component malposition, but most notably, ceramics are brittle, increasing the risk of fracture and implant failure. To improve on this, newer generations of ceramics are being developed in the form of composites. One such composite is zirconia-toughened alumina, wherein ZrO_2 is dispersed throughout a Al_2O_3 matrix, yielding higher strength and toughness than Al_2O_3 alone, which was first commercialized by CeramTec under the name BIOLOX® Delta [42]. Recently, another composite being used as a bearing surface in TJR is silicon nitride, which is a non-oxide ceramic that has shown excellent biocompatibility, toughness, strength and wear resistance, making it a suitable alternative to alumina and zirconia bearings [40], [43]. Ceramic-on-polyethylene (CoP) bearing couples are also in use, but were introduced clinically more recently compared to CoC implants, and therefore, fewer long-term studies on their performance are available [44]. Overall, CoC bearings are recommended for young, active patients owing to the reduced wear rate.

1.4.2.3 Metal Alloys

Metal alloys have long been used in manufacture of TJR components. As mentioned above, the first Charnley hip implant was a metal-on-polyethylene (MoP) bearing. Metallic components of implants are made from cobalt-chromium-molybdenum (CoCrMo) alloys, titanium alloys or stainless-steel. In metal-on-metal (MoM) articulation components, the material of choice is CoCrMo, which is preferred over other metal materials due to better corrosion resistance, strength, lower stiffness and wear resistance [26], [32]. Ti6Al4V, the most common titanium alloy used in TJR, is used to manufacture stem and acetabular components of hip implants due to its mechanical compatibility and biocompatibility with bone. Its primary advantage over CoCrMo is a lower elastic modulus, which helps to reduce stress shielding from the implant, resulting in improved femoral remodelling, in addition to showing superior bone ingrowth over CoCrMo [45]. However, Ti6Al4V is not used in the fabrication of femoral head components due to poor wear resistance [43]. Stainless steel is mostly used in the fabrication of fracture fixation devices like plates and screws rather than orthopaedic implants, as its mechanical properties and biocompatibility are inferior to the aforementioned alloys.

Originally, MoM hip implants were introduced to combat the wear production associated with PE, with the intent of reducing the number of revisions occurring in THR patients. Additionally, MoM hip implants allowed for larger diameter femoral heads, which afforded a wider range of motion, increased stability and reduced dislocation rates [46]. However, clinical studies showing adverse biological effects with MoM hip implants combined with unexpectedly high short-term failure rates, led to this bearing design falling out of favour and it is now rarely used clinically [47]. Attempts to improve MoM bearings and other bearing surface materials in general include surface modifications to enhance the tribological properties of orthopaedic implants. This includes surface texturing and coating, with the latter comprising of various hard and wear resistant coatings including, but not limited to, diamond-like-carbon and metal nitrides [48].

1.5 Implant Failure

1.5.1 Causes of Implant Failure

Ideally, an implant would last indefinitely without succumbing to failure and requiring revision; however, despite most TJR having good clinical outlooks and performance, implant failure remains a cause for concern. A very recent meta-analysis by Evans et al. [49] who reviewed

available THA registry data estimated that 75% of THR last 15-20 years and just over 50% last 25 years. Failure of an implant can be attributed to a variety of reasons and depending on how soon after the arthroplasty the failure occurs, it is categorized as early (within 5 years of the arthroplasty) or late. The most common modes of failure in both THA and TKA are aseptic loosening and instability/dislocation, and while both can occur as early and late failures, instability is more commonly seen with early failures whereas loosening and osteolysis are associated with late failures [50]–[53]. Less common, but still prominent causes of failure are infection and periprosthetic fracture [54]. Revision surgeries are costly and in the case of performing revisions for instability/dislocations, there is a high incidence of failure in addition to deep infections [55].

Late stage failure of implants is mainly due to aseptic loosening resulting from periprosthetic osteolysis. Although there is evidence that it may be multifactorial in nature, with mechanical factors playing a role [56], the overwhelming consensus in literature regarding the catalyst behind aseptic loosening is wear particle production. This triggers a pro-inflammatory response, causing a cascade of events including increased osteoclast differentiation, leading to bone resorption (osteolysis) [30]. Aseptic and septic loosening differ in that there is an absence of bacterial infection with the former. Septic loosening may present as either an acute or chronic infection-induced inflammation causing periprosthetic bone loss and mechanical loosening of the implant [57]. Extensive research has demonstrated the biological response to wear particles from all materials used in implants: PE, metal alloys and ceramics [58]–[66]. Due to the prominence of aseptic loosening in late implant failure and the occurrence with all implant materials, uncovering the exact molecular mechanisms involved and potential therapeutic treatments remains an intense area of research.

1.5.2 Implant Wear

Wear particles produced from the articulating surfaces of implants differ in characteristics like shape and size, and the wear rate varies between materials. As stated above, orthopaedic implants can be manufactured from a selection of materials. However, the work of this thesis focuses specifically on MoM hip implants, and the wear particles produced from this bearing surface. MoM hip implants were introduced as an alternative to MoP bearing surfaces in an attempt to reduce the wear rate and resulting particle generation that is responsible for adverse biological reactions in THR patients. Indeed, MoM wear rates show vast improvement over conventional

MoP in simulator tests. Simulator data with 28 mm femoral heads showed volumetric wear rates of 32.8 mm^3/million cycles (MC) for conventional MoP (with UHMWPE) and between 0.2 mm^3/MC to 2.5 mm^3/MC for MoM [67]–[69]. Despite these reduced wear rates, MoM hip implants still generate wear in both particulate and soluble (ions) form. In addition, all hip implants (including MoM) can undergo corrosion processes at the modular interfaces and bearing surfaces, which increase metal ion levels and present an additional facet in the biological response to wear debris [70]. MoM articulations actually produce more wear particles per year in comparison to MoP bearings; estimations have shown that between 6.7×10^{12} to 2.5×10^{14} metal particles are produced per year in MoM THA patients, which is 13-500 times the 5×10^{11} PE particles from a conventional MoP bearing wearing at 100 mm^3/year [71]. However, when considered in terms of volumetric wear rate, MoM implant rates are lower – this is owed to the majority of wear particles being of nano-scale size, significantly smaller than UHMWPE particles, which are generally in the micron range and rarely smaller than 0.1 μm [72].

Both *in vitro* and *in vivo* studies characterizing wear particles from MoM hip implants have found that the majority of particles produced are chromium oxides, with some CoCrMo particles and very little, if any detectable, Co [73]–[75]. Various oxidation states of Cr exist, including Cr(II), Cr(III) and Cr(VI), with Cr(III) being the most stable. Studies have shown the presence of Cr(III) in metallic wear particles from CoCrMo implants, and despite evidence supporting the detection of Cr(VI) [75], [76], this remains controversial as, to date, no Cr(VI) has been detected in retrieved tissue from patients with either a MoM or MoP implant [77]. As such, for the present study, chromium(III) oxide (Cr_2O_3) and CoCrMo particles were used, with the former being chosen over other oxides for relevancy *in vivo*, stability, and commercial availability. Various characteristics of Cr_2O_3 and CoCrMo particles and wear rates from MoM implants from a selection of studies are summarized in Table 1 and 2, respectively.

Table 1. Characterization of Cr_2O_3 and CoCrMo Wear Particles from MoM Hip Implants.

Composition	Size	Shape
Cr_2O_3 (most prominent composition, majority of smaller particles)	*In vitro* Average between 40 – 50 nm, range from approx. 10 – 250 nm (varies with cycle number and alloy composition) [71], [73]–[75], [78] *In vivo* Average between 30 – 60 nm, range from approx. 10 – 830 nm (varies with time since implantation) [71], [73]–[75], [78]	Mostly round/spherical to oval [71], [78]
CoCrMo (typically, the composition of larger particles)	*In vitro* Averages 50 – 80 nm, range from approx. 20 – 230 nm (varies with cycle number and alloy composition) [71], [73]–[75], [78] *In vivo* Averages 40 – 120 nm, range from approx. 10 nm up to 4 μm (varies with time since implantation) [71], [73], [74], [78], [79]	Mostly needle-shaped, spherical [71], [73], [74], [80]

Table 2. Wear Rates of MoM Hip Implants (MC = million cycles)

Wear Rate	Volumetric wear
In vitro	Run-in period: 3-4 mm^3/MC [81] Steady-state period: 0.119-2 mm^3/MC [82], [83]
In vivo	Volumetric rate of 0.3-5 mm^3/year [84], [85]

1.5.3 Biological Effects of Wear Particles from CoCrMo Alloys

In addition to research focused on characterizing wear particles generated from MoM hip implants, interest in the biological effects of these particles also emerged. The effects of particulate CoCrMo alloy in both the nano- and micrometre range, and Co^{2+} and Cr^{3+} on various cell types have been studied extensively [58], [60], [61], [86]–[92]. Periprosthetic tissues around failed MoM hip implants have shown necrotic and inflammatory changes in response to deposited wear particles, which are postulated to be due to either cytotoxic or delayed hypersensitivity reactions [93]. *In vitro* studies looking at cell viability with CoCrMo alloy particles show that there are dose-dependent cytotoxic effects, but they are specific to particle size, exposure duration and cell type [88], [92]. Indeed, a study by Papageorgiou et al. [94] comparing the effects of nano- and micron-size CoCr particles on fibroblasts reported the nanoparticles to be more toxic compared to the microparticles at equivalent particle masses per cell. This is thought to be due to the fact that nanoparticles have an increased surface area as a result of their smaller size relative to microparticles, leading to faster corrosion or dissolution of the nanoparticles and more prominent acute toxic effects [94]. However, cytotoxic and genotoxic effects were still observed with microparticles, albeit over a longer period of time and to a lesser extent, suggesting that these effects cause damage on a lower level and are more chronic [94]. CoCr particles have also been shown to have genotoxic effects on cells, with microparticles causing DNA damage and both numerical and structural chromosome aberrations in fibroblasts [95]. Additionally, CoCrMo alloy particles induce inflammatory responses both *in vivo* and *in vitro* in a variety of cell types through induction and secretion of proinflammatory cytokines including interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) [58], [89], [96], [97]. With nanoparticles, there is also a higher potential to disseminate systemically, owing to their small size and significant number, which has the possibility of exerting effects at distal sites to the implant [98]. Overall, wear particles from CoCrMo alloys can cause adverse biological reactions in the surrounding implant tissue on the cellular and molecular level, proving a cause for concern with MoM hip implants.

1.5.4 Biological Effects of Ions from CoCrMo Alloys

In addition to the production of wear particles, the effects of metal ions must also be considered in the context of metal implant wear. Ion release can occur through different processes, including

articulation between bearing surfaces, dissolution of wear particles, disruption of the passive oxide layer [60] or corrosion and fretting at the modular interfaces in stem-implant designs, termed 'trunnionosis' [99]. Similarly to metal particles, the biological response to metal ions has been studied in a variety of cell types, with cytotoxic and genotoxic effects being observed [100]–[105]. In the context of CoCrMo-based implants, the ions mainly produced are Cr and Co, with studies showing Co^{2+} to be more toxic than Cr^{3+} [106]. It has been well documented that patients who have undergone MoM hip arthroplasty have elevated metal ion concentrations in their blood and urine [107]. MacDonald et al. [108] compared ion levels in erythrocytes from patients with either a MoM or MoP hip implant at a median 2-year follow-up. In patients with MoM articulations, Co and Cr blood serum levels were 1.10 parts per billion (ppb) and 2.5 ppb, respectively, in contrast to 0.17 ppb and 1.30 ppb, respectively, in patients with MoP articulations. Although these levels are raised compared to control subjects (no implants), where the urine and serum levels of Co and Cr are extremely low or undetectable, they are still within the toxicological limits deemed as safe following industrial exposure to these metals [109]. Nonetheless, another study with follow-ups of up to 10 years found high levels of Co and Cr in MoM implant patients, with Co and Cr levels 7- to 10- times higher than the control population (no implants) in MoM HR patients [46]. As such, the long-term local and systemic effects of elevated ion levels from MoM hip implants are not fully understood, and their implications in adverse tissue reactions remain a cause for concern.

1.6 Early Failure of Metal-on-Metal Hip Implants

The resurgence of MoM hip implants in the 1990's with a second generation of implants was attributed in part to the reduced volumetric wear rates over the conventional MoP bearings, with the thought being that the incidence of aseptic loosening and implant failure would be reduced [43]. However, as the occurrence of early failure became apparent, concerns regarding the efficacy of MoM bearing surfaces led to their reduced use in clinical settings. These failures are associated with adverse reactions to metal debris (ARMD), an umbrella term for arthroplasty failures accompanied by pain, a large, sterile effusion, and/or macroscopic necrosis or metallosis (metallic staining of the surrounding tissue) [110], [111] which include pseudotumors and aseptic lymphocyte-dominated vasculitis-associated lesions (ALVAL). Pandit et al. [112] reported on 17 patients with MoM HR all presenting a soft-tissue mass associated with their implant that was neither malignant nor infective, and that the authors identified as pseudotumors. Pseudotumors

represent a neo-synovial proliferation (soft tissue mass) with or without a variable amount of fluid and/or tissue necrosis [46]. Willert et al. [113] described the lymphocyte-dominated immunological response in periprosthetic tissues retrieved from MoM THR revisions as ALVAL. The production of metal wear particles, along with the accompanying cytotoxic effects and potential hypersensitivity response to these particles, have been proposed as the mechanisms responsible for these clinical presentations of ARMD [114].

Hypersensitivity reactions to metal wear particles involve the adaptive immune system, and histological observations have revealed the presence of T and B lymphocytes, plasma cells, eosinophilic granulocytes and macrophages in some MoM periprosthetic tissues [113]. Metal ions and particles can act as haptens through interactions with proteins, with the former forming organometallic complexes and the latter developing a protein corona (bound or adsorbed proteins to the particle surface), therefore capable of eliciting an adaptive immune response [46]. There have been clinical observations of variability in individual patient responses to similar metal wear and corrosion products, suggesting there are genetic factors influencing the development of a hypersensitivity reaction and pseudotumors [57].

The result of these early failures culminated in federal health regulatory bodies issuing notices regarding the risks surrounding MoM hip implants, product recalls and lawsuits [115]–[119], and as mentioned earlier, effectively led to a substantial reduction in the use of MoM hip implants clinically.

1.7 Periprosthetic Osteolysis and Aseptic Loosening

1.7.1 Particle Disease Theory

A correlation between wear particles and aseptic loosening was first proposed by Willert and Semlitsch in 1977, wherein they examined periprosthetic tissue samples and noted a foreign-body reaction in the presence of macrophages and foreign-body giant cells [120]. In the 1980's, reports emerged of focal progressive or non-progressive osteolysis with well-fixed cemented femoral components, leading to the thought that polymethyl methacrylate (PMMA) bone cement particles were the main culprit, resulting in the term 'cement disease' being coined [121], [122], although this would later be revealed to be an erroneous conclusion. In 1994, Harris revised the loosening and osteolytic model to include particles produced from all implant materials to be responsible, renaming it 'particle disease' [123]. In short, the particle disease theory describes the low-grade

chronic inflammatory response to implant wear particles, resulting in periprosthetic osteolysis and subsequent aseptic implant loosening. Wear particles stimulate the expression of pro-inflammatory and osteoclastic cytokines from periprosthetic cells, promoting osteoclast activity and inhibiting the osteogenic activity of osteoblasts, thus upregulating osteoresorption resulting in bone loss [124]. Aseptic loosening typically takes years to develop, with early symptoms being either absent or mild, causing developing osteolytic lesions to go unnoticed until later stages when loosening and component migration may already have occurred [57]. The underlying molecular pathways involved in periprosthetic osteolysis continue to be studied and remain an active area of research.

1.7.2 Innate Immune Response to Wear Particles

The immune response to wear particles leads to a chronic inflammatory response that is considered non-specific [125]. Many immigrant and resident cells in the periprosthetic environment are involved in the process of osteolysis including osteoclasts (derived from the monocyte/macrophage lineage) and dendritic cells although macrophages play a dominant role [126]. Effectively, small wear particles are phagocytosed predominantly by macrophages (though other cells in the periprosthetic environment are capable of phagocytosis), stimulating the release of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α, among others) and chemokines which in turn attracts additional cells, amplifying the overall inflammatory response [126], [127]. Retrieved samples of synovial fluid and periprosthetic tissues from patients undergoing a THR revision have shown the presence of these signalling molecules [128], [129], which are involved in stimulating mature osteoclast formation (from osteoclast precursor cells) and inhibiting the differentiation and function of osteoblasts, creating a periprosthetic environment favoring bone resorption [130], [131]. The bioreactivity of particles and the degree of the inflammatory response has been shown to depend on particle characteristics: composition, size, dose, and shape. Larger particle doses and elongated/irregular particles induce stronger inflammatory responses [132]. There is a lack of consensus regarding what composition of implant particles is more reactive, but there is growing agreement that metallic particles are more reactive and inflammatory than polymeric particles, although there still remains contradiction from some authors [132], [133].

1.7.3 Macrophage Activation in Response to Wear Particles and Ions

Although a variety of cell types have been implicated in the osteolytic process, cells from the monocyte/macrophage lineage are key mediators in the immune response to wear particles [134]–

[136]. Macrophages are multifunctional cells in the innate immune system that mainly phagocytose microbes and debris and do not require previous exposure to an antigen to initiate a response, working to maintain tissue homeostasis. Moreover, depending on the microenvironment and stimuli, macrophages can undergo a polarization process, which results in the development of a functional phenotype marked by specific surface markers and cytokine secretion [137]. Macrophages are phenotypically either M1 (classically activated), which are pro-inflammatory, or M2 (alternatively activated), which are involved in wound healing and tissue repair and can be further sub-divided into M2a/b/c/d [137].

In the presence of implant wear particles, macrophages phagocytose these particles. The reaction of macrophages to wear particles is in part dependent on the particle size and composition – nanometre particles (<150 nm) are reported to be ingested via pinocytosis whereas larger particles (150 nm to 10 µm) are phagocytosed [132]. When macrophages attempt, but are unable to phagocytose wear particles, they can experience a state of 'frustrated phagocytosis', which can lead to the release of inflammatory content [138]. The particle size range purported to be most biologically active varies, with some studies reporting 0.1 – 1.0 µm, with others including up to 7.2 µm [132], [136]. For particles outside the phagocytosable range (>10 µm), multinucleated giant cells are formed (via the fusion of multiple macrophages) to sequester the large particles [139].

The exact mechanisms underlying the activation of macrophages by wear particles are not yet fully understood and remain an active area of research. The recognition of wear particles by macrophages has been shown to elucidate the activation process. Macrophages are equipped with pattern recognition receptors (PRRs) that are integral to the recognition of evolutionarily conserved microbial patterns (pathogen-associated molecular patterns or PAMPs), or exogenous danger signals, and endogenous (alarmins) danger signals (danger-associated molecular patterns or DAMPs), like cellular products from tissue injury [134]. PRRs are host proteins that are located on the cell surface and in cellular compartments (i.e., endosomal-lysosomal compartment) or the cytosol [140]. Two such PRR families are the membrane-associated toll-like receptors (TLRs) and the cytosolic nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs) [141]. Of particular interest are TLRs, which have increasingly been shown to play a critical role in the inflammatory response to particles [142]. TLRs are involved in the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ), an inducible transcription factor involved in the regulation of various immune and inflammatory responses, including the upregulation of

multiple pro-inflammatory cytokines, including TNF-α and ILs [143], [144]. TLRs have been shown to be activated by various wear products including PMMA particles, alkane polymers generated from UHMWPE, titanium particles and ions [142], [145]–[147]. Additionally, based on clinical findings that subclinical bacterial biofilms exist on implants, it has been proposed that PAMPs from these biofilms bind to wear particles, thus contributing to their recognition via TLRs [56], [148]. The wear particle-induced activation of macrophages also involves the activation of the NOD-, leucine-rich-repeat (LRR)-, pyrin domain (PYD)-containing protein 3 (NLRP3) inflammasome, a multi-protein complex, and an integral component of the innate immune system [149]. The molecular mechanisms through which wear particles and ions from CoCrMo implants induce IL-1β release (and the involvement of the NLRP3 inflammasome) is the focus of this thesis. The current body of knowledge regarding the NLRP3 inflammasome will be discussed in detail below.

1.8 Inflammasomes

1.8.1 Introduction to Inflammasomes

Innate immunity acts as the first line of defense against any 'non-self' entity, and does so by detecting and eliminating potentially harmful agents. Research into the intricate workings of the innate immune system led to the discovery of multiprotein complexes known as inflammasomes, which have been implicated in inflammatory processes. As a result, understanding the mechanisms through which they function has garnered interest from several disciplines, including orthopaedics. Inflammasomes trigger pro-inflammatory responses through the activation of the protease caspase-1, of which the primary substrates are the inactive pro-forms of cytokines from the IL-1 family, such as IL-1β and IL-18, crucial mediators of inflammation in their mature, active forms [150]. Additionally, inflammasome activation has been shown to be involved in pyroptosis, a caspase-1 dependent, inflammatory cell death [151]. The involvement of a pro-inflammatory response in periprosthetic osteolysis and subsequent aseptic loosening highlights the relevancy of inflammasome research in orthopaedic studies.

1.8.2 Inflammasome Structure

Before the formal identification of the inflammasome, caspase-1 was discovered in 1989 [152], [153] and was named IL-1β-converting enzyme (ICE) due to its ability to cleave the 31-kilodalton (kDa) pro-IL-1β into its active 17-kDa IL-1β form [154], although the mechanism behind caspase-

1 activation was unknown. In 2002, Martinon et al. [155] identified the first inflammasome, which was comprised of a complex of caspase-1, caspase-5, an apoptosis-associated speck-like protein (ASC) (adaptor protein containing a caspase recruitment domain [CARD]; also known as PYCARD) and NLRP1, elucidating caspase-1 activation. Subsequently, additional inflammasome complexes have been described, with most containing NLR sensor molecules. Slight variations exist between specific inflammasomes, but the overall configuration is generally the same: a sensor molecule (i.e., NLRP) connects to caspase-1 through the adaptor protein ASC (common to all inflammasomes) [156]. ASC mediates inflammasome assembly via homotypic interactions, with its N-terminal PYD and C-terminal CARD interacting with inflammasome sensor molecules and pro-caspase-1, respectively [157]. Caspase-1 is synthesized as an inactive 45-kDa zymogen (pro-caspase-1), and when recruited by ASC, is autocatalyzed into its active form (caspase-1), which is composed of two subunits: p20 and p10 [158].

1.8.3 NLRP3 Inflammasome

To date, four inflammasome complexes have been well characterized in the literature. Three of these inflammasomes are from the NLR family (of which 23 have been identified in humans), with one from the pyrin and hematopoietic interferon-inducible nuclear (HIN) domain-containing proteins (PYHIN) family: NLRP1, NLRP3 and NLRC4 (NOD-, LRR-, CARD-containing 4; also known as IPAF), and the absent in melanoma 2 (AIM2) inflammasome [140]. NLRC4 differs from the other NLR inflammasomes in that it contains a CARD rather than a PYD domain, and the AIM2 inflammasome lacks any members of the NLR family, rather consisting of a HIN200 domain and PYD [140]. Research into each of these inflammasomes has shown that they appear to be activated by specific stimuli. Studies of the NLRP1 inflammasome have indicated that it is activated by bacterial proteases and the accompanying proteolytic activity, rather than by a pathogenic ligand [159]–[161], whereas the NLRC4 inflammasome has been shown to be activated by a cytosolic PAMP, flagellin, from *Salmonella typhimurium* or *Legionella pneumophila* [162]. However, a study by Suzuki et al. [163] using *Shigella flexneri*, which is a non-flagellated bacterium, showed NLRC4 activation, thus indicating that NLRC4 may recognize additional PAMPs, but this remains to be elucidated. The AIM2 inflammasome binds cytosolic double-stranded (ds)DNA via its HIN200 domain, and has been shown to be activated in response to viral, bacterial, mammalian and synthetic dsDNA [164]–[166].

The most extensively studied inflammasome is the NLRP3 inflammasome, as its involvement has been implicated in several human diseases [167]. It is composed of the NLRP3 sensor molecule, ASC adaptor protein and pro-caspase-1. Upon activation, NLRP3 is oligomerized, and together with the recruited ASC proteins forms a large multiprotein complex that brings pro-caspase-1 monomers into close proximity, triggering their autocatalytic activity and amplifying the activation of caspase-1 [156], [168]. The NLRP3 inflammasome is unique to other inflammasomes in that it can be activated by an extensive variety of stimuli that are distinctly different from one another [167] and not necessarily pathogenic in origin, in contrast with the other identified inflammasomes. These stimuli include, but are not limited to metal particles and ions [58], [62], [169]–[171], viral pathogens [172], [173], cholesterol and uric acid crystals [174]–[176], silica, asbestos and aluminum [177], [178], DAMPs like adenosine triphosphate (ATP), pore-forming toxins [179], [180] and bacterial pathogens [181]–[183]. Given the vast diversity of these stimuli and the lack of congruity structurally and chemically, it is unlikely that they all activate NLRP3 through physical interaction [156]. Accordingly, investigations into a potential common cellular signal induced by NLRP3 stimuli were pursued. Currently, this activation signal is still under debate and yet to be agreed upon, although several mechanisms have been proposed including ion flux, lysosomal damage, reactive oxygen species (ROS) production, and mitochondrial dysfunction [167]. Additionally, subsequent studies into NLRP3 activation revealed the requirement of a 'priming' signal to license full NLRP3 inflammasome activation [184]. Following activation, inflammasomes are cleared via autophagy [185], although notably, active caspase-1 has been shown to be secreted concurrently with IL-1β and IL-18 [186], [187]. More recently, NLRP3 and ASC oligomers have also been identified extracellularly following inflammasome activation, and were shown to be capable of spreading inflammatory signals to surrounding cells through internalization [168]. As mentioned above, the NLRP3 inflammasome has been shown to be involved in orthopaedic implant wear particle-mediated inflammation, thus constituting a focus of this thesis.

1.8.4 NLRP3 Inflammasome Priming

In 2009, Bauernfeind et al. [184] first identified the requirement of priming for NLRP3 inflammasome activation. They found that priming is required as the basal levels of pro-IL-1β, which is not constitutively expressed in cells, and NLRP3 are inadequate for inflammasome formation (unlike ASC and caspase-1 [167]), indicating *de novo* protein synthesis is a limiting step

in NLRP3 inflammasome activation [184]. Additionally, this priming step was shown to be regulated by NF-κβ-dependent signals, and overall, it was determined that for full NLRP3 inflammasome activation two signals are required: first, NLRP3 (and pro-IL-1β) expression needs to be transcriptionally upregulated and second, an activating stimulus is needed to trigger inflammasome assembly post-transcriptionally [184]. The necessity of this priming signal has been proposed to act as a safe-guard mechanism to ensure that the inflammasome activation and the resulting IL-1β release occur in response to legitimate stimuli when necessary [158]. Notably, dysregulation of the NLRP3 inflammasome has shown to be present in auto-inflammatory diseases, further supporting this notion [158].

In essence, the priming stimuli for the NLRP3 inflammasome can consist of any receptor involved in a signalling pathway resulting in NF-κβ activation, such as TLRs, NLRs, IL-1 receptor 1 type 1, TNF receptor 1 and 2 [184], [188], making a number of stimuli responsible for priming *in vivo*. However, for *in vitro* inflammasome studies, lipopolysaccharide (LPS), a bacterial PAMP, is commonly used as a priming stimulus. LPS primes the inflammasome and activates NF-κβ through engaging its receptor, which has been shown to be TLR4 [184], [189]. Of interest, Gurung et al. [190] found that the ability of LPS to prime NLRP3 is temporary, as extended LPS exposure (12 to 24 hours, compared to the typical priming period of ≤ 4 hours) saw its efficacy as a priming stimulus subdued. They also reported that chronic stimulation of TLRs induced IL-10 expression, which reduced NLRP3 inflammasome activation, indicating that prolonged NLRP3 activation may be regulated through negative feedback loops to prevent excessive inflammatory responses. Of note, a study by Juliana et al. [191] demonstrated that incubating macrophages with LPS for only 10 minutes, significantly shorter than the ≥ 2 hours required for upregulation of NLRP3 expression, was capable of priming the NLRP3 inflammasome, suggesting a post-transcriptional regulatory role for priming in NLRP3 activation. Indeed, the authors reported a novel post-translational modification mechanism wherein priming stimulated the deubiquination of NLRP3 at its LRR domain, which is normally ubiquinated in its basal state, thus rendering it inactive and unable to oligomerize. Additionally, high expression levels of NLRP3 did not require LPS priming and could be activated by the addition of ATP alone. The authors postulated that when NLRP3 is highly expressed, it is partially ubiquinated and that the addition of a NLRP3 agonist (i.e., ATP) is sufficient for inflammasome activation. Py et al. [192] further elucidated this post-translational

modification by showing that BRCA1[1]/BRCA2[2]-containing complex subunit 3 (BRCC3), a deubiquitinating enzyme, was responsible for the deubiquination of NLRP3 and that the use of G5, a small-molecule inhibitor of deubiquination, inhibited NLRP3 inflammasome assembly, but did not affect transcriptional priming via LPS/TLR4. It should be noted that additional post-translational modifications regulating NLRP3 have been reported in the literature, including phosphorylation and sumoylation [193]. Overall, the requirement of priming for inflammasome activation has been well established and has been revealed to be quite complex, and the molecular pathways involved continue to be investigated.

1.8.5 NLRP3 Inflammasome Priming and Orthopaedic Wear Particles and Ions

Despite the establishment of priming in inflammasome research, there is some discord in the literature regarding orthopaedics and the role of the inflammasome in response to wear products. Studies by Caicedo et al. [58], [103], [169] on the inflammatory response to metal particles and ions do not describe a priming step in their methodology, notably including their original publication implicating the role of the NLRP3 inflammasome [169]. A subsequent study from the same group [194] reported that Co-alloy particles alone were able to induce IL-1β release in the absence of a priming stimulus and found no significant difference between Co-alloy particles alone and Co-alloy particles/LPS co-challenge in osteolysis *in vivo* using a murine calvaria model. However, an additional study by the same authors showed a significant increase in osteolysis in a murine calvaria model when CoCrMo particles were used in combination with LPS compared to CoCrMo particles alone [195]. These results implied the involvement of TLRs (TLR4 specifically) in the inflammatory response to metal wear particles, and taken together, these results somewhat refute their previous findings.

In contrast, other groups examining the immune response to wear particles used a priming step [56], [148], [196]–[199]. For example, studies from Dr. Greenfield's group have proposed that bacterial endotoxins adhered to wear particles contribute to their immune reactivity and play a role in aseptic loosening [56], [148]. The removal of adherent endotoxin from the surface of titanium particles reduced osteolysis by 50-70% *in vivo*, and the addition of LPS to the particles reinstated their immune reactivity [199]. A study by Manzano et al. [200] found that adherent PAMPs on

[1] BRCA1: Breast cancer 1 DNA repair associated (gene)
[2] BRCA2: Breast cancer 2 DNA repair associated (gene)

wear particles are necessary to prime the NLRP3 inflammasome, which was facilitated by their cognate TLRs. Furthermore, it has been hypothesized that adherent bacterial PAMPs on wear particles, but not endogenous alarmins, are responsible for activating their cognate TLRs during wear-particle mediated osteolysis, although the possibility that alarmins work concurrently with PAMPs and TLRs during aseptic loosening should not be excluded [201]. The role of endotoxin in aseptic loosening is debateable, as there is an assumption that orthopaedic implants are sterile and endotoxin-free prior to implantation, and patients with aseptic loosening are asymptomatic of an infection. Nevertheless, there is evidence supporting potential sources of endotoxin in patients with aseptic loosening [148]. The most likely source is sub-clinical bacterial biofilms on the surface of implants that cause no indication of infection, yet may provide a source of PAMPs that contribute to the immune reactivity of wear particles [57], [148]. All-in-all, despite some uncertainties, most studies looking at the activation of the inflammasome by metal wear particles suggest that priming is required.

1.8.6 Mechanisms of NLRP3 Inflammasome Activation

As previously mentioned, the array of stimuli capable of activating the NLRP3 inflammasome are structurally and chemically diverse, with the probability of all these activators directly binding NLRP3 being quite low [156]. The more likely scenario is that these various activators trigger effects upstream of the inflammasome that result in a few select, or possibly single, unifying downstream mechanism(s) leading to NLRP3 activation [167]. Despite continued research into the mechanisms of inflammasome activation, the final signal leading to NLRP3 activation remains under debate, although the most prominent proposed mechanisms are ion fluxes, lysosomal damage, ROS production, and mitochondrial dysfunction [167].

1.8.6.1 Ion Flux

There is large agreement in the literature regarding the role of potassium efflux as a potential upstream signal of NLRP3 activation. This was demonstrated by a study pre-dating the discovery of the inflammasome, showing that the microbial toxin nigericin, a K^+/H^+ ionophore antiporter, and ATP promoted mature IL-1β release through depletion of intracellular K^+ in cells stimulated by LPS [202]. Subsequent studies investigating mechanisms of NLPR3 activation in response to pathogenic and particulate stimuli confirmed the necessity of K^+ efflux, whereby low cytosolic K^+ was sufficient for NLPR3 activation [172], [203]. Additionally, high levels of extracellular K^+

prevent NLRP3 inflammasome activation, though not the activation of AIM2 or NLRC4 inflammasomes [204], [205]. However, recent studies showing NLRP3 inflammasome activation independent of K^+ efflux have called into question whether K^+ efflux is the common upstream denominator of all NLRP3 activators [206], [207].

In addition to K^+, calcium signalling has also been linked to NLRP3 inflammasome activation. Various NLRP3 stimuli induce Ca^{2+} mobilization during inflammasome activation, and inhibition of Ca^{2+} signalling has been shown to impair NLRP3 inflammasome activation, but not of the AIM2 and NLRC4 inflammasomes [208], [209]. Ca^{2+} mobilization can occur through two mechanisms: flux through membrane channels, or from endoplasmic reticulum (ER)-linked intracellular Ca^{2+} stores that release Ca^{2+} into the cytosol [193]. In fact, inhibition of inositol 1,4,5-trisphosphate receptor, a Ca^{2+}-release channel on the ER, diminishes Ca^{2+} mobilization and NLRP3 activation [208]. The mechanism by which Ca^{2+} mobilization triggers NLRP3 inflammasome activation is not fully understood, although it has been suggested that excessive release of Ca^{2+} from the ER causes mitochondrial Ca^{2+} overload, resulting in mitochondrial dysfunction and ROS production, a potential NLRP3 inflammasome activation mechanism [209]. Similar to K^+, there is contradicting evidence regarding the role of Ca^{2+} in NLRP3 inflammasome activation. Katsnelson et al. [210] found that Ca^{2+} signalling was dispensable for NLRP3 inflammasome activation, revealing that BAPTA, a chelator of cytosolic Ca^{2+}, inhibited NLRP3 activation independent of its expected antagonistic effects on Ca^{2+} signalling.

The question of whether both K^+ and Ca^{2+} movement are required for inflammasome activation has yet to be answered. The possibility exists that NLRP3 activation depends only on the flux of one of these ions, and that movement of the other is the corresponding reciprocal flux maintaining ionic balance [211]. This scenario was demonstrated in studies showing that ATP-induced intracellular shifts of Ca^{2+} and high extracellular Ca^{2+} activation of the inflammasome were blocked in the presence of high extracellular K^+ [203], [209]. Overall, the role of ion fluxes in NLRP3 activation remains a topic of debate, and more research is required to fully uncover the mechanisms potentially linking ion flux and inflammasome activation.

1.8.6.2 Lysosomal Damage

Phagocytosis of indigestible particulate matter such as metal alloy particles [58], silica and aluminum salt [177], [212] causes lysosomal damage and eventual rupture, releasing the

particulate matter and lysosomal enzymes into the cytoplasm. This lysosomal damage has been suggested as a possible mechanism of NLRP3 inflammasome activation [177]. The release of cathepsins, specifically cathepsin B (a cysteine protease), from the lysosomal compartment has been linked to NLRP3 activation following lysosomal rupture [58], [177], [213]. The use of a cathepsin B inhibitor, CA-074-Me, has shown to be effective at inhibiting NLRP3 activation and IL-1β release in response to crystals [177], [214], as well as CoCrMo particles [58]. However, macrophages deficient in cathepsin B showed little to no difference in IL-1β secretion and NLRP3 activation in response to particulate matter [215], and the genetic deletion of various cathepsins, including cathepsin B, also had minimal effect on NLRP3 activation [214]. This suggests that multiple, redundant cathepsins, which can be inhibited by off-target effects of CA-074-Me, play a role in NLRP3 inflammasome activation [214]. Of note, lysosomal damage by particulate matter triggers K^+ efflux, which can lead to NLRP3 inflammasome activation [203], although no mechanism linking lysosomal rupture and K^+ efflux has been determined [187]. In the context of metal implant particles, Caicedo et al. [58] investigated the potential role of lysosomal damage in NLRP3 activation in response to micrometre-size CoCrMo particles. They found that lysosomal destabilization, and the resulting leakage of cathepsin B, led to NLRP3 activation and IL-1β release. Additionally, larger and irregularly-shaped particles induced more IL-1β release in comparison to smaller, spherical particles, implying that the larger, irregular particles had a higher propensity to cause lysosomal damage.

1.8.6.3 ROS Production and Mitochondrial Dysfunction

The involvement of ROS and the mitochondria in NLRP3 inflammasome activation was first suggested by Hornung et al. [216] while linking lysosomal damage and activation. A subsequent study by Zhou et al. [217] corroborated the association between ROS production and NLRP3 activation. They highlighted that mitochondria were responsible for generating the ROS necessary for inflammasome activation, and that inhibiting autophagy (specifically mitophagy) led to the accumulation of damaged mitochondria, enhancing NLRP3 inflammasome activation. Moreover, they linked NLRP3 inflammasome activation to mitochondrial dysfunction, which was evidenced by a decrease in mitochondrial membrane potential and by the fact that knockdown of voltage-dependent anion channels prevented NLRP3 activation and ROS generation in response to NLRP3 agonists [217]. Furthermore, a study by Sang et al. [218] found that Vitamin C, a ROS scavenger, inhibited activation of the NLRP3 inflammasome through scavenging mitochondrial ROS.

Additional studies continued to look at the role of mitochondrial dysfunction in NLRP3 inflammasome activation, revealing further interactions [193], [211], [217], [219]–[221]. For example, Nakahira et al. [219] suggested that the release of mitochondrial DNA (mtDNA) into the cytosol from dysfunctional mitochondria in response to LPS combined with ATP contributed to IL-1β release and NLRP3 inflammasome activation. However, the pathway through which this led to NLRP3 activation was not fully understood, as NLRP3-deficient cells had impaired membrane potential loss and mtDNA release but were still able to generate ROS. These results then imply that NLRP3 is a downstream target of ROS, but is required upstream of mitochondrial dysfunction [211]. Interestingly, another study found that oxidized mtDNA itself is capable of binding NLRP3 to trigger inflammasome activation [220]. Additionally, increasing evidence shows that the mitochondria may act as a site for inflammasome assembly. Indeed, while inactive NRLP3 is associated with the ER, NLRP3 localizes and associates with the mitochondria and mitochondrial-associated membrane (MEM) upon activation [217], [221]. Additionally, at least three proteins have been suggested as potential adaptors between NLRP3 and mitochondria: cardiolipin (mitochondrial phospholipid), mitochondrial antiviral signalling protein ([MAVS], an adaptor protein for RNA sensing) and mitofusin 2 [193]. However, further research needs to be done to clarify the possible roles of these proteins in NLRP3 activation.

Despite the evidence implicating ROS generation and mitochondrial dysfunction as potential activators of the NLRP3 inflammasome, whether they are truly a triggering mechanism still remains a topic of debate. ROS generation by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) was originally proposed as the common cellular signal leading to NLRP3 activation [178]. However, additional studies brought this into question, as it was shown that NOX-deficient cells still had functioning NLRP3 inflammasome since IL-1β release was not affected [177], [222]. Also, some models of inflammasome activation have indicated that both mitochondrial ROS-dependent and -independent pathways are possible for NLRP3 activation [223], [224], indicating that ROS generation and mitochondrial dysfunction are not necessarily mutually-exclusive activating signals for NLRP3. As an example, Iyer et al. [224] showed that linezolid, an antibiotic, triggered NLRP3 inflammasome activation through mitochondrial dysfunction, but independently of mitochondrial ROS production. Bauernfeind et al. [225] showed that ROS inhibitors interfered with the induction of NLRP3 expression during priming, but NLRP3 inflammasome activation was not affected, evidently showing that ROS generation may not be a

requirement for inflammasome activation. In conclusion, the pathways through which ROS and mitochondrial dysfunction may trigger NLRP3 inflammasome activation are complex and remain incompletely characterized, and their individual or synchronized roles remain to be fully elucidated.

1.9 Inflammasome-Independent IL-1β Release

Although the processing of pro-IL-1β by inflammasomes to produce active IL-1β has been extensively characterized in the literature, evidence has emerged that mature IL-1β can be produced independently of the inflammasome. The fact that enzymes other than caspase-1 may be capable of processing pro-IL-1β into its active form emerged in part due to conflicting results between *in vitro* and *in vivo* studies. Using an *in vivo* model of aseptic inflammation induced by turpentine, Fantuzzi et al. [226] compared the responses (specifically, the circulating levels and local production of IL-1α, IL-1β, IL-6 and TNF-α as well as variations in body weight and the production of protein serum amyloid) of caspase-$1^{-/-}$ mice with the responses of IL-$1\beta^{-/-}$ mice. The authors found that there was no difference in the inflammatory response or levels of mature IL-1β between wild type mice and those deficient in caspase-1, but the inflammatory response in IL-$1\beta^{-/-}$ mice was completely abrogated. Additionally, in an *in vivo* model of lung inflammation using diesel exhaust particles (DEP), Provoost et al. [227] reported that NLRP3 and caspase-1 knock-out mice showed similar IL-1β release and inflammation to wild type mice after DEP exposure, suggesting other proteases may be involved in the activation of IL-1β. Indeed, studies looking at a diversity of inflammatory models, including *Mycobacterium tuberculosis* infection, osteomyelitis, ulcerative colitis and sterile inflammation, have substantial caspase-1-independent (and by extension inflammasome-independent), but IL-1β-dependent components [228]–[231].

While caspase-1 is known to cleave pro-IL-1β, investigations into whether additional enzymes could activate the pro-form of the cytokine were subsequently undertaken. These investigations resulted in the identification of additional enzymes that are capable of processing IL-1β independent of the inflammasome, with the majority being neutrophil- and macrophage-derived serine proteases including: elastase and chymase [232], granzyme A [233], [234] and proteinase 3 [235], [236]. Additionally, matrix metalloproteinases (MMPs) [237], cathepsins [230], [238] and bacterial protease [239] have also been shown to be able to process pro-IL-1β. Notably, these enzymes all cleave pro-IL-1β at different locations from caspase-1, resulting in active IL-1β

fragments differing slightly in size from the 17-kDa produced from the inflammasome-dependent/caspase-1 pathway [232]. The discrepancy between results observed *in vivo* and *in vitro* regarding inflammasome involvement in IL-1β activation has mostly been attributed to which cell population is most prominent in an inflammatory infiltrate [240]–[242]. For example, inflammatory responses dominated by neutrophil recruitment could explain the inflammasome-independent IL-1β production since many neutrophil serine proteases have been shown to activate IL-1β [240], [241]. Overall, it has become apparent that various immune cells can process IL-1β through different pathways, which could have implications for potential therapeutic treatments. Indeed, the dominant immune cells in the inflammatory infiltrate in a given disease should be considered when devising potential pharmacological modulators [242].

From the enzymes listed above that are capable of activating IL-1β, of particular interest are MMPs as they have been identified in macrophages exposed to orthopaedic wear particles [243]–[245]. MMPs are a group of zinc proteases that are typically involved in the degradation and remodelling of the extracellular matrix (ECM), of which 23 have been identified in humans [246]. However, additional research into these proteinases has revealed a role for them in additional physiological processes, including inflammation and innate immunity. MMPs have been shown to play a role in the repair of epithelium, microbial defenses, chemokine regulation and cytokine activity [247]. Indeed, in 1998, Schönbeck et al. [237] reported a novel IL-1β processing pathway via MMPs. They found that MMP-3, -2 and -9 (also known as stromelysin-1, gelatinase A and gelatinase B, respectively) were capable of processing human pro-IL-1β into its biologically active form. Additionally, they found that each of these MMPs had varying effectiveness in their ability to produce active IL-1β, with MMP-9 capable of cleaving pro-IL-1β within minutes and yielding a stable, bioactive 17 kDa fragment, while MMP-3 and MMP-2 were less effective [237]. Interestingly, MMP-3 was able to completely degrade mature IL-1β during prolonged incubation [237]. In the context of orthopaedic biomaterials, studies have shown that MMPs, including MMP-2 and -9, are produced and expressed by macrophages in response to implant wear particles including Ti alloy and PE [243]–[245]. There is also evidence that inhibiting some of these MMPs can down-regulate pro-inflammatory cytokine release. Notably, inhibition of MMP-9 and -3 was shown to reduce the production of TNF-α, the expression of IL-1β (among other pro-inflammatory cytokines) and the activity of NF-κβ in LPS-stimulated microglial cells [248], and inhibition of MMP-9 has also been linked to a reduction in wear particle-induced osteolysis *in vivo* [249]. These

results highlight the potential for inflammasome-independent pathways of IL-1β production, indicating that the immune response to implant wear particles could potentially involve additional mechanisms other than, or in concert with, the NLRP3 inflammasome.

2 Objectives and Hypotheses

The overall objective of this research project was to identify molecular mechanisms leading to IL-1β release in the presence of wear and corrosion products from CoCrMo-based implants, with a focus placed on NLRP3 inflammasome activation pathways. Pharmaceutical agents that target mechanisms potentially involved in NLRP3 inflammasome activation were used (a caspase-1 inhibitor and an antioxidant), and the subsequent IL-1β release from bone marrow-derived macrophages (BMDM) exposed to particles and ions was measured. Elucidating these mechanisms will provide a better understanding of the inflammatory response to implant wear and corrosion products, and potentially aid in the development of therapeutic treatments for aseptic loosening of implants.

2.1 Objectives:

The primary objectives of this thesis were:

1. Determine if, in murine macrophages, IL-1β release induced by micrometre-sized CoCrMo particles and nanometre-sized Cr_2O_3 particles is caspase-1-dependent;
2. Determine if, in murine macrophages, IL-1β release induced by micrometre-sized CoCrMo particles and nanometre-sized Cr_2O_3 particles is reduction-oxidation (redox)-dependent;
3. Determine if, in murine macrophages, IL-1β release induced by micrometre-sized CoCrMo particles and nanometre-sized Cr_2O_3 particles is NLRP3 inflammasome-dependent.

Additionally, the effects of metal ions (Co^{2+}, Cr^{3+}, and Ni^{2+}) on NLRP3 inflammasome activation were analyzed as a continuation of previous studies conducted in our laboratory using these ions. Finally, the effects of MMP inhibition on IL-1β release induced by micrometre-size CoCrMo particles were analyzed following the results obtained in Objective 3.

2.2 Hypotheses:

The hypotheses of this thesis were:

1. IL-1β release in macrophages exposed to micrometre-size CoCrMo particles and nanometre-size Cr_2O_3 particles is caspase-1-dependent;
2. IL-1β release in macrophages exposed to micrometre-size CoCrMo particles, but not Cr_2O_3 particles, is redox-dependent;
3. IL-1β release in macrophages exposed to micrometre-size CoCrMo particles and nanometre-size Cr_2O_3 particles is NLRP3 inflammasome-dependent.

3 Materials and Methods

3.1 Particles

CoCrMo and Cr_2O_3 particles were obtained commercially, in the phagocytosable size range: 3.4 μm (average diameter) for CoCrMo particles (Praxair Surface Technologies, Indianapolis, IN) and 60-nm (Sigma-Aldrich, MO) as well as 700-nm (Acros, Geel, Belgium) for Cr_2O_3 particles. Particles were sterilized for 1h in sterile-filtered 70% (v/v) ethanol, followed by a 15-minute incubation in a sonicator bath (35 kHz) to disperse any clumps. Particles were then washed three times in Dulbecco's phosphate buffered saline (DPBS; Sigma-Aldrich) to remove any traces of ethanol (particles were sonicated for 10 minutes between each wash) prior to incubation with cells. Particles were resuspended in Dulbecco's Modified Eagle Medium with 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, L-glutamine and sodium pyruvate (DMEM; Wisent, Saint-Jean-Baptiste, QC), supplemented with 8% heat-inactivated ultra-low endotoxin fetal bovine serum (FBS; NorthBio, Toronto, ON) and 100 U/mL Penicillin-Streptomycin (Thermo Fisher, Waltham, MA) to prepare stock solutions. These solutions were then serially diluted and an aliquot (150 μL) was added to each sample containing the cells (300 μL) to achieve the following final concentrations:

- CoCrMo: 7, 17, 33, 67, 133 particles per macrophage
- Cr_2O_3 (60 nm): 0.33, 1, 1.7 and 2.3 million particles per macrophage
- Cr_2O_3 (700 nm): 333, 667, 1000 and 1333 particles per macrophage

Both the CoCrMo and Cr_2O_3 particle concentrations (based on a previous study [250]) were selected to test the full range of cytotoxic effects. The mass of particles required for a desired concentration was calculated based on the density of each particle type (ρ = 4.86 g/cm^3 and 5.21 g/cm^3 for CoCrMo and Cr_2O_3 particles, respectively) and particle volume (assuming spherical shape). Particles were tested for endotoxins using the ToxinSensor™ Chromogenic LAL (limulus amebocyte lysate) Endotoxin Assay Kit (Genscript, Piscataway, NJ), following the manufacturer's protocol. Briefly, particles were sterilized and washed as above, and then serially diluted in 1 mL of kit-provided LAL reagent water. Samples (100 μL) of these solutions were then used in the assay. Absorbance was read at 545 nm with a microplate reader (Synergy™ 4; BioTek, Winooski, VT), and the value obtained for the blank (LAL reagent water) was subtracted from each

measurement. The minimum sensitivity of the assay is 0.01 endotoxin units (EU)/mL, as per the manufacturer's specifications. Sterilized particles contained ≤0.04 EU/mL of endotoxin.

3.2 Ions

Stock solutions of Co^{2+}, Cr^{3+}, and Ni^{2+} were prepared as described elsewhere [251]. Briefly, pre-determined masses of $CoCl_2•6H_2O$ (≥99.5% purity; Fisher Scientific, Hampton, NH), $CrCl_3•6H_2O$ (≥99.2% purity; Sigma), and $NiCl_2•6H_2O$ (≥99.999% purity; Sigma) powders were dissolved in cell culture-grade water (Lonza, Walkersville, MD) and sterilized via filtration through 0.2-μm pore size cellulose acetate syringe filters (VWR, Mississauga, ON). These stock solutions were then serially diluted and an aliquot (6 μL) was added to each sample containing the cells (300 μL) to achieve the following final concentrations: 18 parts per million (ppm) (Co^{2+}), 300 ppm (Cr^{3+}) and 48 ppm (Ni^{2+}), which were based on a previous study [62].

3.3 Cell Culture

Cells from bone marrow extracted from the tibiae, femora, and pelvic bones of 5- to 16-week-old female C57BL/6 mice (Charles River, Wilmington, MA) were differentiated into macrophages. Procedures (Protocol ME-2350-R4) were approved by the University of Ottawa Animal Care Committee. Mice were euthanized by CO_2 gas asphyxiation to minimize suffering, followed by cervical dislocation. Immediately prior to dissection, mice were soaked in 70% (v/v) ethanol. Following excision of the bones, a 27G x ½" needle-syringe (BD Biosciences, Durham, NC) filled with DMEM supplemented with 8% FBS and 100 U/mL Penicillin-Streptomycin (complete growth medium; CGM) was used to flush out the bone marrow, which was subsequently filtered through a 100-μm nylon mesh cell strainer (Fisher Scientific). The strained bone marrow cell suspension was then centrifuged at 150***g*** for 10 minutes at room temperature (RT) and resuspended in CGM. Suspension dishes (100-mm diameter; Greiner Bio-One, Monroe, NC) were coated with 50 μL of CGM containing 50 ng of recombinant macrophage-colony stimulating factor (M-CSF; R&D Systems, Minneapolis, MN), and then seeded with cell suspension containing 1.5 x 10^6 cell/mL. CGM was added to bring the final volume to 10 mL. β-mercaptoethanol (β-ME) (10 μL) was then added to each dish. The seeded dishes were finally incubated for 6 days at 37 °C and 5% CO_2 in a humidified environment. At the end of the incubation, the medium was aspirated from the dishes, which were rinsed with 5 mL of CGM. Mature, adherent BMDM were harvested from the plates by gentle pressure washing of the disk surface using a 10-mL Class A volumetric pipette

(SIBATA Scientific Technology, Saitama, Japan). The detached BMDM were collected by centrifugation at 300***g*** for 10 minutes at RT, and resuspended at 1.5 x 10^6 cells/mL in CGM. Cell viability was determined via dye-exclusion hemocytometry. Twenty-four-well tissue culture plates (Greiner Bio-One) were seeded with 0.3 mL of cell suspension and incubated for 3h to allow cell attachment. Following this incubation, cell supernatants were aspirated and the adherent BMDM were primed with 500 ng/mL LPS (Sigma) for 3h under cell culture conditions, prior to any treatment using pharmaceutical agents and the addition of particles or ions.

3.4 Treatments Using Pharmaceutical Agents

3.4.1 Caspase-1 Inhibition and ROS Scavenging

The effects of capase-1 inhibition and ROS scavenging on IL-1β release induced by CoCrMo particles and Cr_2O_3 particles were tested. Following the LPS-priming incubation, the cell supernatants were aspirated and, depending on which treatment was being analyzed, replaced with fresh CGM (300 μL) supplemented with either:

- 0 or 20 μM Z-WEHD-FMK (R&D Systems, Minneapolis, MN), an irreversible caspase-1 inhibitor; or
- 0 or 2 mM L-Ascorbic acid (L-AA; Sigma), an antioxidant.

Cells were incubated with the pharmaceutical agent for 1h under cell culture conditions. Aliquots (150 μL) of CoCrMo particles (7 to 133 particles per macrophage), 60-nm Cr_2O_3 particles (0.33 to 2.3 million particles per macrophage) or 700-nm Cr_2O_3 particles (333 to 1333 particles per macrophage) were then added to the culture supernatants, and the cells were incubated an additional 18h. Nigericin (5 μM) (Cayman Chemical, Ann Arbor, MI) or fresh CGM were added to the culture supernatants (instead of particles) for the positive and negative controls, respectively. At the end of the incubation, supernatants were collected, centrifuged at 300***g*** for 10 minutes at 4°C, snap-frozen in liquid nitrogen, and stored at -80°C. Supernatants were analyzed later for IL-1β release by enzyme-linked immunosorbent assay (ELISA).

3.4.2 MMP Inhibition

The effect of MMP inhibition on IL-1β release induced by CoCrMo particles was tested. Following the LPS-priming incubation, the cell supernatants were aspirated and replaced with fresh CGM supplemented with either:

- 0, 50, 100 or 200 μM MMP-2/MMP-9 Inhibitor I (M2/9I; Cayman Chemical); or
- 200, 400 or 800 μM NNGH (Abcam, Cambridge, UK)

M2/9I is a potent MMP-2 and MMP-9 inhibitor, while NNGH is an MMP-3 inhibitor with broad spectrum MMP inhibition. Cells were incubated with the inhibitors for 1h under cell-culture conditions. Aliquots (150 μL) of CoCrMo particles (67 particles per macrophage) were then added to the culture supernatants, and the cells were incubated an additional 18h. The same positive and negative controls were used as described above. At the end of the incubation, supernatants were collected and analyzed as above.

3.5 NLRP3 Inhibition Using NLRP3$^{-/-}$ Mice

The effect of NLRP3 inhibition (using NLRP3$^{-/-}$ cells) on IL-1β release induced by CoCrMo particles, Cr_2O_3 particles and Co^{2+}, Cr^{3+}, or Ni^{2+} was tested. Cells from bone marrow extracted from the tibiae, femora, and pelvic bones of 6- to 17- week-old female NLRP3$^{-/-}$ and wild type (WT) C57BL/6J mice (The Jackson Laboratory, Bar Harbour, ME) were differentiated into macrophages following the same procedure as described above. Following the LPS-priming incubation, cell supernatants were aspirated and replaced with fresh CGM (300 μL). CoCrMo particles (7 to 133 particles per macrophage), 60-nm Cr_2O_3 particles (0.33 to 2.3 million particles per macrophage), 700-nm Cr_2O_3 particles (333 to 1333 particles per macrophage), Co^{2+} (18 ppm), Cr^{3+} (300 ppm), or Ni^{2+} (48 ppm) were then added to the culture supernatants, and the cells were incubated an additional 18h. Nigericin (5 μM) (Cayman Chemical), fresh CGM or cell-culture grade water (ion solvent) were added to the culture supernatants (instead of particles or ions, respectively) for the positive (Nigericin) and negative (CGM and cell-culture grade water) controls. At the end of the incubation, supernatants were collected, centrifuged at 300***g*** for 10 minutes at 4°C, snap-frozen in liquid nitrogen and stored at -80°C. Supernatants were analyzed later for IL-1β release by ELISA.

3.6 Cytokine Analysis

Concentrations of IL-1β in culture supernatants were measured by ELISA using the IL-1β Mouse Uncoated ELISA Kit (Thermo Fisher), following the manufacturer's instructions. Supernatants were thawed and gently mixed prior to use. A microplate reader (Synergy™ 4) was used to measure absorbance at 450 nm, with the absorbance at a reference wavelength of 570 nm

subtracted from the measured values. The minimum sensitivity of the assay is 8 pg/mL, as per the manufacturer's specifications.

3.7 Statistical Analysis

Statistical analysis was performed using models constructed in R (version 3.5.2). Treatments (Z-WEHD-FMK, L-AA, and MMP inhibitors), NLRP3$^{-/-}$ results, and cell mortality (with CoCrMo particles) were analyzed using a mixed linear model with pharmaceutical agent and/or particle concentration or ion concentration as fixed factors and experiment as a random factor. Experiments with a single replicate (cell mortality with Cr_2O_3 particles and all experiments conducted with WT BMDM obtained from The Jackson Laboratory) were analyzed with either a univariate or bivariate linear model, with pharmaceutical agent and/or particle concentration or ion concentration as fixed factors. Pairwise comparisons were performed using Tukey post-hoc tests in all models. Data are presented either as means ± standard error of the mean (SEM) or means ± standard deviation (SD), respectively.

4 Results

4.1 Effects of Caspase-1 Inhibition on IL-1β Release

Overall, results showed a particle concentration-dependent increase in IL-1β release with all three particles tested. The presence of a caspase-1 inhibitor, Z-WEHD-FMK, induced a partial inhibition of IL-1β release with both CoCrMo and Cr_2O_3 particles.

More specifically, IL-1β release increased with CoCrMo particle concentrations, by up to 324% with 33 particles per macrophage, relative to the negative control (cells with no particles and no Z-WEHD-FMK) ($p<0.001$; Fig. 1A). Of note, IL-1β release decreased with higher particle concentrations (67 and 133 particles per macrophage), likely due to higher toxicity of the particles. However, IL-1β release remained significantly higher than that in the negative control ($p<0.001$ in both cases; Fig. 1A). The presence of 20 μM Z-WEHD-FMK induced an 83% decrease in IL-1β release in the negative control ($p=0.003$), and a decrease of 43% to 78% with all CoCrMo particle concentrations ($p<0.001$ in all cases; Fig. 1A).

IL-1β release also increased with Cr_2O_3 particle concentrations, by up to 630% and 845% with 2.3 million particles per macrophage (60-nm particles) and 1333 particles per macrophage (700-nm particles), respectively, relative to the negative control (cells with no particles and no Z-WEHD-FMK) ($p<0.001$ in both cases; Fig. 1B and C). Similarly to the results with CoCrMo particles, the presence of 20 μM Z-WEHD-FMK decreased IL-1β release with the Cr_2O_3 particles. Specifically, IL-1β release decreased by 95% in the negative control ($p<0.001$; Fig. 1B), by 62% to 98% with all 60-nm particle concentrations ($p\leq0.008$ in all cases; Fig. 1B), and by 57% to 74% with 700-nm particles at a concentration equal to or higher than 667 particles per macrophage ($p<0.001$ in all cases; Fig. 1C). The concentration of Z-WEHD-FMK tested (20 μM) was previously found to have minimal cytotoxic effects [62].

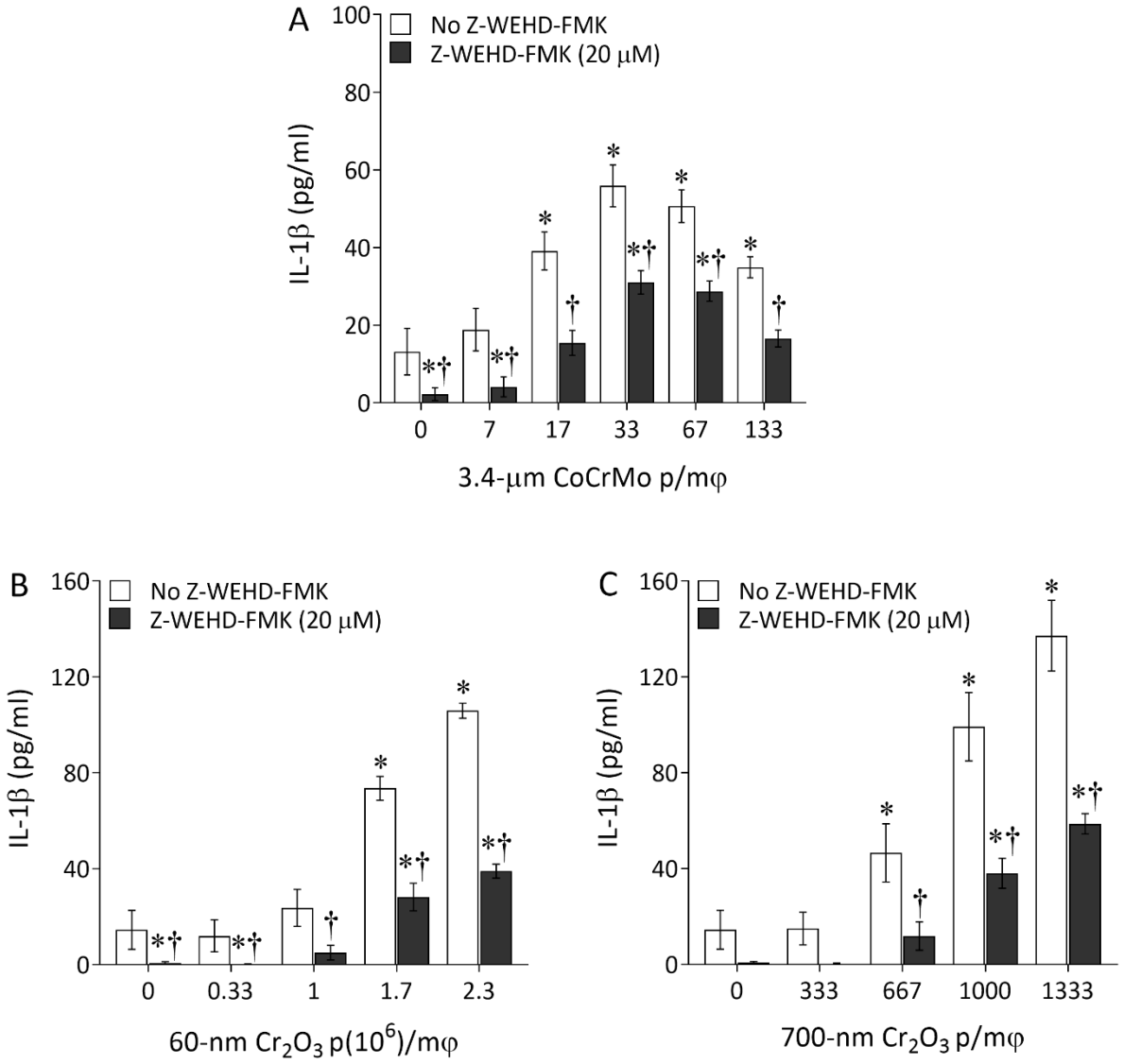

Figure 1. Interleukin-1β (IL-1β) release by bone marrow-derived macrophages (BMDM) after exposure to: (A) CoCrMo particles; (B) 60-nm Cr_2O_3 particles; or (C) 700-nm Cr_2O_3 particles, with or without Z-WEHD-FMK. Cells were primed for 3h with 500 ng/mL lipopolysaccharide (LPS), and then incubated for 18h under cell culture conditions with the indicated particle concentrations and with or without 20 μM Z-WEHD-FMK (caspase-1 inhibitor). IL-1β release was analyzed by ELISA. Statistical analysis was performed using a mixed linear model with particle concentration and inhibitor as fixed factors and experiment as a random factor. An asterisk (*) indicates a significant difference (p<0.05) between a given particle concentration with or without Z-WEHD-FMK and the negative control (no particles, no Z-WEHD-FMK). A dagger (†) indicates a significant difference (p<0.05) between the results obtained with and without Z-WEHD-FMK at a given particle concentration. Data are presented as means ± SEM of 3-5 independent experiments performed in triplicate. p/mφ: particles per macrophage.

4.2 Effects of ROS Scavenging on IL-1β Release

Similarly to the results obtained when analyzing the effects of caspase-1 inhibition, IL-1β release increased with particle concentration, by up to 525%, 607%, and 745% with 67 CoCrMo particles per macrophage, 2.3 million Cr_2O_3 particles (60 nm) per macrophage, and 1333 Cr_2O_3 particles (700 nm) per macrophage, respectively, relative to the negative control (cells with no particles and no L-AA) ($p<0.001$ in all cases; Fig. 2A, B and C).

Results also showed that the presence of 2 mM L-AA with CoCrMo particles induced a partial decrease in IL-1β release, while it had a minimal effect with Cr_2O_3 particles. More specifically, the presence of 2 mM L-AA with CoCrMo particles induced a 40% to 51% decrease in IL-1β release with a particle concentration equal to or higher than 17 particles per macrophage ($p\leq0.002$ in all cases; Fig. 2A). The presence of 2 mM L-AA had no significant effect on IL-1β release with either the 60-nm or 700-nm Cr_2O_3 particles, at all concentrations analyzed (Fig. 2B and C). The concentration of L-AA tested (2 mM) was previously found to have minimal cytotoxic effects [62].

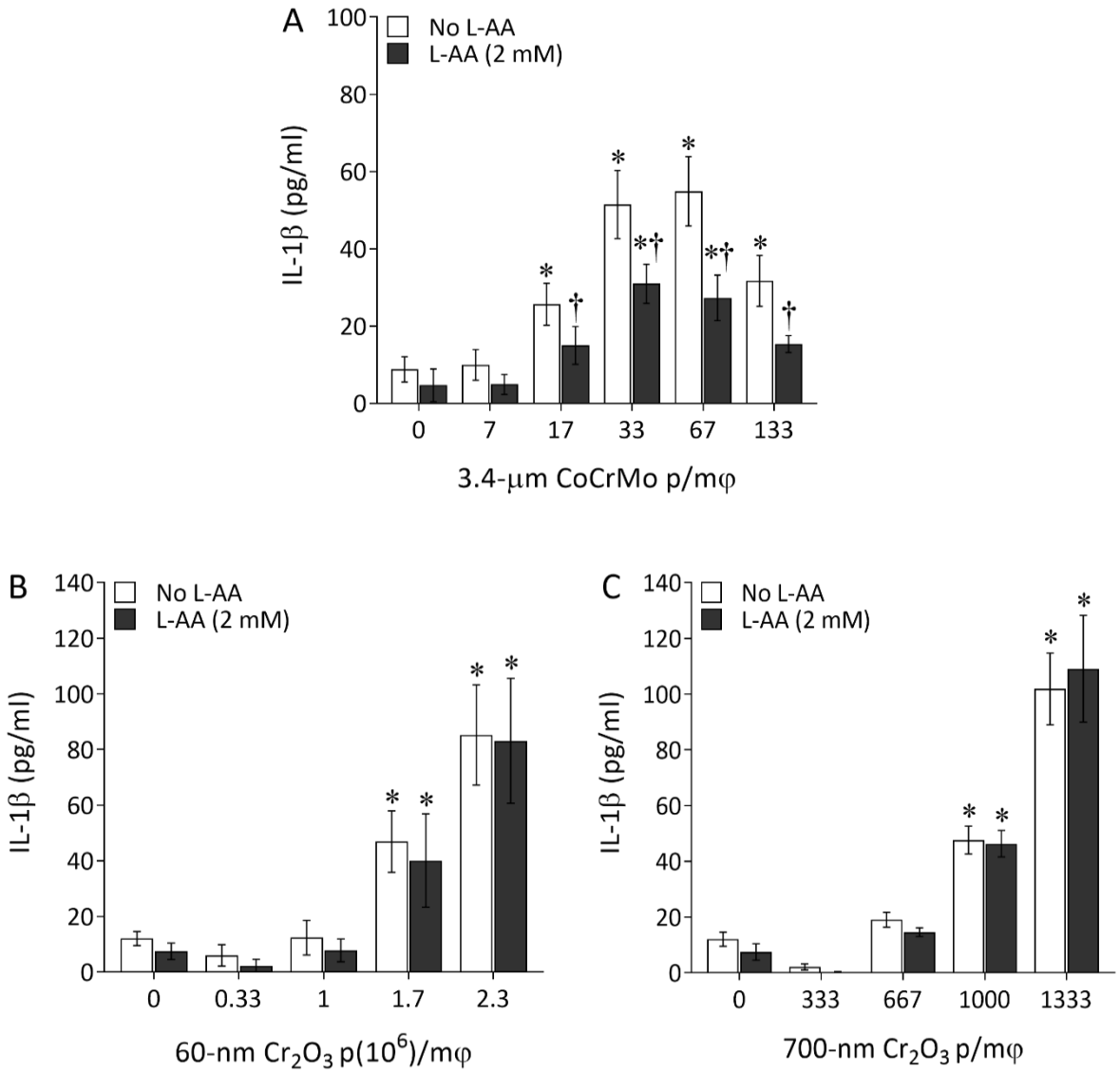

Figure 2. Interleukin-1β (IL-1β) release by bone marrow-derived macrophages (BMDM) after exposure to: (A) CoCrMo particles; (B) 60-nm Cr_2O_3 particles; or (C) 700-nm Cr_2O_3 particles, with or without L-Ascorbic acid (L-AA). Cells were primed for 3h with 500 ng/mL lipopolysaccharide (LPS), and then incubated for 18h under cell culture conditions with the indicated particle concentrations and with or without 2 mM L-AA (antioxidant). IL-1β release was analyzed by ELISA. Statistical analysis was performed using a mixed linear model with particle concentration and antioxidant as fixed factors and experiment as a random factor. An asterisk (*) indicates a significant difference (p<0.05) between a given particle concentration with or without L-AA and the negative control (no particles, no L-AA). A dagger (†) indicates a significant difference (p<0.05) between the results obtained with and without L-AA at a given particle concentration. Data are presented as means ± SEM of 3 independent experiments performed in triplicate. p/mφ: particles per macrophage.

4.3 Effects of NLRP3 Inhibition on IL-1β Release

Results showed that exposure of NLRP3$^{-/-}$ BMDM to CoCrMo particles led to a particle concentration-dependent increase in IL-1β release, by up to 2496% with 67 particles per macrophage, relative to the negative control (p<0.001; Fig. 3A).

IL-1β release remained low with all 60-nm and 700-nm Cr_2O_3 particle concentrations analyzed, and similar to that in the negative control (Fig. 3B and C).

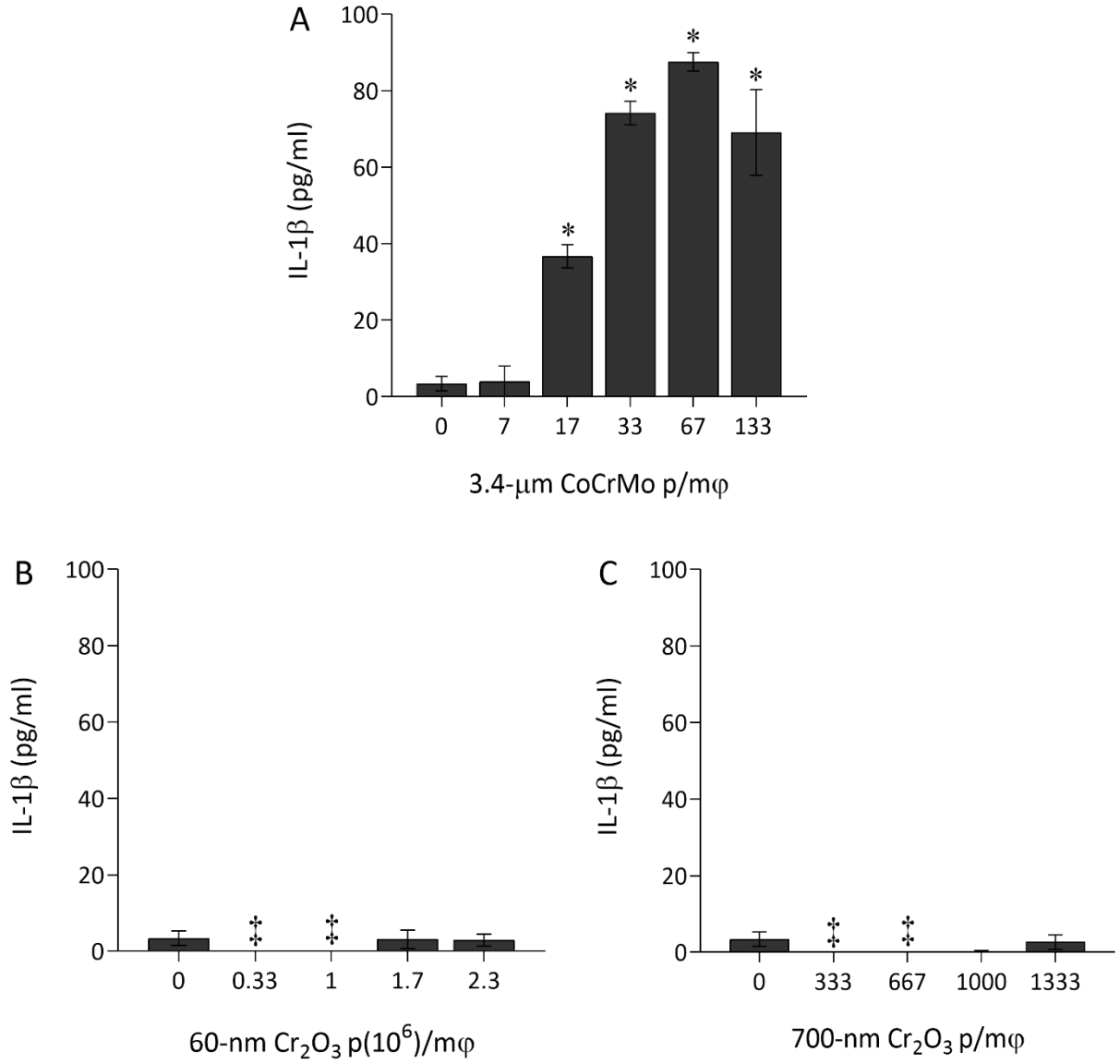

Figure 3. Interleukin-1β (IL-1β) release by NLRP3$^{-/-}$ bone marrow-derived macrophages (BMDM) after exposure to: (A) CoCrMo particles; (B) 60-nm Cr_2O_3 particles; or (C) 700-nm Cr_2O_3 particles. Cells were primed for 3h with 500 ng/mL lipopolysaccharide (LPS), and then incubated for 18h under cell culture conditions with the indicated particle concentrations. IL-1β release was analyzed by ELISA. Statistical analysis was performed using a mixed linear model with particle concentration as a fixed factor and experiment as a random factor. An asterisk (*) indicates a significant difference (p<0.05) between a given particle concentration and the negative control (no particles). A double dagger (‡) indicates that the measurement was below the detection limit. Data are presented as means ± SEM of 3 independent experiments performed in triplicate. p/mφ: particles per macrophage.

Experiments using ions were performed as a continuation of previous studies conducted in our laboratory, which used these ions and mice from The Jackson Laboratory. In WT BMDM exposed to ions, results showed an increase in IL-1β release of 1146% with 18 ppm Co^{2+} and 635% with 300 ppm Cr^{3+}, relative to the negative control (p=0.002 and p<0.001, respectively; Fig. 4A). The highest increase in IL-1β release (2265% relative to the negative control) was observed with 48 ppm Ni^{2+} (p<0.001; Fig. 4A). IL-1β release by NLRP3$^{-/-}$ BMDM exposed to 18 ppm Co^{2+} and 48 ppm Ni^{2+} increased by 3094% and 3243%, respectively, relative to the negative control (p<0.001 in both cases; Fig. 4B). IL-1β release also increased by 223% with 300 ppm Cr^{3+}, relative to the negative control, although this increase was not significant (Fig. 4B).

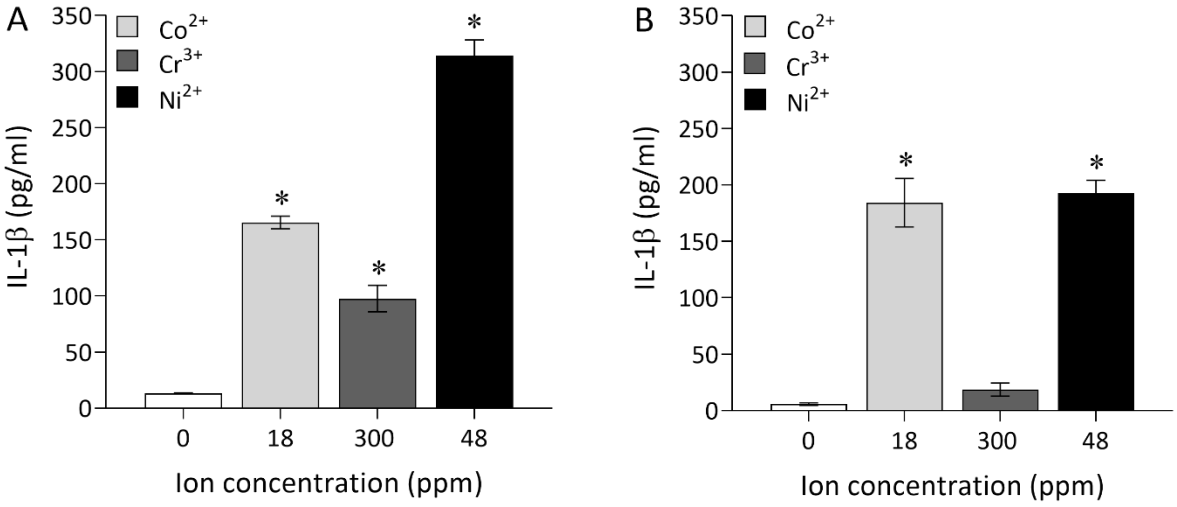

Figure 4. Interleukin-1β (IL-1β) release by bone marrow-derived macrophages (BMDM) obtained from: (A) Wild type; or (B) NLRP3$^{-/-}$ mice, after exposure to Co^{2+}, Cr^{3+}, or Ni^{2+}. Cells were primed for 3h with 500 ng/mL lipopolysaccharide (LPS), and then incubated for 18h under cell culture conditions with the indicated particle concentrations. IL-1β release was analyzed by ELISA. Statistical analysis of results from wild type BMDM was performed using a univariate linear model with ion concentration as a fixed factor. Analysis of results from NLRP3$^{-/-}$ BMDM was performed using a mixed linear model with ion concentration as a fixed factor and experiment as a random factor. An asterisk (*) indicates a significant difference (p<0.05) between a given particle concentration and the negative control (no particles). Data in Figure A are presented as means ± SD of a single experiment performed in triplicate. Data in Figure B are presented as means ± SEM of 3 independent experiments performed in triplicate.

4.4 Effects of MMP Inhibition on IL-1β Release

Results showed that the presence of either MMP-2/9 inhibitor I (M2/9I) or NNGH (MMP-3 inhibitor with broad spectrum MMP inhibition) induced a partial inhibition of IL-1β release with CoCrMo particles (analyzed at the single concentration of 67 particles/macrophage). This decrease was dependent on the inhibitor concentration.

More specifically, while IL-1β release increased by 403% with 67 particles per macrophage without the inhibitor, relative to the negative control (cells with no particles and no M2/9I) ($p<0.001$; Fig. 5A), the presence of M2/9I decreased IL-1β release by up to 49% with the highest inhibitor concentration tested (200 μM) ($p<0.001$; Fig. 5A).

Similarly, while IL-1β release increased by 379% with 67 particles per macrophage without the inhibitor, relative to the negative control (cells with no particles and no NNGH) ($p<0.001$; Fig. 5B), the presence of NNGH decreased IL-1β release by up to 52% with the highest inhibitor concentration tested (800 μM) ($p<0.001$; Fig. 5B).

The concentrations of both M2/9I and NNGH tested were found to have minimal cytotoxic effects (see Appendix 2).

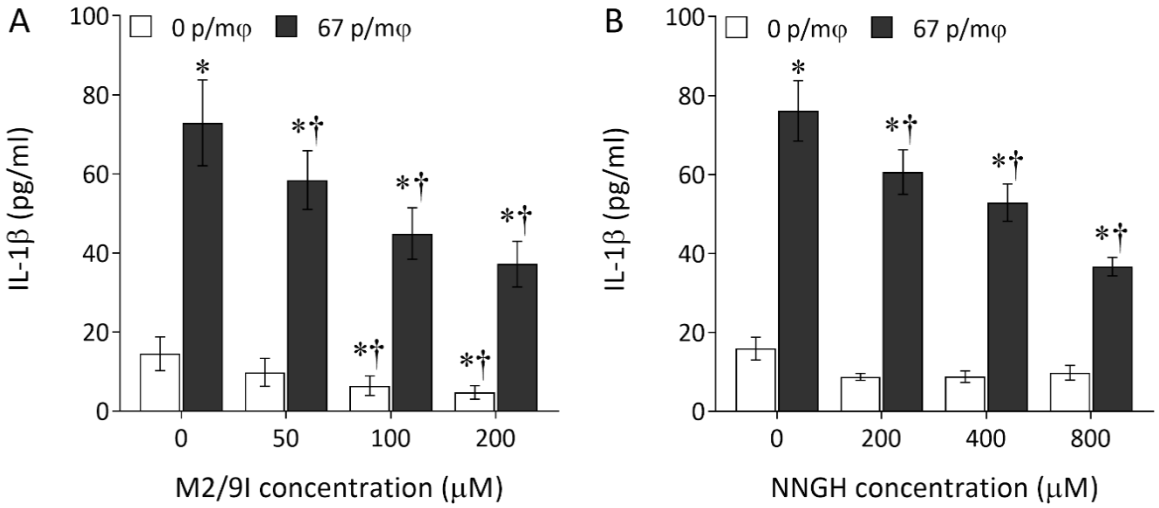

Figure 5. Interleukin-1β (IL-1β) release by bone marrow-derived macrophages (BMDM) after exposure to CoCrMo particles with or without: (A) MMP-2/9 inhibitor I (M2/9I); or (B) NNGH. Cells were primed for 3h with 500 ng/mL lipopolysaccharide (LPS), and then incubated for 18h under cell culture conditions with the indicated particle concentration and with or without 50 to 200 μM M2/9I (MMP-2 and MMP-9 inhibitor) or 200 to 800 μM NNGH (MMP-3 and broad spectrum MMP inhibitor). IL-1β release was analyzed by ELISA. Statistical analysis was performed using a mixed linear model with particle and inhibitor concentration as fixed factors and experiment as a random factor. An asterisk (*) indicates a significant difference (p<0.05) between a given particle concentration with or without different concentrations of M2/9I or NNGH and the negative control (no particles, no M2/9I or NNGH). A dagger (†) indicates a significant difference (p<0.05) between a given particle concentration with and without different concentrations of M2/9I or NNGH. Data are presented as means ± SEM of 3 independent experiments performed in triplicate. p/mφ: particles per macrophage.

5 Discussion

5.1 Experimental Parameters

5.1.1 Project Focus

Both ions and wear particles from CoCrMo-based implants (including MoM hip implants) have been shown to induce inflammatory responses *in vitro* [62], [169], [196], which has been implicated as a principal cause of periprosthetic osteolysis resulting in aseptic loosening of the implant [124], and as such these wear products are a cause for concern. The focus of this thesis was to investigate molecular mechanisms involved in IL-1β release in response to wear products and corrosion from CoCrMo-based implants. Specifically, the effects of micrometre-size CoCrMo and nanometre-size Cr_2O_3 particles on IL-1β release by BMDM were examined, with a focus placed on the roles of caspase-1 (likely indicator of NLRP3 inflammasome activation) (Objective 1), ROS (Objective 2) (potential activator of the NLRP3 inflammasome), and the NLRP3 inflammasome itself (Objective 3). Additionally, the role of the NLRP3 inflammasome in IL-1β release in response to Co^{2+}, Cr^{3+}, and Ni^{2+} was examined. Finally, the involvement of MMPs in the release of IL-1β in response to CoCrMo particles was also investigated.

5.1.2 Particles and Ions

Commercially available, round CoCrMo (average size 3.4 μm) and Cr_2O_3 (60 and 700 nm) particles were used in the present study. The characteristics (shape, size and composition) of these particles are similar to those isolated from MoM periprosthetic tissues *in vivo* and hip simulators *in vitro* [71], [73]–[75], [78]–[80], with the 60-nm Cr_2O_3 particles having the closest resemblance. As such, these particles can be considered clinically relevant. Additionally, the selected size of the CoCrMo particles was to ensure they were within the phagocytosable range [132]. CoCrMo and Cr_2O_3 particle concentrations (of which the latter were based on a previous study [250]) were selected to test a range of cytotoxic effects. Co^{2+} and Cr^{3+} were analyzed as these ions are released from the dissolution of wear particles and from corrosion of CoCrMo-based implants [107], [146]. Ni constitutes a small proportion of CoCrMo alloys (<0.5%) and is not a major concern with CoCrMo-based implants. However, it is present in larger amounts in other metal alloys, such as stainless steels (which are also used for orthopaedic applications), and as such, Ni^{2+} is also of interest. The ion concentrations were determined previously [62], and based on previous *in vitro* studies investigating their cytotoxic effects and cytokine release in macrophages [60], [252], [253].

The concentrations of particles and ions used in the present study are likely higher than concentrations expected *in vivo*; however, these selections were based on the assumption that macrophages require increased concentrations of stimulating agents *in vitro* compared to *in vivo* where cells are subject to multiple, simultaneous stimulating factors [62], [250]. Additionally, while metal ion levels in the blood and urine of patients with a MoM hip implant have been reported to be in the ppb range [108], [254], [255], concentrations in periprosthetic tissues are likely higher than those in body fluids where they are diluted [252]. The analysis of the particle cytotoxicity is included in Appendix 1, and ion cytotoxicity was assessed previously in our laboratory [62].

5.1.3 Cells

Macrophages have been shown to be key mediators in the immune response to wear products [134]–[136], and have been shown to release inflammatory cytokines in response to wear particles and ions [142], [145], [146]. Murine BMDM were chosen for the present study, as primary cells are preferential over cell lines due to their superior physiological relevance [256]. Additionally, compared to other sources of primary macrophages, such as peritoneal and alveolar macrophages, a much higher yield of BMDM can be obtained from a single mouse, and as such, BMDM are typically favored for macrophage models in immunological studies [257]. Murine cells were chosen over human as mice provide a more sustainable source of cells for the number required to meet experimental needs. Responses from murine cells are also less varied compared to human cells. Lastly, murine BMDM have previously been used to investigate metal ion-induced inflammasome activation [62].

5.2 Analysis of IL-1β Release after Exposure to CoCrMo Wear and Corrosion Products

The present study analyzed molecular mechanisms involved in IL-1β release by macrophages in response to wear particles and ions from CoCrMo-based implants, with a focus placed on NLRP3 inflammasome activation pathways. Pharmaceutical agents that target mechanisms potentially involved in NLRP3 inflammasome activation were used (a caspase-1 inhibitor and an antioxidant), and the subsequent IL-1β release from primed murine BMDM in the presence of particles or ions was measured, as inflammasome activation leads to IL-1β release [150]. It should be noted that L-AA, the antioxidant used in the present study, exerts a variety of pharmacological effects as it is

involved in other biochemical processes *in vivo*. Additionally, the effects on MMP inhibition on IL-1β release induced by CoCrMo particles were analyzed.

5.2.1 IL-1β Release after Exposure to CoCrMo Particles

Since metal particles have been shown to stimulate the NLRP3 inflammasome [169], [170], [196], and inflammasome activation results in the activation of caspase-1 [158] which cleaves pro-IL-1β into active IL-1β [154], the role of caspase-1 in IL-1β release in response to micrometre-size CoCrMo particles was investigated as a likely indicator of inflammasome activation. In the absence of a caspase-1 inhibitor, results showed a particle concentration-dependent increase in IL-1β release from BMDM exposed to CoCrMo particles. This is in agreement with a previous study by Caicedo et al. [58], who found that IL-1β release increased with increasing concentrations of 6-μm CoCrMo particles. IL-1β release decreased with higher particle concentrations, likely due to the increased toxicity of the particles, but still remained significantly higher than that in the negative control (Figure 1A). The presence of a caspase-1 inhibitor, Z-WEHD-FMK, led to a partial decrease in IL-1β release with CoCrMo particles. This suggests that IL-1β release was partially dependent on caspase-1 cleavage of pro-IL-1β, but additional molecular mechanisms may be involved.

The propensity of the NLRP3 inflammasome to be activated by a wide variety of stimuli [167] that are chemically and structurally diverse makes it unlikely that they all activate NLRP3 through physical interaction [156]. Instead, it has been suggested that these stimuli trigger effects upstream of the inflammasome which result in a few, or potentially single, unifying inflammasome activation mechanism(s) [167]. One such proposed mechanism is ROS production [258]. It has also been shown that the presence of an NADPH inhibitor decreased inflammasome-dependent IL-1β release induced by CoCrMo particles in THP-1 cells [169]. Accordingly, the role of ROS in IL-1β release after exposure to CoCrMo particles was investigated. Results from experiments using L-AA, an antioxidant, showed a partial decrease in IL-1β release after exposure to CoCrMo particles. Interestingly, the level of reduction in IL-1β release was similar with each particle concentration tested in the presence of either Z-WEHD-FMK or L-AA, suggesting that the activation of caspase-1 in response to CoCrMo particles may be redox-dependent.

BMDM obtained from NLRP3$^{-/-}$ mice were then exposed to CoCrMo particles to determine whether IL-1β release in response to these particles was dependent on the NLRP3 inflammasome.

Results showed high levels of IL-1β, similar to those released by WT BMDM exposed to the same particles (Figure 3A and A3A). These results suggest that IL-1β release in murine BMDM exposed to micrometre-size CoCrMo particles is independent of the NLRP3 inflammasome. Interestingly, together with the results from the experiments using Z-WEHD-FMK and L-AA, these results suggest that IL-1β release in response to CoCrMo particles may involve caspase-1 and ROS, but independently from the NLRP3 inflammasome. Therefore, caspase-1 could be involved in pathways that lead to IL-1β release other than the canonical NLRP3 inflammasome pathway.

Interestingly, studies by Caicedo et al. [58], [169] reported that IL-1β release in response to CoCrMo particles was mediated by the NLRP3 inflammasome and dependent on caspase-1. Additionally, the reported levels of IL-1β were much higher than in the present study. Possibilities for the discrepancies observed between the present study and those by Caicedo et al. [58], [169] may be due to differences in cell type (murine vs. human) and/or differences in experimental design. In the present study, murine BMDM were used while Caicedo et al. used both human primary macrophages (derived from peripheral blood mononuclear cells isolated from donor blood samples) and THP-1 monocytes (cell line) that were differentiated into macrophages using a phorbol ester. Differences in experimental design included the use of LPS for priming and NLPR3$^{-/-}$ mice in the present study, whereas Caicedo et al. did not employ a priming step, and used RNA interference (RNAi) to knock-down the gene expression of NLRP3 and ASC. Finally, these authors used Z-VAD-FMK to inhibit caspase-1; however this inhibitor has been shown to be a pan-caspase inhibitor with broad spectrum activity [259], and it is therefore possible that there may have been unintended off-target effects of this inhibitor.

No definitive mechanisms of inflammasome-independent caspase-1 activation have been proposed to date [260]. However, it has been suggested that ROS may directly activate caspase-1 via extracellular signal-regulated kinases (ERK) 1/2 [261]. Moreover, emerging evidence has shown that enzymes other than capsase-1 are capable of processing pro-IL-1β into mature IL-1β, suggesting the possibility of caspase-1-independent (and presumably inflammasome-independent) processing of IL-1β [240], [242]. One group of enzymes identified as being able to cleave pro-IL-1β into IL-1β are MMPs. Schönbeck et al. [237] found that IL-1β was predominately cleaved by MMP-9, with MMP-3 and MMP-2 also capable, but with lower efficacy. Nakashima et al. [244] found that primary human macrophages predominately expressed MMP-9, but also MMP-1 and -2 in response to titanium alloy and PMMA particles, in a dose- and time-dependent manner.

Additionally, Nawrocki et al. [245] found that MMP-2 and its activator MT1-MMP were expressed in macrophages and multinucleated giant cells containing phagocytosed polyethylene debris. These findings, coupled with those from the present study showing that CoCrMo particles do not appear to release IL-1β through the NRLP3 inflammasome, postulated the idea that MMPs may be a potential mechanism leading to mature IL-1β release from BMDM in the presence of CoCrMo particles, independent of the NLRP3 inflammasome. Of interest were MMP-2 and -9, as the studies previously mentioned reported that these MMPs were expressed in the presence of orthopaedic wear products. As such, an inhibitor specific to MMP-2 and -9, as well as an MMP-3 inhibitor with broad spectrum MMP inhibition were added to BMDM exposed to CoCrMo particles.

Results showed that the presence of either M2/9I or NNGH induced a partial inhibition of IL-1β release. These results suggest that MMPs are involved in the release of IL-1β in response to micrometre-size CoCrMo particles in BMDM. Although both inhibitors (at the highest concentration) reduced IL-1β release by approximately half, the concentration of NNGH required was much higher than that of M2/9I (800 μM vs. 200 μM, respectively). Therefore, it is possible that MMP-2 and -9 are involved in IL-1β release in response to CoCrMo particles more so than MMP-3 or other MMPs, as the inhibition seen with NNGH may be due to broad spectrum MMP inhibition, although this would need to be investigated further. These results are similar to those of Woo et al. [248], who found that inhibition of MMP-3 and MMP-9 suppressed mRNA expression of proinflammatory cytokines (including IL-1β) in LPS-stimulated murine microglial cells. The exact mechanism through which MMPs lead to IL-1β release in response to CoCrMo particles, or whether they play a role in activating caspase-1 remains to be elucidated. Of interest, a study by Wu et al. [262] using thoracic smooth muscle cells (SMC) treated with palmitic acid (PA) found that recombinant caspase-1 directly cleaved recombinant MMP-9. The authors also found that MMP-9 binds to NLRP3, ASC and caspase-1 through co-immunoprecipitation of protein lysate from PA-treated SMCs. This suggests an interaction may exist between MMP-9 and caspase-1/NLRP3 inflammasome.

Overall, the results from the present study suggest that micrometre-size CoCrMo particles induce IL-1β release through: 1. pathway(s) involving caspase-1 that may be redox-dependent, but not NLRP3 inflammasome-dependent; and/or 2. a pathway that does not involve caspase-1 (since IL-1β release was not fully inhibited in presence of caspase-1 inhibitor). Additionally, results showed

that MMPs are involved in the release of IL-1β, but whether MMPs directly process pro-IL-1β into active IL-1β, or are involved in a possible caspase-1-dependent pathway remains to be seen.

5.2.2 IL-1β Release after Exposure to Cr_2O_3 Particles

As with the CoCrMo particles, experiments were performed to determine if IL-1β release in response to Cr_2O_3 particles is caspase-1- and/or redox-dependent. Results showed a particle concentration-dependent increase in IL-1β release in BMDM exposed to nanometre-size Cr_2O_3 particles. These results are similar to those from previous findings [263], [264]. Horie et al. [263] found that the gene expression of IL-1β and IL-8 was enhanced in THP-1 cells exposed to 60-nm Cr_2O_3 particles, although the authors did not examine cytokine release as in the present study. Andujar et al. [264] found that IL-1β release significantly increased in THP-1 macrophages exposed to 15 nm CrOOH (chromium oxide mineral) particles. These authors reported significantly higher levels of IL-1β release than those found in the present study, which may be due to differences in cell type (human vs. murine) and differences in particle characteristics (size and composition).

The addition of a caspase-1 inhibitor, Z-WEHD-FMK, induced a greater than 50% decrease in IL-1β release by WT BMDM obtained from Charles River mice and exposed to either 60- or 700-nm Cr_2O_3 particles. Interestingly, in WT BMDM obtained from The Jackson Laboratory mice, the presence of Z-WEHD-FMK reduced IL-1β release to levels at or below that of the negative control (no particles, no Z-WEHD-FMK) with 60-nm Cr_2O_3 particles at all concentrations tested (see Appendix 3; Figure A3B). This suggests that IL-1β release in response to Cr_2O_3 particles could be fully caspase-1-dependent. Additional experiments would need to be performed to confirm this hypothesis, especially since the experiment with the WT BMDM from The Jackson Laboratory mice was only performed once, and this result was observed only with one size (60 nm) of Cr_2O_3 particles.

Results from experiments using L-AA, an antioxidant, showed that L-AA had no significant effect on IL-1β release with either 60-nm or 700-nm Cr_2O_3 particles at any concentration analyzed, suggesting that ROS are not involved in IL-1β release induced by nanometre-size Cr_2O_3 particles. Lastly, BMDM obtained from NLRP3$^{-/-}$ mice exposed to 60- or 700-nm Cr_2O_3 particles released very low levels of IL-1β that remained similar to that in the negative control (no particles) (Figure 3).

Overall, these results suggest that IL-1β release by BMDM exposed to nanometre-size Cr_2O_3 particles is NLRP3 inflammasome-dependent, and that caspase-1 is involved. Additionally, inflammasome activation in response to Cr_2O_3 particles did not appear to be redox-dependent. These findings corroborate those of previous studies, which have shown that nanometre-size ceramic particles, such as titanium dioxide (titania; TiO_2), activate the NLRP3 inflammasome, and lead to IL-1β release in a caspase-1-dependent manner [265], [266]. The authors however showed that NLRP3 inflammasome activation was ROS-dependent. Although the present study found IL-1β release induced by Cr_2O_3 particles to be ROS-independent, this could be due to difference in particle composition.

5.2.3 IL-1β Release after Exposure to Ions

Previous studies have shown IL-1β release in response to Co^{2+}, Cr^{3+}, and Ni^{2+} purportedly through NLRP3 inflammasome activation [169], [171]; however, the exact mechanisms through which IL-1β is released in response to these ions has not been fully elucidated.

Results from NLRP3$^{-/-}$ BMDM exposed to these ions showed that IL-1β release in response to Co^{2+} was significantly higher than that in the negative control. In addition, IL-1β release was similar between the WT and NLRP3$^{-/-}$ BMDM (165 pg/ml vs. 184 pg/ml, respectively; Figure 4), suggesting that IL-1β release induced by Co^{2+} is independent of the NLRP3 inflammasome. On the other hand, IL-1β release by NLRP3$^{-/-}$ BMDM in response to Cr^{3+} was very low and similar to that in the negative control, suggesting that Cr^{3+}-induced IL-1β release is NLRP3 inflammasome-dependent. Finally, IL-1β release by NLRP3$^{-/-}$ BMDM in response to Ni^{2+} was significantly higher than that in the negative control, but partially reduced compared to the release from WT BMDM, suggesting that Ni^{2+}-induced IL-1β release is partially NLRP3 inflammasome-dependent. Results with BMDM obtained from a WT mouse (N=1) showed that Ni^{2+} induced the most IL-1β release, followed by Co^{2+} and Cr^{3+}.

Overall, these results corroborate findings from a previous study in our laboratory [62], although IL-1β release was highest in response to Cr^{3+}, followed by Ni^{2+} and Co^{2+}. Discrepancies in results may be due to differences in experimental design (i.e., RPMI for cell medium vs. DMEM in the present study). Interestingly, a study by Caicedo et al. [169] using THP-1 cells found that 0.1 mM (approximately 6 ppm) Ni^{2+} induced the highest IL-1β release, while Co^{2+} and Cr^{3+} induced similar levels, although subsequent experiments within the same study showed variation in which ion

induced the highest IL-1β release. Additionally, other studies have reported no IL-1β release in response to Co^{2+} and Cr^{3+} in THP-1 cells [267], [268]. These results suggest that IL-1β release in response to ions is highly variable both across and within studies.

5.3 Priming

The necessity to prime for NLRP3 inflammasome activation has been well established in the literature, and is commonly achieved using LPS *in vitro* [184], [190], [269]. Interestingly, in the present study, priming BMDM with LPS prior to exposure with CoCrMo particles, Cr_2O_3 particles or ions was necessary to detect IL-1β release (initial experiments performed using non-primed BMDM exposed to CoCrMo or Cr_2O_3 particles did not show any IL-1β release). Similarly, other studies examining the immune response to orthopaedic wear particles have employed the use of a priming step [196], [197]. Furthermore, studies from the Greenfield group [148], [200] found that adherent endotoxin on titanium particles increases their bioactivity in murine macrophages [148], and that these adherent endotoxins, or PAMPs, are required to prime the NLRP3 inflammasome through interaction with their cognate TLRs [200]. It has been proposed that sub-clinical bacterial biofilms on implant surfaces, which do not cause an infection, provide a source of PAMPs which contribute to the immune reactivity of wear particles [57], [148]. As such, *in vivo*, priming may be achieved through PAMPs (including LPS) derived from sub-clinical bacterial biofilms on implant surfaces or through endogenous alarmins that may or may not be working concurrently with PAMPs [201].

5.4 Conclusions from the Present Study

5.4.1 Role of Caspase-1 in BMDM Response to CoCrMo and Cr_2O_3 Particles

The role of caspase-1 in IL-1β release induced by CoCrMo and Cr_2O_3 particles was analyzed by exposing BMDM to varying concentrations of CoCrMo, 60-nm or 700-nm Cr_2O_3 particles, with or without a caspase-1 inhibitor (Z-WEHD-FMK). Results showed that caspase-1 inhibition induced a partial decrease in IL-1β release with all particles tested, suggesting that IL-1β release in response to CoCrMo particles or Cr_2O_3 particles is partially caspase-1 dependent.

5.4.2 Role of ROS in BMDM Response to CoCrMo and Cr_2O_3 Particles

The role of ROS in IL-1β release induced by CoCrMo and Cr_2O_3 particles was analyzed by exposing BMDM to varying concentrations of CoCrMo, 60-nm or 700-nm Cr_2O_3 particles with or

without an antioxidant (L-AA). Results showed that ROS scavenging induced a partial decrease in IL-1β release with CoCrMo particles, while it had minimal effect with Cr_2O_3 particles. These results suggest that IL-1β release in response to CoCrMo particles, but not Cr_2O_3 particles, is partially redox-dependent.

5.4.3 Role of NLRP3 Inflammasome in BMDM Response to CoCrMo and Cr_2O_3 Particles, as well as Co^{2+}, Cr^{3+}, and Ni^{2+}

To determine if IL-1β release in response to CoCrMo particles, Cr_2O_3 particles, and Co^{2+}, Cr^{3+}, and Ni^{2+} is NLRP3 inflammasome-dependent, BMDM obtained from NLRP3$^{-/-}$ mice were treated with the aforementioned agents.

Results showed elevated IL-1β release by NLRP3$^{-/-}$ BMDM in response to CoCrMo particles and Co^{2+}, similar to the release by WT BMDM, suggesting that IL-1β release in response to CoCrMo particles and Co^{2+} is NLRP3 inflammasome-independent. In contrast, IL-1β release by NLRP3$^{-/-}$ BMDM in response to Cr_2O_3 particles and Cr^{3+} remains at the level of the negative control (no particles and no ions, respectively) and was much lower than the release by WT BMDM treated with the same particles or ions. These results suggest that IL-1β release in response to Cr_2O_3 particles and Cr^{3+} is NLRP3 inflammasome-dependent. Finally, IL-1β release by NLRP3$^{-/-}$ BMDM in response to Ni^{2+} remained significantly higher than that in the negative control (same cells with no ions), but was lower than the release by WT BMDM. This suggests that IL-1β release in response to Ni^{2+} may be partially NLRP3 inflammasome-dependent but other mechanisms may be involved.

5.4.4 Role of MMPs in BMDM Response to CoCrMo Particles

To determine if IL-1β release induced by CoCrMo particles is MMP-dependent, BMDM were exposed to CoCrMo particles (67 particles/macrophage) with or without varying concentrations of an MMP-2/9 inhibitor (M2/9I), or an MMP-3 inhibitor with broad spectrum MMP inhibition (NNGH). Results showed that MMP inhibition partially reduced IL-1β release, suggesting that IL-1β release in response to CoCrMo particles is partly MMP-dependent. Furthermore, a much higher concentration of NNGH was required to achieve the same level of inhibition as M2/9I, suggesting that MMP-2/9 may be more relevant over other MMPs.

5.4.5 Summary

The molecular mechanisms leading to the release of IL-1β in response to wear and corrosion products from CoCrMo-based implants were investigated in the present study. Overall, the findings provide new insights into these molecular mechanisms, and are illustrated in Figure 6. These findings may help lead to the development of potential therapeutic interventions or prevention of the inflammatory response responsible for aseptic loosening.

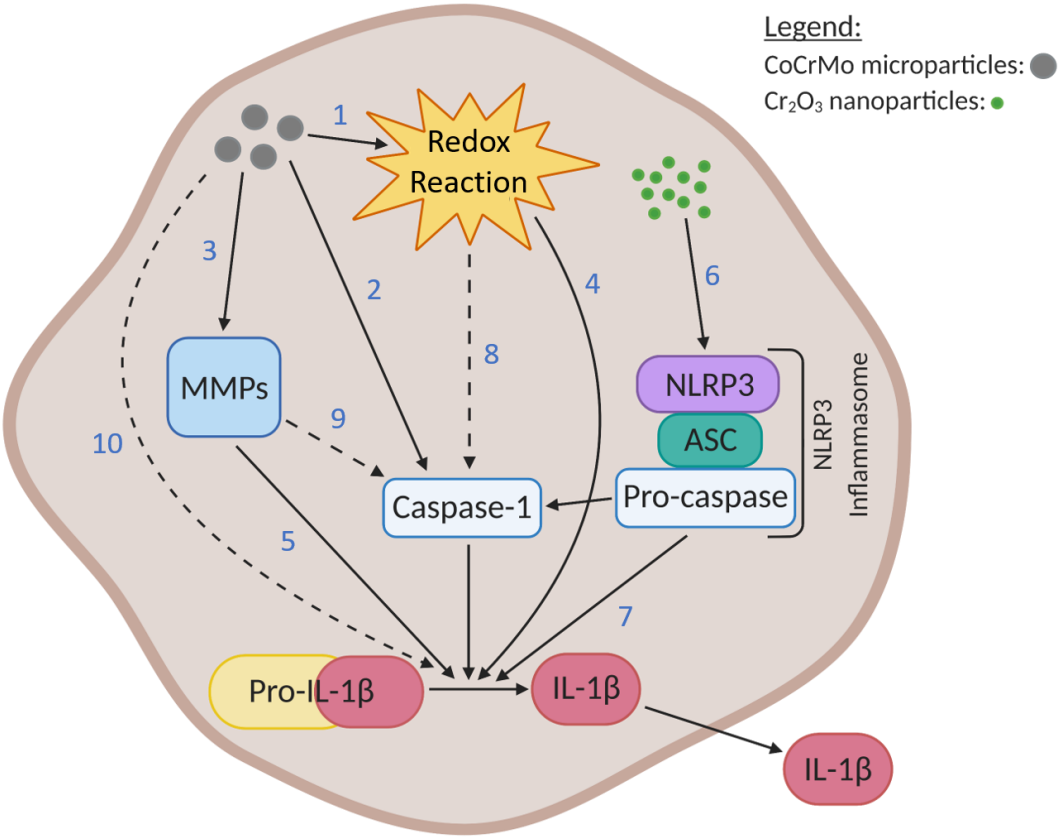

Figure 6. Proposed model of the molecular mechanisms involved in interleukin-1β (IL-1β) release after exposure to CoCrMo and Cr_2O_3 particles in bone marrow-derived macrophages (BMDM). IL-1β release induced by CoCrMo particles is partially caspase-1-, redox- and MMP-dependent, but NLRP3 inflammasome-independent (pathways 2, 1 → 4, and 3 → 5). Hypothetical pathways for IL-1β release induced by CoCrMo particles include: caspase-1 dependence on redox (pathway 1 → 8) or on MMPs (pathway 3 → 9). IL-1β release induced by Cr_2O_3 particles is NLRP3 inflammasome-dependent (pathway 6), but an additional pathway through which the NLRP3 inflammasome activates additional caspases or other molecules (pathway 7) may exist. Pathway 10 is a hypothetical pathway independent of those illustrated above. Solid lines represent pathways based on results from the present study. Dashed lines represent hypothetical pathways.

5.5 Future Studies

5.5.1 IL-1β Release by BMDM from Caspase-1$^{-/-}$ Mice

The findings from the present study with micrometre-size CoCrMo particles suggest that these particles induce IL-1β release through: 1. pathway(s) involving caspase-1 that may be redox-dependent but NLRP3 inflammasome-dependent; and/or 2. pathway(s) that do(es) not involve caspase-1. As such, a future study may involve the use of BMDM obtained from caspase-1$^{-/-}$ mice with or without an antioxidant to ascertain if IL-1β induced by CoCrMo particles is indeed caspase-1-dependent, and if so, determine if this pathway is redox-dependent (pathway 8 in Figure 6). Additionally, caspase-1$^{-/-}$ BMDM could be used with or without MMP inhibitors to determine if caspase-1 is dependent on MMPs (pathway 9 in Figure 6). This could also be extended to the other wear and corrosion products used in the present study (Cr_2O_3 particles, Co^{2+}, Cr^{3+}, or Ni^{2+}).

5.5.2 Other Pathways Leading to IL-1β Release

The present study showed that IL-1β release in response to micrometre-size CoCrMo particles is partly MMP-dependent. Interestingly, previous studies have shown that proteases other than caspase-1 and MMPs are capable of processing pro-IL-1β [270]. In addition, caspase-8 has been shown to be capable of directly cleaving pro-IL-1β into active IL-1β [271], [272]. Moreover, caspase-8 can act as both a positive and negative regulator of the NLRP3 inflammasome [273]. As such, future studies could investigate the involvement of other proteases, or caspase-8, in IL-1β release and inflammasome activation in response to CoCrMo particles (potential pathway 10 in Figure 6). Similar experiments could also be expanded to other wear and corrosion products from CoCrMo-based implants (like the Cr_2O_3 and metal ions described in the present study), or possibly wear products from other implant materials.

Lastly, future studies could investigate the effects of other wear products such as Ti6Al4V wear particles (another common alloy used in orthopaedic implant components), and include an *in vivo* animal model.

6 References

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Appendix 1: Cytotoxicity of CoCrMo and Cr_2O_3 particles

The method used to assess CoCrMo and Cr_2O_3 particle cytotoxicity (trypan blue dye-exclusion hemocytometry) required detaching adherent cells from 24-well plates. However, cell detachment was difficult and resulted in low cell recovery (particularly at high particle concentrations). As such, the results are presented here as they may have been affected by the low cell recovery.

Methods

The cytotoxicity of the particles was evaluated by measuring cell mortality after exposure to the particles. Following the LPS-priming incubation (described in Section 3.3), the cell supernatants were aspirated and replaced with fresh CGM (300 μL in each well). Aliquots (150 μL) of CoCrMo particles (7 to 133 particles per macrophage), 60-nm Cr_2O_3 particles (0.33 to 2.3 million particles per macrophage) or 700-nm Cr_2O_3 particles (333 to 1333 particles per macrophage) were then added to each sample, and the cells were incubated an additional 18h. At the end of the incubation, the cell culture supernatants were removed. Adherent cells were first washed with 400 μL/well of ice-cold DPBS (Sigma) and then incubated with 300 μL of ice-cold Accutase® (Millipore Sigma, Burlington, MA) for 15 minutes at RT. After the 15-minute incubation, cells were detached by gentle pipetting using a P1000 manual single-channel pipette (Mettler Toledo, Columbus, OH). Cell mortality was assessed by dye-exclusion hemocytometry under phase contrast microscopy using trypan blue (0.04% [w/v] final concentration; Sigma) and an improved Neubauer hemocytometer (Hausser Scientific, Horsham, PA).

Results

Results showed a CoCrMo particle concentration-dependent increase in cell mortality, up to 52% with 133 particles per macrophage ($p<0.001$ when compared to the negative control; Fig. A1A). The total cell number (live and dead) decreased as particle concentration increased, by up to 94% with 133 CoCrMo particles per macrophage, relative to the negative control ($p<0.001$; Fig. A1B).

Similarly, a particle concentration-dependent increase in cell mortality was observed with both 60- and 700-nm Cr_2O_3 particles, up to 43% and 50%, respectively ($p<0.001$ when compared to the negative control; Fig. A2A and C). The total cell number decreased with increasing Cr_2O_3 particle concentration, by up to 92% and 93% with 60- and 700-nm Cr_2O_3, respectively, relative to the negative control ($p<0.001$ in both cases; Fig. A2B and D).

Discussion

As mentioned above, the method used to measure mortality required first the detachment of adhered cells, which may have caused mechanical damage and loss of cells. Of note is that cell recovery in the negative control was between 30-40%. Additionally, at high particle concentrations, cells were likely in a compromised state and would be more sensitive to manipulation. It is therefore possible that many cells may have burst upon detachment, thereby excluding them from the total number of recovered cells (which is most evident at the highest particle concentrations tested). Accordingly, conclusions drawn from these results are subject to the caveat that cell recovery was quite low and not fully representative of all cells in each sample.

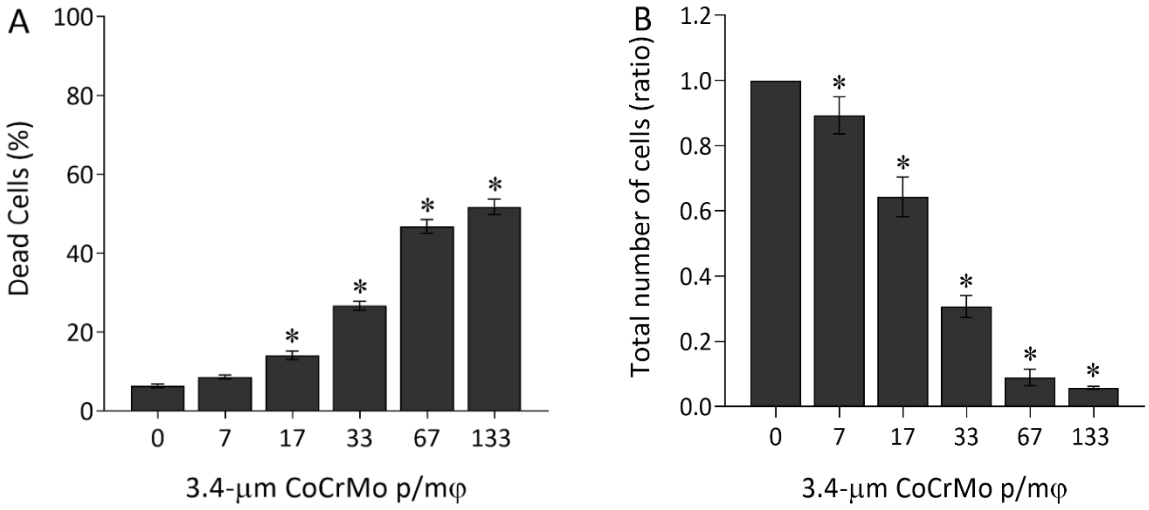

Figure A1. Mortality of bone marrow-derived macrophages (BMDM) after exposure to CoCrMo particles: (A) Percentage of dead cells; and (B) total number of cells (live and dead). Cells were primed for 3h with 500 ng/mL lipopolysaccharide (LPS), and then incubated for 18h under cell culture conditions with the indicated particle concentrations. Cells were counted using a hemocytometer and mortality was determined using trypan blue dye-exclusion. Total cell numbers (live and dead) are expressed as a ratio of the negative control (cells with no particles). Statistical analysis was performed using a mixed linear model with particle concentration as a fixed factor and experiment as a random factor. An asterisk (*) indicates a significant difference (p<0.05) between a given particle concentration and the negative control. Data are presented as means ± SEM of 3 independent experiments performed in triplicate. p/mφ: particles per macrophage.

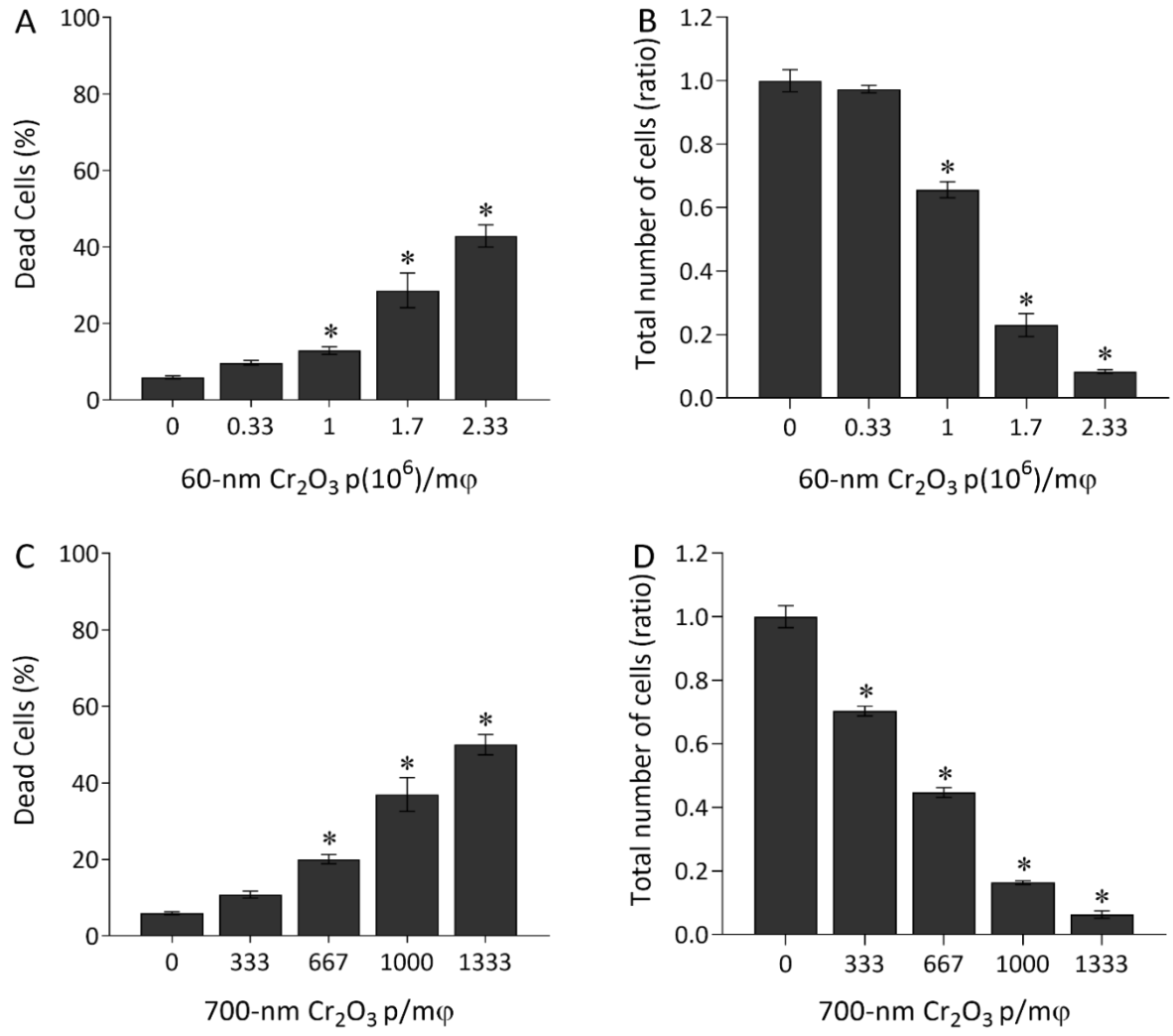

Figure A2. Mortality of bone marrow-derived macrophages (BMDM) after exposure to 60- or 700-nm Cr_2O_3 particles: (A, C) Percentages of dead cells; and (B, D) total numbers of cells (live and dead). Cells were primed for 3h with 500 ng/mL lipopolysaccharide (LPS), and then incubated for 18h under cell culture conditions with the indicated particle concentrations. Cells were counted using a hemocytometer and mortality was determined using trypan blue dye-exclusion. Total cell numbers (live and dead) are expressed as a ratio of the negative control (cells with no particles). Statistical analysis was performed using a univariate linear model with particle concentration as a fixed factor. An asterisk (*) indicates a significant difference (p<0.05) between a given particle concentration and the negative control. Data are presented as means ± (SD) of a single experiment performed in triplicate. p/mφ: particles per macrophage.

Appendix 2: Cytotoxicity of the MMP Inhibitors

Data collection and analysis of the cytotoxicity of the MMP inhibitors were performed by another student in our laboratory, Morgane Lignereux. The potential cytotoxic effects of the two MMP inhibitors tested in the present study were assessed by measuring BMDM mortality in the presence of the highest concentration used for each inhibitor (i.e., 200 μM and 800 μM for M2/9I and NNGH, respectively) using trypan blue dye-exclusion. Following incubation with the MMP inhibitors (as described in Section 3.5.2), cell supernatants were aspirated and replaced with pre-warmed (37°C) DPBS (300 μL). An aliquot of trypan blue dye solution (0.04% [w/v] final concentration) was added to the DPBS and immediately mixed by swirling. Cells were incubated for 30 seconds at RT. The dye solution was aspirated and replaced with fresh DPBS (300 μL) and cells were immediately imaged with brightfield or phase contrast microscopy at 100X magnification and counted. A minimum of 300 cells were counted in each sample. Results showed that both 200 μM M2/9I and 800 μM NNGH had minimal cytotoxic effects (< 5% mortality).

Appendix 3: Comparison of Cells from Mice Supplied by The Jackson Laboratory and Charles River Laboratories

Mice from The Jackson Laboratory were used for all experiments using NLRP3$^{-/-}$ BMDM, as well as for all experiments with Co^{2+}, Cr^{3+}, and Ni^{2+} (both WT and NLRP3$^{-/-}$) so that the results would be more directly comparable with previous studies conducted in our laboratory with these ions and using mice from this supplier. However, BMDM used to analyze the effects of caspase-1 inhibition, ROS scavenging, and MMP inhibition were obtained from mice purchased from Charles River Laboratories. Additional experiments were therefore performed to determine if there were any inherent differences in the cell response (IL-1β release) to the wear particles when using BMDM derived from mice from both suppliers. Accordingly, BMDM derived from The Jackson Laboratory WT mice were exposed to CoCrMo or Cr_2O_3 particles in the presence or absence of caspase-1 inhibitor Z-WEHD-FMK (Figure A3), and the results were compared to those obtained when using BMDM from Charles River mice (Figure 1 in Section 4.1).

Results showed that IL-1β release in the absence of Z-WEHD-FMK was similar when using mice from either supplier; however, the highest level of IL-1β released after exposure to 700-nm Cr_2O_3 particles was much higher in BMDM derived from The Jackson Laboratory mice. It should be noted, however, that the results from The Jackson Laboratory WT mice are from single experiments. The presence of 20 μM Z-WEHD-FMK induced a similar decrease (in percentages) in IL-1β release in mice from both suppliers after exposure to all particles tested.

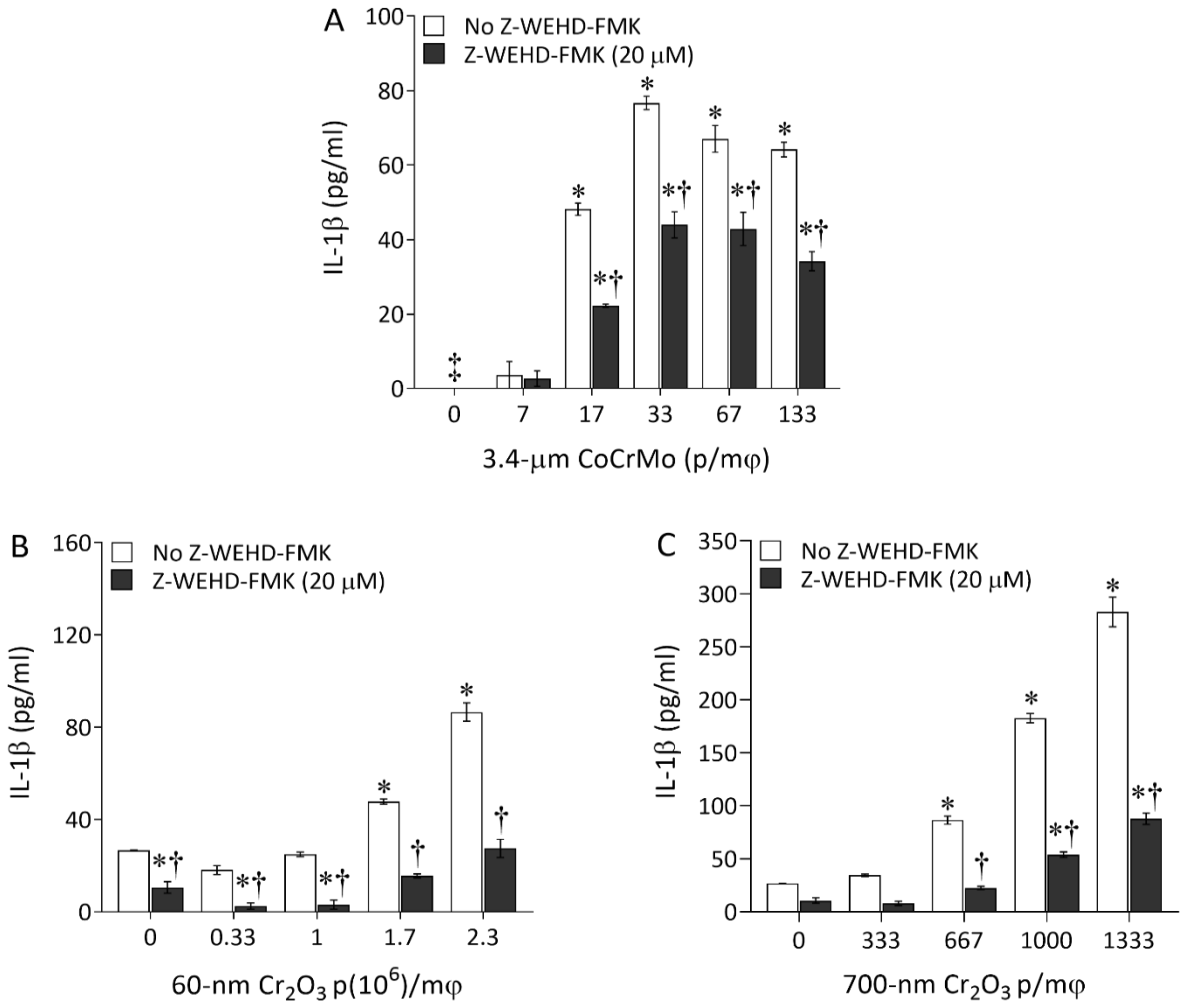

Figure A3. Interleukin-1β (IL-1β) release by bone marrow-derived macrophages (BMDM) from Jackson WT mice after exposure to: (A) CoCrMo particles; (B) 60-nm Cr_2O_3 particles; or (C) 700-nm Cr_2O_3 particles, with or without Z-WEHD-FMK. Cells were primed for 3h with 500 ng/mL lipopolysaccharide (LPS), and then incubated for 18h under cell culture conditions with the indicated particle concentrations and with or without 20 μM Z-WEHD-FMK (caspase-1inhibitor). IL-1β release was analyzed by ELISA. Statistical analysis was performed using a bivariate linear model with particle concentration and inhibitor as fixed factors. An asterisk (*) indicates a significant difference (p<0.05) between a given particle concentration with or without Z-WEHD-FMK and the negative control (no particles, no Z-WEHD-FMK). A dagger (†) indicates a significant difference (p<0.05) between the results obtained with and without Z-WEHD-FMK at a given particle concentration. Data are presented as means ± SD of single experiments performed in triplicate. p/mφ: particles per macrophage.