Analysis and Modulation of In Vitro Cell Response to Metal Ions From CoCrMo Alloys Used in Orthopaedic Applications

by

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Acknowledgements

Anybody who has been seriously engaged in scientific work of any kind realizes that over the entrance to the gates of the temple of science are written the words: 'Ye must have faith.'

Max Planck

I offer my most sincere gratitude to those who have enabled, facilitated, and contributed to making this project possible.

First, I express my sincere thanks to my thesis supervisor, Dr. Isabelle Catelas, who took me in for an extended project in the lab and without whose motivation and encouragement I would not have begun my graduate experience in biomedical engineering. She provided no limitation on my exposure and involvement to any of the other projects going on in the lab allowing me to branch out significantly and broaden my research and analytical skills. Her guidance into the world of cell culture and biochemistry made for a far less intimidating transition for an engineering student with no lab experience. Next, to Dr. Eric A. Lehoux, Research Associate in the lab, whose expertise and patience through hours of brainstorming and manuscript revisions were a life-saver. I cannot thank him enough for filling in as an extra pair of hands during important and lengthy experiments and for passing along excellent pointers to improve my lab competence. Lastly, I would like to thank the other members of my committee, Dr. Rosalind Labow (BMI), Dr. Sidney Omelon (Chemical and Biological Engineering), and Dr. Fabio Variola (Mechanical Engineering) for their assistance and enthusiasm in reviewing my thesis.

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Lastly I would also like to acknowledge the Hans K. Uhthoff Fellowship award from the Division of Orthopaedic Surgery for personal funding in 2011.

Thesis Organization

Where is the wisdom we have lost in knowledge? Where is the knowledge we have lost in information?

T.S. Eliot

This thesis is submitted as a compilation of two manuscripts and is divided into four main parts.

Part 1 is divided into two chapters which introduce the context of the project and the hypothesis and objectives of this thesis.

Part 2 is a literature review focusing on hip implants; pharmaceuticals proposed to increase implant longevity; macrophage and lymphocyte biology; and previous *in vitro* studies of metal ion-induced biological effects.

Part 3 is the compilation of two manuscripts. The first manuscript (being finalized for submission to the Journal of Orthopaedic Research) focusses on the effects of metal ions (Co^{2+} and Cr^{3+}) on monocytes/macrophages release of pro-inflammatory cytokines, chemokines, and direct/indirect lymphocyte migration. The second manuscript (published in the Journal of Biomaterials and Tissue Engineering, Vol. 4, No. 11, pp.981-991, Nov. 2014) focusses on the use of simvastatin to modulate TNF–α and chemokines released by macrophages exposed to trivalent chromium ions.

Part 4 is the final part of the thesis which discusses the conclusions from the thesis, the clinical importance of the findings, technical considerations, and future work.

Statement of Author Contributions

It is a profound and necessary truth that the deep things in science are not found because they are useful; they are found because it was possible to find them.

J. Robert Oppenheimer

Results from work performed throughout this thesis have been grouped into 2 studies presented here as independent manuscripts. All experimental design work, assay planning, and finalized result interpretation in these studies were designed by myself and Dr. Isabelle Catelas (thesis supervisor) with advice from Dr. Eric A. Lehoux (Research Associate in Dr. Catelas' laboratory).

CONTRIBUTORS TO MANUSCRIPT ONE

All work concerning the data acquisition, analysis, and interpretation was carried out primarily by myself, with the following acknowledgements to: Siu-Yan Lee (summer undergraduate student) who assisted in some of the experiment preparation and acquisition for the cytokine quantification assays; Dr. Eric A. Lehoux who assisted with the preparation and collection of the migration experiment samples as well as the sample dilutions during the cytokine quantification assays when Siu-Yan Lee was unavailable; and Dr. Subash Sad (Professor in the Department of Biochemistry, Microbiology and Immunology (BMI) at the Faculty of Medicine) who generously supplied the mouse spleens (coordinated through Dr. Julie Joseph, a Postdoctoral Fellow in Dr. Sad's laboratory).

The manuscript was written by myself and reviewed by Dr. Eric A. Lehoux and Dr. Isabelle Catelas. Dr. Paul E. Beaulé (orthopaedic surgeon at The Ottawa Hospital) contributed to the clinical interpretation of the chemokine release portion of this work during the preparation of a conference submission.

CONTRIBUTORS TO MANUSCRIPT TWO

All work concerning the data acquisition, analysis, and interpretation was carried out primarily by myself, with the following acknowledgements to: Dr. Eric A. Lehoux who assisted with the acquisitions of the micrographs; and Dr. Fiona McMurray and Ghadi Antoun of the laboratory of Dr. Mary-Ellen

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The manuscript was written by myself and reviewed extensively by Dr. Eric A. Lehoux and Dr. Isabelle Catelas. Prior to submission, Dr. Paul E. Beaulé gave a cursory review contributing to the clinical interpretation of the work.

This manuscript underwent a peer-review procedure during the fall of 2014 and is now published in the Journal of Biomaterials and Tissue Engineering, Vol. 4, No. 11, pp.981-991, Nov. 2014. Its reproduction in this thesis was approved by the publishers.

Journal Publications and Conference Abstracts

Somewhere, something incredible is waiting to be known.

Carl Sagan

While working towards my Master's degree in Biomedical Engineering, several of the studies I contributed to yielded results that have been included in manuscripts for publication in peer-reviewed journals [1–3] or in abstracts included in peer-reviewed conference proceedings, as detailed below.

The first manuscript (included in full in Part III Chapter 1) focusses on the effects of divalent cobalt and trivalent chromium ions on macrophage chemokine release and lymphocyte migration *in vitro* and is being finalized for submission to the Journal of Orthopaedic Research [1]. Initial work included in this manuscript was presented at the 57th Annual Meeting of the Orthopaedic Research Society (ORS) in 2011 [4].

The second manuscript (included in full in Part III Chapter 2) focusses on the modulation of inflammatory mediators in macrophages stimulated with trivalent chromium ions and was published in the Journal of Biomaterials and Tissue Engineering [2]. Initial work included in this manuscript was presented at the 57th Annual Meeting of the ORS in 2011 [5].

In addition, I contributed to the statistical analysis of the data included in a manuscript on the immunophenotypic analysis of peripheral blood lymphocytes. The manuscript is now in press for publication in the journal Clinical Orthopaedics and Related Research [3], but because it was not part of my thesis project, it is not reproduced in this thesis. I was also involved in the statistical analysis of data from other studies on immunophenotypic analysis [6–10], as well as studies on proteomics [11–13] and implant tribology [14, 15], presented at various national and international conferences.

Finally, I was the first author of an abstract on a side project on osteoclastogenesis that was presented at the 9th World Biomaterials Congress in 2012 [16].

LIST OF JOURNAL PUBLICATIONS AND CONFERENCE ABSTRACTS

[1] S. J. Baskey, E. A. Lehoux, S. Lee, P. E. Beaulé, and I. Catelas, "Effects of cobalt and chromium ions on CC chemokine release from macrophages and migration of lymphocytes *in vitro*," Journal of Orthopaedic Research (in preparation).

[2] S. J. Baskey, P. E. Beaulé, E. A. Lehoux, and I. Catelas, "Simvastatin modulates the release of TNF–α and CC chemokines from macrophages exposed to trivalent chromium ions," *J. Biomater. Tissue Eng.*, vol. 4, no. 11, pp. 981–991, Nov. 2014.

[3] I. Catelas, E. A. Lehoux, I. Hurda, S. J. Baskey, L. Gala, R. Foster, P. R. Kim, and P. E. Beaulé, "Do patients with a failed metal-on-metal hip implant with a pseudotumor present differences in their peripheral blood lymphocyte subpopulations?" Clinical Orthopaedics and Related Research (in press).

[4] S. Baskey, S. Lee, P. Beaulé, and I. Catelas, "Chemokine release by macrophages exposed to cobalt and chromium ions *in vitro*," *57th Annual Meeting of the Orthopaedic Research Society*, Long Beach, CA, USA, Jan. 2011.

[5] S. Baskey, P. Beaulé, and I. Catelas, "Modulation of TNF-alpha and chemokine release by macrophages exposed to chromium ions using simvastatin," *57th Annual Meeting of the Orthopaedic Research Society*, Long Beach, CA, USA, Jan. 2011.

[6] I. Hurda, P. E. Beaulé, S. J. Baskey, E. A. Lehoux, and I. Catelas, "Comparison of peripheral blood lymphocyte phenotypes in patients with failed metal-on-metal and metal-on-polyethylene hip implants," *9th World Biomaterials Congress*, Chengdu, China, Jun. 2012.

[7] E. Guira, X. Xiang, I. Hurda, E. A. Lehoux, P. E. Beaulé, S. J. Baskey, and I. Catelas, "Analysis of peripheral blood lymphocyte phenotypes in patients with failed metal-on-metal hip implants," *30th Annual Meeting of the Canadian Biomaterials Society*, Ottawa, ON, Canada, May 2013.

[8] E. A. Lehoux, X. Xiang, M. O. Lemay, E. Guira, I. Hurda, S. J. Baskey, P. E. Beaulé, and I. Catelas, "Immunophenotypic analysis of peripheral blood lymphocytes in patients with failed metal-on-metal hip implants associated with a pseudotumor," *60th Annual Meeting of the Orthopaedic Research Society*, New Orleans, LA, USA, Mar. 2014.

[9] X. Xiang, E. Guira, I. Hurda, E. A. Lehoux, M. O. Lemay, S. J. Baskey, P. E. Beaulé, and I. Catelas, "Comparison of peripheral blood lymphocyte phenotypes in patients with failed metal-on-metal hip implants with and without a pseudotumor," *American Orthopaedic Association (AOA) / Canadian Orthopaedic Association (COA) Combined Meeting*, Montréal, QC, Canada, Jun. 2014.

[10] E. A. Lehoux, I. Hurda, S. J. Baskey, P. E. Beaulé, and I. Catelas, "Immunophenotypic analysis of peripheral blood lymphocytes in patients with hip implant-related metal hypersensitivity," *61st Annual Meeting of the Orthopaedic Research Society*, Las Vegas, NV, USA, Mar. 2015.

[11] E. A. Lehoux, Z. Ning, P. E. Beaulé, D. Figeys, S. J. Baskey, and I. Catelas, "Comparative proteomic analysis of synovial fluid from patients with failed metal-on-metal and metal-on-polyethylene hip implants," *59th Annual Meeting of the Orthopaedic Research Society*, San Antonio, TX, USA, Jan. 2013.

[12] E. A. Lehoux, Z. Ning, P. E. Beaulé, D. Figeys, S. J. Baskey, and I. Catelas, "Proteomic analysis of synovial fluid from patients with failed hip implants," *30th Annual Meeting of the Canadian Biomaterials Society*, Ottawa, ON, Canada, May 2013.

[13] E. A. Lehoux, Z. Ning, P. E. Beaulé, D. Figeys, S. J. Baskey, and I. Catelas, "Identification of proteins differentially represented in the synovial fluid of patients with failed metal-on-metal and metal-on-polyethylene hip implants," *American Orthopaedic Association (AOA) / Canadian Orthopaedic Association (COA) Combined Meeting*, Montréal, QC, Canada, Jun. 2014.

[14] G. McAllister, P. Beaulé, S. J. Baskey, M. Nganbe, and I. Catelas, "Mapping of surface roughness and corrosion of necks from failed modular hip implants," *2nd International Conference on BioTribology*, Toronto, Ontario, Canada, May 2014.

[15] G. McAllister, P. E. Beaulé, S. J. Baskey, M. Nganbe, and I. Catelas, "Surface analysis of modular necks and failed hip implants," *31st Annual Meeting of the Canadian Biomaterials Society*, Halifax, NS, Canada, Jun. 2014.

[16] S. J. Baskey, E. A. Lehoux, and I. Catelas, "Stimulation of macrophages by chromium (III) ions induces an increase of TRAP expression in vitro," *9th World Biomaterials Congress*, Chengdu, China, Jun. 2012.

Thesis advisor: Isabelle Catelas, P.Eng., Ph.D. Stephen J. Baskey

Analysis and Modulation of *In Vitro* Cell Response to Metal Ions From CoCrMo Alloys Used in Orthopaedic Applications

Abstract

Despite the high success rates of hip replacements, implant-wear mediated periprosthetic osteolysis remains the most prominent cause of long-term implant failure. Other adverse tissue reactions including hypersensitivity reactions and pseudotumors have also recently been reported as a cause for short-term implant failures. The objectives of this thesis were: 1.) To analyze the effects of Co^{2+} and Cr^{3+} released from CoCrMo alloys used in hip implants on macrophage chemokine release; 2.) To determine if Co^{2+}, Cr^{3+}, and the chemokines in cultures of macrophages exposed to Co^{2+} and Cr^{3+} can induce migration of T and B lymphocytes; and 3) To analyze the potential modulation of macrophage response to Cr^{3+} using simvastatin as an anti-inflammatory agent. Results showed that the release of TNF–α and CC chemokines were ion-specific and dose-dependent. Results also suggested that Co^{2+} and Cr^{3+} may be capable of directly stimulating the migration of T cells, but not that of B cells, suggesting the potential of these ions to create a micro-environment that would favour a T cell-mediated response *in vivo*. Results also showed that simvastatin was capable of decreasing chemokine release in macrophages exposed to Cr^{3+}, suggesting its potential to modulate the Cr^{3+}-induced inflammatory response. Together, these studies improve the understanding of the role metal ions play in ion-mediated adverse tissue reactions and potential therapies that may modulate the immune response to metal ions.

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List of Acronyms

Like all sweet dreams, it will be brief, but brevity makes sweetness, doesn't it?

STEPHEN KING, 11/22/63

Term	Abbreviation
3-hydroxy-3-methylglutaryl-coenzyme A	HMG-CoA
adverse local tissue reactions	ALTR
adverse reactions to metallic debris	ARMD
analysis of variance	ANOVA
antigen presenting cell	APC
aseptic lymphocyte-dominated vasculitis-associated lesions	ALVAL
basic fibroblast growth factor	bFGF
bone morphogenetic protein	BMP
bovine serum albumin	BSA
calcium cations	Ca^{2+}
Canadian Joint Replacement Registry	CJRR
carbon dioxide	CO_2
ceramic-on-ceramic	CoC
ceramic-on-polyethylene	CPE
chromium chloride	$CrCl_3$
chromium chloride hexahydrate	$CrCl_3 \cdot 6H_2O$
cluster of differentiation	CD
cobalt carbonate	$CoCO_3$
cobalt chloride	$CoCl_2$
cobalt-chromium-molybdenum	CoCrMo
computed tomography	CT
cyclooxygenase	COX
cysteine-cysteine	CC
cytotoxic T cells	Tc
dendritic cells	DC
Dulbecco's modified Eagle medium	DMEM
enzyme linked immuno-sorbent assay	ELISA
equilibrated medium	EQM
fetal bovine serum	FBS
fluorescein isothiocyanate	FITC
glucocorticoid	GC

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Term	Abbreviation
granulocyte-macrophage colony-stimulating factor	GM–CSF
guanosine triphosphatases prenylation	GTPases
high density polyethylene	HDPE
highly cross-linked polyethylene	HXPE
hip arthroplasty	HA
hospital morbidity database	HMD
hydrogen peroxide	H_2O_2
inductively coupled plasma-mass spectrometry	ICPMS
insulin-like growth factor	IGF
interferon gamma	IFN–γ
interleukin	IL
lipopolysaccharide	LPS
list mode data	LMD
low-density lipoprotein	LDL
lymphocyte migration buffer	LMB
lymphocyte separation medium	LSM
macrophage colony-stimulating factor	
see also CSF–1	M–CSF
macrophage inflammatory protein	MIP
magnesium cations	Mg^{2+}
magnetic resonance imaging	MRI
major histocompatibility complex	MHC
matrix metalloproteinase	MMP
metal-on-metal	MM
metal-on-polyethylene	MPE
mitogen-activated protein kinase	MAPK
monocyte chemotactic protein 1 (CCL2)	MCP–1
natural killer	NK
nitrogen oxide	NO
nonsteroidal anti-inflammatory drug	NSAID
nuclear factor kappa-B	NFκB
osteoprotegerin	OPG
oxygen	O_2
parathyroid hormone	PTH
phosphate buffered saline	PBS
phycoerythrin	PE
poly(methyl methacrylate)	PMMA
polyethylene terephthalate	PET
propidium iodide	PI
prostaglandin E2	PGE_2
reactive nitrogen species	RNS

Continued on next page

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Term	Abbreviation
reactive oxygen species	ROS
regulated on activation, normal T cell expressed and secreted (CCL5)	RANTES
reverse transcription polymerase chain reaction	RT-PCR
Roswell Park Memorial Institute	RPMI
sodium azide	NaN_3
sodium bicarbonate	$NaHCO_3$
stromal cell-derived factor	SDF
surface replacement	SR
synovial-like interface membrane	SLIM
T helper cells	Th
T-cell receptor	TCR
titanium-6-aluminum-4-vanadium	Ti6Al4V
total hip replacement	THR
transforming growth factor	TGF
trivalent chromium ions	Cr^{3+}
tumor necrosis factor	TNF
U.S. Food and Drug Administration	FDA
ultra-high molecular weight polyethylene	UHMWPE
	Concluded

Index of Suppliers

An expert is a person who has made all the mistakes that can be made in a very narrow field.

Niels Bohr

Supplier	Location	Pages
American Type Culture Collection (ATCC)	Manassas, Virginia	33, 50
BD Biosciences	Durham, North Carolina	33, 34
BD Diagnostics	Franklin Lakes, New Jersey	33
Beckman Coulter	Indianapolis, Indiana	34
BioTek	Winooski, Vermont	34, 52
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VWR	Mississauga, Ontario	34
Wisent	St. Bruno, Québec	33, 35, 50

Part I

Thesis Introduction

Part I — Chapter 1

Introduction

> *"Begin at the beginning," the King said gravely, "and go on till you come to the end: then stop."*
>
> Lewis Carroll, *Alice in Wonderland*

Hip implants have a long history of innovation, re-evaluation, and clinical success. They are used when a patient's range of motion becomes limited or painful. Surgeons have a wide variety of implants to choose from with different combinations of component size and shape, bearing materials, fixation methods, and surgical techniques. Having a wide variety of options available is helpful to customize an implant for the needs of a particular patient. It is estimated that over 800,000 hip arthroplasties (HAs) are performed worldwide every year [1]. In an aging population, this number will continue to increase leading to tens of millions of hip implants in the global population, the trend for which is already evident in recent reports of numerous national registries. Despite the success of these surgeries, patients frequently require a revision surgery to replace one or more implant components. While long-term implant failure remains a concern that will affect a large percentage of the patient population, early implant failure tends to be associated with more dire consequences.

The most common cause of long-term implant failure is periprosthetic osteolysis (bone loss around the implant), which is generally caused by the inflammatory reaction of the body's immune system to wear products generated by wear and corrosion including particles and metal ions. Inflammatory mediators such as tumor necrosis factor–α (TNF–α) have been shown to be involved in periprosthetic osteolysis, by causing an imbalance in the regular process of bone remodelling through stimulated osteoclast function coupled with inhibited osteoblast function. Osteolysis involves intricate and complex inter- and intra-cellular reactions between macrophages and other cells of the immune system. A concern with the presence of abnormal soft tissue reactions referred to as pseudotumors, often leading to early implant failure, has also recently emerged. These reactions can be symptomatic or asymptomatic, and although their causes and mechanisms remain largely unknown, some studies have suggested that they may be related to corrosion leading to elevated levels of cobalt and chromium ions [2–4]. Metal ions are believed to be one of the major causes of early implant failure in some types of implants [5, 6].

Considerable effort goes into the design and development of the implants to minimize the release of wear and corrosion products in the attempt to limit adverse reactions. Another approach is to consider pharmaceutical intervention to modulate various pathways activated by wear particles and metal ions.

Since many drugs have serious side effects (including immunosuppression), it is important to find a target that can be modulated by a drug with tolerable side effects. The mevalonate pathway, also known as the isoprenoid pathway, is an important fundamental pathway, largely involved in cell metabolism, and has been of interest as a target for pharmaceutical intervention to mitigate the *in vivo* response to wear products using 3-hydroxy-3-methylglutaryl-coenzyme A (HMG–CoA) reductase inhibitors, collectively known as statins. Simvastatin, a synthetic statin approved by the U.S. Food and Drug Administration (FDA) in 1991 to decrease low-density lipoprotein (LDL) cholesterol levels in the blood, has recently been shown to lower the risk of revision surgery in patients with a total hip replacement (THR) after receiving statin therapy [7, 8]. More specifically, simvastatin has been shown to decrease the release of inflammatory mediators [9, 10], which could potentially reduce the inflammatory response leading to periprosthetic osteolysis and other adverse tissue reactions. Simvastatin has also been shown to increase bone formation from osteoblasts [11, 12] and decrease bone resorption from osteoclasts [13], which overall could potentially reduce periprosthetic osteolysis directly.

Hypotheses and Objectives

Science may be described as the art of systematic oversimplification.

Karl Popper

2.1 RATIONALE

The shared goal of *in vitro* and *in vivo* studies related to the effects of wear and corrosion products, such as metal ions, is to increase the understanding of the local and systemic consequences on the various cells and organs in the body as well as the underlying mechanisms, in order to better comprehend implant failure. Understanding and modulating the biological effects of wear particles and metal ions may allow increasing the longevity of the implants which would allay the need of revision surgeries and improve the quality of life of patients. In particular, studying the *in vitro* effects of macrophages in response to ions from metals commonly used in orthopaedic applications is an important area of research as *in vitro* models can be developed to explain complex responses and contributing factors behind implant failure. In particular, increased pro-inflammatory cytokine and chemokine release are indicators of inflammation, a key immune process having direct consequences on the development of osteolytic lesions and other adverse tissue reactions. The improved understanding of the direct consequences of metal ions is important not just because it means we can more accurately predict the immune response to wear and corrosion products from specific types of implants, but also it can lead to the identification of suitable therapeutic targets to modulate the immune response.

2.2 HYPOTHESES

Cobalt and chromium are the main constituents of the cobalt-chromium-molybdenum (CoCrMo) alloys used in orthopaedic applications, and in particular in metal-on-metal (MM) hip implant bearings, and cobalt and chromium ions being released through implant wear and corrosion have been associated with inflammation and adverse tissue reactions [6]. Specifically, previous *in vitro* studies using Co^{2+} and Cr^{3+} have shown that these ions affect the viability of macrophages and up-regulate the release of pro-inflammatory cytokines [14–16]. The hypotheses of the present study were:

1. Co^{2+} and Cr^{3+} induce the release of pro-inflammatory chemokines from macrophages *in vitro*;
2. These chemokines are chemoattractants capable of inducing lymphocyte migration; and
3. Statins, specifically simvastatin, can modulate the inflammatory response of macrophages to Co^{2+} and Cr^{3+}.

2.3 OBJECTIVES

The long term goal of this research is to improve implant longevity by modulating macrophage response to metal ions released from hip implants, specifically those made of CoCrMo alloys, subjected to wear and/or corrosion in order to decrease inflammation that can also lead to other adverse tissue reactions. The specific objectives of this thesis were:

1. To analyze the effects of Co^{2+} and Cr^{3+} on chemokine release by J774A.1 monocytes/macrophages;
2. To determine if Co^{2+}, Cr^{3+}, and the chemokines in cultures of J774A.1 monocytes/macrophages exposed to Co^{2+} and Cr^{3+} can induce migration of T and B lymphocytes; and
3. To analyze the potential modulation of J774A.1 monocyte/macrophage response to Cr^{3+} using simvastatin as an anti-inflammatory agent. Specifically, the effects of simvastatin on macrophage mortality and morphology as well as on the release of chemokines by macrophages were analyzed.

Results provide a better understanding of the macrophage response to metal ions, the migration of T and B lymphocytes, and the potential of simvastatin as a pharmaceutical agent to modulate the ion-induced inflammation *in vitro*. This work may lead to the development of clinical options for the treatment of metal ion-induced inflammation in patients with hip implants.

References

[1] K. E. Alvarez, *Total hip replacement*, L. L. Hench, J. R. Jones, and M. B. Fenn, Eds. London, UK: Imperial College Press, 2012.

[2] Y. Kwon, S. J. Ostlere, P. McLardy-Smith, N. A. Athanasou, H. S. Gill, and D. W. Murray, "Asymptomatic pseudotumors after metal-on-metal hip resurfacing arthroplasty: prevalence and metal ion study," *J. Arthroplasty*, vol. 26, no. 4, pp. 511–8, Jun. 2011.

[3] R. M. Urban, M. J. Tomlinson, D. J. Hall, and J. J. Jacobs, "Accumulation in liver and spleen of metal particles generated at nonbearing surfaces in hip arthroplasty," *J. Arthroplasty*, vol. 19, no. 8 Supp, pp. 94–101, Dec. 2004.

[4] H. Willert, G. H. Buchhorn, A. Fayyazi, F. Renata, M. Windler, G. Köster, and C. H. Lohmann, "Metal-on-metal bearings and hypersensitivity in patients with artificial hip joints. A clinical and histomorphological study," *J. Bone Joint Surg. Am.*, vol. 87, no. 1, pp. 28–36, Jan. 2005.

[5] R. E. Andrews, K. M. Shah, J. M. Wilkinson, and A. Gartland, "Effects of cobalt and chromium ions at clinically equivalent concentrations after metal-on-metal hip replacement on human osteoblasts and osteoclasts: Implications for skeletal health," *Bone*, vol. 49, no. 4, pp. 717–23, Oct. 2011.

[6] A. J. Hart, P. D. Quinn, F. Lali, B. Sampson, J. A. Skinner, J. J. Powell, J. Nolan, K. Tucker, S. Donell, A. Flanagan, and J. F. W. Mosselmans, "Cobalt from metal-on-metal hip replacements may be the clinically relevant active agent responsible for periprosthetic tissue reactions," *Acta Biomater.*, vol. 8, no. 10, pp. 3865–73, Oct. 2012.

[7] A. Lübbeke, G. Garavaglia, K. J. Rothman, A. Bonvin, C. Roussos, H. Miozzari, and P. Hoffmeyer, "Statins may reduce femoral osteolysis in patients with total hip arthroplasty," *J. Orthop. Res.*, vol. 31, no. 5, pp. 814–820, May 2013.

[8] T. M. Thillemann, A. B. Pedersen, F. Mehnert, S. P. Johnsen, and K. Søballe, "The risk of revision after primary total hip arthroplasty among statin users: a nationwide population-based nested case-control study," *J. Bone Joint Surg. Am.*, vol. 92, no. 5, pp. 1063–72, May 2010.

[9] B. P. Leung, N. Sattar, A. Crilly, M. Prach, D. W. McCarey, H. Payne, R. Madhok, C. Campbell, J. A. Gracie, F. Y. Liew, and I. B. McInnes, "A novel anti-inflammatory role for simvastatin in inflammatory arthritis," *J. Immunol.*, vol. 170, no. 3, pp. 1524–1530, Feb. 2003.

[10] G. Weitz-Schmidt, "Statins as anti-inflammatory agents," *Trends Pharmacol. Sci.*, vol. 23, no. 10, pp. 482–6, Oct. 2002.

[11] T. Maeda, A. Matsunuma, I. Kurahashi, T. Yanagawa, H. Yoshida, and N. Horiuchi, "Induction of osteoblast differentiation indices by statins in MC3T3-E1 cells," *J. Cell. Biochem.*, vol. 92, no. 3, pp. 458–71, Jun. 2004.

[12] G. Vallés, C. Pérez, A. Boré, F. Martín-Saavedra, L. Saldaña, and N. Vilaboa, "Simvastatin prevents the induction of interleukin-6 gene expression by titanium particles in human osteoblastic cells," *Acta Biomater.*, vol. 9, no. 1, pp. 4916–25, Jan. 2013.

[13] J. Woo, H. Nakagawa, A. M. Krecic, K. Nagai, A. D. Hamilton, S. M. Sebti, and P. H. Stern, "Inhibitory effects of mevastatin and a geranylgeranyl transferase i inhibitor (GGTI-2166) on mononuclear osteoclast formation induced by receptor activator of NFκB ligand (RANKL) or tumor necrosis factor-α (TNF-α)," *Biochem. Pharmacol.*, vol. 69, no. 1, pp. 87–95, Jan. 2005.

[14] I. Catelas, A. Petit, D. J. Zukor, and O. L. Huk, "Cytotoxic and apoptotic effects of cobalt and chromium ions on J774 macrophages - Implication of caspase-3 in the apoptotic pathway," *J. Mater. Sci.: Mater. Med.*, vol. 12, no. 10-12, pp. 949–53, 2001.

[15] I. Catelas, A. Petit, D. J. Zukor, J. Antoniou, and O. L. Huk, "TNF-alpha secretion and macrophage mortality induced by cobalt and chromium ions in vitro-qualitative analysis of apoptosis," *Biomaterials*, vol. 24, no. 3, pp. 383–91, Feb. 2003.

[16] I. Catelas, A. Petit, H. Vali, C. Fragiskatos, R. Meilleur, D. J. Zukor, J. Antoniou, and O. L. Huk, "Quantitative analysis of macrophage apoptosis vs. necrosis induced by cobalt and chromium ions in vitro," *Biomaterials*, vol. 26, no. 15, pp. 2441–53, May 2005.

Part II

Literature Review

You have to look behind you. The present and the future are built on the past. I know that you will want to find where you came from before you'll know where to go.

Jessica Brockmole, *Letters from Skye*

1 INTRODUCTION TO HIP ARTHROPLASTY

1.1 Anatomy

Hip arthroplasties (HAs) are a class of successful orthopaedic surgeries that are performed worldwide to restore joint functionality and alleviate pain. Modern day HA use a ball-and-socket articulation to mimic the functionality of the biological joint. The hip joint is a deep enarthrodial joint situated on either side of the pelvic girdle capable of flexion, extension, adduction, abduction, circumduction, and rotation making it one of the most free-moving joints in the body. It is the second largest joint in the body [1] and is responsible for locomotion and providing support to the human body in a variety of postures. The hip joint consists of a deep cup-like bony feature called the acetabulum which provides a secure socket for the articulation of the femoral head. When undamaged, the surfaces of the acetabulum and the femoral head are lined with a layer of cartilage which protects the bone and provides an exceptionally low coefficient of friction during loading and articulation. The natural joint is held together by a complex system of ligaments around the superior femoral features both inside and outside of the acetabulum. These ligaments form the joint capsule which provides strength and stability to the joint, limitations to the joint's range of motion, and a container for the synovial fluid.

1.2 Clinical assessment

HAs are typically recommended when a patient's pain over a variety of activities becomes excessive and a corresponding decrease in range of motion is evident [2]. Although the primary cause of pain and impaired function comes from degenerative diseases such as osteoarthritis and rheumatoid arthritis, hip implants can also be used to repair special cases of bone defects including fracture, impingement, and osteonecrosis [3, 4]. It may be possible to restore functionality and eliminate pain with surgical reshaping, which may be indicated in cases such as impingement. However, in specific cases when damage is too severe or a reshaping technique is contraindicated, the use of a total joint replacement may be considered if beneficial to the patient [3]. Total hip replacements (THRs) and surface replacements (SRs) are man-made implants designed to replace the ball shaped top of the femoral head and the cup shaped acetabulum.

1.3 Current Canadian clinical significance of total hip replacements

31 666 THRs, 817 SRs, and 10 230 partial hip replacements were performed during a 12 month period in Canada in 2010-2011, which represent a five year increase of 10.6% since 2006-2007 [4]. Of these 42 713 hip surgeries, there were 5319 (12.5%) procedures performed in patients under the age of 55 [4]. This represents a significant number of patients with potentially higher activity levels and life expectancies. The increased implementation of hip implants in younger patients and the quantity of surgeries performed are indicative of the success of HA in alleviating pain and restoring joint function despite the potential need for revision later in the patient's life [5, 6]. Interestingly, gender demographics in Canada, in 2010-2011, followed different trends. There were 34% more men under the age of 55 receiving a hip implant than females, but no difference for males or females between the age of 55 to 64, and there were 77% more females above the age of 65 [4]. It has been proposed that the primary reason for hip implants being more prevalent in women is related to their significantly higher instance of osteopathologies related to bone loss which tend to increase with age [1]. Indeed, in the Canadian registry, the most common cause for hip replacements was degenerative osteoarthritis, hip fracture, and osteonecrosis (82.1, 6.3, and 3.5 % respectively).

Between 1996-1997 and 2010-2011, there was an estimated 77% increase in the total number of hip replacement surgeries. This 77% increase was interpolated from data originating in two recent consecutive Canadian joint replacement registry (CJRR) reports [4, 7]. Data compiled in the CJRR reports is supplemented with data from the hospital morbidity database (HMD). It should be noted that numbers presented from the CJRR are not as complete as those from the HMD, as it is known that not all eligible surgeons participated in the CJRR data collection for the reports in question, and there is no independent verification that surgeons submitted complete data for all relevant procedures that were performed during the requested periods. Coverage of submitted procedures in the 2013 annual CJRR report is estimated at 43.8% based on comparisons with the HMD. Despite the lack of complete patient coverage in the CJRR, it still provides useful details about specific levels of patient distinction including age, gender, clinical scenario, and implant details that is interpreted as representative of the trends/proportions in performed procedures. One of the most important implant details is whether the surgery is a primary or a revision surgery. Between 2010-2011, 10% of the HA procedures were revisions. While this indicates that hip replacements are quite successful, this still represents nearly 1748 patients in Canada, a significant number of individuals whose quality of life could still be improved.

Continuing efforts to improve implant longevity and patient quality of life focus on the optimization of implant designs, development of new bearing materials, improvement in the understanding of wear-mediated immune responses, and identification of immunological targets which could be modulated pharmacologically.

1.4 Design of implant systems

Modern day HA typically make use of either a THR or a SR. THRs and SRs differ primarily in the amount of bone-preservation and thus, must be evaluated for their applicability in patient specific clinical scenarios. Health of bone stock is only one of the factors considered when selecting a suitable joint replacement for a patient. Careful consideration is given to numerous physiological and clinical factors and to previous levels of success and reliability concerning implant performance. Patient specific and surgically relevant factors include gender, weight, activity level, previous surgery, and state of the current bone [8, 9]. Implant selection is also dependent on anthropomorphically matched implant characteristics including head size, stem length, stem curvature, surface finish, mechanical additions such as collars for load distribution, and patient compatibility with bone cement [10].

Modern day hip implants use four or five identifiable functional components designed to work in concert which may be further categorized as femoral components and acetabular components. Customarily, the femoral components of THR implants include a stem fixed inside the femur and a ball or larger curved surface to support loaded articulation while the acetabular components may include a metal shell fixed into the acetabulum and a cup liner made of a wide variety of materials including ultra-high molecular weight polyethylene (UHMWPE), highly cross-linked polyethylene (HXPE), cobalt-chromium-molybdenum (CoCrMo), titanium-6-aluminum-4-vanadium (Ti6Al4V), or a ceramic, which cradles the ball replacing the femoral head [1, 11]. Implant fixation of THRs may introduce a fifth facultative component such pins, screws, or a lining of bone cement, while SRs only utilize press fits. Additionally, in modern day SR, the femoral and acetabular components are both made from CoCrMo.

Hip implants are under considerable loads during everyday activities and for the implants to function well it is crucial that the implant is fixed securely in the host bone. Numerous techniques are available and may be combined for implant fixation including pins, screws, press fits, and/or cement for both the stem and the cup. It is interesting to note that pins and screws are also used with fracture plates [12] and as part of femoral neck fracture treatments [13]. They are typically made of stainless steel, CoCrMo, or Ti6Al4V alloys and are designed for long term implantation [14]. However, over the course of their implantation, pins and screws are subject to corrosion and ion release. Considerable scientific and clinical studies are undertaken to determine the effects that fixation methods have on implant longevity and which clinical scenarios bone cement is most applicable to. Some of these studies argue that it is the bone cement that led to high success rates of 85–95% after 15 years of implantation using early versions of modern-day joint replacements [10, 15]. However, for studies drawing comparisons, it is crucial that the groups are well paired to ensure that erroneous conclusions are not drawn from study ambiguities. Nevertheless, cemented implants (with or without the use of press fits or screws) currently represent

only 17% of the hip replacements in Canada as their use decreased over the last 10 years [4]. Many models of cementless implants are secured using biological fixation techniques based on the implant surface coating with a rough porous layer of material such as hydroxyapatite, the main mineral component of bone, to encourage bone in-growth and therefore improve osseointegration. Nevertheless, not all surface coatings are porous, and thus cannot withstand tensile loads and rely solely on shear strength to provide strength at the interface [16]. Making use of cementless techniques has clinical benefit because bone cement is a brittle material and subject to wear, and like other types of wear particles, is of significant consequence to cells of the immune system.

2 HISTORY OF JOINT REPLACEMENTS: CONCEPTS AND TRIALS LEADING TO THE SUCCESS OF MODERN TOTAL HIP ARTHROPLASTIES

Degenerative hip diseases are not new conditions. However, an increasing emphasis on quality of life has created a focus on understanding and treating these diseases. Studies into the paleopathology of osteoarthritis have revealed that, as a disease, it hasn't changed much in 100 million years. Osteoarthritis has been present in dinosaurs and in dozens of human civilizations including Neanderthal and Cro-Magnon man; ancient Egyptians and Nubians; and Neolithic, Mediaeval, and Saxon civilizations [17, 18].

During periods of ancient medicine, when only a primitive understanding of internal medicine existed, numerous civilizations were still cognizant of the difference between dislocation and fracture [19]. Studies on Egyptian skeletons showed considerable incidence of fracture and successfully reduced (treated) dislocations [20]. Historic records between 3000 to 450 BC in China, Egypt, India, and Greece document cases of pelvic and acetabular treatment using knifes as cutting instruments (manufactured from stone, bronze, or iron), massage, splints (typically made of naturally occurring and readily available materials such as tree bark, animal hair, and mud), traction, compression bandages, abscess drainage, dressings, wound irrigation, and general debridement with antiseptics. Medical advances in joint repair remained largely unchanged until prerequisite advances in microbiology and antisepsis, anaesthesiology, and medical imaging (through the use of x-rays) resulted in decreases in post-operative infection, increases in the safety of significant surgical procedures, and improvements in non-invasive studies of internal medicine [19]. Not all of these concepts were fully employed during the first attempts to repair joint defects. Of the first seven published hip surgeries of the 19th century, surgeon Anthony White performed the only operation on the hip that was considered successful in 1822 [21]. Excisions of hip joints, practiced by some surgeons on dead subjects, removed a diseased or damaged portion of the bone (frequently from a fall or gunshot) to alleviate pain and impeded mobility, but since the operation didn't repair and preserve any of the damaged bone, joint stability was

not improved [21, 22]. At this time, a successful surgery was one that didn't end with the patient's immediate death [21]. Survivorship of these surgeries was very short — patients frequently died of complications due to the surgery within days or weeks. The landmark operation in 1822 (as reported by Chelius and South from Anthony White's personal notes) only lasted for five years [21], despite Mr. Anthony White's obituary claiming the patient survived for twelve years [23]. The next successful excision of a hip joint was not until March 1845, but by this time, a new approach to restoring joint functionality was emerging.

2.1 Origins of joint replacements

The concept of restoring joint functionality by replacing the surfaces in the damaged joint dates back all the way to 1840, when New York surgeon John Murray Carnochan repaired a maxillary joint using a wooden block and cotton as the material in an interpositional arthroplasty [24–27]. During the following 80 years, interpositional arthroplasties using nearly a dozen different biological and synthetic materials were documented in the literature published by surgeons worldwide. It was during this time that a few innovative surgeons and scientists began testing materials in animal joints before utilizing them in their patients [24–27]. Among the most successful interpositional materials used in re-establishing motion to an ankylosed joint were muscle and fascia. This combination was first used by Auguste Stanislas Verneuil in 1860 and although it wasn't immediately popular, in 1882 Heinrich Helferich presented muscular interposition as a treatment for afflicted joints to the German Surgical Congress [25], after which it began to become adapted and recommended by numerous other surgeons [24, 25]. Similarly, pig bladder was proposed by the French surgeon Foedre in the early 1900s and was made popular by William Steven Baer in 1918 while Sir Robert Jones covered a reconstructed femoral head with gold foil which returned the patient's motion in the joint for over twenty-one years [25]. Following this pioneering, and somewhat successful, work to restore joint functionality, more comprehensive solutions to replace the joint were conceived.

The origin of hip, knee, and shoulder replacements with a specially designed implant is generally traced back to surgeon Themistocles Glück's work in trying to guide tissue regeneration or provide replacements, complete with fixation, for bone and joint defects. Glück's philosophy to implant design was that in the design of man-made joint replacements "we must aim for smaller volume and lower weight, together with strength and the most efficient shape, basing our efforts on the structural principles of the human skeleton." He tested a variety of materials including glass, wood, ivory, and metals including aluminum and nickel plated steel using animals and skeletons.

In 1890, Glück worked with and presented cemented *ex vivo* implants made of ivory in most of the major joints of the body including the shoulder, hip, ankle, knee, and wrist, and he performed fourteen

total joint surgeries implementing his ivory implant *in vivo* in a variety of joints including the knee, hip, wrist, and elbow [25–31]. It is reported that the first surgery was a hinged ivory knee replacement in a seventeen year old girl suffering with a tuberculosis knee operated on in May of 1890. Glück reported on five of these replacement procedures (three knees, one wrist, and one elbow) in patients suffering from tuberculosis and chronic infection. These procedures were all initially quite successful and may have been the impetus for treating joints completely destroyed by tuberculosis with a joint replacement. However, based on his experiences, Glück acknowledged that prior joint infection contraindicates joint arthroplasty and of the five reported cases he removed the implants from all but one knee patient [28, 29].

2.2 Progression of material selection

Although Glück's colleagues disagreed with him and attempted to discredit him [28, 29], his principles are recognized as valid and laid the foundation for hip replacements. In the following century, incredible advances were made in surgical techniques and material science making HAs one of the most successful surgeries in the world. The next significant attempt to implement a functional femoral implant came in 1919 by Pierre Delbet while performing a hemiarthroplasty using a rubber implant [25, 26]. Conversely, the first acetabular replacement for hemiarthroplasty was performed in 1925 by Marius Smith-Petersen in the form of a moulded glass cup which provided a smooth surface for the articulation of the femoral head. Unfortunately, the glass mould could not withstand the *in vivo* loads and shattered, causing a catastrophic failure [25, 32]. Encouraged by the biocompatibility of the glass, Smith-Petersen experimented with new materials such as celluloid, Pyrex, Bakelite, and Vitallium. During a period of 10 years, Smith-Petersen implanted 500 Vitallium moulds which had clinically acceptable success as a bearing surface in this era of interpositional arthroplasty [25, 31].

The first practical THR was made out of stainless steel and fixed to the bone with bolts and screws [31, 32]. Despite some similarities between the materials selected and fixation method with materials and techniques used in modern THR, this design, attributed to Philip Wiles of the London Middlesex hospital in 1938, did not perform satisfactorily [25]. While success was limited for the surgeons of the early 20th century, their efforts in material selection and design were crucial to build the foundation of joint replacements for arthritis treatments [31].

With the success of hip arthroplasties beginning, new ideas in design and material use were proposed and tested by doctors around the world. In this new age of hip arthroplasty, one of the most acclaimed early implants was the short acrylic stem-only prosthesis, used in hemiarthroplasty to treat arthritic femoral heads, designed in 1948 by the medical doctors and brothers, Robert and Jean Judet. Despite high wear susceptibility as well as high and rapid rates of failure, the Judet brothers' prosthesis was a

source of inspiration and a starting point for many others in the orthopaedic community [25]. In particular, three surgeons, namely Frederick Röeck Thompson, Harold R. Böhlman, and Austin Moore, refined Judets' design between 1940 and 1952 with several versions of a Vitallium device with a considerably larger stem, a unique collar for the implant head, and a fenestrated stem designed to promote bone in-growth [25]. Implant designs using a metal bearing surface were further developed by George McKee and John Watson-Farrar in 1953 with their introduction of the modified Thompson prosthesis coupled with an acetabular cup fixed with screws. This was followed by a cementless approach with a similar metal-on-metal (MM) articulation applied in 1964 by Peter Ring. While implant longevity studies have shown these implants to be performing relatively well, there were incidents that demonstrated acetabular loosening at five years in some patients and impingement caused by design flaws in the head-neck-ratio [25, 33].

Significant progress was made in the field of orthopaedics during the mid-1950s when medical doctor Sir John Charnley began his work on the refinement of friction in the THR through improved bearing surfaces which would have the same low friction level of cartilage [34]. Charnley's first implant drew on the McKee-Farrar MM hip, and used Teflon for the cup liner to reduce the coefficient of friction. Unfortunately, Teflon wear products had poor biocompatibility and induced a very severe inflammatory reaction leading to loosening of the hip implant. Further work by Charnley involved poly(methyl methacrylate) (PMMA) bone cement and RCH 1000 high density polyethylene [35]. This material is currently classified as UHMWPE because high density polyethylene (HDPE) is now defined as having a molecular weight less than 2×10^5 and UHMWPE molecular weights are between 3.5×10^6 to 6×10^6 [34, 35]. Both PMMA and UHMWPE are materials still used today because of their success in clinical implementation. Regardless of the success of polyethylene bearings, long term failure remained a cause for concern with Charnley's implants, which has caused other materials to be revisited, improved upon, and combined with new materials for the articulating load bearing surfaces and new implant designs.

3 *IMPLANT WEAR: CONSIDERATIONS AND PROBLEMS WITH MATERIAL SELECTION*

Material selection for optimal clinical use as an artificial bearing surface to replace the natural cartilage requires pairing materials with a low coefficient of friction, low volumetric wear, high biocompatibility, and physical properties congruous with those of bone. As presented in section 2 (History of total hip replacements), numerous materials have been pursued because of what was understood at the time concerning specific properties including biocompatibility, brittleness, chemical stability, coefficient of friction, hardness, modulus of elasticity, volumetric wear (overall accumulation and particle volume), and wettability. In most cases, bearing surfaces with high wear resistance are desired in an effort to

decrease implant wear. However, the surgeons must also consider patient-implant suitability, surgical techniques, and other patient specific physiological and clinical factors in order to maximize implant longevity.

3.1 Metal-on-metal hip implants and associated wear products

As observed from the previously described history of hip implants, it is of interest that the first successful THR bearing was made of a CoCrMo alloy. This material is still used today for MM bearings because of its good resistance to corrosion and wear. The linear rate of corrosion of CoCrMo alloys is approximately 50 nm/year [36]. Despite the decrease in the use of the first generation MM bearings after the introduction of Charnley's metal-on-polyethylene (MPE) THR, MM bearings were the subject of renewed interest in the 1990s because of their lower volumetric wear. It is estimated that currently, over 1 million patients world-wide have received a MM hip implant (THR or SR) since the 1980s [37].

The first generation of MM hip implants exhibited wear rates around 1 to 6 mm^3/year [38]. One important factor influencing wear is considered to be diametral clearance [38], which is being studied and optimized through modelling, simulation, and improvements in manufacturing tolerances. In addition to bearing surface wear, MM bearings and other metal components of implants are subjected to friction, bending, micro-movement, fretting, third body wear, fatigue, oxidation, ion release, crevice corrosion, pitting, stress, mechanically assisted fretting, fretting corrosion, and galvanic corrosion [1, 14, 39–43]. MM hip implants and the neck tapers of modular implants have been shown to release metal ions through a variety of mechanisms *in vivo* [39, 43–47].

The surface of CoCrMo alloys used in MM hip implants is manufactured with a passive oxidation layer of chromium oxide, which reduces but doesn't eliminate the corrosion of the CoCrMo alloy. During wear, subsidence, and loading, the surface re-oxidizes, thereby forming a new passive oxidation layer [46]. Studies identifying the complex states of MM wear products demonstrated that the particle composition in periprosthetic tissues includes chromium oxide [48–50] and chromium phosphate [46].

As previously mentioned, the increased quantity of metal corrosion products released by MM hip implants, mainly cobalt and chromium ions, is of great clinical concern. The primary sources of metal ions are the aforementioned implant wear and corrosion.

3.2 Metal-on-polyethylene hip implants and associated wear particles

Since its inception over sixty years ago, the MPE bearing, which consists of a metal ball articulating on a polyethylene cup liner, has been one of the most popular and successful material combinations

accounting for 50–75% of the HA surgeries world-wide [4, 51–53]. Polyethylene has come a long way since its first introduction in orthopaedics. Material stability and wear characteristics have seen numerous improvements through advancements including heat pressing, chemical cross-linking, and working with longer polymer chains increasing the molecular weight of the polyethylene [35, 54].

3.2.1 *Conventional ultra-high-molecular-weight polyethylene*

Volumetric wear from conventional UHMWPE cups has been reported to be upwards of 0.2 mm/year [55]. Because of the presence of large amounts of wear particles that have led to periprosthetic osteolysis surrounding conventional MPE bearings [54, 56], other articulating surfaces have been considered. Nevertheless, conventional UHMWPE still accounts for 6–8% of THRs in many countries [4, 51, 53]. Polyethylene wear is also dependent on the material properties of the corresponding implant head. A study of MPE and ceramic-on-polyethylene (CPE) implants showed that the annual wear rate of the CPE implants was significantly lower than that of the MPE over a 10-year implantation time [57].

3.2.2 *Highly cross-linked ultra-high-molecular-weight polyethylene*

HXPE was introduced into clinical use in 1998 to improve wear rates as well as to decrease polyethylene oxidation and the resulting release of free radicals *in vivo* [58, 59]. It is estimated that there are over 1 million patients with HXPE implants in the United States alone [59]. HXPE exhibits reasonable resistance to fatigue and fracture, with lower oxidation and volumetric wear compared to UHMWPE. An implant retrieval study, demonstrated that the wear rate of the HXPE implants was below the osteolysis threshold of 0.1 mm/year [60]. Introducing annealing cycles to the fabrication process of HXPE is expected to further decrease the release of free radicals and improve wear rates [58] in second generation HXPEs.

3.3 Ceramic-on-ceramic hip implants and associated wear particles

Ceramics are a more recent material when compared to the history behind metals and polyethylene and currently have very specific clinical scenarios to which they are applicable. Ceramic-on-ceramic (CoC) articulations make up approximately 10% of the utilized bearing materials [4]. Ceramic components can also be paired with other bearing surface materials already mentioned in the form of CPE, ceramic-on-metal, and metal-on-ceramic (although metal-on-ceramic articulations are much more rare [61]).

Studies into the wear of implants with one or more ceramic components actually demonstrate that ceramic components produce two very different types of particles from bearing wear versus wear associated with micro-separation of the head and cup, rim contact, and inter- and intra- granular fracture [62, 63]. Additionally, wear particles from the bearing surfaces tend to be smaller than those resulting from micro-separation, as demonstrated by wear-simulator studies attempting to replicate clinically relevant wear rates, patterns, and particles *in vitro* [62, 63].

3.4 Implant modularity

Customizing an implant to a patient's specific requirements is greatly benefited by using modular implants compatible with a wide array of components [39, 41, 64]. The concept of modular implants not only allows for greater customization of implants for a specific patient, they also permit easier implant assembly during surgery and component substitution/replacement during revision surgery when a replaceable component has failed. Nevertheless, there is currently considerable clinical concern in increasing modularity as the modular junctions that connect distinct components are subject to stress, wear, fretting, corrosion, and cold welding [41, 64].

4 ETIOLOGY OF IMPLANT FAILURE

Although hip implants are effective at restoring the joint function and removing pain, implant failure remains a significant cause for concern both in the long term, and unfortunately the short term, as even low revision rates will affect a significant number of hip implant patients. Understanding the mechanisms behind failure is important because the patient demographic for hip implants is varied and multitudinous. MPE implant failure rates have been reported at up to 20% during the first ten years [65]. In the case of MM implants, head size, micro-separation (head offset), as well as head and cup inclination/anteversion angles, each can increase wear rates and may lead to increased rates of implant failure [66, 67]. Some studies with sufficient patient numbers and longevity have demonstrated that failure due to metal-related pathologies is more common with MM bearings [51, 68]. Therefore, there are concerns about MM hip implant longevity, especially since lower failure rates due to osteolysis have not been reported with MM hip implants compared to MPE hip implants with similar characteristics [55, 69].

4.1 Causes of implant failure

Aseptic loosening due to periprosthetic osteolysis (bone loss around the implant) was first identified by Dr. John Charnley in [70]. Periprosthetic osteolysis is primarily caused by wear particles and metal ions which generate a complex immune response adversely affecting bone remodelling. Periprosthetic osteolysis can lead to aseptic loosening, which accounts for approximately three-quarters of revisions, making it the leading cause of implant failure [71]. Additionally, through both synergistic and independent mechanisms, mechanical factors such as synovial fluid pressure and stress shielding also contribute to aseptic implant failure [54, 72, 73]. In the last couple of decades, it has also been noted that implant failure due to aseptic loosening may be associated with other symptomatic or asymptomatic adverse soft tissue reactions [74, 75], including metallosis, hypersensitivity and pseudotumors. Most of these reactions suffer from poor consensus in the literature with regards to the underlying mechanisms. Additional causes for failure include infection (septic failure), recurrent dislocation, fracture (both of the periprosthetic bone, or the implant), and general problems arising from technical errors during surgery [71].

4.2 Mechanisms of wear- and corrosion-mediated implant failure

As previously mentioned, MM hip implants were introduced as alternatives to conventional MPE implants in order to decrease the incidence of periprosthetic osteolysis. Implant retrieval studies have demonstrated lower volumetric wear, smaller wear particles [76], and delayed wear [50]. *Ex vivo* analysis of periprosthetic tissues has demonstrated that periprosthetic tissues of MM implants may be characterized by lower numbers of giant multinucleated cells [76] and histiocytes [50], a lower number of cells producing cytokines (interleukin–1β (IL–1β), IL–6, and TNF–α) [77], and lower overall periprosthetic tissue reactions [50]. In general, differences between MPE and MM implants point towards a lower inflammatory reaction with MM implants.

However, there are additional histological studies that have portrayed numerous cases with lymphocytic (predominantly T cell) infiltrates, necrosis, and soft tissue lesions in the presence of metal wear products [74, 76, 78–80] which can be the result of hypersensitivity reactions. Nevertheless, similar histological features have also been observed in some patients with MPE implants [50].

Since periprosthetic osteolysis, the most common cause of failure, is primarily driven by the immune response to wear particles and metal ions, it is important to understand the contributions and interactions between the cells involved and how failure characteristics are changed across different implant systems (*i.e.*, implant fixation and bearing surfaces in particular). A major contributor to bone resorption is the non-specific inflammatory response to wear particles and metal ions [74, 81–84] involving

the activation of the nuclear factor kappa-B (NFκB) signalling pathway. One of the primary mechanisms by which the NFκB pathway is able to potentiate osteolysis and eventually implant aseptic loosening is through the upregulation of macrophage production and secretion of pro-inflammatory mediators including regulators of tissue homeostasis, nitrogen oxide (NO) catalyzing enzymes, and pro-inflammatory cytokines [74, 85, 86]. Histological studies have shown elevated levels of cytokines including TNF–α, monocyte chemotactic protein–1 (MCP–1), macrophage inflammatory protein–1α (MIP–1α), IL–1α, IL–1β, IL–6, IL–8, IL–11, transforming growth factor–β (TGF–β), prostaglandin E2 (PGE_2), macrophage colony-stimulating factor (M–CSF), granulocyte-macrophage colony-stimulating factor (GM–CSF), and matrix metalloproteinases (MMPs) in periprosthetic tissues of failed hip implants [74, 77, 78, 87–90]. Many of these pro-inflammatory cytokines are known to have direct and specific effects on recruiting and inducing the differentiation of osteoclast precursors, maturation of osteoclasts, and even enhancing the activity of functional osteoclasts [71, 74, 87, 91, 92].

While most of these pro-inflammatory cytokines are known to be released from macrophages, it has been reported that fibroblasts release similar factors. Therefore, aseptic loosening may be facilitated by macrophages and fibroblasts [74]. Macrophages and fibroblasts are also important when characterizing the macroscopic changes in the periprosthetic membrane adjacent to the bone. Observations of this periprosthetic membrane generally characterize the tissues as inflamed, layered with heterogeneous distributions of synovial-like cells, macrophages, fibroblasts, foreign body giant cells, polymorphonuclear leukocytes, osteoclasts, and osteoblasts. There is also typically the presence of hyaluronan-containing synovial fluid and a synovial lining which appears frequently in the literature as a synovial-like interface membrane (SLIM) [74].

Metal ions from implant wear and/or corrosion may further complicate the immunological response because they are known to complex with proteins [93–96], thereby forming haptens that may contribute to the production of the aforementioned pro-inflammatory mediators, and may also induce further tissue damage through other immune responses, including hypersensitivity and pseudotumors [54, 74, 94, 97, 98]. Pseudotumors are soft-tissue lesions made up of dense connective tissue, macrophages, lymphocytes, and other cells that are known to exist in periprosthetic tissues such as plasma cells, eosinophils, and neutrophils, with extensive amounts of necrosis [99]. It is interesting to observe that macrophages, lymphocytes, and wear particles frequently exist at the heart of these cystic masses [99]. Depending on the patient, these reactions may be symptomatic or asymptomatic and can lead to early implant failure. Their exact mechanisms remain unclear and of tremendous clinical concern. In a recent review of histopathological studies, evidence was presented that increased adverse tissue reactions correlated with elevated levels of implant wear. Interestingly however, there is insufficient information to formulate a relationship between the amount of wear in the tissues and

the severity of the local immune response, indicating that more information about the congruence of patient, implant, and surgical factors is required [100].

It has been reported that metal ion levels in the serum, synovial fluid, and whole blood of patients with a joint replacement are significantly higher than the corresponding levels in patients with no implant [38, 86, 92, 101–104]. These ions are found in the form of soluble ions, oxides, insoluble metal salt precipitates, and may also be found chelated with organic anions forming organometallic complexes [36]. Metal ions also persist inside the cells (having been transported into the cells through ion channels) or bound to cell membranes [36]. Elevated levels of metal ions have been observed in periprosthetic cells, tissues, and fluids of patients with failed hip implants. It has also been measured that metal ions circulate in the blood and are transported systemically to organs throughout the body resulting in metal ion accumulation in organs such as the liver, spleen, kidney, lymphatic tissues, and bone marrow [36, 105, 106]. Thus, the effects of these metal ions are wide spread and of considerable clinical importance [54]. While elevated levels of cobalt have been reported in some organs of patients with failed hip implants [36], it has also been observed that, cobalt ions undergo minimal uptake by cells and tend to be rapidly expelled in the urine [105].

It has been difficult, however, to identify patient/implant factors that contribute to increased levels of ions in the blood. Patient activity levels are one of the suspected factors that may lead to changes in elevated levels of metal ions, but this has been a theory that has not been confirmed with strong clinical data [107, 108]. Nevertheless, more recent studies have been able to correlate patient age with increased metal ion levels. This age correlation is presumed to be directly related to the expected activity spectrum of the patient [109]. Another hypothesis is that larger head diameters would lead to increased levels of metal ions, exposed surface area, and alterations to stress distributions under loading. However, the head sizes studied were not found to contribute differently [101]. Despite the inherent logical consistency of some of the above theories in predicting serum ion levels, isolating factors responsible for elevated metal ion levels may be more complicated than anticipated. The levels of metal ions in the blood have long been hypothesized to have a correlation to early implant failure and adverse tissue reactions [110], although the reliability of ion levels to predict the development of adverse tissue reactions and implant failure remains questionable, especially considering the variability in ion results between laboratories [111].

While it is known mechanisms of cell-protein-ion interactions are complicated and not well understood, clinical studies have shown that adverse local tissue reactions (ALTR) or adverse reactions to metallic debris (ARMD), including hypersensitivity and pseudotumors, may be associated with elevated levels of cobalt and chromium ions and other corrosion products [75, 112–115]. More importantly, adverse tissue reactions with elevated metal ion levels are not limited to patients with MM hip implants and have also been observed in patients with modular neck implants (independent of the

bearing materials) due to fretting and crevice corrosion [43, 64, 112–116]. Many of the metals employed in orthopaedic implants, including chromium, cobalt, nickel, tantalum, titanium, and vanadium are known to be metal sensitizers (*i.e.*, metals that act as hapten moieties in antigens and proteins) [94, 117]. Depending on the material composition, the ions released may not cause metal-sensitivity reactions but may still activate the immune system by forming complexes with native proteins [94]. Specifically, cobalt and chromium are considered to be strong metal sensitizers, and CoCrMo alloys used in MM hip implants contain large quantities of both of these metals [94].

4.3 Diagnostic techniques

Given the clinical importance of addressing implant failure, an accurate understanding behind the etiology of the different immune reactions to better predict a patient's risk factors and better identify the symptoms of implant failure, is of the utmost importance. A current area of research in clinical orthopaedics pertains to the early detection of symptoms that will lead to implant failure. There is great interest in identifying relationships between implant wear, stability, positioning, tissue health, and immune state, all with increased risk of failure. X-rays, computed tomography (CT), magnetic resonance imaging (MRI), and ultrasound are currently used by some clinicians as a non-invasive diagnostic technique for assessing the implant wear, alignment, osseointegration or quality of the bone-cement interface, bone density, degree of osteolysis, and presence of soft-tissue lesions including pseudotumors [98, 99, 118–121]. These diagnostics are important to determine whether the implant is stable and if the tissues around the bone are manifesting symptoms of an adverse reaction that will culminate in the requirement for an implant revision.

5 *IN VITRO* STUDIES ON CELL RESPONSE TO METAL IONS

In vitro studies of single cell-type cultures present an informative way of developing and testing theories about the etiology of implant failure and the specific role that wear products play as instigators in adverse immune response. Because ion release is not unique to MM hip implants (there are metal components in MPE and CoC implants as well), the biological consequences of producing excess metal ions is very important to understand.

Previous *in vitro* studies on multiple types of cells found in periprosthetic tissues have demonstrated that divalent cobalt (Co^{2+}) has greater toxicity than trivalent chromium (Cr^{3+}) [44, 122–126], may induce apoptosis and necrosis in time- and dose-dependent manners, and elicit the release of pro-inflammatory mediators including cytokines, chemokines, and costimulatory molecules [44, 123, 127–131]. These studies have led to greater clinical concern and suggestions that cobalt may

be the more clinically relevant active agent associated with periprosthetic tissue reactions [46]. *In vitro* studies have also demonstrated that Cr^{3+} can also induce apoptosis and necrosis in time- and dose-dependent manners and stimulate the release of pro-inflammatory mediators including cytokines and chemokines from multiple types of cells found in periprosthetic tissues [44, 123, 131–133].

Other studies examine the effects of metal ions directly on osteoclasts and osteoblasts to study the implications metal ions have on skeletal health, bone remodelling, and osteolysis. One study using Co^{2+} and Cr^{3+} demonstrated that forming osteoclasts are affected differently than mature osteoclasts [134]. Co^{2+} and Cr^{3+} were observed to have a mild stimulatory effect on bone resorption functionality by forming osteoclasts compared to mature osteoclasts, which displayed a reduction in cell number and resorption functionality. On the other hand, Cr^{6+} was observed to reduce cell survival and functionality indicating the extreme toxicity of Cr^{6+} [134]. Osteoblasts appear less sensitive to metal ions such as cobalt and chromium at low, sublethal clinically relevant concentrations, as studies have demonstrated negligible change to cell survival and functionality [134, 135]. Some studies demonstrated that high concentrations of Co^{2+} and Cr^{3+} induced an increase in oxidative stress and a decrease in osteoblast survival and activity [136].

6 CELLS OF INTEREST IN IMPLANT FAILURE

6.1 Macrophages

Macrophages are of considerable interest in wear-mediated tissue reactions such as periprosthetic osteolysis because they have previously been shown to be the dominant cell type found in interface membranes of failed MPE THRs [137] and in inflamed periprosthetic tissues [78]. They are the primary type of phagocytizing and antigen-presenting cells (APCs) involved in type-IV hypersensitivity reactions [138] and are crucial players in many inflammatory pathways [92] that are known to affect bone homeostasis. Macrophages are derived from the hematopoietic stem cell-derived myeloid lineage. Briefly, in order to obtain a macrophage, pluripotent hematopoietic stem cells differentiate within the bone marrow into myeloid stem cells, which yield lineage-specific colony-forming unit cells that are specific to the production of neutrophils, monocytes (which differentiate into macrophages in peripheral tissue compartments such as the breast, lungs/alveoli, tonsils, spleen, placental tissue, lymph nodes, serous cavities, outside the intestines, or subdermal tissues [139, 140] having been transported through the blood to the tissues which they reach by migrating across the blood vessel wall [141]), eosinophils, basophils, mast cells, megakaryocytes, and erythrocytes [142]. Macrophages are capable of further specialization and differentiation in the liver where they are then known as Kupffer cells, the brain where they are known as microglia, the skin where they are known as Langerhans cells, and in

bone marrow where they differentiate changing from a mononuclear cell to a multinuclear osteoclast [140].

Macrophages are extremely versatile cells capable of changing function depending on the level of activation. They are known to exist in three stages of activation or readiness. The first, termed resting, covers macrophages that have a life span of a few months and are found persisting lethargically in the tissues, with nominal debris/antigen removal [139]. Upon receiving a priming signal of cytokines, such as interferon–γ (IFN–γ), MCP–1, and/or stimulation through hypoxic conditions [143], the macrophage enters an activated state where it upregulates the expression of class II major histocompatibility complex (MHC) molecules, which display fragments of foreign bodies ingested by macrophages converting them effectively to an APC for T helper (Th) cells. Macrophages may also be activated directly by molecules from generic components of pathogens that bind directly to their membranes. Directly activated macrophages are referred to as hyperactivated and are efficient dispatchers of ingested bacterium (increased internal lysosomal function/numbers) and large external multicellular parasites that are too large to phagocytize (lysosomal deposits) [139, 142]. In this state of hyperactivation, macrophages swell to ingest large bodies and produce TNF cytokines that are capable of killing tumor and virus-infected cells as well as activating other cells of the immune system [139]. These activation states are collectively referred to as M1 or classically activated macrophage phenotypes. In general, M1 are the cells recognized for all the pro-inflammatory and phagocytic capacities previously mentioned [143]. Other anti-inflammatory cytokines such as IL–4, IL–10, IL–13, TGF–β, and glucocorticoids (GCs) induce a different macrophage phenotype known as the M2 or alternatively activated macrophages. M2 macrophages resist re-activation and they present enhanced selective phagocytic capabilities, enhanced antigen presentation, increased production of anti-inflammatory cytokines, and inhibited production of pro-inflammatory cytokines [143]. Although the dichotomy of M1 and M2 phenotypes of macrophage activation efficiently divide the two into pro- and anti-inflammatory functionalities, the effect of different stimuli and the ensuing differences in macrophage response, morphology, and function have been well documented and reviewed [143–146].

6.2 Lymphocytes

The changes in periprosthetic lymphocyte populations have been identified as one of the most significantly altered aspects of the periprosthetic cellular profile in aseptic lymphocyte-dominated vasculitis-associated lesions (ALVAL) and and in many cases of pseudotumors. T cells and B cells make up the two main types of lymphocytes. These cells are typically quiescent, only becoming active during antigen stimulation when they are needed to protect the host environment [139]. Lymphocytes come

from lymphoid stem cells which differentiate in the bone marrow from pluripotent hematopoietic stem cells [142].

T cells are lymphoid progenitors that have matured in the thymus or the tonsils [139]. Their activation usually requires surface T-cell receptor (TCR) binding to a cognate peptide presented on the class II molecules of an MHC molecule and a costimulatory molecule [139]. There are many important subtypes of T cells including: Th cells, cytotoxic T (Tc) cells, and natural killer T (NKT) cells. Th cells, and their differentiated subsets, are crucial to the function of B cells and activation of macrophages and Tc cells because active Th cells proliferate and release numerous cytokines including IL–2 and IFN–γ [139]. Tc cells are important because they destroy cells, such as infected or cancer cells, upon recognition of Class I MHC molecules on the surface of APCs [139]. Lastly, NKT are important because they perform functions attributed to each NK, Th, and Tc cells [139].

Since B cells can be APCs and are key producers of antibodies, they can facilitate both the innate and adaptive immune response [139]. B cell response to innate immunological signals is not homogeneous across the subsets of B cells. B cell subsets include B-1 cells and B-2 cells [147]. B-1 cells are the primary source for naturally produced antibodies and are crucial in maintaining tissue homeostasis and protecting the host from mucosal pathogens [147]. B-2 cells include phenotypically distinct populations of mature B cells known as follicular zone B cells and marginal zone B cells and are considered to contribute through T cell-independent and innate-like immune responses [147]. Follicular B cells are the largest population of B cells developing from progenitors in the bone marrow and are found in peripheral blood, spleen follicles, and lymph nodes [147]. Marginal zone B cells are also found in the spleen and peripheral blood and are known to differentiate into antibody-secreting cells upon antigen recognition [147].

7 CYTOKINES AND CHEMOKINES OF INTEREST IN IMPLANT FAILURE

There are many important molecules in the inflammatory pathways leading to periprosthetic osteolysis in response to implant wear particles and metal ions. The NFκB pathway is a critical pathway in inflammation and osteoclastogenesis and is responsible for the production of numerous pro-inflammatory cytokines and chemokines [71, 92]. One of the more important cytokines in implant-wear-mediated periprosthetic osteolysis is TNF–α. TNF–α is of significant importance in osteolysis as it is a regulatory cytokine for numerous other pro-inflammatory cytokines for IL–1, IL–6, IL–8, and GM–CSF [71] and is part of a synergistic mechanism with receptor activator of NFκB ligand (RANKL) that results in the differentiation of osteoclast precursors into functional osteoclasts and NFκB activation [71].

Chemokines are a family of cytokines that provide vital cell signalling, activation, and trafficking functionality targeted to specific cell populations during different stages of both innate and adaptive inflammatory immune responses, as well as fibrosis, and angiogenesis [148–150]. They are released by numerous cells, including monocytes, macrophages, dendritic cells (DC), peripheral lymphocytes, endothelial cells, fibroblasts, eosinophils, basophils, neutrophils, T cells, and NK cells [149]. Increased levels of some of these pro-inflammatory cytokines and chemokines have been shown in periprosthetic tissues using reverse transcription polymerase chain reaction (RT-PCR) and/or immunohistochemistry [74, 77, 78, 87–90]. The use of RT-PCR may actually underestimate the effects of implant wear products on the production of these signalling molecules because it has recently been shown, in the context of chronic inflammation, that gene regulation of chemokines may be predominantly controlled post-translationally [151].

Cysteine-cysteine (CC) chemokines are an important group of chemokines characterized by two adjacent cysteine amino acids. CC chemokines make up the largest of the four groups of chemokines and include MCP–1, MIP–1α, MIP–1β, and regulated on activation, normal T cell expressed and secreted (RANTES), which are believed to be important molecules in adverse immune responses because they are known to target different types of leukocytes by stimulating changes in cell function and migration [83, 90, 149]. Specifically, MCP–1 is known to stimulate an increase of IL–1 and IL–6 release and regulate the migration and accumulation of monocytes/macrophages, DC, NK and memory T lymphocytes [149]. Of particular interest in osteolysis is the observation that osteoblasts may produce functional MCP–1 under inflammatory conditions causing local monocyte recruitment [152].

MIP–1α also stimulates the production of IL–1, IL–6, and TNF–α, and regulates migration of monocytes/macrophages, B cells, Tc lymphocytes, NK cells, and eosinophils, while MIP–1β regulates chemotaxis for Th lymphocytes [149]. Both MIP–1α and MIP–1β have been found to be osteoclastic factors capable of increasing the expression of receptor activator of NFκB ligand (RANKL) and may be two proteins with significant impact on osteoclast activation [153, 154] . MIP–1α is of particular interest because some studies have found increased osteoclast differentiation [153], formation [155], and migration [156] in the presence of MIP–1α.

RANTES, a CC chemokine related to MIP–1α and MIP–1β has also been reported to be produced by various cells, including Th lymphocytes, monocytes, and DC, and is a strong chemoattractant for macrophages, eosinophils, and mature Th cells [149, 157]. Similar to MIP–1α, RANTES has been shown to increase osteoclast size, motility, and differentiation of osteoclast precursors [158].

Numerous other chemokines including stromal cell-derived factor–1 (SDF–1) and MIP–3β are known to be bone marrow-expressed chemokines and are known to be important the migration and

development of in T cell precursors [150]. This may have a compounding effect on T cell-mediated (type-IV hypersensitivity) reactions.

8 PATHWAYS AND PHARMACEUTICALS OF CLINICAL INTEREST IN MITIGATING THE EFFECTS OF WEAR PRODUCT-INDUCED PERIPROSTHETIC OSTEOLYSIS

Despite the success of HAs, it is widely understood that the greatest amelioration for increasing implant longevity will come from the development and integration of new and improved bearing surfaces, prosthetic design, and clinical understanding of the implants and confounding patient factors. Regardless, mechanical and chemical implant wear is an inevitable outcome of any implant design and thus, there is clinical interest in limiting the severity of the cellular response.

Pharmaceutical strategies to modulate wear product-induced inflammation and osteolysis have long focused on the inflammatory pathways, specifically through local or systemic administration of anti-inflammatory agents, growth factors, and osteoclast inhibitors. The use of drugs already approved for bone etiologies, including joint pain, inflammation, and bone loss (*i.e.*, osteoporosis, rheumatoid arthritis, osteoarthritis, and psoriatic arthritis), were considered as possible candidates for periprosthetic osteolysis treatments due to their shared symptomology. However, the efficacy of these drugs as viable treatments for periprosthetic osteolysis has not been demonstrated in clinical trials, and thus has not been approved for widespread clinical use for patients with hip implants.

There are numerous pathways and targets of interest in controlling the response to wear products including: 1.) pathways promoting cell-death via apoptosis rather than necrosis, the former being preferable because it is regulated through conserved biological pathways that limit inflammatory reactions (*i.e.*, the release of cytokines [44] and the level of released intracellular contents including intracellular cytokines from bursting cells [123]); 2.) modulating the inflammation by attenuating or inhibiting the production and release of pro-inflammatory cytokines, oxidase enzymes, reactive oxygen species (ROS), and other contributors to oxidative stress; 3.) modulating osteoclast formation, function, signalling, and activity by controlling activation of pathways including RANK-RANKL-OPG, mitogen-activated protein kinase (MAPK), NFκB, mevalonate, and inflammasome; and 4.) promoting bone formation using other osteogenic bone and growth factors [71, 159–161].

8.1 Anti-inflammatory agents

Numerous different pro-inflammatory cytokines may be suitable targets for the modulation of the inflammatory reaction leading to periprosthetic osteolysis. One of the most important pro-inflammatory

mediators involved in periprosthetic osteolysis is TNF–α which has led to proposals using TNF antagonists and anti-TNF-gene therapy to limit the excessive levels of inflammation and consequently decreasing osteolytic consequences. IL–1 inhibitors were also considered despite the more significant regulatory effects that TNF–α displays on numerous cytokines including IL–1, IL–6, IL–8, and GM–CSF [159, 160].

An additional crucial family involved in the inflammatory cascade are the eicosanoids, which include leukotrienes, lipoxins, prostaglandins, prostacycline, and thromboxane. PGE_2 is a preeminent pro-inflammatory mediator which can be inhibited through the use of nonsteroidal anti-inflammatory drugs (NSAIDs) and cyclooxygenase (COX) inhibitors [159]. Numerous COX inhibitors are U.S. Food and Drug Administration (FDA)-approved for treating rheumatoid arthritis and osteoarthritis [160, 161]. Unfortunately, many types of anti-inflammatory drugs present strong immunosuppressive and antianabolic effects [160] and therefore, despite the existence of *in vivo* animal studies successfully suppressing wear-debris induced osteolysis, very few have progressed to human trials [162].

Some therapies have also used anti-inflammatory cytokines, such as IL–4 and IL–10, to increase the production of additional anti-inflammatory cytokines such as IL-1ra, to decrease the release of TNF–α, IL–1, IL–6, IL–8, PGE_2, and GM–CSF, as well as to decrease the production of hydrogen peroxide (H_2O_2) and NO intermediates [71, 159].

It has also been reported that inflammation may be exacerbated by ROS and/or reactive nitrogen species (RNS) [163], which may therefore represent an additional target for pharmacological intervention using antioxidants such as pyconogenol, ascorbic acid, N-acetylcysteine, pyrrolidine dithiocarbamic acid, and nitrosoprocainamide [161].

8.2 Anti-resorptive and anti-osteolytic agents

Whether achieved through modulation of osteoclast signalling or direct inhibition of osteoclast function, reducing functional osteoclastic bone resorption is of great interest in limiting osteolysis [159–161]. Ideally, anti-resorptive and anti-osteolytic agents would be specific in their attenuation of osteoclast function, and therefore would have a minimal influence on the overall immune system. One of the specific targets of interest in decreasing osteolysis and implant-wear-mediated inflammation is NFκB. It is known that NFκB is crucial to cytokine and chemokine expression [164], is involved in osteoclast cell function, is known to be activated in macrophages and osteoclasts upon exposure to implant wear products, and has numerous possible targets for inhibition [165].

It has been proposed that a prophylactic administration of antiresorptive agents could improve osseointegration due to an increased presence of bone stock [160]. Bisphosphonates and receptor activator

of NFκB ligand (RANKL) antagonists are two such drugs that have been proposed to decrease or reverse periprosthetic osteolysis. Bisphosphonates are currently approved for use in patients with osteoporosis, Paget's disease, and hypercalcemia [71, 160]. However, recent efforts to treat patients with rheumatoid arthritis or periprosthetic osteolysis using bisphosphonates have not returned promising results [160]. There are *in vivo* animal studies which have demonstrated inhibitions in particle-induced osteolysis, but there are still inconsistent observations between distinct studies with different animal models [71, 159, 161]. Receptor activator of NFκB ligand (RANKL) antagonists, on the other hand, are of great clinical interest due to their specificity in targeting the skeletal system rather than the immune system, which alleviates concerns of possible undesirable immunosuppressive effects [160].

8.3 Osteogenic and anabolic agents

Since it has been established that many of the same instigators (such as TNF–α) of enhanced bone resorption also inhibit bone formation, the dynamic process of bone remodelling is subject to a significant imbalance in the periprosthetic environment containing wear products, eventually leading to osteolysis. Therefore, a valuable approach may be to improve and restore osteoblast function. This is important because osteoblasts/stromal cells are critical cells behind the production of osteoprotegerin (OPG) [166], which is important for regulating osteoclast formation and activity.

Proposed pharmaceuticals that could re-establish normal or enhanced osteogenic behaviour have included: recombinant human parathyroid hormone (PTH), which is an anabolic agent found to improve bone formation and consequently osseointegration after surgery; and growth factors such as TGF–β, bone morphogenetic proteins (BMPs), insulin-like growth factors (IGFs) including IGF–1, IGF–2, and basic fibroblast growth factor (bFGF) (specifically bFGF–2), which regulate osteoblast differentiation and proliferation leading to increased bone formation and decreased numbers of osteoclast-like-cells [159–161].

8.4 Statins, and more specifically simvastatin

Since many of the drugs listed in the approaches described above have serious side effects (including strong anabolic and immunosuppressive effects), it is important to find a target (or multiple targets) that can be modulated by a pleiotropic drug with tolerable side effects. Alternatively, a combination of different therapeutic agents may also be used, but future pharmaceutical investigations should focus on optimizing the benefits of a particular drug, while minimizing its side effects.

Possible drugs with potentially milder side effects are 3-hydroxy-3-methylglutaryl-coenzyme A (HMG–CoA) reductase inhibitors, collectively known as statins. Simvastatin, a synthetic statin ap-

proved by the FDA in 1991 to decrease low-density lipoprotein (LDL) cholesterol level in the blood, has recently been shown to lower the risk of revision surgery after a total hip arthroplasty (THA) [167, 168]. More specifically, simvastatin has been shown to enhance macrophage phagocytosis by altering the lipid content in the cell membrane [169] and to decrease the release of inflammatory mediators [170, 171], which could potentially reduce the inflammatory response to implant wear particles and metal ions.

Other interesting properties of simvastatin relevant to orthopaedic applications, and specifically periprosthetic osteolysis, are its demonstrated ability to inhibit osteoclast-mediated bone resorption by decreasing osteoclast function [172] and to stimulate bone formation by enhancing osteoblast maturation and function [173]. *In vivo*, simvastatin has been shown to improve osseointegration of orthopaedic implants in animal trials [174, 175] and to decrease osteolysis induced by polyethylene particles in mouse calvaria [176, 177] and rabbit models [178]. *In vitro*, studies have also demonstrated the applicability of simvastatin treatments for the attenuation of titanium wear particle-induced IL-6 expression and production [179].

The modulation of the inflammatory response by simvastatin may involve the geranylgeranylpyrophosphate pathway [180] through the prevention of the conversion of HMG-CoA to mevalonic acid, thereby inhibiting the production of cholesterol and prenylated proteins. The inhibition of protein prenylation (specifically guanosine triphosphatases prenylation (GTPases)) changes the lipid structure/content in the cellular membrane [172]. The reduction in prenylated signalling molecules, which are required by osteoclasts, also results in the inhibition of osteoclast-mediated resorption [172, 181] and is part of the same geranylgeranylpyrophosphate pathway proposed to be responsible for the beneficial effects of statins on osteoblasts [179, 182].

Hence, simvastatin may be a potential pleiotropic agent for modulating periprosthetic osteolysis due to its anti-inflammatory properties, ability to inhibit bone resorption, and ability to stimulate bone formation.

9 REFERENCES

[1] K. E. Alvarez, *Total hip replacement*, L. L. Hench, J. R. Jones, and M. B. Fenn, Eds. London, UK: Imperial College Press, 2012.

[2] K. E. Dreinhöfer, P. Dieppe, T. Stürmer, D. Gröber-Grätz, M. Flören, K. P. Günther, W. Puhl, and H. Brenner, "Indications for total hip replacement: comparison of assessments of orthopaedic surgeons and referring physicians," *Ann. Rheum. Dis.*, vol. 65, no. 10, pp. 1346–50, Oct. 2006.

[3] P. E. Beaulé, D. J. Allen, J. C. Clohisy, P. Schoenecker, and M. Leunig, "The young adult with hip impingement: deciding on the optimal intervention," *J. Bone Joint Surg. Am.*, vol. 91, no. 1, pp. 210–21, Jan. 2009.

[4] Canadian Joint Replacement Registry (CJRR), "Hip and knee replacements in canada: Canadian joint replacement registry 2013 annual report," Canadian Institute for Health Information, Tech. Rep., 2013.

[5] M. A. Adelani, J. A. Keeney, A. Palisch, S. A. Fowler, and J. C. Clohisy, "Has total hip arthroplasty in patients 30 years or younger improved? a systematic review," *Clin. Orthop. Relat. Res.*, vol. 471, no. 8, pp. 2595–601, Aug. 2013.

[6] J. Daniel, P. B. Pynsent, and D. J. W. McMinn, "Metal-on-metal resurfacing of the hip in patients under the age of 55 years with osteoarthritis," *J. Bone Joint Surg. Br.*, vol. 86, no. 2, pp. 177–84, Mar. 2004.

[7] Canadian Joint Replacement Registry (CJRR), "Hip and knee replacements in canada 2008-2009 annual report," Canadian Institute for Health Information, Tech. Rep., 2009.

[8] P. E. Beaulé, F. J. Dorey, M. J. Le Duff, M. LeDuff, T. Gruen, and H. C. Amstutz, "Risk factors affecting outcome of metal-on-metal surface arthroplasty of the hip," *Clin. Orthop. Relat. Res.*, no. 418, pp. 87–93, Jan. 2004.

[9] A. J. Shimmin, J. Bare, and D. L. Back, "Complications associated with hip resurfacing arthroplasty," *Orthop. Clin. North Am.*, vol. 36, no. 2, pp. 187–93, ix, Apr. 2005.

[10] J. W. Nicholson, *Synthetic Materials in Medicine*. The Royal Society of Chemistry, 2002, ch. 1, pp. 1–24.

[11] H. C. Amstutz, P. Campbell, N. Kossovsky, and I. C. Clarke, "Mechanism and clinical significance of wear debris-induced osteolysis," *Clin. Orthop. Relat. Res.*, vol. 276, pp. 7–18, Mar. 1992.

[12] R. M. Urban, M. J. Tomlinson, D. J. Hall, and J. J. Jacobs, "Accumulation in liver and spleen of metal particles generated at nonbearing surfaces in hip arthroplasty," *J. Arthroplasty*, vol. 19, no. 8 Supp, pp. 94–101, Dec. 2004.

[13] J. Tidermark, S. Ponzer, O. Svensson, A. Söderqvist, and H. Törnkvist, "Internal fixation compared with total hip replacement for displaced femoral neck fractures in the elderly. A randomised, controlled trial," *J. Bone Joint Surg. Br.*, vol. 85, no. 3, pp. 380–388, Apr. 2003.

[14] J. W. Nicholson, *Metals*. The Royal Society of Chemistry, 2002, ch. 4, pp. 107–147.

[15] L. I. Havelin, O. Robertsson, A. M. Fenstad, S. r. Overgaard, G. Garellick, and O. Furnes, "A Scandinavian experience of register collaboration: the Nordic Arthroplasty Register Association (NARA)," *J. Bone Joint Surg. Am.*, vol. 93 Suppl 3, pp. 13–9, Dec. 2011.

[16] D. L. Bartel, D. T. Davy, and T. M. Keaveny, *Orthopaedic Biomechanics: Mechanics and Design in Musculoskeletal Systems*, H. Stark, Ed. New Jersey: Pearson Education, 2007.

[17] J. Dequeker and F. Luyten, “The history of osteoarthritis-osteoarthrosis,” *Ann. Rheum. Dis.*, vol. 67, no. 1, pp. 5–10, Jan. 2008.

[18] T. L. Dupras, L. J. Williams, H. Willems, and C. Peeters, “Pathological skeletal remains from ancient egypt: the earliest case of diabetes mellitus?” *Pract. diabetes int.*, vol. 27, no. 8, pp. 358–63a, Oct. 2010.

[19] N. Prevezas, “Evolution of pelvic and acetabular surgery from ancient to modern times,” *Injury*, vol. 38, no. 4, pp. 397–409, Apr. 2007.

[20] P. Salib, “Orthopaedic and traumatic skeletal lesions in ancient Egyptians,” *J. Bone Joint Surg. Br.*, vol. 44-B, no. 4, pp. 944–947, Nov. 1962.

[21] J. M. Chelius and J. F. South, *Of excision of the hip-joint*. Philadelphia, USA: Lea & Blanchard, 1847, vol. III, ch. IV, pp. 735–739, translated from the german, and accompanied with additional notes and observations by John F. South.

[22] P. Hernigou, “Earliest times before hip arthroplasty: from John Rhea Barton to Themistocles Glück,” *Int. Orthop.*, vol. 37, no. 11, pp. 2313–8, Nov. 2013.

[23] Lancet (Br. ed.), “Obituary. Mr. Anthony White,” *The Lancet*, vol. 53, no. 1334, pp. 324 – 5, Mar. 1849, originally published as Volume 1, Issue 1334.

[24] N. Allison and B. Brooks, “The Classic: Symposium on Arthroplasty: Arthroplasty: Experimental and Clinical Methods,” *Clin. Orthop. Relat. Res.*, vol. 468, no. 1, pp. 6–11, Jan. 2010.

[25] P. F. Gomez and J. A. Morcuende, “Early attempts at hip arthroplasty: 1700s to 1950s,” *Iowa Orthop. J.*, vol. 25, pp. 25–9, 2005.

[26] N. Heybeli and E. F. Mumcu, “Total hip arthroplasty (history and development),” *Süleyman Demirel Üniversitesi Tip Fakültesi Dergisi*, vol. 6, no. 4, pp. 21–7, 1999.

[27] J. B. Murphy, “The Classic: Ankylosis: Arthroplasty-Clinical and Experimental,” *Clin. Orthop. Relat. Res.*, vol. 466, no. 11, pp. 2573–8, Nov. 2008.

[28] R. A. Brand, M. A. Mont, and M. M. Manring, “Biographical Sketch: Themistocles Glück (1853-1942),” *Clin. Orthop. Relat. Res.*, vol. 469, no. 6, pp. 1525–27, Jun. 2011.

[29] N. Eynon-Lewis, D. Ferry, and M. Pearse, “Themistocles Gluck: an unrecognised genius,” *Br. Med. J.*, vol. 305, no. 6868, pp. 1534–6, Dec. 1992.

[30] T. Gluck, “Report on the positive results obtained by the modern surgical experiment regarding the suture and replacement of defects of superior tissue, as well as the utilization of re-absorbable and living tamponade in surgery. 1891,” *Clin. Orthop. Relat. Res.*, vol. 469, no. 6, pp. 1528–35, Jun. 2011.

[31] I. D. Learmonth, C. Young, and C. Rorabeck, “The operation of the century: total hip replacement,” *Lancet*, vol. 370, no. 9597, pp. 1508–19, Oct. 2007.

[32] S. R. Knight, R. Aujla, and S. P. Biswas, “Total hip arthroplasty-over 100 years of operative history,” *Orthop. Rev. (Pavia)*, vol. 3:e16, no. 2, pp. 72–4, Sep. 2011.

[33] W. T. Long, L. D. Dorr, and V. Gendelman, “An american experience with metal-on-metal total hip arthroplasties: a 7-year follow-up study,” *J. Arthroplasty*, vol. 19, no. 8 Suppl 3, pp. 29–34, Dec. 2004.

[34] S. M. Donald, “Sir John Charnley (1911-1982): inspiration to future generations of orthopaedic surgeons,” *Scott. Med. J.*, vol. 52, no. 2, pp. 43–6, 2007.

[35] J. W. Nicholson, *Polymers*. The Royal Society of Chemistry, 2002, ch. 2, pp. 25–62.

[36] R. Chiu and S. B. Goodman, *Biological response of osteoblasts and osteoprogenitors to orthopaedic wear debris*. InTech, 2012, ch. 8, pp. 183–222.

[37] Y. Kwon, A. V. Lombardi, J. J. Jacobs, T. K. Fehring, C. G. Lewis, and M. E. Cabanela, "Risk stratification algorithm for management of patients with metal-on-metal hip arthroplasty: consensus statement of the American Association of Hip and Knee Surgeons, the American Academy of Orthopaedic Surgeons, and the Hip Society," *J. Bone Joint Surg. Am.*, vol. 96, no. 1, p. e4, Jan. 2014.

[38] A. G. Cobb and T. P. Schmalzreid, "The clinical significance of metal ion release from cobalt-chromium metal-on-metal hip joint arthroplasty," *Proc. Inst. Mech. Eng., H J. eng. med.*, vol. 220, no. 2, pp. 385–98, Feb. 2006.

[39] J. D. Bobyn, M. Tanzer, J. J. Krygier, A. R. Dujovne, and C. E. Brooks, "Concerns with modularity in total hip arthroplasty," *Clin. Orthop. Relat. Res.*, vol. 298, pp. 27–36, Jan. 1994.

[40] H. J. Cooper, "The local effects of metal corrosion in total hip arthroplasty," *Orthop. Clin. North Am.*, vol. 45, no. 1, pp. 9–18, Jan. 2014.

[41] J. L. Gilbert, C. A. Buckley, and J. J. Jacobs, "In vivo corrosion of modular hip prosthesis components in mixed and similar metal combinations. the effect of crevice, stress, motion, and alloy coupling," *J. Biomed. Mater. Res.*, vol. 27, no. 12, pp. 1533–44, Dec. 1993.

[42] A. Panagiotidou, J. Meswania, J. Hua, S. Muirhead-Allwood, A. Hart, and G. Blunn, "Enhanced wear and corrosion in modular tapers in total hip replacement is associated with the contact area and surface topography," *J. Orthop. Res.*, vol. 31, no. 12, pp. 2032–9, Dec. 2013.

[43] R. Pivec, R. M. Meneghini, W. J. Hozack, G. H. Westrich, and M. A. Mont, "Modular taper junction corrosion and failure: how to approach a recalled total hip arthroplasty implant," *J. Arthroplasty*, vol. 29, no. 1, pp. 1–6, Jan. 2014.

[44] I. Catelas, A. Petit, D. J. Zukor, J. Antoniou, and O. L. Huk, "TNF-alpha secretion and macrophage mortality induced by cobalt and chromium ions in vitro-qualitative analysis of apoptosis," *Biomaterials*, vol. 24, no. 3, pp. 383–91, Feb. 2003.

[45] A. J. Hart, P. D. Quinn, B. Sampson, A. Sandison, K. D. Atkinson, J. A. Skinner, J. J. Powell, and J. F. W. Mosselmans, "The chemical form of metallic debris in tissues surrounding metal-on-metal hips with unexplained failure," *Acta Biomater.*, vol. 6, no. 11, pp. 4439–46, Nov. 2010.

[46] A. J. Hart, P. D. Quinn, F. Lali, B. Sampson, J. A. Skinner, J. J. Powell, J. Nolan, K. Tucker, S. Donell, A. Flanagan, and J. F. W. Mosselmans, "Cobalt from metal-on-metal hip replacements may be the clinically relevant active agent responsible for periprosthetic tissue reactions," *Acta Biomater.*, vol. 8, no. 10, pp. 3865–73, Oct. 2012.

[47] J. J. Jacobs, A. K. Skipor, L. M. Patterson, N. J. Hallab, W. G. Paprosky, J. Black, and J. O. Galante, "Metal release in patients who have had a primary total hip arthroplasty. A prospective, controlled, longitudinal study," *J. Bone Joint Surg. Am.*, vol. 80, no. 10, pp. 1447–58, Oct. 1998.

[48] I. Catelas, J. D. Bobyn, J. J. Medley, D. J. Zukor, A. Petit, and O. L. Huk, "Effects of digestion protocols on the isolation and characterization of metal-metal wear particles. II. Analysis of ion release and particle composition," *J. Biomed. Mater. Res.*, vol. 55, no. 3, pp. 330–7, Jun. 2001.

[49] I. Catelas, J. D. Bobyn, J. B. Medley, J. J. Krygier, D. J. Zukor, and O. L. Huk, "Size, shape, and composition of wear particles from metal-metal hip simulator testing: effects of alloy and number of loading cycles," *J. Biomed. Mater. Res. A*, vol. 67, no. 1, pp. 312–27, Oct. 2003.

[50] I. Catelas, P. A. Campbell, J. D. Bobyn, J. B. Medley, and O. L. Huk, "Wear particles from metal-on-metal total hip replacements: effects of implant design and implantation time," *Proc. Inst. Mech. Eng., H J. eng. med.*, vol. 220, no. 2, pp. 195–208, Feb. 2006.

[51] Australian Orthopaedic Association National Joint Replacement Registry, "Hip and knee arthroplasty: annual report 2013," Australian Orthopaedic Association (AOA), Tech. Rep., 2013.

[52] National Joint Registry (NJR), "10th annual report of the national joint registry for england, wales and northern ireland," National Joint Registry for England, Wales and Northern Ireland, Tech. Rep., 2013.

[53] G. Garellick, J. Kärrholm, C. Rogmark, and P. Herberts, "Swedish hip arthroplasty register: Annual report 2010," Swedish Hip Arthroplasty Register, Tech. Rep., 2010.

[54] I. Catelas and J. J. Jacobs, "Biologic activity of wear particles," *Instr. Course Lect.*, vol. 59, pp. 3–16, Jan. 2010.

[55] L. D. Dorr, Z. Wan, D. B. Longjohn, B. Dubois, and R. Murken, "Total hip arthroplasty with use of the Metasul metal-on-metal articulation. four to seven-year results," *J. Bone Joint Surg. Am.*, vol. 82, no. 6, pp. 789–98, Jun. 2000.

[56] T. P. Schmalzried, M. Jasty, and W. H. Harris, "Periprosthetic bone loss in total hip arthroplasty. Polyethylene wear debris and the concept of the effective joint space," *J. Bone Joint Surg. Am.*, vol. 74, no. 6, pp. 849–63, Jul. 1992.

[57] S. Wang, S. Zhang, and Y. Zhao, "A comparison of polyethylene wear between cobalt-chrome ball heads and alumina ball heads after total hip arthroplasty: a 10-year follow-up," *J. Orthop. Surg. Res.*, vol. 8, no. 20, pp. 1–4, Jul. 2013.

[58] S. B. Goodman, E. Gómez Barrena, M. Takagi, and Y. T. Konttinen, "Biocompatibility of total joint replacements: A review," *J. Biomed. Mater. Res. A*, vol. 90, no. 2, pp. 603–618, Aug. 2009.

[59] S. Kurtz and M. Manley, *Cross-Linked Polyethylene*, first edit ed. Elsevier, 2009, ch. 62, pp. 456–467.

[60] G. E. R. Thomas, D. J. Simpson, S. Mehmood, A. Taylor, P. McLardy-Smith, H. S. Gill, D. W. Murray, and S. Glyn-Jones, "The seven-year wear of highly cross-linked polyethylene in total hip arthroplasty: a double-blind, randomized controlled trial using radiostereometric analysis," *J. Bone Joint Surg. Am.*, vol. 93, no. 8, pp. 716–22, Apr. 2011.

[61] Australian Orthopaedic Association National Joint Replacement Registry, "Metal and ceramic bearing surface in total conventional hip arthroplasty," Australian Orthopaedic Association (AOA), Tech. Rep., 2013.

[62] A. Hatton, J. E. Nevelos, A. A. Nevelos, R. E. Banks, J. Fisher, and E. Ingham, "Alumina-alumina artificial hip joints. Part I: a histological analysis and characterisation of wear debris by laser capture microdissection of tissues retrieved at revision," *Biomaterials*, vol. 23, no. 16, pp. 3429–40, Aug. 2002.

[63] J. L. Tipper, A. Hatton, J. E. Nevelos, E. Ingham, C. Doyle, R. Streicher, A. B. Nevelos, and J. Fisher, "Alumina-alumina artificial hip joints. Part II: characterisation of the wear debris from in vitro hip joint simulations," *Biomaterials*, vol. 23, no. 16, pp. 3441–8, Aug. 2002.

[64] H. Krishnan, S. P. Krishnan, G. Blunn, J. A. Skinner, and A. J. Hart, "Modular neck femoral stems," *Bone. Joint. J.*, vol. 95-B, no. 8, pp. 1011–21, Aug. 2013.

[65] E. M. Schwarz, D. Campbell, S. Totterman, A. Boyd, R. J. O'Keefe, and R. J. Looney, "Use of volumetric computerized tomography as a primary outcome measure to evaluate drug efficacy in the prevention of peri-prosthetic osteolysis: a 1-year clinical pilot of etanercept vs. placebo," *J. Orthop. Res.*, vol. 21, no. 6, pp. 1049–55, Nov. 2003.

[66] B. J. R. F. Bolland, D. J. Culliford, D. J. Langton, J. P. S. Millington, N. K. Arden, and J. M. Latham, "High failure rates with a large-diameter hybrid metal-on-metal total hip replacement: clinical, radiological and retrieval analysis," *J. Bone Joint Surg. Br.*, vol. 93, no. 5, pp. 608–15, May 2011.

[67] I. J. Leslie, S. Williams, G. Isaac, E. Ingham, and J. Fisher, "High cup angle and microseparation increase the wear of hip surface replacements," *Clin. Orthop. Relat. Res.*, vol. 467, no. 9, pp. 2259–65, Sep. 2009.

[68] National Joint Registry (NJR), "6th annual report of the national joint registry for england, wales and northern ireland," National Joint Registry for England, Wales and Northern Ireland, Tech. Rep., 2009.

[69] D. Naudie, C. P. Roeder, J. Parvizi, D. J. Berry, S. Eggli, and A. Busato, "Metal-on-metal versus metal-on-polyethylene bearings in total hip arthroplasty: a matched case-control study," *J. Arthroplasty*, vol. 19, no. 7 Suppl 2, pp. 35–41, Oct. 2004.

[70] J. Charnley, "Fracture of femoral prostheses in total hip replacement. a clinical study," *Clin. Orthop. Relat. Res.*, vol. 111, pp. 105–20, Sep. 1975.

[71] G. Holt, C. Murnagham, J. Reilly, and R. M. D. Meek, "The biology of aseptic osteolysis," *Clin. Orthop. Relat. Res.*, vol. 460, pp. 240–52, Jul. 2007.

[72] H. C. Amstutz, *Hip resurfacing principles, indications, technique and results*. Philadelphia: Saunders Elsevier, 2008.

[73] D. R. Haynes, T. N. Crotti, and H. Zreiqat, "Regulation of osteoclast activity in peri-implant tissues," *Biomaterials*, vol. 25, no. 20, pp. 4877–85, Sep. 2004.

[74] J. Gallo, J. Vaculova, S. B. Goodman, Y. T. Konttinen, and J. P. Thyssen, "Contributions of human tissue analysis to understanding the mechanisms of loosening and osteolysis in total hip replacement," *Acta Biomater.*, vol. 10, no. 6, pp. 2354–2366, Jun. 2014.

[75] Y. Kwon, S. J. Ostlere, P. McLardy-Smith, N. A. Athanasou, H. S. Gill, and D. W. Murray, "Asymptomatic pseudotumors after metal-on-metal hip resurfacing arthroplasty: prevalence and metal ion study," *J. Arthroplasty*, vol. 26, no. 4, pp. 511–8, Jun. 2011.

[76] P. F. Doorn, J. M. Mirra, P. A. Campbell, and H. C. Amstutz, "Tissue reaction to metal on metal total hip prostheses," *Clin. Orthop. Relat. Res.*, vol. 329 Suppl, pp. S187–205, Aug. 1996.

[77] I. Catelas, P. A. Campbell, F. Dorey, A. Frausto, B. G. Mills, and H. C. Amstutz, "Semi-quantitative analysis of cytokines in MM THR tissues and their relationship to metal particles," *Biomaterials*, vol. 24, no. 26, pp. 4785–97, Nov. 2003.

[78] P. A. Campbell, M. Wang, H. C. Amstutz, and S. B. Goodman, "Positive cytokine production in failed metal-on-metal total hip replacements," *Acta Orthop. Scand.*, vol. 73, no. 5, pp. 506–12, Oct. 2002.

[79] P. Campbell, E. Ebramzadeh, S. Nelson, K. Takamura, K. De Smet, and H. C. Amstutz, "Histological features of pseudotumor-like tissues from metal-on-metal hips," *Clin. Orthop. Relat. Res.*, vol. 468, no. 9, pp. 2321–7, Sep. 2010.

[80] H. Willert, G. H. Buchhorn, A. Fayyazi, F. Renata, M. Windler, G. Köster, and C. H. Lohmann, "Metal-on-metal bearings and hypersensitivity in patients with artificial hip joints. A clinical and histomorphological study," *J. Bone Joint Surg. Am.*, vol. 87, no. 1, pp. 28–36, Jan. 2005.

[81] D. R. Haynes, T. N. Crotti, a. E. Potter, M. Loric, G. J. Atkins, D. W. Howie, and D. M. Findlay, "The osteoclastogenic molecules RANKL and RANK are associated with periprosthetic osteolysis," *J. Bone Joint Surg. Br.*, vol. 83, no. 6, pp. 902–11, Aug. 2001.

[82] G. M. Keegan, I. D. Learmonth, and C. P. Case, "A systematic comparison of the actual, potential, and theoretical health effects of cobalt and chromium exposures from industry and surgical implants," *Crit. Rev. Toxicol.*, vol. 38, no. 8, pp. 645–74, 2008.

[83] T. Lin, Y. Tamaki, J. Pajarinen, H. A. Waters, D. K. Woo, Z. Yao, and S. B. Goodman, "Chronic inflammation in biomaterial-induced periprosthetic osteolysis: NF-κB as a therapeutic target," *Acta Biomater.*, vol. 10, no. 1, pp. 1–10, Jan. 2014.

[84] Y. Nakashima, D. H. Sun, M. C. Trindade, W. J. Maloney, S. B. Goodman, D. J. Schurman, and R. L. Smith, "Signaling pathways for tumor necrosis factor-alpha and interleukin-6 expression in human macrophages exposed to titanium-alloy particulate debris in vitro," *J. Bone Joint Surg. Am.*, vol. 81, no. 5, pp. 603–615, May 1999.

[85] S. B. Goodman, "Wear particles, periprosthetic osteolysis and the immune system," *Biomaterials*, vol. 28, no. 34, pp. 5044–5048, Dec. 2007.

[86] N. J. Hallab, M. Caicedo, K. McAllister, A. Skipor, H. Amstutz, and J. J. Jacobs, "Asymptomatic prospective and retrospective cohorts with metal-on-metal hip arthroplasty indicate acquired lymphocyte reactivity varies with metal ion levels on a group basis," *J. Orthop. Res.*, vol. 31, no. 2, pp. 173–82, Feb. 2013.

[87] E. Ingham and J. Fisher, "The role of macrophages in osteolysis of total joint replacement," *Biomaterials*, vol. 26, no. 11, pp. 1271–1286, Apr. 2005.

[88] N. Ishiguro, T. Kojima, T. Ito, S. Saga, H. Anma, K. Kurokouchi, Y. Iwahori, T. Iwase, and H. Iwata, "Macrophage activation and migration in interface tissue around loosening total hip arthroplasty components," *J. Biomed. Mater. Res.*, vol. 35, no. 3, pp. 399–406, Jun. 1997.

[89] P. Koulouvaris, K. Ly, L. B. Ivashkiv, M. P. Bostrom, B. J. Nestor, T. P. Sculco, and P. E. Purdue, "Expression profiling reveals alternative macrophage activation and impaired osteogenesis in periprosthetic osteolysis," *J. Orthop. Res.*, vol. 26, no. 1, pp. 106–116, Jan. 2008.

[90] Y. Nakashima, D. H. Sun, M. Trindade, L. E. Chun, Y. Song, S. B. Goodman, D. J. Schurman, W. J. Maloney, and R. L. Smith, "Induction of macrophage C-C chemokine expression by titanium alloy and bone cement particles," *J. Bone Joint Surg. Br.*, vol. 81, no. 1, pp. 155–62, Jan. 1999.

[91] E. M. Greenfield, Y. Bi, A. A. Ragab, V. M. Goldberg, and R. R. Van De Motter, "The role of osteoclast differentiation in aseptic loosening," *J. Orthop. Res.*, vol. 20, no. 1, pp. 1–8, Jan. 2002.

[92] N. J. Hallab and J. J. Jacobs, "Biologic effects of implant debris," *Bull. NYU Hosp. Jt. Dis.*, vol. 67, no. 2, pp. 182–8, 2009.

[93] C. Bresson, C. Darolles, A. Carmona, C. Gautier, N. Sage, S. Roudeau, R. Ortega, E. Ansoborlo, and V. Malard, "Cobalt chloride speciation, mechanisms of cytotoxicity on human pulmonary cells, and synergistic toxicity with zinc," *Metallomics*, vol. 5, no. 2, pp. 133–43, Feb. 2013.

[94] N. J. Hallab, K. Mikecz, C. Vermes, A. Skipor, and J. J. Jacobs, "Orthopaedic implant related metal toxicity in terms of human lymphocyte reactivity to metalprotein complexes produced from cobalt-base and titanium-base implant alloy degradation," *Mol. Cell. Biochem.*, vol. 222, pp. 127–36, Mar. 2001.

[95] C. Tkaczyk, O. L. Huk, F. Mwale, J. Antoniou, D. J. Zukor, A. Petit, and M. Tabrizian, "The molecular structure of complexes formed by chromium or cobalt ions in simulated physiological fluids," *Biomaterials*, vol. 30, no. 4, pp. 460–7, Feb. 2009.

[96] J. Yang and J. Black, "Competitive binding of chromium, cobalt and nickel to serum proteins," *Biomaterials*, vol. 15, no. 4, pp. 262–268, Mar. 1994.

[97] S. Natu, R. P. Sidaginamale, J. Gandhi, D. J. Langton, and A. V. F. Nargol, "Adverse reactions to metal debris: histopathological features of periprosthetic soft tissue reactions seen in association with failed metal on metal hip arthroplasties," *J. Clin. Pathol.*, vol. 65, no. 5, pp. 409–18, May 2012.

[98] V. Nikolaou, S. G. Bergeron, O. L. Huk, D. J. Zukor, and J. Antoniou, "Evaluation of persistent pain after hip resurfacing," *Bull. NYU Hosp. Jt. Dis.*, vol. 67, no. 2, pp. 168–172, 2009.

[99] H. Pandit, S. Glyn-Jones, P. McLardy-Smith, R. Gundle, D. Whitwell, C. L. M. Gibbons, S. Ostlere, N. Athanasou, H. S. Gill, and D. W. Murray, "Pseudotumours associated with metal-on-metal hip resurfacings," *J. Bone Joint Surg. Br.*, vol. 90, no. 7, pp. 847–851, Jul. 2008.

[100] P. A. Campbell, M. S. Kung, A. R. Hsu, and J. J. Jacobs, "Do retrieval analysis and blood metal measurements contribute to our understanding of adverse local tissue reactions?" *Clin. Orthop. Relat. Res.*, vol. Epub ahead of print, pp. 1–10, Aug. 2014.

[101] J. Antoniou, D. J. Zukor, F. Mwale, W. Minarik, A. Petit, and O. L. Huk, "Metal ion levels in the blood of patients after hip resurfacing: a comparison between twenty-eight and thirty-six-millimeter-head metal-on-metal prostheses," *J. Bone Joint Surg. Am.*, vol. 90 Suppl 3, pp. 142–8, Aug. 2008.

[102] H. S. Dobbs and M. Minski, "Metal ion release after total hip replacement," *Biomaterials*, vol. 1, no. 4, pp. 193–198, Oct. 1980.

[103] A. Hartmann, F. Hannemann, J. Lützner, A. Seidler, H. Drexler, K.-P. Günther, and J. Schmitt, "Metal ion concentrations in body fluids after implantation of hip replacements with metal-on-metal bearing–systematic review of clinical and epidemiological studies," *PLoS One*, vol. 8, no. 8 e70359, pp. 1–25, Aug. 2013.

[104] A. K. Skipor, P. A. Campbell, L. M. Patterson, H. C. Anstutz, T. P. Schmalzried, and J. J. Jacobs, "Serum and urine metal levels in patients with metal-on-metal surface arthroplasty," *J. Mater. Sci. Mater. Med.*, vol. 13, no. 12, pp. 1227–34, Dec. 2002.

[105] K. Merritt and S. A. Brown, "Effects of metal particles and ions on the biological system," *Techniques in Orthopaedics*, vol. 8, no. 4, pp. 228–236, 1993.

[106] P. C. Grevatt, "Toxicological Review of Trivalent Chromium (CAS No. 16065-83-1) In Support of Summary Information on the Integrated Risk Information System (IRIS)," U.S. Environmental Protection Agency, Washington, DC, Tech. Rep., Aug. 1998.

[107] R. G. Bitsch, M. Zamorano, T. Loidolt, C. Heisel, J. J. Jacobs, and T. P. Schmalzried, "Ion production and excretion in a patient with a metal-on-metal bearing hip prosthesis. a case report," *J. Bone Joint Surg. Am.*, vol. 89, no. 12, pp. 2758–63, Dec. 2007.

[108] C. Heisel, M. Silva, A. K. Skipor, J. J. Jacobs, and T. P. Schmalzreid, "The relationship between activity and ions in patients with metal-on-metal bearing hip prostheses," *J. Bone Joint Surg. Am.*, vol. 87, no. 4, pp. 781–7, Apr. 2005.

[109] M. Meftah, A. M. Haleem, M. B. Burn, K. M. Smith, and S. J. Incavo, "Early corrosion-related failure of the rejuvenate modular total hip replacement," *J. Bone Joint Surg. Am.*, vol. 96, no. 6, pp. 481–487, Mar. 2014.

[110] D. J. Langton, R. P. Sidaginamale, T. J. Joyce, S. Natu, P. Blain, R. D. Jefferson, S. Rushton, and A. V. F. Nargol, "The clinical implications of elevated blood metal ion concentrations in asymptomatic patients with MoM hip resurfacings: a cohort study," *BMJ open*, vol. 3, no. 3, p. e001541, Mar. 2013.

[111] J. J. Jacobs, A. K. Skipor, P. A. Campbell, N. J. Hallab, R. M. Urban, and H. C. Amstutz, "Can metal levels be used to monitor metal-on-metal hip arthroplasties?" *J. Arthroplasty*, vol. 19, no. 8 Suppl 3, pp. 59–65, Dec. 2004.

[112] R. B. Cook, B. J. R. F. Bolland, J. A. Wharton, S. Tilley, J. M. Latham, and R. J. K. Wood, "Pseudotumour formation due to tribocorrosion at the taper interface of large diameter metal on polymer modular total hip replacements," *J. Arthroplasty*, vol. 28, no. 8, pp. 1430–6, Sep. 2013.

[113] H. Cooper, C. Della Valle, R. Berger, M. Tetreault, W. Paprosky, S. Sporer, and J. Jacobs, "Corrosion at the head-neck taper as a cause for adverse local tissue reactions after total hip arthroplasty," *J. Bone Joint Surg. Am.*, vol. 94, no. 18, pp. 1655–61, Sep. 2012.

[114] H. J. Cooper, R. M. Urban, R. L. Wixson, R. M. Meneghini, and J. J. Jacobs, "Adverse local tissue reaction arising from corrosion at the femoral neck-body junction in a dual-taper stem with a cobalt-chromium modular neck," *J. Bone Joint Surg. Am.*, vol. 95, no. 10, pp. 865–72, May 2013.

[115] I. P. S. Gill, J. Webb, K. Sloan, and R. J. Beaver, "Corrosion at the neck-stem junction as a cause of metal ion release and pseudotumour formation," *J. Bone Joint Surg. Br.*, vol. 94, no. 7, pp. 895–900, Jul. 2012.

[116] G. B. Higgs, J. A. Hanzlik, D. W. MacDonald, J. L. Gilbert, C. M. Rimnac, and S. M. Kurtz, "Is increased modularity associated with increased fretting and corrosion damage in metal-on-metal total hip arthroplasty devices?: a retrieval study," *J. Arthroplasty*, vol. 28, no. 8 Suppl, pp. 2–6, Sep. 2013.

[117] N. Hallab, K. Merritt, and J. J. Jacobs, "Metal sensitivity in patients with orthopaedic implants," *J. Bone Joint Surg. Am.*, vol. 83-A, no. 3, pp. 428–36, Mar. 2001.

[118] J. G. Cahir, A. P. Toms, T. J. Marshall, J. Wimhurst, and J. Nolan, "CT and MRI of hip arthroplasty," *Clin. Radiol.*, vol. 62, no. 12, pp. 1163–71; discussion 1172–3, 2007.

[119] D. S. Garbuz, B. A. Hargreaves, C. P. Duncan, B. A. Masri, D. R. Wilson, and B. B. Forster, "The John Charnley Award: Diagnostic accuracy of MRI versus ultrasound for detecting pseudotumors in asymptomatic metal-on-metal THA," *Clin. Orthop. Relat. Res.*, vol. 472, no. 2, pp. 417–423, Feb. 2014.

[120] R. J. Looney, A. Boyd, S. Totterman, G. Seo, J. Tamez-Pena, D. Campbell, L. Novotny, C. Olcott, J. Martell, F. A. Hayes, R. J. O'Keefe, and E. M. Schwarz, "Volumetric computerized tomography as a measurement of periprosthetic acetabular osteolysis and its correlation with wear," *Arthritis Res.*, vol. 4, no. 1, pp. 59–63, Oct. 2002.

[121] A. Mistry, J. Cahir, S. T. Donell, J. Nolan, and A. P. Toms, "MRI of asymptomatic patients with metal-on-metal and polyethylene-on-metal total hip arthroplasties," *Clin. Radiol.*, vol. 66, no. 6, pp. 540–545, Jun. 2011.

[122] I. Catelas, A. Petit, D. J. Zukor, and O. L. Huk, "Cytotoxic and apoptotic effects of cobalt and chromium ions on J774 macrophages - Implication of caspase-3 in the apoptotic pathway," *J. Mater. Sci.: Mater. Med.*, vol. 12, no. 10-12, pp. 949–53, 2001.

[123] I. Catelas, A. Petit, H. Vali, C. Fragiskatos, R. Meilleur, D. J. Zukor, J. Antoniou, and O. L. Huk, "Quantitative analysis of macrophage apoptosis vs. necrosis induced by cobalt and chromium ions in vitro," *Biomaterials*, vol. 26, no. 15, pp. 2441–53, May 2005.

[124] C. Fleury, A. Petit, F. Mwale, J. Antoniou, D. J. Zukor, M. Tabrizian, and O. L. Huk, "Effect of cobalt and chromium ions on human MG-63 osteoblasts in vitro: morphology, cytotoxicity, and oxidative stress," *Biomaterials*, vol. 27, no. 18, pp. 3351–60, Jun. 2006.

[125] N. J. Hallab, C. Vermes, C. Messina, K. A. Roebuck, T. T. Glant, and J. J. Jacobs, "Concentration- and composition-dependent effects of metal ions on human MG-63 osteoblasts," *J. Biomed. Mater. Res.*, vol. 60, no. October, pp. 420–33, Jun. 2002.

[126] O. L. Huk, I. Catelas, F. Mwale, J. Antoniou, D. J. Zukor, and A. Petit, "Induction of apoptosis and necrosis by metal ions in vitro," *J. Arthroplasty*, vol. 19, no. 8, pp. 84–7, Dec. 2004.

[127] M. S. Caicedo, R. Desai, K. McAllister, A. Reddy, J. J. Jacobs, and N. J. Hallab, "Soluble and particulate Co-Cr-Mo alloy implant metals activate the inflammasome danger signaling pathway in human macrophages: a novel mechanism for implant debris reactivity," *J. Orthop. Res.*, vol. 27, no. 7, pp. 847–54, Jul. 2009.

[128] B. M. Devitt, J. M. Queally, M. Vioreanu, J. S. Butler, D. Murray, P. P. Doran, and J. M. O'Byrne, "Cobalt ions induce chemokine secretion in a variety of systemic cell lines," *Acta Orthop.*, vol. 81, no. 6, pp. 756–64, Dec. 2010.

[129] H. Lawrence, D. Deehan, J. Holland, J. Kirby, and A. Tyson-Capper, "The immunobiology of cobalt: demonstration of a potential aetiology for inflammatory pseudotumours after metal-on-metal replacement of the hip," *Bone Joint J*, vol. 96-B, no. 9, pp. 1172–1177, Sep. 2014.

[130] J. M. Queally, B. M. Devitt, J. S. Butler, A. P. Malizia, D. Murray, P. P. Doran, and J. M. O'Byrne, "Cobalt ions induce chemokine secretion in primary human osteoblasts," *J. Orthop. Res.*, vol. 27, no. 7, pp. 855–64, Jul. 2009.

[131] J. Wang, B. Wicklund, R. Gustilo, and D. Tsukayama, "Titanium, chromium and cobalt ions modulate the release of bone-associated cytokines by human monocytes/macrophages in vitro," *Biomaterials*, vol. 17, no. 23, pp. 2233–40, Dec. 1996.

[132] S. J. Baskey, P. E. Beaulé, E. A. Lehoux, and I. Catelas, "Simvastatin modulates the release of TNF–α and CC chemokines from macrophages exposed to trivalent chromium ions," *J. Biomater. Tissue Eng.*, vol. 4, no. 11, pp. 981–991, Nov. 2014.

[133] D. Granchi, E. Verri, G. Ciapetti, L. Savarino, E. Cenni, A. Gori, and A. Pizzoferrato, "Effects of chromium extract on cytokine release by mononuclear cells," *Biomaterials*, vol. 19, no. 1-3, pp. 283–91, Jan-Feb 1998.

[134] R. E. Andrews, K. M. Shah, J. M. Wilkinson, and A. Gartland, "Effects of cobalt and chromium ions at clinically equivalent concentrations after metal-on-metal hip replacement on human osteoblasts and osteoclasts: Implications for skeletal health," *Bone*, vol. 49, no. 4, pp. 717–23, Oct. 2011.

[135] G. J. Thompson and D. A. Puleo, "Effects of sublethal metal ion concentrations on osteogenic cells derived from bone marrow stromal cells," *J. Appl. Biomater.*, vol. 6, no. 4, pp. 249–258, 1995.

[136] W. P. Zijlstra, S. K. Bulstra, J. J. A. M. van Raay, B. M. van Leeuwen, and R. Kuijer, "Cobalt and chromium ions reduce human osteoblast-like cell activity in vitro, reduce the OPG to RANKL ratio, and induce oxidative stress," *J. Orthop. Res.*, vol. 30, no. 5, pp. 740–747, May 2012.

[137] O. L. Huk, D. J. Zukor, W. Ralston, A. Lisbona, and A. Petit, "Apoptosis in interface membranes of aseptically loose total hip arthroplasty," *J Mater Sci Mater Med*, vol. 12, no. 7, pp. 653–658, Jul. 2001.

[138] N. J. Hallab, K. Mikecz, and J. J. Jacobs, "A triple assay technique for the evaluation of metal-induced, delayed-type hypersensitivity responses in patients with or receiving total joint arthroplasty," *J. Biomed. Mater. Res.*, vol. 53, no. 5, pp. 480–9, Sep. 2000.

[139] L. Sompayrac, *How the immune system works*, 3rd ed. Blackwell Publishing, 2008.

[140] C. Thomas, Ed., *Taber's cyclopedic medical dictionary*, 18th ed., ser. Taber's Cyclopedic Medical Dictionary. Philadelphia: F.A. Davis Company, 1997, also traced: Clarence Wilbur Taber.

[141] R. S. Tuan, F. Y. Lee, Y. T. Konttinen, J. M. Wilkinson, and R. L. Smith, "What are the local and systemic biologic reactions and mediators to wear debris, and what host factors determine or modulate the biologic response to wear particles?" *J. Am. Acad. Orthop. Surg.*, vol. 16 Suppl 1, pp. S42–S48, 2008.

[142] D. D. Chaplin, "Overview of the immune response," *J. Allergy Clin. Immunol.*, vol. 125, no. 2 Suppl 2, pp. S3–23, Feb. 2010.

[143] J. S. Duffield, "The inflammatory macrophage: a story of Jekyll and Hyde," *Clin Sci (Lond)*, vol. 104, no. 1, pp. 27–38, Jan. 2003.

[144] S. Gordon, "Alternative activation of macrophages," *Nat. Rev. Immunol.*, vol. 3, no. 1, pp. 23–35, Jan. 2003.

[145] S. Gordon and F. O. Martinez, "Alternative activation of macrophages: mechanism and functions," *Immunity*, vol. 32, no. 5, pp. 593–604, May 2010.

[146] F. O. Martinez and S. Gordon, "The M1 and M2 paradigm of macrophage activation: time for reassessment," *F1000Prime Rep*, vol. 6, p. 13, 2014.

[147] N. Baumgarth, "The double life of a B-1 cell: self-reactivity selects for protective effector functions," *Nat. Rev. Immunol.*, vol. 11, no. 1, pp. 34–46, Jan. 2011.

[148] J. Fan, N. M. Heller, M. Gorospe, U. Atasoy, and C. Stellato, "The role of post-transcriptional regulation in chemokine gene expression in inflammation and allergy," *Eur. Respir. J.*, vol. 26, no. 5, pp. 933–947, Nov. 2005.

[149] S. B. Goodman and T. Ma, "Cellular chemotaxis induced by wear particles from joint replacements," *Biomaterials*, vol. 31, no. 19, pp. 5045–50, 2010.

[150] D. Rossi and A. Zlotnik, "The biology of chemokines and their receptors," *Annu. Rev. Immunol.*, vol. 18, pp. 217–242, 2000.

[151] C. A. Piccirillo, E. Bjur, I. Topisirovic, N. Sonenberg, and O. Larsson, "Translational control of immune responses: from transcripts to translatomes," *Nat. Immunol.*, vol. 15, no. 6, pp. 503–511, Jun. 2014.

[152] A. Yadav, V. Saini, and S. Arora, "MCP-1: chemoattractant with a role beyond immunity: a review," *Clin. Chim. Acta*, vol. 411, no. 21-22, pp. 1570–1579, Nov. 2010.

[153] M. Abe, K. Hiura, J. Wilde, K. Moriyama, T. Hashimoto, S. Ozaki, S. Wakatsuki, M. Kosaka, S. Kido, D. Inoue, and T. Matsumoto, "Role for macrophage inflammatory protein (MIP)-1α and MIP-1β in the development of osteolytic lesions in multiple myeloma," *Blood*, vol. 100, no. 6, pp. 2195–2202, 2002.

[154] U. Heider, L. C. Hofbauer, I. Zavrski, M. Kaiser, C. Jakob, and O. Sezer, "Novel aspects of osteoclast activation and osteoblast inhibition in myeloma bone disease," *Biochem. Biophys. Res. Commun.*, vol. 338, no. 2, pp. 687–693, Dec. 2005.

[155] S. J. Choi, J. C. Cruz, F. Craig, H. Chung, R. D. Devlin, G. D. Roodman, and M. Alsina, "Macrophage inflammatory protein 1-alpha is a potential osteoclast stimulatory factor in multiple myeloma." *Blood*, vol. 96, no. 2, pp. 671–675, Jul. 2000.

[156] K. Fuller, J. M. Owens, and T. J. Chambers, "Macrophage inflammatory protein-1 alpha and il-8 stimulate the motility but suppress the resorption of isolated rat osteoclasts," *J. Immunol.*, vol. 154, no. 11, pp. 6065–6072, Jun. 1995.

[157] K. Conlon, A. Lloyd, U. Chattopadhyay, N. Lukacs, S. Kunkel, T. Schall, D. Taub, C. Morimoto, J. Osborne, J. Oppenheim, H. Young, D. Kelvin, and J. Ortaldo, "CD8+ and CD45RA+ human peripheral blood lymphocytes are potent sources of macrophage inflammatory protein 1 alpha, interleukin-8 and RANTES," *Eur. J. Immunol.*, vol. 25, no. 3, pp. 751–6, Mar. 1995.

[158] X. Yu, Y. Huang, P. Collin-Osdoby, and P. Osdoby, "CCR1 chemokines promote the chemotactic recruitment, RANKL development, and motility of osteoclasts and are induced by inflammatory cytokines in osteoblasts," *J. Bone Miner. Res.*, vol. 19, no. 12, pp. 2065–2077, Dec. 2004.

[159] S. B. Goodman, M. Trindade, T. Ma, M. Genovese, and R. L. Smith, "Pharmacologic modulation of periprosthetic osteolysis," *Clin. Orthop. Relat. Res.*, vol. 430, pp. 39–45, Jan. 2005.

[160] E. M. Schwarz, "What potential biologic treatments are available for osteolysis?" *J. Am. Acad. Orthop. Surg.*, vol. 16, no. (suppl 1), pp. S72–S75, 2008.

[161] M. L. Wang, P. F. Sharkey, and R. S. Tuan, "Particle bioreactivity and wear-mediated osteolysis," *J. Arthroplasty*, vol. 19, no. 8, pp. 1028–1038, Dec. 2004.

[162] K. Ren, A. Dusad, Y. Zhang, and D. Wang, "Therapeutic intervention for wear debris-induced aseptic implant loosening," *Acta Pharmaceutica Sinica B*, vol. 3, no. 2, pp. 76–85, Apr. 2013.

[163] M. L. Wang, P. V. Hauschka, R. S. Tuan, and M. J. Steinbeck, "Exposure to particles stimulates superoxide production by human THP-1 macrophages and avian HD-11EM osteoclasts activated by tumor necrosis factor-alpha and PMA," *J. Arthroplasty*, vol. 17, no. 3, pp. 335–346, Apr. 2002.

[164] T. Lawrence, "The nuclear factor NF-kappaB pathway in inflammation," *Cold Spring Harb Perspect Biol*, vol. 1, no. 6, p. a001651, Dec. 2009.

[165] S. B. Goodman, E. Gibon, J. Pajarinen, T. Lin, M. Keeney, P. Ren, C. Nich, Z. Yao, K. Egashira, F. Yang, and Y. T. Konttinen, "Novel biological strategies for treatment of wear particle-induced periprosthetic osteolysis of orthopaedic implants for joint replacement," *J. R. Soc. Interface*, vol. 11, no. 93, p. 20130962, Apr. 2014.

[166] N. Udagawa, N. Takahashi, H. Yasuda, A. Mizuno, K. Itoh, Y. Ueno, T. Shinki, M. T. Gillespie, T. J. Martin, K. Higashio, and T. Suda, "Osteoprotegerin produced by osteoblasts is an important regulator in osteoclast development and function," *Endocrinology*, vol. 141, no. 9, pp. 3478–3484, Sep. 2000.

[167] A. Lübbeke, G. Garavaglia, K. J. Rothman, A. Bonvin, C. Roussos, H. Miozzari, and P. Hoffmeyer, "Statins may reduce femoral osteolysis in patients with total hip arthroplasty," *J. Orthop. Res.*, vol. 31, no. 5, pp. 814–820, May 2013.

[168] T. M. Thillemann, A. B. Pedersen, F. Mehnert, S. P. Johnsen, and K. Søballe, "The risk of revision after primary total hip arthroplasty among statin users: a nationwide population-based nested case-control study," *J. Bone Joint Surg. Am.*, vol. 92, no. 5, pp. 1063–72, May 2010.

[169] M. Djaldetti, H. Salman, M. Bergman, and H. Bessler, "Effect of pravastatin, simvastatin and atorvastatin on the phagocytic activity of mouse peritoneal macrophages," *Exp. Mol. Pathol.*, vol. 80, no. 2, pp. 160–164, Apr. 2006.

[170] B. P. Leung, N. Sattar, A. Crilly, M. Prach, D. W. McCarey, H. Payne, R. Madhok, C. Campbell, J. A. Gracie, F. Y. Liew, and I. B. McInnes, "A novel anti-inflammatory role for simvastatin in inflammatory arthritis," *J. Immunol.*, vol. 170, no. 3, pp. 1524–1530, Feb. 2003.

[171] G. Weitz-Schmidt, "Statins as anti-inflammatory agents," *Trends Pharmacol. Sci.*, vol. 23, no. 10, pp. 482–6, Oct. 2002.

[172] A. Hughes, M. J. Rogers, A. I. Idris, and J. C. Crockett, "A comparison between the effects of hydrophobic and hydrophilic statins on osteoclast function in vitro and ovariectomy-induced bone loss in vivo," *Calcif. Tissue Int.*, vol. 81, no. 5, pp. 403–13, Nov. 2007.

[173] G. Mundy, "Inflammatory mediators and the destruction of bone," *J. Periodontal Res.*, vol. 26, no. 3 Pt 2, pp. 213–7, May 1991.

[174] Y. Ayukawa, Y. Ogino, Y. Moriyama, I. Atsuta, Y. Jinno, M. Kihara, Y. Tsukiyama, and K. Koyano, "Simvastatin enhances bone formation around titanium implants in rat tibiae," *J. Oral Rehabil.*, vol. 37, no. 2, pp. 123–30, Feb. 2010.

[175] K. Başarir, B. Erdemli, A. Can, E. Erdemli, and T. Zeyrek, "Osseointegration in arthroplasty: can simvastatin promote bone response to implants?" *Int. Orthop.*, vol. 33, no. 3, pp. 855–9, Jul. 2009.

[176] F. von Knoch, C. Wedemeyer, A. Heckelei, G. Saxler, G. Hilken, J. Brankamp, T. Sterner, S. Landgraeber, F. Henschke, F. Löer, and M. von Knoch, "Promotion of bone formation by simvastatin in polyethylene particle-induced osteolysis," *Biomaterials*, vol. 26, no. 29, pp. 5783–9, Oct. 2005.

[177] F. von Knoch, A. Heckelei, C. Wedemeyer, G. Saxler, G. Hilken, F. Henschke, F. Löer, and M. von Knoch, "The effect of simvastatin on polyethylene particle-induced osteolysis," *Biomaterials*, vol. 26, no. 17, pp. 3549–55, Jun. 2005.

[178] T. Tanigo, R. Takaoka, and Y. Tabata, "Sustained release of water-insoluble simvastatin from biodegradable hydrogel augments bone regeneration," *J. Controlled Release*, vol. 143, no. 2, pp. 201–6, May 2010.

[179] G. Vallés, C. Pérez, A. Boré, F. Martín-Saavedra, L. Saldaña, and N. Vilaboa, "Simvastatin prevents the induction of interleukin-6 gene expression by titanium particles in human osteoblastic cells," *Acta Biomater.*, vol. 9, no. 1, pp. 4916–25, Jan. 2013.

[180] N. R. Veillard, V. Braunersreuther, C. Arnaud, F. Burger, G. Pelli, S. Steffens, and F. Mach, "Simvastatin modulates chemokine and chemokine receptor expression by geranylgeranyl isoprenoid pathway in human endothelial cells and macrophages," *Atherosclerosis*, vol. 188, no. 1, pp. 51–8, Sep. 2006.

[181] J. Woo, H. Nakagawa, A. M. Krecic, K. Nagai, A. D. Hamilton, S. M. Sebti, and P. H. Stern, "Inhibitory effects of mevastatin and a geranylgeranyl transferase i inhibitor (GGTI-2166) on mononuclear osteoclast formation induced by receptor activator of NFκB ligand (RANKL) or tumor necrosis factor-α (TNF-α)," *Biochem. Pharmacol.*, vol. 69, no. 1, pp. 87–95, Jan. 2005.

[182] T. Maeda, A. Matsunuma, I. Kurahashi, T. Yanagawa, H. Yoshida, and N. Horiuchi, "Induction of osteoblast differentiation indices by statins in MC3T3-E1 cells," *J. Cell. Biochem.*, vol. 92, no. 3, pp. 458–71, Jun. 2004.

Part III

Manuscripts

Part III — Chapter 1

Effects of Cobalt and Chromium Ions on CC Chemokine Release from Macrophages and Migration of Lymphocytes *In Vitro*

A thinker sees his own actions as experiments and questions–as attempts to find out something. Success and failure are for him answers above all.

Friedrich Nietzsche

1.1 FOREWORD

As described in great detail in the literature review, the mechanisms by which Co^{2+} and Cr^{3+} in can mediate adverse tissue reactions, such as T cell-mediated type-IV hypersensitivity, remain poorly understood. The first two objectives of this thesis were to analyze the effects of Co^{2+} and Cr^{3+} on chemokine release by J774A.1 monocytes/macrophages and determine if Co^{2+}, Cr^{3+}, and the chemokines in cultures of J774A.1 monocytes/macrophages exposed to Co^{2+} and Cr^{3+} can induce the migration of T and B lymphocytes. In order to achieve these objectives, the effect of Co^{2+} and Cr^{3+} on macrophage CC chemokine release (MCP–1, MIP–1α, and RANTES) and on lymphocyte migration were studied. This study has formed the subject of a manuscript being finalized to be submitted to the Journal of Orthopaedic Research and is entitled:

"Effects of cobalt and chromium ions on CC chemokine release from macrophages and migration of lymphocytes *in vitro*", by Stephen J. Baskey, Eric A. Lehoux, Siu-Yan Lee, Paul E. Beaulé, and Isabelle Catelas.

1.2 ABSTRACT

Metal-on-metal bearings are the subject of clinical concern because of adverse tissue reactions including periprosthetic osteolysis, metal hypersensitivity, and pseudotumors. Previous *ex vivo* studies have observed chemokines such as MCP–1 and MIP–1α in periprosthetic tissues. Metal-related hypersensitivity is thought to be a type-IV hypersensitivity reaction and has been observed in some patients with CoCrMo implant components. However, the role of metal ions in initiating this reaction remains

largely unknown. Therefore, the objectives of this study were to analyze the effects of Co^{2+} (2–48 ppm) and Cr^{3+} (25–500 ppm) on the release of pro-inflammatory mediators (*i.e.*, TNF–α, MCP–1, MIP–1α, and RANTES) and on lymphocyte migration. Results showed an increase in TNF–α release up to 13-fold with 48 ppm Co^{2+} and 2.3-fold with 250 ppm Cr^{3+}. Results also demonstrated that the release of CC chemokines was ion-specific and dose-dependent. Specifically, MIP–1α release increased up to 1.5-fold with 24 ppm Co^{2+} and up to 1.9-fold with 150 ppm Cr^{3+}, while MCP–1 and RANTES release decreased, relative to the negative control, in the presence of Co^{2+} or Cr^{3+}. Finally, the number of migrated $TCR\beta^{+}$ lymphocytes (T cells) was significantly higher in the culture supernatants of macrophages exposed to Co^{2+} and Cr^{3+} than in the medium control (*i.e.*, cell culture medium without Co^{2+} and Cr^{3+}). However, it was similarly high in equilibrated medium containing Co^{2+} or Cr^{3+}, suggesting that the migration was directly stimulated by Co^{2+} and Cr^{3+} rather than the released chemokines. There was no significant migration of B cells in any of the conditions analyzed. Overall, results suggest the potential for Co^{2+} and Cr^{3+} to induce a T cell-dominated response, characteristic of a type-IV hypersensitivity reaction, as observed in periprosthetic tissues of some patients with CoCrMo implant components.

1.3 INTRODUCTION

Aseptic loosening due to periprosthetic osteolysis, primarily induced by the inflammatory response to implant wear and corrosion products, is the most common cause of hip implant failure [1]. Metal-on-metal (MM) hip implants have been considered as an alternative to conventional metal-on-polyethylene (MPE) implants because of their higher wear resistance, which was expected to correlate with a decrease in inflammation [2–7]. Since the mid-1980s, over one million patients worldwide have received a MM hip implant [8]. However, the greater overall amount of metal products, especially metal ions, released by MM implants compared to MPE implants, is a major cause for concern [9–16]. Some histological studies of MM periprosthetic tissues have shown evidence of metallic wear debris from corrosion, abrasion, and surface fatigue [17], along with elevated levels of cytokines and chemokines [18–24], and, in some cases, early adverse tissue reactions, including hypersensitivity and pseudotumors [20, 25–27]. Nevertheless, these adverse tissue reactions are not limited to MM bearings and have also been observed in patients with modular MPE implants (*i.e.*, head/taper and neck/stem) exhibiting fretting and crevice corrosion [13, 16, 28–32].

Higher levels of metal ions have been reported in the serum, synovial fluid, and whole blood of patients with a joint replacement compared to subjects with no implant [33–39]. While the mechanisms behind the complex inter- and intra-cellular communications, specifically the interactions of cells, proteins, and metal ions, are not well understood, the potential for metal ions to induce adverse tissue re-

actions has raised clinical concern [26, 28–31]. Specifically, chromium ions are known to accumulate in periprosthetic tissues. They may also accumulate in other tissues/organs, such as the liver, spleen, and bone marrow, because chromium ions circulate in the blood [40, 41]. Cobalt ions, on the other hand, have been observed to undergo minimal uptake by cells and tend to be rapidly expelled in the urine and therefore do not persist for long periods of time in tissues [40]. Nevertheless, cobalt has been shown to exhibit greater toxicity than chromium [3, 42–46], and may therefore be the more clinically relevant active agent associated with adverse tissue reactions [47].

Previous *in vitro* studies have shown that high concentrations of either Co^{2+} or Cr^{3+} can induce macrophage mortality by necrosis [3, 42, 43, 46]. In addition, both Co^{2+} and Cr^{3+} can induce the release of pro-inflammatory mediators including cytokines and chemokines from multiple cell types [3, 48–54]. Chemokines are a family of cytokines involved in cellular signalling, cellular activation, and the trafficking of specific cell populations [55, 56]. They are released by numerous cells found in periprosthetic tissues, including monocytes, macrophages, dendritic cells (DC), peripheral lymphocytes, endothelial cells, fibroblasts, eosinophils, basophils, neutrophils, T cells, and natural killer (NK) cells [56]. Specifically, monocyte chemotactic protein–1 (MCP–1) is known to stimulate an increase of IL–1 and IL–6 release and regulate the migration and accumulation of monocytes/macrophages, DC, NK and memory T lymphocytes [56]. Osteoblasts may also produce functional MCP–1 under inflammatory conditions, which could lead to additional monocyte recruitment [53, 57] and consequently, an increase in periprosthetic osteolysis. MIP–1α also stimulates the production of IL–1, IL–6, and TNF–α, and regulates migration of monocytes/macrophages, B cells, cytotoxic T (Tc) lymphocytes, NK cells, and eosinophils, while MIP–1β regulates chemotaxis for T helper (Th) lymphocytes [56]. Interestingly, previous studies have demonstrated that MIP–1α and regulated on activation, normal T cell expressed and secreted (RANTES) increased size, motility, and differentiation of osteoclasts [58–61].

Finally, previous *in vitro* studies have shown that Cr^{6+} and Co^{2+} can induce T cell apoptosis and limit proliferation, IL–2 release, and lymphocyte function [62]. However, little is known about the effects of Cr^{3+} and Co^{2+} on lymphocyte recruitment and migration.

Therefore, the objective of this study was to determine the effects of Co^{2+} and Cr^{3+} ions on macrophage CC chemokine release (MCP–1, MIP–1α, and RANTES) and on lymphocyte migration. While the focus of the study was on the release of specific CC chemokines induced by Co^{2+} and Cr^{3+}, the release of TNF–α was also measured as a marker of inflammation. Furthermore, since metal implant-related hypersensitivity reactions are thought to be T cell-mediated, type-IV hypersensitivity reactions [63], this study analyzed the chemotactic potency of Co^{2+}, Cr^{3+}, and culture supernatants from J774A.1 monocytes/macrophages stimulated with Co^{2+} and Cr^{3+}, on T cell migration.

1.4 MATERIALS AND METHODS

1.4.1 Macrophages

The J774A.1 monocyte/macrophage mouse cell line (American Type Culture Collection (ATCC), Manasas, VA) was maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2, in medium A composed of Dulbecco's Modified Eagle Medium (DMEM; Wisent, St. Bruno, QC) with 4.5 g/L glucose, 110 mg/L pyruvic acid, and supplemented with 5% (v/v) premium grade heat-inactivated fetal bovine serum (FBS; Wisent). Cells were harvested for experiments by scraping, or by pipetting with a Class A volumetric glass pipette, and allowed to recover for 3–4 hours under cell culture conditions with periodic mixing to minimize cell attachment, as previously described [48]. The harvested macrophages were then washed and resuspended in medium A.

1.4.2 Lymphocytes

Lymphocytes were isolated from the spleens of 5–10 week old wild type C57BL6/J mice (The Jackson Laboratory, Bar Harbor, ME). Animals were cared for and housed (at the Animal Care Facility of the University of Ottawa) in accordance with Canadian Council on Animal Care guidelines. Protocols and procedures were approved and monitored by the University of Ottawa Animal Care Committee. Mice were kept under a 12 hour:12 hour light:dark photoperiod with *ad libitum* access to food and water. Animals were euthanized by CO_2 asphyxiation followed by cervical dislocation. Euthanized mice were soaked with 70% (v/v) ethanol, and spleens were harvested by careful dissection. The harvested spleens were immediately placed in pre-cooled medium B (Roswell Park Memorial Institute (RPMI)-1640 medium (Wisent) supplemented with 10% FBS) and kept on ice. Each spleen was dissociated under aseptic conditions by grinding between two frosted glass microscope slides (Fisher Scientific, Fairlawn, NJ), as previously described [64]. The resulting dissociated cells were resuspended in medium B, filtered through a 70-μm nylon mesh cell strainer (BD Biosciences, Durham, NC), collected by centrifugation (420×g for 7 min at room temperature), and resuspended in 2 mL of fresh medium B.

Lymphocytes were purified by density gradient centrifugation using lymphocyte separation medium (LSM) (Wisent). Briefly, 3 mL of LSM was introduced slowly under the 2 mL of cell suspension using a 5-mL syringe secured to a 21 gauge 1.5 inch hypodermic needle (BD, Franklin Lakes, NJ), and the layered sample was centrifuged (400×g for 15 min at room temperature), as per the manufacturer's instructions. The isolated lymphocytes (buffy coat) were collected by aspiration, along with the layer of medium B, and the top half of the LSM layer (as per the manufacturer's instructions). The lymphocytes were immediately washed by dilution with 10 mL of medium B and collected by centrifugation

(420×g for 7 min at room temperature). This wash step was repeated twice, and the lymphocyte concentration was adjusted to 5 × 10^6 cells/mL in lymphocyte migration buffer (LMB: DMEM supplemented with 0.5% (w/v) bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO)). Cells were counted with a Cytomics FC 500 Series flow cytometer (Beckman Coulter, Indianapolis, IN) using a calibrated suspension of fluorospheres (CountBright™ Absolute Counting Beads; Life Technologies, Burlington, ON), as per the manufacturer's instructions.

1.4.3 Cobalt and Chromium ions

Stock solutions of Co^{2+} and Cr^{3+} were freshly prepared by dissolving $CoCl_2$ (Fisher Scientific) and $CrCl_3$ (Sigma-Aldrich) in DMEM, respectively, and sterile-filtered using 0.2-μm pore size cellulose acetate syringe filters (Catalogue No. 28145-477; VWR, Mississauga, ON). Sterile-filtration of these solutions did not cause a detectable loss of Co^{2+} or Cr^{3+}, as determined by inductively coupled plasma-mass spectrometry [48].

1.4.4 TNF–α and chemokine release

For each experimental condition, 0.5 × 10^6 macrophages were resuspended in 1 mL of medium A containing Co^{2+} and Cr^{3+} at different concentrations: 2, 4, 8, 16, 24, 32, and 48 ppm or 25, 50, 100, 150, 250, 350, and 500 ppm, respectively. Macrophages incubated with neither Co^{2+} nor Cr^{3+} served as negative controls, and macrophages incubated with 1 μg/mL of lipopolysaccharide (LPS) from *E. coli* O55:B5 (Catalogue No. L6529; Sigma-Aldrich) were used as pro-inflammatory controls for TNF–α and CC chemokine production (data not shown), as previously described [65–67].

All samples, in loosely capped 5-mL untreated polystyrene culture tubes (Catalogue No. 352003; BD Biosciences), were incubated at 37 °C for 24 hours. At the end of the incubation, the cell suspensions were centrifuged (150×g for 6 min at room temperature), and the supernatants were frozen/stored at −80 °C for cytokine release measurements by enzyme-linked immunosorbent assay (ELISA).

Freshly thawed culture supernatants were gently mixed, and concentrations of TNF–α, MCP–1, MIP–1α, and RANTES were measured by ELISA with the following kits: Mouse TNF–α Quantikine® ELISA; Mouse CCL2/JE/MCP–1 Quantikine® ELISA; Mouse CCL3/MIP–1α Quantikine® ELISA; and Mouse CCL5/RANTES Quantikine® ELISA; respectively, as per manufacturer's instructions (R&D Systems, Minneapolis, MN). Absorbance measurements were performed at 450 nm using a hybrid microplate reader (Synergy™ 4; BioTek, Winooski, VT). The nominal minimum concentrations of TNF–α, MCP–1, MIP–1α, and RANTES detectable by ELISA were 5.1, 2, 1.5, and 2 pg/mL, respec-

tively, and the kits were specific enough to avoid cross-reactivity with other cytokines (as per manufacturer's specifications).

1.4.5 Lymphocyte migration assay

Culture supernatants were prepared as described above, for TNF–α and chemokine release, re-centrifuged (150×g for 6 min at room temperature), snap frozen in liquid nitrogen, and stored at −80 °C for use as chemoattractants in lymphocyte migration experiments.

Experimental conditions included: (1) medium with Co^{2+} (equilibrated medium (EQM; medium A incubated under cell culture conditions for 24 hours) containing 8 ppm Co^{2+}); (2) medium with Cr^{3+} (EQM containing 100 ppm Cr^{3+}); (3) culture supernatant with Co^{2+} (culture supernatant from macrophages exposed to 8 ppm Co^{2+} for 24 hours); (4) culture supernatant with Cr^{3+} (culture supernatant from macrophages exposed to 100 ppm Cr^{3+} for 24 hours); (5) medium (chemokinesis) control (EQM); (6) a culture supernatant control (culture supernatant from macrophages incubated in medium A for 24 hours); and (7) a pro-inflammatory culture supernatant control (culture supernatant from macrophages stimulated with 1 μg/mL of LPS).

Migration experiments were performed in 24-well suspension cell culture multiwell plates (Greiner Bio-One, Frickenhausen, Baden-Württemberg, Germany) using modified Boyden chambers (*i.e.*, hanging cell culture inserts with a 5-μm pore size, polyethylene terephthalate (PET) membrane; Millicell®; Millipore, Billerica, MA). Briefly, 600-μL aliquots of freshly thawed control solutions and culture supernatants, for the conditions detailed above, were added to each well. A hanging cell culture insert containing 1×10^6 lymphocytes resuspended in 200 μL of LMB was then introduced into each well and the multiwell plates were incubated undisturbed for 3.5 hours under cell culture conditions.

At the end of the incubation, cells that migrated into the wells were immediately transferred into 5-mL polystyrene tubes (Greiner Bio-One), centrifuged (420×g for 7 min at room temperature), and resuspended in 100 μL cold (4 °C) staining buffer (phosphate buffered saline (PBS; Wisent) without Ca^{2+} and Mg^{2+} supplemented with 2% (v/v) heat inactivated FBS and 0.01% (w/v) NaN_3). The resuspended cells were then stained for 30 minutes in the dark at 4 °C with 2 μg/mL phycoerythrin (PE)-labelled anti-mouse T-cell receptor–β (TCRβ) (clone H57-597; eBioscience, San Diego, CA) and 1 μg/mL fluorescein isothiocyanate (FITC)-labelled anti-mouse cluster of differentiation 19 (CD19) (clone ebio1D3; eBioscience) to identify T and B lymphocytes, respectively. The stained cells were diluted immediately to 500 μL with staining buffer containing 0.05 μg/mL propidium iodide (PI; Biotium, Hayward, CA) and 20 μL of a calibrated suspension of fluorospheres (see above). The cells were analyzed, without delay, by flow cytometry. Flow cytometry data was extracted from individual

list mode data (LMD) files using Bioconductor version 3, flowCore R package version 1.30.7 and was analyzed using R-3.1.0.

1.4.6 Statistical analysis

Data are presented as means ± S.E.M. (with propagated error). Statistical analysis implemented in R-3.1.0 was performed using a linear mixed-effects model and multiple-comparison Tukey contrasts using sample as a fixed effect and experiment as a random effect. A p-value less than 0.05 was considered significant.

1.5 RESULTS

1.5.1 Effect of Co^{2+} and Cr^{3+} on TNF–α release by J774A.1 macrophages

ELISA results revealed a Co^{2+} concentration-dependent increase in TNF–α release reaching 13-fold, relative to the negative control (cells with no metal ions), with 48 ppm Co^{2+} ($p<0.001$; Figure 1A). A significant increase in TNF–α release, reaching 1.7-fold ($p<0.02$) and 2.3-fold ($p<0.001$), relative to the negative control, was also observed with 150 and 250 ppm Cr^{3+}, respectively. A decrease relative to the maximum measured Cr^{3+}-induced TNF–α release was observed with higher concentrations of Cr^{3+} (*i.e.*, 350 and 500 ppm; Figure 2A).

1.5.2 Effect of Co^{2+} and Cr^{3+} on CC chemokine release by J774A.1 macrophages

1.5.2.1 MCP–1

ELISA results revealed a decrease in MCP–1 release with all concentrations of Co^{2+} or Cr^{3+} tested, relative to the negative control (up to 4.2-fold with 48 ppm Co^{2+} ($p<0.001$; Figure 1B) and 8.3-fold with 500 ppm Cr^{3+} ($p<0.001$; Figure 2B)).

1.5.2.2 MIP–1α

Results revealed that both Co^{2+} and Cr^{3+} induced an increase in MIP–1α release (Figures 1C and 2C). Co^{2+} induced a significant increase reaching 1.5-fold, relative to the negative control, with 24 ppm Co^{2+} ($p<0.001$). A significant increase in MIP–1α release reaching 1.9-fold, relative to the negative control, was also observed with 150 ppm Cr^{3+} ($p<0.001$). A significant decrease was observed with

higher concentrations of Co^{2+} (32 and 48 ppm) and Cr^{3+} (350 and 500 ppm) down to 3.0-fold and 10-fold, relative to the negative control, with 48 ppm Co^{2+} and 500 ppm Cr^{3+}, respectively ($p<0.001$).

1.5.2.3 RANTES

Results revealed a concentration-dependent decrease in RANTES release with all concentrations of Co^{2+} or Cr^{3+} tested, relative to the negative control (up to 4.2-fold with 48 ppm Co^{2+} ($p<0.001$; Figure 1D) and 50-fold with 500 ppm Cr^{3+} ($p<0.001$; Figure 2D)).

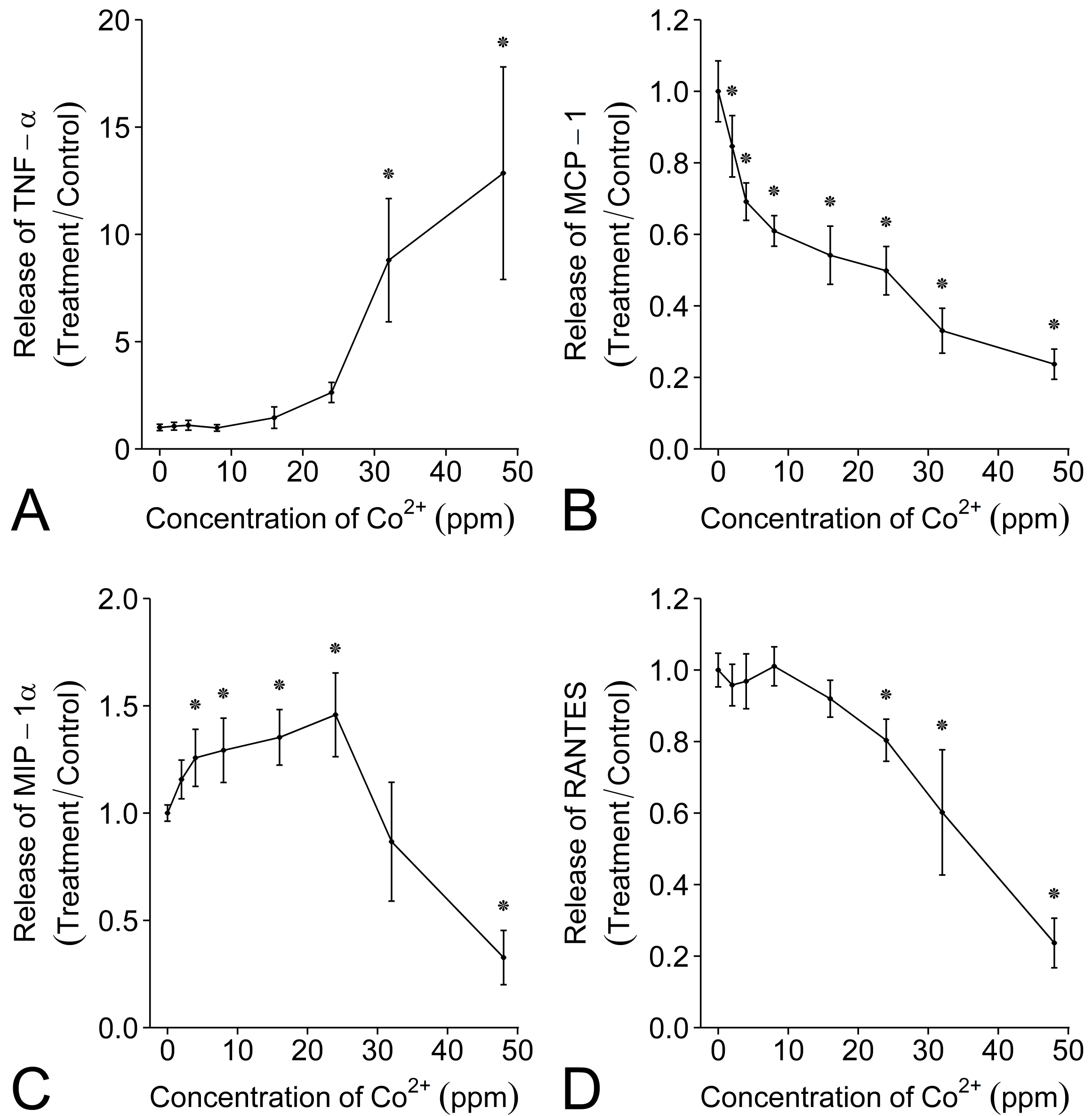

Figure 1: Effect of Co^{2+} on TNF–α and CC chemokine release by J774A.1 macrophages. (A) Tumor necrosis factor–α (TNF–α); (B) Monocyte chemotactic protein–1 (MCP–1); (C) Macrophage inflammatory protein–1α (MIP–1α); and (D) Regulated on activation, normal T cell expressed and secreted (RANTES). An asterisk (*) indicates a significant difference ($p<0.05$) between an experimental condition and the negative control (no Co^{2+}). Cells were incubated 24-hours with the indicated concentrations of Co^{2+} at 37 °C. TNF–α and CC chemokine release were measured by ELISA. Data are presented as means ± S.E.M. of 4 independent experiments performed in triplicate.

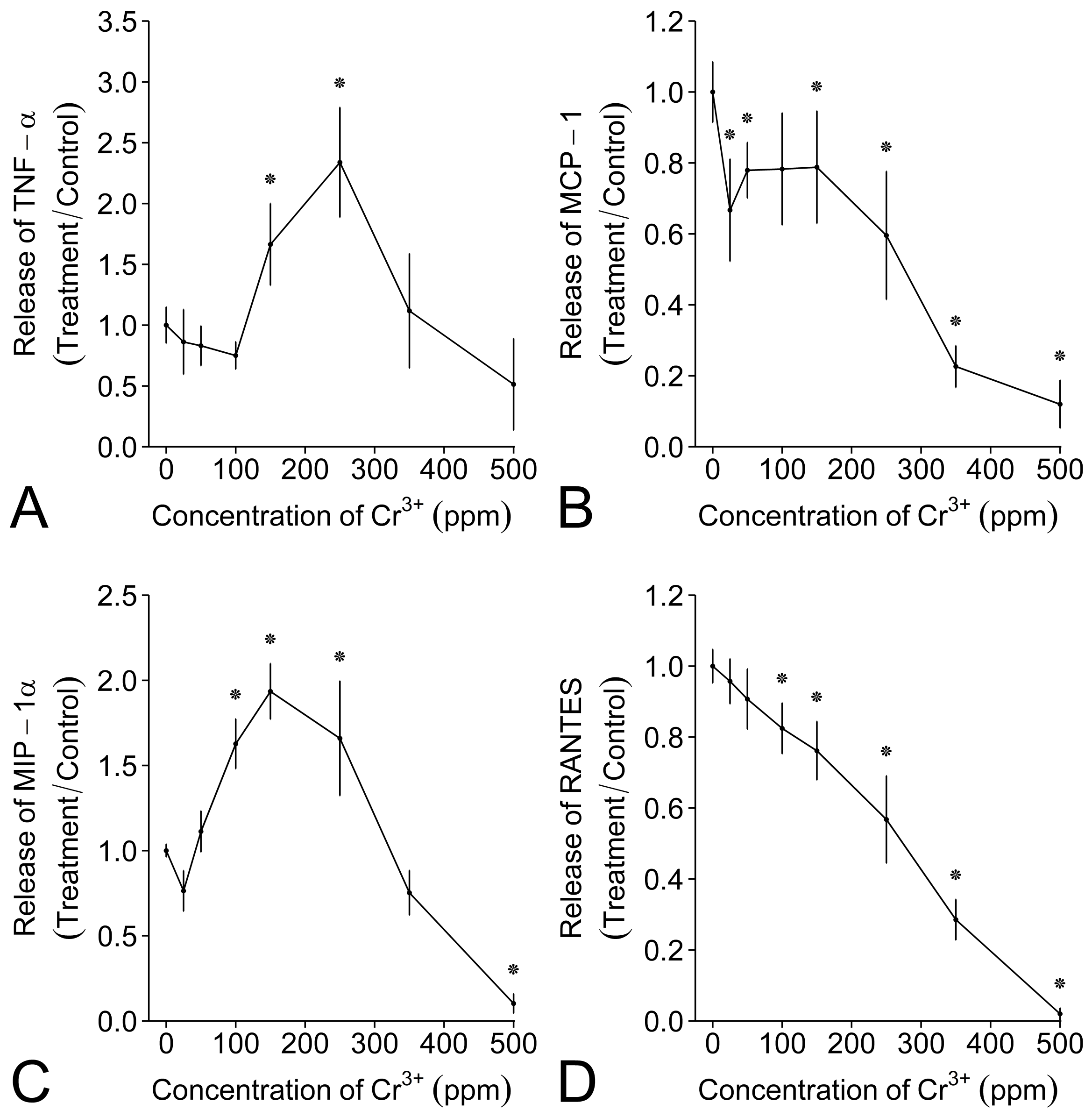

Figure 2: Effect of Cr^{3+} on TNF–α and CC chemokine release by J774A.1 macrophages. (A) Tumor necrosis factor–α (TNF–α); (B) Monocyte chemotactic protein–1 (MCP–1); (C) Macrophage inflammatory protein–1α (MIP–1α); and (D) Regulated on activation, normal T cell expressed and secreted (RANTES). An asterisk (*) indicates a significant difference ($p<0.05$) between an experimental condition and the negative control (no Cr^{3+}). Cells were incubated 24-hours with the indicated concentrations of Cr^{3+} at 37 °C. TNF–α and CC chemokine release were measured by ELISA. Data are presented as means ± S.E.M. of 3-4 independent experiments performed in triplicate.

1.5.3 Effect of Co^{2+} and Cr^{3+} on lymphocyte migration

1.5.3.1 T cell migration

The migration of TCRβ^{+} cells (*i.e.*, T cells) was greater in all experimental conditions than in the medium control (EQM without Co^{2+} and Cr^{3+} *i.e.*, chemokinesis control). Interestingly, the number of migrated TCRβ^{+} cells in the culture supernatant control (supernatant from the culture of untreated J774A.1 cells) was also higher than in the culture supernatants with Co^{2+} and Cr^{3+} (1.3-fold and 1.4-fold, respectively; $p<0.001$; Figure 3A). There was no notable difference in the number of migrated TCRβ^{+} cells between respective ion-specific comparisons of medium and culture supernatants for each of Co^{2+} and Cr^{3+}.

1.5.3.2 B cell migration

The number of migrated TCRβ^{-} cells was significantly lower in culture supernatants with Co^{2+} than in the medium control, the culture supernatant control, and the medium with Co^{2+} (1.3, 1.3, and 1.4-fold, respectively; $p<0.014$). Similarly, the number of migrated TCRβ^{-} cells was also significantly lower in culture supernatants with Cr^{3+} than in the medium control, the culture supernatant control, and the medium with Cr^{3+} (1.8, 1.8, and 1.7-fold, respectively; $p<0.001$). Interestingly, there was no significant difference in the number of migrated TCRβ^{-} cells in the medium control and in the culture supernatant control (Figure 3B).

The decrease in the number of migrated CD19^{+} cells (*i.e.*, B cells; Figure 3C), a subpopulation of TCRβ^{-} cells, was lower in all culture supernatant conditions compared to the number of migrated TCRβ^{-} cells in the corresponding conditions (Figure 3B). The number of migrated CD19^{+} cells was lower in the culture supernatants with Co^{2+} than in the medium control and the medium with Co^{2+} (1.8-fold and 1.9-fold, respectively; $p<0.001$). Similarly, a decrease in the number of migrated CD19^{+} cells was also observed between the culture supernatants with Cr^{3+} and each of the medium control and the medium with Cr^{3+} (4.0-fold and 4.2-fold, respectively; $p<0.001$). In contrast to what was observed with the TCRβ^{-} cells, the number of migrated CD19^{+} cells was significantly lower in the culture supernatant control than in the medium control (1.5-fold; $p<0.001$; Figure 3C).

Interestingly, there were no differences in the number of migrated TCRβ^{-} or CD19^{+} cells between the medium control, the medium with Co^{2+}, and the medium with Cr^{3+} (Figures 3B and 3C).

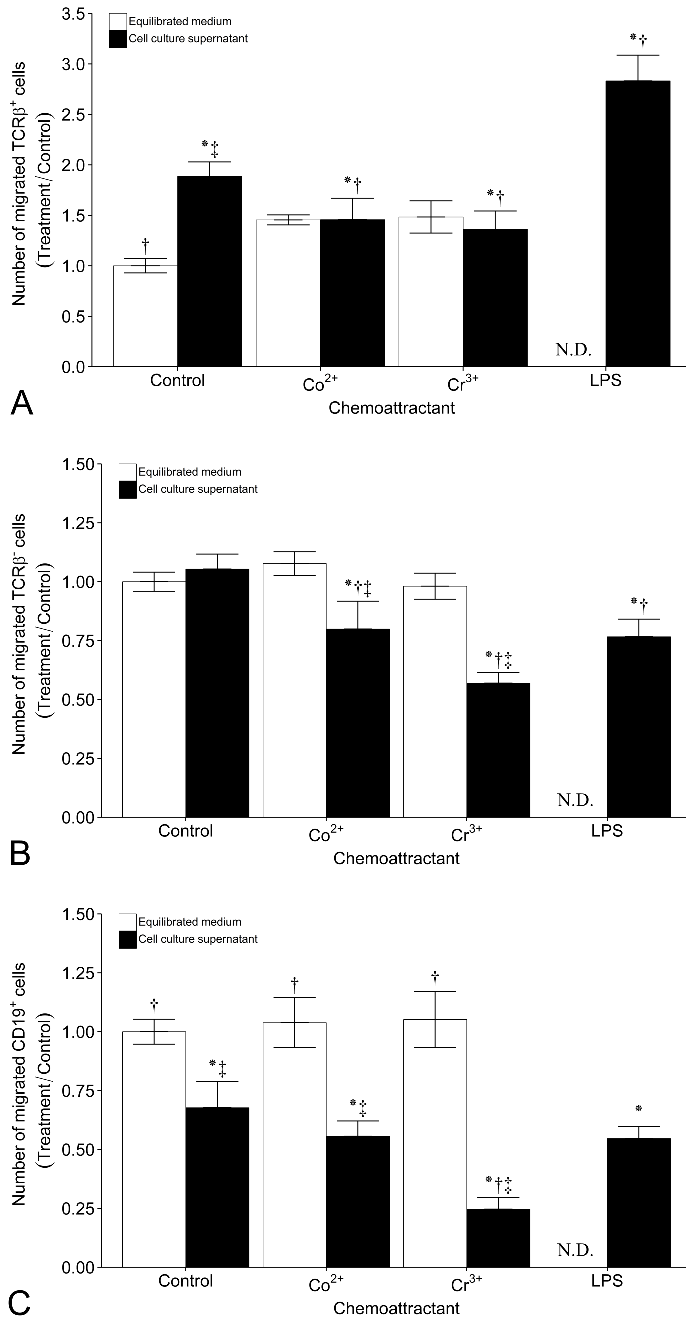

Figure 3: Effects on lymphocyte migration of Co^{2+}, Cr^{3+}, and culture supernatants from J774A.1 macrophages exposed to Co^{2+} and Cr^{3+} for 24 hours. (A) Migrated TCRβ^{+} cells; (B) Migrated TCRβ^{-} cells; and (C) Migrated CD19^{+} cells. An asterisk (*) indicates a significant difference ($p<0.05$) between the number of migrated cells in an experimental condition and the medium control. A dagger (†) indicates a significant difference ($p<0.05$) between the number of migrated cells in an experimental condition and the culture supernatant control (culture supernatant from untreated J774A.1 cells). A double dagger (‡) indicates a significant difference ($p<0.05$) between the number of migrated cells in an experimental condition with J774A.1 culture supernatant and the equilibrated medium for the same treatment. The cells were incubated undisturbed for 3.5-hours at 37 °C. The migrated cells were analyzed by flow cytometry. Data are presented as means ± S.E.M. of 3 independent experiments with 3 to 6 sample replicates, with the exception of the data for the conditions with medium and Co^{2+} or Cr^{3+} in panels A and B, and the entire panel C, which are from a single experiment.

1.6 DISCUSSION

The present study analyzed the effects of Co^{2+} and Cr^{3+} on the release of TNF–α and specific CC chemokines (MCP–1, MIP–1α, and RANTES) from macrophages, as well as the chemotactic response of T cells to Co^{2+}, Cr^{3+}, and culture supernatants of macrophages exposed to Co^{2+} and Cr^{3+}.

This study focused on the effects of Co and Cr ions because cobalt-chromium-molybdenum (CoCrMo) and stainless steel, two alloys used extensively in orthopaedic applications, contain large proportions of Co and Cr (approximately 51 to 66% Co and 19 to 30% Cr in CoCrMo alloys and approximately 17 to 19% Cr in stainless steel alloys) [68]. *In vivo*, these metal alloys undergo wear and corrosion that can lead to a physiologically significant release of Co and Cr ions. These ions are of considerable clinical interest because of their pervasive effects on cells and their putative role in adverse tissue reactions. While cobalt exists in two common oxidation states +2 and +3, divalent Co ions (Co^{2+}) were used in this study because trivalent Co ions (Co^{3+}) rapidly reduce to Co^{2+} in aqueous environments [69, 70]. Similarly, while Cr also exists in two common oxidation states, +3 and +6, trivalent Cr ions (Cr^{3+}) were used in this study because hexavalent chromium ions (Cr^{6+}) rapidly reduce to Cr^{3+} under physiological conditions [71]. The selection of relatively high Co^{2+} and Cr^{3+} concentrations for this *in vitro* study was based on previous studies using the same ions and culture model, the assumption that ion levels are higher in periprosthetic tissues than in body fluids (excluding synovial fluid), and the observation that the stimulation of macrophages requires higher concentrations of stimulating agent *in vitro* than it does *in vivo* where the cells are subjected simultaneously to multiple stimulating factors [48]. However, the highest concentrations of Co^{2+} used in this study were almost five times higher than in previous studies [3, 42, 43, 46] to explore macrophage response to Co^{2+} over a wider range of toxicity.

The J774A.1 murine monocyte/macrophage cell line was used as a macrophage model to analyze the effects of Co^{2+} and Cr^{3+} on the release of TNF–α and specific CC chemokines by macrophages (Figures 1 and 2). The reasons for the selection of this cell line and a short exposure time (24 hours) to Co^{2+} and Cr^{3+} have been reviewed elsewhere [48].

The release of TNF–α was measured primarily as an indicator of the intensity of the pro-inflammatory response of the macrophages and to facilitate comparisons with other studies. The effects of Co^{2+} on the release of TNF–α, were consistent with, but overall more modest than, those reported by Catelas *et al.*, with the same cell line [3]. The more modest effects observed in this study may be due to the use of a higher concentration of sodium bicarbonate ($NaHCO_3$) which can effectively decrease the concentration of Co^{2+} by forming cobalt carbonate ($CoCO_3$) complexes that precipitate [72]. This explanation is consistent with the observation that the release of TNF–α reported by Catelas *et al.*, (2.4-fold with 10 ppm Co^{2+}, the highest concentration of Co^{2+} tested in that study) corresponds to the release observed

with 24 ppm Co^{2+} in this study (2.6-fold, relative to the negative control). In contrast, the release of TNF–α from macrophages exposed to Cr^{3+} was similar in both studies with a maximum release observed with 250 ppm Cr^{3+}, and a similar but non-significant release (relative to the negative control) with 350 ppm Cr^{3+}. These results are also in agreement with those of our recent study on the effects of Cr^{3+} on the release of TNF–α from macrophages maintained in rotation [48]. Overall, the observed release of TNF–α confirms that Co^{2+} and Cr^{3+} induced a significant pro-inflammatory response by the macrophages. *In vivo*, this pro-inflammatory response could play a significant role in the recruitment of immune cells to periprosthetic tissues through both the associated release of chemokines and the positive chemotactic effects of TNF–α on Tc cells [73].

The negative effect of high concentrations of Co^{2+} and Cr^{3+} on the release by macrophages of TNF–α and CC chemokines (see below) may reflect the cytotoxicity of these metal ions (rapid cell death and/or interference with the production and/or release of chemokines) [3, 48]. However, this apparent negative effect may also be (at least in part) an artefact due to the interference of Co^{2+} and Cr^{3+} with the ELISA used to measure cytokine concentration. Both Co^{2+} and Cr^{3+} are known to bind proteins in a dose-dependent way [74] and may therefore interfere with antibody recognition of the target molecule.

The production of MIP–1α by macrophages has been shown to be upregulated by both titanium and poly(methyl methacrylate) (PMMA) particles [24] and, more recently, by Cr^{3+} [48]. In the present study, the analysis of chemokine release demonstrated that the upregulation of MIP–1α release induced by Co^{2+} and Cr^{3+} was similar, albeit somewhat higher with Cr^{3+} than Co^{2+}. Interestingly, the Cr^{3+}-induced release of MIP–1α closely mirrored that of TNF–α, in agreement with our recent study with macrophages maintained in rotation [48]. As with TNF–α, the significant decrease in the release of MIP–1α induced by high concentrations of both Co^{2+} and Cr^{3+} may be attributed to cytotoxicity and/or the interference of Co^{2+} and Cr^{3+} with the MIP–1α assay. Overall, the observed Co^{2+}- and Cr^{3+}-induced release of MIP–1α suggests that this chemokine plays a significant role in the inflammatory response observed in periprosthetic tissues, including the recruitment of the following immune cells: monocytes/macrophages; Tc cells; B cells; and NK cells [56].

Unlike CoCrMo, titanium, ultra-high molecular weight polyethylene (UHMWPE), and PMMA particles which have been reported to increase the release of MCP–1 from macrophages after a 24-hour exposure [24, 75], both Co^{2+} and Cr^{3+} decreased the release of MCP–1 in a dose-dependent way. This suggests that these wear particles and Co^{2+} and/or Cr^{3+} act through different macrophage activation mechanisms/pathways. Despite differences in the experimental design, both the present study and that of Nakashima *et al.*, demonstrate that none of the wear products studied induced the release of RANTES, which may explain why this chemokine has not been observed in granulomas [24].

Interestingly, in a recent study we reported a small increase in the release of MCP–1 and RANTES from J774A.1 macrophages in rotation exposed to low concentrations of Cr^{3+} [48]. The decrease of MCP–1 and RANTES release observed in the present study suggests that the previously reported increase was either induced by rotation rather than Cr^{3+} or induced by the synergistic effect of rotation and exposure to Cr^{3+}. Therefore, we cannot exclude the possibility that Cr^{3+} might upregulate the release of MCP–1 and RANTES *in vivo*, where multiple stimulating factors can be present simultaneously. It is also possible that longer incubations with low concentrations of Co^{2+} or Cr^{3+} would lead to increases in Co^{2+}- and/or Cr^{3+}-induced release of MCP–1 and RANTES in the absence of rotation. However, the results of the present study suggest that neither the Co^{2+}- nor Cr^{3+}-induced release of these chemokines by macrophages play a key role in the tissues surrounding CoCrMo implant components.

Lymphocytes isolated from the spleen of C57BL/6 mice, a common murine lymphocyte model, were used to investigate the chemotactic effects on T cells and B cells of Co^{2+}, Cr^{3+}, and the cocktail of chemokines secreted by macrophages exposed to Co^{2+} or Cr^{3+}. Preliminary chemotactic experiments using J774A.1 macrophages were also performed. However, in an apparent disagreement with some published studies [76–79], it was found that these murine macrophages were too large for the pores (8-μm diameter) of the modified Boyden chambers and became trapped inside them (unpublished data). The relatively low concentrations of Co^{2+} (8 ppm) and Cr^{3+} (100 ppm) used for the chemotaxis experiments were selected as a compromise between minimizing cytotoxicity and maximizing the release of chemokines (TNF–α and MIP–1α were used as indicators of chemokine release).

The number of migrated TCRβ$^+$ lymphocytes (T cells; Figure 3A) was significantly higher in the culture supernatant of macrophages exposed to Co^{2+} and Cr^{3+} than in the medium control (*i.e.*, cell culture medium without Co^{2+} and Cr^{3+}). This *per se* suggests a chemotactic effect on TCRβ$^+$ lymphocytes from the chemokines released by the macrophages exposed to Co^{2+} and Cr^{3+}. However, the number of migrated TCRβ$^+$ lymphocytes was similarly high in EQM containing Co^{2+} and Cr^{3+}. Therefore, the migration of the TCRβ$^+$ lymphocytes cannot be attributed to released chemokines, but rather appears to be directly stimulated by Co^{2+} and Cr^{3+}. A positive chemotactic response to metal cations such as Co^{2+} and Cr^{3+} is surprising since metal salts such as gold, copper, aluminum, and iron typically inhibit chemotaxis [80]. However, beryllium cations (Be^{2+}) have been reported to stimulate the chemotactic migration of lymphocytes *in vitro* [80]. This is especially interesting, since Be, like Co and Cr, is associated with type-IV hypersensitivity reactions resulting in 'granuloma' formation [27, 80]. As pointed out by Sawyer *et al.* [80], the metal-induced attraction of blood lymphocytes may be a key feature in the recruitment of immune cells in affected tissues and the promotion of granuloma formation. However, as with Be, we cannot eliminate the possibility that Co^{2+} and Cr^{3+} stimulated chemokinesis rather than chemotaxis. Interestingly, a previous study demonstrated that neither Co^{2+} nor Cr^{3+} induced

neutrophil migration in a protein-free environment [81]. This may reflect a difference between lymphocytes and neutrophils, or may indicate that proteins are required for the stimulation of migration by Co^{2+} and Cr^{3+}, possibly to form metal ion-protein complexes. Metals may act with serum proteins to crosslink receptors and non-specifically activate T cells without the presence of an antigen-presenting cell (APC) [82].

Interestingly, the number of migrated TCRβ^{+} cells was lower in culture supernatant with Co^{2+} or Cr^{3+} than in the culture supernatant control (supernatant without Co^{2+} or Cr^{3+}). This may be due to Co^{2+} or Cr^{3+} interfering with the production and/or release of specific lymphocyte chemoattractants, as observed with RANTES. It may also be due to Co^{2+} or Cr^{3+} interference with the stimulation of lymphocytes by chemoattractants released in the presence of Co^{2+} or Cr^{3+} (*e.g.*, MIP–1α), through the interaction of these metal ions with the chemokines and/or their receptors. None of these possibilities are mutually exclusive. However, *in vivo*, the negative effects of Co^{2+} or Cr^{3+} on the response of lymphocytes to chemoattractants are probably tempered by the more limited availability of these metal ions. The concentrations of Co^{2+} or Cr^{3+} used in this study are higher than those expected in periprosthetic tissues (where the concentrations cannot be accurately measured). Additionally, complexation and/or sequestration of Co^{2+} or Cr^{3+} is also expected to be more extensive *in vivo*. Furthermore, lymphocytes are expected to be more responsive to chemoattractants *in vivo* because they are subjected simultaneously to multiple stimulating factors. Therefore, *in vivo* Co^{2+}, Cr^{3+}, and chemokines may act in parallel or synergistically to recruit lymphocytes to the periprosthetic tissues. Finally, since the migration of the TCRβ^{+} cell population was inhibited by the presence of Co^{2+} or Cr^{3+} in the culture supernatants, the present study did not attempt to define T-cell subpopulations.

The presence of Co^{2+} or Cr^{3+} in medium did not significantly affect the number of migrated CD19^{+} lymphocytes (B cells; Figure 3C), relative to the medium control (*i.e.*, cell culture medium without Co^{2+} and Cr^{3+}). In contrast, the presence of Co^{2+} or Cr^{3+} in culture supernatant decreased, by up to 4-fold the number of migrated CD19^{+} lymphocytes, relative to the medium control. Similar trends were observed with TCRβ^{-} lymphocytes (mostly B cells; Figure 3B). This lack of B-cell migration is consistent with the T-cell dominated response observed in periprosthetic tissues and is characteristic of a type-IV hypersensitivity reaction [27, 83].

Finally, a larger proportion of TCRβ^{-} lymphocytes than CD19^{+} lymphocytes migrated in the culture supernatant with and without Co^{2+} or Cr^{3+}, relative to the corresponding medium conditions. This indicates the presence of a migrating subpopulation(s) of TCRβ^{-}CD19^{-} lymphocytes, possibly NKT cells.

1.7 CONCLUSION

In conclusion, the present study demonstrated that relatively low concentrations of Co^{2+} and Cr^{3+} can induce selective CC chemokine release, and that MIP–1α may play a significant role in the inflammatory response to Co^{2+} and Cr^{3+} in periprosthetic tissues. Moreover, our results raise the possibility that Co^{2+} and Cr^{3+} can directly stimulate the migration of T cells, but not that of B cells. This is consistent with the T-cell dominated response, characteristic of a type-IV hypersensitivity reaction, observed in periprosthetic tissues of some patients with CoCrMo implant components.

1.8 ACKNOWLEDGEMENTS

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1.9 REFERENCES

[1] G. Holt, C. Murnagham, J. Reilly, and R. M. D. Meek, "The biology of aseptic osteolysis," *Clin. Orthop. Relat. Res.*, vol. 460, pp. 240–52, Jul. 2007.

[2] P. E. Beaulé, I. Catelas, and J. B. Medley, *Current controversies, Bearing surface: metal-on-metal*, first edit ed. Elsevier, 2009, ch. 62, pp. 468–477.

[3] I. Catelas, A. Petit, D. J. Zukor, J. Antoniou, and O. L. Huk, "TNF-alpha secretion and macrophage mortality induced by cobalt and chromium ions in vitro-qualitative analysis of apoptosis," *Biomaterials*, vol. 24, no. 3, pp. 383–91, Feb. 2003.

[4] I. Catelas and J. J. Jacobs, "Biologic activity of wear particles," *Instr. Course Lect.*, vol. 59, pp. 3–16, Jan. 2010.

[5] P. F. Doorn, J. M. Mirra, P. A. Campbell, and H. C. Amstutz, "Tissue reaction to metal on metal total hip prostheses," *Clin. Orthop. Relat. Res.*, vol. 329 Suppl, pp. S187–205, Aug. 1996.

[6] L. D. Dorr, Z. Wan, D. B. Longjohn, B. Dubois, and R. Murken, "Total hip arthroplasty with use of the Metasul metal-on-metal articulation. four to seven-year results," *J. Bone Joint Surg. Am.*, vol. 82, no. 6, pp. 789–98, Jun. 2000.

[7] T. P. Schmalzried, P. C. Peters, B. T. Maurer, C. R. Bragdon, and W. H. Harris, "Long-duration metal-on-metal total hip arthroplasties with low wear of the articulating surfaces," *J. Arthroplasty*, vol. 11, no. 3, pp. 322–31, Apr. 1996.

[8] Y. Kwon, A. V. Lombardi, J. J. Jacobs, T. K. Fehring, C. G. Lewis, and M. E. Cabanela, "Risk stratification algorithm for management of patients with metal-on-metal hip arthroplasty: consensus statement of the American Association of Hip and Knee Surgeons, the American Academy of Orthopaedic Surgeons, and the Hip Society," *J. Bone Joint Surg. Am.*, vol. 96, no. 1, p. e4, Jan. 2014.

[9] K. E. Alvarez, *Total hip replacement*, L. L. Hench, J. R. Jones, and M. B. Fenn, Eds. London, UK: Imperial College Press, 2012.

[10] J. D. Bobyn, M. Tanzer, J. J. Krygier, A. R. Dujovne, and C. E. Brooks, "Concerns with modularity in total hip arthroplasty," *Clin. Orthop. Relat. Res.*, vol. 298, pp. 27–36, Jan. 1994.

[11] H. J. Cooper, "The local effects of metal corrosion in total hip arthroplasty," *Orthop. Clin. North Am.*, vol. 45, no. 1, pp. 9–18, Jan. 2014.

[12] J. L. Gilbert, C. A. Buckley, and J. J. Jacobs, "In vivo corrosion of modular hip prosthesis components in mixed and similar metal combinations. the effect of crevice, stress, motion, and alloy coupling," *J. Biomed. Mater. Res.*, vol. 27, no. 12, pp. 1533–44, Dec. 1993.

[13] H. Krishnan, S. P. Krishnan, G. Blunn, J. A. Skinner, and A. J. Hart, "Modular neck femoral stems," *Bone. Joint. J.*, vol. 95-B, no. 8, pp. 1011–21, Aug. 2013.

[14] J. W. Nicholson, *Metals*. The Royal Society of Chemistry, 2002, ch. 4, pp. 107–147.

[15] A. Panagiotidou, J. Meswania, J. Hua, S. Muirhead-Allwood, A. Hart, and G. Blunn, "Enhanced wear and corrosion in modular tapers in total hip replacement is associated with the contact area and surface topography," *J. Orthop. Res.*, vol. 31, no. 12, pp. 2032–9, Dec. 2013.

[16] R. Pivec, R. M. Meneghini, W. J. Hozack, G. H. Westrich, and M. A. Mont, "Modular taper junction corrosion and failure: how to approach a recalled total hip arthroplasty implant," *J. Arthroplasty*, vol. 29, no. 1, pp. 1–6, Jan. 2014.

[17] G. Perino, B. F. Ricciardi, S. A. Jerabek, G. Martignoni, G. Wilner, D. Maass, S. R. Goldring, and P. E. Purdue, "Implant based differences in adverse local tissue reaction in failed total hip arthroplasties: a morphological and immunohistochemical study," *BMC Clin. Pathol.*, vol. 14, no. 1, p. 39, 2014.

[18] P. A. Campbell, M. Wang, H. C. Amstutz, and S. B. Goodman, "Positive cytokine production in failed metal-on-metal total hip replacements," *Acta Orthop. Scand.*, vol. 73, no. 5, pp. 506–12, Oct. 2002.

[19] I. Catelas, P. A. Campbell, F. Dorey, A. Frausto, B. G. Mills, and H. C. Amstutz, "Semi-quantitative analysis of cytokines in MM THR tissues and their relationship to metal particles," *Biomaterials*, vol. 24, no. 26, pp. 4785–97, Nov. 2003.

[20] J. Gallo, J. Vaculova, S. B. Goodman, Y. T. Konttinen, and J. P. Thyssen, "Contributions of human tissue analysis to understanding the mechanisms of loosening and osteolysis in total hip replacement," *Acta Biomater.*, vol. 10, no. 6, pp. 2354–2366, Jun. 2014.

[21] E. Ingham and J. Fisher, "The role of macrophages in osteolysis of total joint replacement," *Biomaterials*, vol. 26, no. 11, pp. 1271–1286, Apr. 2005.

[22] N. Ishiguro, T. Kojima, T. Ito, S. Saga, H. Anma, K. Kurokouchi, Y. Iwahori, T. Iwase, and H. Iwata, "Macrophage activation and migration in interface tissue around loosening total hip arthroplasty components," *J. Biomed. Mater. Res.*, vol. 35, no. 3, pp. 399–406, Jun. 1997.

[23] P. Koulouvaris, K. Ly, L. B. Ivashkiv, M. P. Bostrom, B. J. Nestor, T. P. Sculco, and P. E. Purdue, "Expression profiling reveals alternative macrophage activation and impaired osteogenesis in periprosthetic osteolysis," *J. Orthop. Res.*, vol. 26, no. 1, pp. 106–116, Jan. 2008.

[24] Y. Nakashima, D. H. Sun, M. Trindade, L. E. Chun, Y. Song, S. B. Goodman, D. J. Schurman, W. J. Maloney, and R. L. Smith, "Induction of macrophage C-C chemokine expression by titanium alloy and bone cement particles," *J. Bone Joint Surg. Br.*, vol. 81, no. 1, pp. 155–62, Jan. 1999.

[25] P. A. Campbell, M. S. Kung, A. R. Hsu, and J. J. Jacobs, "Do retrieval analysis and blood metal measurements contribute to our understanding of adverse local tissue reactions?" *Clin. Orthop. Relat. Res.*, vol. Epub ahead of print, pp. 1–10, Aug. 2014.

[26] Y. Kwon, S. J. Ostlere, P. McLardy-Smith, N. A. Athanasou, H. S. Gill, and D. W. Murray, "Asymptomatic pseudotumors after metal-on-metal hip resurfacing arthroplasty: prevalence and metal ion study," *J. Arthroplasty*, vol. 26, no. 4, pp. 511–8, Jun. 2011.

[27] G. Mahendra, H. Pandit, K. Kliskey, D. Murray, H. S. Gill, and N. Athanasou, "Necrotic and inflammatory changes in metal-on-metal resurfacing hip arthroplasties," *Acta Orthop.*, vol. 80, no. 6, pp. 653–659, Dec. 2009.

[28] R. B. Cook, B. J. R. F. Bolland, J. A. Wharton, S. Tilley, J. M. Latham, and R. J. K. Wood, "Pseudotumour formation due to tribocorrosion at the taper interface of large diameter metal on polymer modular total hip replacements," *J. Arthroplasty*, vol. 28, no. 8, pp. 1430–6, Sep. 2013.

[29] H. Cooper, C. Della Valle, R. Berger, M. Tetreault, W. Paprosky, S. Sporer, and J. Jacobs, "Corrosion at the head-neck taper as a cause for adverse local tissue reactions after total hip arthroplasty," *J. Bone Joint Surg. Am.*, vol. 94, no. 18, pp. 1655–61, Sep. 2012.

[30] H. J. Cooper, R. M. Urban, R. L. Wixson, R. M. Meneghini, and J. J. Jacobs, "Adverse local tissue reaction arising from corrosion at the femoral neck-body junction in a dual-taper stem with a cobalt-chromium modular neck," *J. Bone Joint Surg. Am.*, vol. 95, no. 10, pp. 865–72, May 2013.

[31] I. P. S. Gill, J. Webb, K. Sloan, and R. J. Beaver, "Corrosion at the neck-stem junction as a cause of metal ion release and pseudotumour formation," *J. Bone Joint Surg. Br.*, vol. 94, no. 7, pp. 895–900, Jul. 2012.

[32] G. B. Higgs, J. A. Hanzlik, D. W. MacDonald, J. L. Gilbert, C. M. Rimnac, and S. M. Kurtz, "Is increased modularity associated with increased fretting and corrosion damage in metal-on-metal total hip arthroplasty devices?: a retrieval study," *J. Arthroplasty*, vol. 28, no. 8 Suppl, pp. 2–6, Sep. 2013.

[33] J. Antoniou, D. J. Zukor, F. Mwale, W. Minarik, A. Petit, and O. L. Huk, "Metal ion levels in the blood of patients after hip resurfacing: a comparison between twenty-eight and thirty-six-millimeter-head metal-on-metal prostheses," *J. Bone Joint Surg. Am.*, vol. 90 Suppl 3, pp. 142–8, Aug. 2008.

[34] A. G. Cobb and T. P. Schmalzreid, "The clinical significance of metal ion release from cobalt-chromium metal-on-metal hip joint arthroplasty," *Proc. Inst. Mech. Eng., H J. eng. med.*, vol. 220, no. 2, pp. 385–98, Feb. 2006.

[35] H. S. Dobbs and M. Minski, "Metal ion release after total hip replacement," *Biomaterials*, vol. 1, no. 4, pp. 193–198, Oct. 1980.

[36] N. J. Hallab and J. J. Jacobs, "Biologic effects of implant debris," *Bull. NYU Hosp. Jt. Dis.*, vol. 67, no. 2, pp. 182–8, 2009.

[37] N. J. Hallab, M. Caicedo, K. McAllister, A. Skipor, H. Amstutz, and J. J. Jacobs, “Asymptomatic prospective and retrospective cohorts with metal-on-metal hip arthroplasty indicate acquired lymphocyte reactivity varies with metal ion levels on a group basis,” *J. Orthop. Res.*, vol. 31, no. 2, pp. 173–82, Feb. 2013.

[38] A. Hartmann, F. Hannemann, J. Lützner, A. Seidler, H. Drexler, K.-P. Günther, and J. Schmitt, “Metal ion concentrations in body fluids after implantation of hip replacements with metal-on-metal bearing–systematic review of clinical and epidemiological studies,” *PLoS One*, vol. 8, no. 8 e70359, pp. 1–25, Aug. 2013.

[39] A. K. Skipor, P. A. Campbell, L. M. Patterson, H. C. Anstutz, T. P. Schmalzried, and J. J. Jacobs, “Serum and urine metal levels in patients with metal-on-metal surface arthroplasty,” *J. Mater. Sci. Mater. Med.*, vol. 13, no. 12, pp. 1227–34, Dec. 2002.

[40] K. Merritt and S. A. Brown, “Effects of metal particles and ions on the biological system,” *Techniques in Orthopaedics*, vol. 8, no. 4, pp. 228–236, 1993.

[41] P. C. Grevatt, “Toxicological Review of Trivalent Chromium (CAS No. 16065-83-1) In Support of Summary Information on the Integrated Risk Information System (IRIS),” U.S. Environmental Protection Agency, Washington, DC, Tech. Rep., Aug. 1998.

[42] I. Catelas, A. Petit, D. J. Zukor, and O. L. Huk, “Cytotoxic and apoptotic effects of cobalt and chromium ions on J774 macrophages - Implication of caspase-3 in the apoptotic pathway,” *J. Mater. Sci.: Mater. Med.*, vol. 12, no. 10-12, pp. 949–53, 2001.

[43] I. Catelas, A. Petit, H. Vali, C. Fragiskatos, R. Meilleur, D. J. Zukor, J. Antoniou, and O. L. Huk, “Quantitative analysis of macrophage apoptosis vs. necrosis induced by cobalt and chromium ions in vitro,” *Biomaterials*, vol. 26, no. 15, pp. 2441–53, May 2005.

[44] C. Fleury, A. Petit, F. Mwale, J. Antoniou, D. J. Zukor, M. Tabrizian, and O. L. Huk, “Effect of cobalt and chromium ions on human MG-63 osteoblasts in vitro: morphology, cytotoxicity, and oxidative stress,” *Biomaterials*, vol. 27, no. 18, pp. 3351–60, Jun. 2006.

[45] N. J. Hallab, C. Vermes, C. Messina, K. A. Roebuck, T. T. Glant, and J. J. Jacobs, “Concentration- and composition-dependent effects of metal ions on human MG-63 osteoblasts,” *J. Biomed. Mater. Res.*, vol. 60, no. October, pp. 420–33, Jun. 2002.

[46] O. L. Huk, I. Catelas, F. Mwale, J. Antoniou, D. J. Zukor, and A. Petit, “Induction of apoptosis and necrosis by metal ions in vitro,” *J. Arthroplasty*, vol. 19, no. 8, pp. 84–7, Dec. 2004.

[47] A. J. Hart, P. D. Quinn, F. Lali, B. Sampson, J. A. Skinner, J. J. Powell, J. Nolan, K. Tucker, S. Donell, A. Flanagan, and J. F. W. Mosselmans, “Cobalt from metal-on-metal hip replacements may be the clinically relevant active agent responsible for periprosthetic tissue reactions,” *Acta Biomater.*, vol. 8, no. 10, pp. 3865–73, Oct. 2012.

[48] S. J. Baskey, P. E. Beaulé, E. A. Lehoux, and I. Catelas, “Simvastatin modulates the release of TNF–α and CC chemokines from macrophages exposed to trivalent chromium ions,” *J. Biomater. Tissue Eng.*, vol. 4, no. 11, pp. 981–991, Nov. 2014.

[49] M. S. Caicedo, R. Desai, K. McAllister, A. Reddy, J. J. Jacobs, and N. J. Hallab, “Soluble and particulate Co-Cr-Mo alloy implant metals activate the inflammasome danger signaling pathway in human macrophages: a novel mechanism for implant debris reactivity,” *J. Orthop. Res.*, vol. 27, no. 7, pp. 847–54, Jul. 2009.

[50] B. M. Devitt, J. M. Queally, M. Vioreanu, J. S. Butler, D. Murray, P. P. Doran, and J. M. O'Byrne, "Cobalt ions induce chemokine secretion in a variety of systemic cell lines," *Acta Orthop.*, vol. 81, no. 6, pp. 756–64, Dec. 2010.

[51] D. Granchi, E. Verri, G. Ciapetti, L. Savarino, E. Cenni, A. Gori, and A. Pizzoferrato, "Effects of chromium extract on cytokine release by mononuclear cells," *Biomaterials*, vol. 19, no. 1-3, pp. 283–91, Jan-Feb 1998.

[52] H. Lawrence, D. Deehan, J. Holland, J. Kirby, and A. Tyson-Capper, "The immunobiology of cobalt: demonstration of a potential aetiology for inflammatory pseudotumours after metal-on-metal replacement of the hip," *Bone Joint J*, vol. 96-B, no. 9, pp. 1172–1177, Sep. 2014.

[53] J. M. Queally, B. M. Devitt, J. S. Butler, A. P. Malizia, D. Murray, P. P. Doran, and J. M. O'Byrne, "Cobalt ions induce chemokine secretion in primary human osteoblasts," *J. Orthop. Res.*, vol. 27, no. 7, pp. 855–64, Jul. 2009.

[54] J. Wang, B. Wicklund, R. Gustilo, and D. Tsukayama, "Titanium, chromium and cobalt ions modulate the release of bone-associated cytokines by human monocytes/macrophages in vitro," *Biomaterials*, vol. 17, no. 23, pp. 2233–40, Dec. 1996.

[55] J. Fan, N. M. Heller, M. Gorospe, U. Atasoy, and C. Stellato, "The role of post-transcriptional regulation in chemokine gene expression in inflammation and allergy," *Eur. Respir. J.*, vol. 26, no. 5, pp. 933–947, Nov. 2005.

[56] S. B. Goodman and T. Ma, "Cellular chemotaxis induced by wear particles from joint replacements," *Biomaterials*, vol. 31, no. 19, pp. 5045–50, 2010.

[57] A. Yadav, V. Saini, and S. Arora, "MCP-1: chemoattractant with a role beyond immunity: a review," *Clin. Chim. Acta*, vol. 411, no. 21-22, pp. 1570–1579, Nov. 2010.

[58] M. Abe, K. Hiura, J. Wilde, K. Moriyama, T. Hashimoto, S. Ozaki, S. Wakatsuki, M. Kosaka, S. Kido, D. Inoue, and T. Matsumoto, "Role for macrophage inflammatory protein (MIP)-1α and MIP-1β in the development of osteolytic lesions in multiple myeloma," *Blood*, vol. 100, no. 6, pp. 2195–2202, 2002.

[59] S. J. Choi, J. C. Cruz, F. Craig, H. Chung, R. D. Devlin, G. D. Roodman, and M. Alsina, "Macrophage inflammatory protein 1-alpha is a potential osteoclast stimulatory factor in multiple myeloma." *Blood*, vol. 96, no. 2, pp. 671–675, Jul. 2000.

[60] K. Fuller, J. M. Owens, and T. J. Chambers, "Macrophage inflammatory protein-1 alpha and il-8 stimulate the motility but suppress the resorption of isolated rat osteoclasts," *J. Immunol.*, vol. 154, no. 11, pp. 6065–6072, Jun. 1995.

[61] X. Yu, Y. Huang, P. Collin-Osdoby, and P. Osdoby, "CCR1 chemokines promote the chemotactic recruitment, RANKL development, and motility of osteoclasts and are induced by inflammatory cytokines in osteoblasts," *J. Bone Miner. Res.*, vol. 19, no. 12, pp. 2065–2077, Dec. 2004.

[62] M. Akbar, J. M. Brewer, and M. H. Grant, "Effect of chromium and cobalt ions on primary human lymphocytes in vitro," *Journal of Immunotoxicology*, vol. 8, no. 2, pp. 140–149, Jun. 2011.

[63] S. B. Goodman, "Wear particles, periprosthetic osteolysis and the immune system," *Biomaterials*, vol. 28, no. 34, pp. 5044–5048, Dec. 2007.

[64] A. B. Klein, S. G. Witonsky, S. A. Ahmed, S. D. Holladay, R. Gogal, Jr, L. Link, and C. M. Reilly, "Impact of different cell isolation techniques on lymphocyte viability and function," *J. Immunoassay Immunochem.*, vol. 27, no. 1, pp. 61–76, 2006.

[65] A. Prabhu, C. E. Shelburne, and D. F. Gibbons, "Cellular proliferation and cytokine responses of murine macrophage cell line J774A.1 to polymethylmethacrylate and cobalt-chrome alloy particles," *J. Biomed. Mater. Res.*, vol. 42, no. 4, pp. 655–63, Dec. 1998.

[66] M. Taira, T. Kagiya, H. Harada, M. Sasaki, S. Kimura, T. Narushima, T. Nezu, and Y. Araki, "Microscopic observations and inflammatory cytokine productions of human macrophage phagocytising submicron titanium particles," *J. Mater. Sci.: Mater. Med.*, vol. 21, no. 1, pp. 267–75, Jan. 2010.

[67] R. VanOs, L. L. Lildhar, E. A. Lehoux, P. E. Beaulé, and I. Catelas, "In vitro macrophage response to nanometer-size chromium oxide particles," *J. Biomed. Mater. Res. B Appl. Biomater.*, vol. 102, no. 1, pp. 149–59, Aug. 2014.

[68] G. M. Keegan, I. D. Learmonth, and C. P. Case, "Orthopaedic metals and their potential toxicity in the arthroplasty patient: A review of current knowledge and future strategies," *J. Bone Joint Surg. Br.*, vol. 89, no. 5, pp. 567–573, May 2007.

[69] W. H. Emmons, *The enrichment of ore deposits*. US Government Printing Office, 1917, vol. 625.

[70] E. A. Greenwood, Norman.N., "Cobalt, rhodium and iridium," *Chemistry of the Elements*, 2nd ed. Elsevier, 1997, ch. 26, p. 1116.

[71] A. Sargeant and T. Goswami, "Hip implants - paper VI - ion concentrations," *Mater. Des.*, vol. 28, no. 1, pp. 155–71, 2007.

[72] C. Bresson, C. Darolles, A. Carmona, C. Gautier, N. Sage, S. Roudeau, R. Ortega, E. Ansoborlo, and V. Malard, "Cobalt chloride speciation, mechanisms of cytotoxicity on human pulmonary cells, and synergistic toxicity with zinc," *Metallomics*, vol. 5, no. 2, pp. 133–43, Feb. 2013.

[73] E. C. Ebert, "Tumour necrosis factor-alpha enhances intraepithelial lymphocyte proliferation and migration," *Gut*, vol. 42, no. 5, pp. 650–655, May 1998.

[74] J. Yang and J. Black, "Competitive binding of chromium, cobalt and nickel to serum proteins," *Biomaterials*, vol. 15, no. 4, pp. 262–268, Mar. 1994.

[75] A. M. Kaufman, C. I. Alabre, H. E. Rubash, and A. S. Shanbhag, "Human macrophage response to UHMWPE, TiAlV, CoCr, and alumina particles: analysis of multiple cytokines using protein arrays," *J. Biomed. Mater. Res. A*, vol. 84, no. 2, pp. 464–74, Feb. 2008.

[76] J. Hwang, I. D. C. Fraser, S. Choi, X. Qin, and M. I. Simon, "Analysis of C5a-mediated chemotaxis by lentiviral delivery of small interfering RNA," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 101, no. 2, pp. 488–493, Jan. 2004.

[77] R. F. Tsuji, G. P. Geba, Y. Wang, K. Kawamoto, L. A. Matis, and P. W. Askenase, "Required early complement activation in contact sensitivity with generation of local C5-dependent chemotactic activity, and late T cell interferon gamma: a possible initiating role of B cells," *J. Exp. Med.*, vol. 186, no. 7, pp. 1015–1026, Oct. 1997.

[78] R. F. Tsuji, I. Kawikova, R. Ramabhadran, M. Akahira-Azuma, D. Taub, T. E. Hugli, C. Gerard, and P. W. Askenase, "Early local generation of C5a initiates the elicitation of contact sensitivity by leading to early T cell recruitment," *J. Immunol.*, vol. 165, no. 3, pp. 1588–1598, Aug. 2000.

[79] L. V. Yang, C. G. Radu, L. Wang, M. Riedinger, and O. N. Witte, “Gi-independent macrophage chemotaxis to lysophosphatidylcholine via the immunoregulatory GPCR G2A,” *Blood*, vol. 105, no. 3, pp. 1127–1134, Feb. 2005.

[80] R. T. Sawyer, D. E. Doherty, B. A. Schumacher, and L. S. Newman, “Beryllium-stimulated in vitro migration of peripheral blood lymphocytes,” *Toxicology*, vol. 138, no. 3, pp. 155–163, Nov. 1999.

[81] A. Remes and D. Williams, “Chemotaxis and the inhibition of chemotaxis of human neutrophils in reponse to metal ions,” *J. Mater. Sci.: Mater. Med.*, vol. 1, no. 1, pp. 26–32, 1990.

[82] N. J. Hallab, K. Mikecz, C. Vermes, A. Skipor, and J. J. Jacobs, “Orthopaedic implant related metal toxicity in terms of human lymphocyte reactivity to metalprotein complexes produced from cobalt-base and titanium-base implant alloy degradation,” *Mol. Cell. Biochem.*, vol. 222, pp. 127–36, Mar. 2001.

[83] P. A. Revell, “The combined role of wear particles, macrophages and lymphocytes in the loosening of total joint prostheses,” *J. R. Soc. Interface*, vol. 5, no. 28, pp. 1263–78, Nov. 2008.

Simvastatin Modulates the Release of TNF–α and CC Chemokines from Macrophages Exposed to Trivalent Chromium Ions

The man of science has learned to believe in justification, not by faith, but by verification.

Thomas Henry Huxley

2.1 FOREWORD

As shown in the previous study, both Co^{2+} and Cr^{3+} are capable of increasing the release of MIP–1α, a key chemokine in bone homeostasis, as well inducing the migration of T cells. These results are consistent with the elevated levels of chemokines and T cells observed in periprosthetic tissues of some patients with CoCrMo implants, suggesting a type-IV hypersensitivity reaction. The final objective of this thesis was to analyze the potential modulation of J774A.1 monocyte/macrophage response to Cr^{3+} ions using simvastatin as an anti-inflammatory agent. Specifically, in order to achieve this objective, the effects of simvastatin on macrophage mortality and morphology as well as on the release of chemokines by macrophages were analyzed. This study may lead to the development of clinical options for the treatment of metal ion-induced inflammation in patients. The results have been the subject of a manuscript published in the Journal of Biomaterials and Tissue Engineering (Vol. 4, No. 11, pp.981-991, Nov. 2014) and is entitled:

"Simvastatin modulates the release of TNF–α and CC chemokines from macrophages exposed to trivalent chromium ions", by Stephen J. Baskey, Paul E. Beaulé, Eric A. Lehoux, and Isabelle Catelas.

2.2 ABSTRACT

Previous *in vitro* studies have shown that trivalent chromium ions (Cr^{3+}) can induce cell death and the release of pro-inflammatory cytokines. In addition, chemokines such as MCP–1 and MIP–1α have been observed in the presence of implant wear particles. Therapeutic approaches to minimize the inflammatory response to metal ions leading to periprosthetic osteolysis and other adverse tissue re-

actions could help mitigate some of the concerns associated with metal ions from hip replacements. Therefore, the objective of this study was to analyze the effects of Cr^{3+} on macrophage mortality and morphology, as well as on the release of TNF–α, MCP–1, MIP–1α, MIP–1β, and RANTES, with and without simvastatin. J774A.1 macrophages were exposed to 50–500 ppm Cr^{3+} with and without 5 or 10 μM simvastatin for 24h, in rotation. Mortality results showed that Cr^{3+} induced macrophage mortality in a dose-dependent manner. In addition, simvastatin at 10 μM effectively prevented Cr^{3+}-induced vacuolization and cell swelling. Results also showed that while high concentrations of Cr^{3+} (particularly 500 ppm) may be too toxic to stimulate TNF–α and CC chemokine release, lower concentrations of Cr^{3+} are capable of inducing TNF–α and selective CC chemokine release, with a predominance of MIP–1α (up to 100% increase). Release of MCP–1 and RANTES were increased with up to 150 ppm Cr^{3+}, but to a lower extent. The effects of simvastatin were dose-dependent, except for TNF–α, and reduced the release of TNF–α as well as that of all chemokines at all Cr^{3+} concentrations analyzed, thereby demonstrating the potential of simvastatin to reduce metal ion-induced inflammatory response leading to periprosthetic osteolysis and other adverse tissue reactions.

2.3 INTRODUCTION

In the 1980s, metal-on-metal (MM) hip implants with cobalt-chromium-molybdenum (CoCrMo) bearing surfaces were reintroduced as an alternative to conventional metal-on-polyethylene (MPE) implants because of their lower volumetric wear rates, delayed wear, and the expected associated decrease of periprosthetic inflammation.[1–6] It is estimated that since the mid-1980s, more than 1 million patients worldwide have received a MM hip implant.[7] However, the greater amount of metal products, especially metal ions, released by MM implants compared to MPE implants, is a major cause for concern.

The long-term effects of these metal products are a concern because of the local and systemic effects of the ions.[3] It has also been reported that the levels of metal ions in the serum, synovial fluid, and whole blood of patients with a joint replacement are significantly higher than the corresponding levels in patients with no implant.[8–14] More importantly, adverse tissue reactions with elevated levels of metal ions are not limited to MM bearings and have also been observed in patients with modular MM and MPE hip implants (i.e., head/taper and neck/stem) due to fretting and crevice corrosion.[15–21] While the mechanisms of cell-metal ion interactions are still poorly understood, clinical studies have shown that adverse tissue reactions, including hypersensitivity and pseudotumors, may be associated with elevated levels of cobalt and chromium ions and other corrosion products.[15–18,22]

Previous *in vitro* studies have demonstrated that Co^{2+} and Cr^{3+} can induce cell mortality by apoptosis at low concentrations, and necrosis at higher concentrations.[23,24] In addition, Co^{2+} and Cr^{3+} have been

shown to induce the release of pro-inflammatory cytokines from monocytes/macrophages *in vitro*, such as TNF–α, IL–1β, IL–6, and transforming growth factor–β1 (TGF–β1).[2,25,26] Finally, the release of chemokines, specifically monocyte chemotactic protein–1 (MCP–1) has also been observed in the presence of Co^{2+} in osteoblast cultures.[27,28]

Chemokines are a family of cytokines that provide critical cell signaling and trafficking functionality targeted to specific cell populations.[29] They are released by numerous cells, including monocytes, dendritic cells, peripheral lymphocytes, endothelial cells, fibroblasts, eosinophils, basophils, neutrophils, T cells, and natural killer (NK) cells.[29] MCP–1 and macrophage inflammatory protein–1 (MIP–1) are important molecules in adverse immune responses because they are known to target different types of leukocytes and stimulate changes in cell function and migration.[29–32] Specifically, MCP–1 is known to stimulate an increase of IL–1 and IL–6 release and regulate the migration and accumulation of monocytes/macrophages, dendritic cells, NK cells, and memory T cells.[29] MIP–1α also stimulates the production of IL–1, IL–6, and TNF–α, and regulates migration of monocytes/macrophages, B cells, cytotoxic T cells, NK cells, and eosinophils, while MIP–1β regulates chemotaxis for T-helper cells.[29] RANTES (regulated on activation, normal T cell expressed and secreted), reported to be produced by various cells, including T-helper cells, monocytes, and dendritic cells, is a strong chemoattractant for macrophages, eosinophils, and mature T-helper cells.[29,33] Increased levels of some of these pro-inflammatory cytokines and chemokines have been shown in periprosthetic tissues.[30] In addition, previous studies have shown that Co^{2+} induced an upregulation of IL–8 and MCP–1 in osteoblasts.[27,28] However, little is known about the effects of chromium ions on chemokine production.

Different therapeutic approaches have been suggested to modulate periprosthetic osteolysis resulting from wear product-induced inflammation, including the use of: antiresorptive agents such as bisphosphonates and RANKL antagonists; anabolic agents; and anti-inflammatory drugs such as traditional nonsteroidal anti-inflammatory drugs (NSAIDs), cyclooxygenase (COX) inhibitors, TNF antagonists, and IL–1 antagonists.[34] However, the side effects of these drugs, in particular the antianabolic effects of traditional NSAIDs and COX–2 inhibitors, and the immunosuppressive effects of all the anti-inflammatory drugs, represent a significant concern.[34] An alternative to these drugs with potentially milder side effects are 3-hydroxy-3-methylglutaryl-coenzyme A (HMG–CoA) reductase inhibitors, collectively known as statins. Simvastatin, a synthetic statin approved by the U.S. Food and Drug Administration (FDA) in 1991 to decrease low-density lipoprotein (LDL) cholesterol level in the blood, has recently been shown to lower the risk of revision surgery after total hip replacement.[35,36] More specifically, simvastatin has been shown to decrease the release of inflammatory mediators,[37,38] which could potentially reduce the inflammatory response leading to periprosthetic osteolysis and other adverse tissue reactions.

The objective of this study was to analyze the effects of Cr^{3+} on macrophage mortality and morphology, as well as on the release of TNF–α and CC chemokines (MCP–1, MIP–1α, MIP–1β, and RANTES), with and without simvastatin. While the focus of the study was on the release of specific CC chemokines induced by Cr^{3+} and their potential downregulation by simvastatin, cell mortality, cell morphology, and the release of TNF–α, a commonly-used marker of inflammation, were also analyzed to further evaluate the potential of simvastatin to decrease Cr^{3+} cytotoxicity and inflammatory effects.

2.4 *MATERIALS AND METHODS*

2.4.1 Cells

The J774A.1 monocyte/macrophage mouse cell line American Type Culture Collection (ATCC) was maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2, in complete growth medium composed of Dulbecco's Modified Eagle Medium (DMEM; Catalogue No. 319-005-CL; Wisent) supplemented with premium grade heat-inactivated fetal bovine serum (FBS; Catalogue No. 080-150; Wisent) to a final concentration of 5% (v/v).

Cells were harvested for experiments by scrapping, or by pipetting with a Class A volumetric pipette, and allowed to recover for 3–4 hours under cell culture conditions with periodic mixing. The harvested macrophages were then washed and resuspended in complete growth medium.

2.4.2 Trivalent chromium ions

Stock Cr^{3+} solutions were freshly prepared by dissolving a chromium hexahydrate salt ($CrCl_3 \cdot 6H_2O$; Sigma-Aldrich) in DMEM without serum, and were sterile-filtered using 0.2-μm pore size cellulose acetate membrane syringe filters (28145-477). Sterile-filtration of the stock solutions did not cause a detectable loss of Cr^{3+}, as determined by inductively coupled plasma-mass spectrometry (ICPMS) (London Laboratory Services Group, London Health Sciences Centre, London, ON) (data not shown).

2.4.3 Simvastatin

Simvastatin was activated using the protocol of Sadeghi et al.,[39] as recommended by the supplier (Catalogue No. S6196; Sigma-Aldrich). Briefly, simvastatin (4 mg) was completely dissolved in absolute ethanol (100 μL) in glass endotoxin-free vials (GenScript, Piscataway, NJ), and the solution was alkalinized with 0.1 N sodium hydroxide (150 μL). Simvastatin was activated by a 2-hour incubation in an oven at 50 °C (to convert the drug into its pharmacologically active free-acid form). At the end of the

incubation, the solution was thermally equilibrated to room temperature, and the pH was adjusted to 7.6 with 1 N hydrochloric acid.

2.4.4 Experimental protocol

For each experimental condition, 0.5×10^6 macrophages were resuspended in 1 mL of complete growth medium containing Cr^{3+} at different concentrations: 50, 150, 250, 350, and 500 ppm, with and without 5 or 10 μM simvastatin. Macrophages incubated without Cr^{3+} served as negative controls, and macrophages incubated with 1 μg/mL of lipopolysaccharides (LPS) from *E. coli* O55:B5 (L6529) were used as positive controls (data not shown) for cell activation and cytokine/chemokine production, as previously described.[40–42] Macrophages incubated with simvastatin, but without Cr^{3+}, were used as additional experimental controls to test the effects of the drug alone.

All samples, in tightly capped 5-mL untreated polystyrene culture tubes (Corning, Tewksbury, MA), were incubated at 37 °C for 24 hours with constant rotation (8 rpm). At the end of the incubation, the cell suspensions were analyzed for cell mortality and centrifuged ($150\times g$ for 6 min at room temperature). Supernatants were frozen/stored at −80 °C for cytokine release measurements by enzyme-linked immunosorbent assay (ELISA).

The rate of oxygen consumption by macrophages (0.5×10^6 per mL) incubated at 37 °C in complete growth medium without Cr^{3+} nor simvastatin was measured using an Oxygraph-2k respirometer (Oroboros Instruments, Innsbruck, Austria) calibrated as described by Soltysinska et al.[43]. The initial rate of oxygen consumption, approximately 50 pmol of oxygen (O_2) per second per 10×10^6 cells, indicated that the macrophages in the tightly-capped culture tubes were not under hypoxic stress during the 24-hour incubations since less than 10% of the total oxygen available in the tubes was consumed by the end of the incubation.

2.4.5 Cell mortality

Cell mortality was analyzed by measuring the percentages of dead cells (identified by trypan blue staining) and the total number of cells (viable and dead) remaining in the cell suspension at the end of the incubation. Cells were counted using an improved Neubauer hemocytometer under phase contrast microscopy.

2.4.6 Cell morphology

The effects of Cr^{3+} on cell morphology with and without simvastatin were observed under light microscopy. For photomicrography, the cell suspensions were transferred into poly-d-lysine-coated 35-mm glass-bottom culture dishes (MatTek Corporation, Ashland, MA) at the end of the 24-hour incubation. Micrographs were taken with a Nikon Digital Sight DS-Ri1 camera mounted on a Nikon Eclipse Ti inverted microscope system using NIS Elements Basic Research imaging software version 3.20.01 and a 60x plan apochromatic water immersion objective (Melville, NY).

2.4.7 TNF–α and CC chemokine release

Freshly thawed culture supernatants were gently mixed and concentrations of TNF–α, MCP–1, MIP–1α, MIP–1β, and RANTES were measured by ELISA with the following kits: Mouse TNF–α Quantikine® ELISA, Mouse CCL2/JE/MCP–1 Quantikine® ELISA, Mouse CCL3/MIPIalpha Quantikine® ELISA, Mouse CCL4/MIP–1β Quantikine® ELISA, and Mouse CCL5/RANTES Quantikine® ELISA, as per manufacturer's instructions (R&D Systems). Absorbance measurements were performed at 450 nm using a hybrid microplate reader (Synergy™ 4; BioTek). The nominal minimum concentrations of TNF–α, MCP–1, MIP–1α, MIP–1β, and RANTES detectable by ELISA were 5.1, 2, 1.5, 3, and 2 pg/mL, respectively, and the kits were specific enough to avoid cross-reactivity with other cytokines (as per manufacturer's specifications).

2.4.8 Statistical analysis

Data are presented as mean $\pm$ S.E.M. (with propagated error) of 3 to 7 experiments. Statistical analysis implemented in R-3.1.0 was performed using a pairwise additive two-way analysis of variance (ANOVA) with sample as a fixed effect and experiment as a random effect. A p-value less than 0.05 was considered significant.

2.5 *RESULTS*

2.5.1 Effects of Cr^{3+} on the mortality of J774A.1 macrophages with and without simvastatin

2.5.1.1 Percentage of dead cells

Dye-exclusion analysis revealed a Cr^{3+} concentration-dependent increase in the percentages of dead macrophages (Figure 1A). A significant increase was observed with all Cr^{3+} concentrations analyzed, up to 46% with 500 ppm ($p<0.001$).

Simvastatin at 5 μM had a tendency to increase the percentage of dead cells although a significant increase was observed only in the negative control (macrophages with no Cr^{3+}) and with 50 ppm Cr^{3+} (4% in both cases; $p<0.02$). Increasing the concentration of simvastatin from 5 μM to 10 μM further increased the percentage of dead cells by 4% with 50 ppm Cr^{3+} ($p=0.008$), and also induced a significant increase in both the negative control and with 350 ppm Cr^{3+} compared to the same condition without simvastatin (8% in both cases; $p<0.03$) (Figure 1A).

2.5.1.2 Total number of cells

Total (viable and dead) cell counts revealed a Cr^{3+}-induced decrease of 15% to 25% in the total number of cells, relative to the negative control (macrophages with no Cr^{3+}), with all Cr^{3+} concentrations analyzed ($p<0.001$) (Figure 1B).

With the exception of a 13% decrease observed in the negative control with 5 μM simvastatin, relative to the same condition without the drug ($p<0.001$) (Figure 1B), simvastatin at both 5 and 10 μM had a tendency to increase the total number of cells with all Cr^{3+} concentrations, although a significant increase was observed only at 10 μM simvastatin with 150 ppm and 250 ppm Cr^{3+} (15% and 18%, respectively; $p<0.04$).

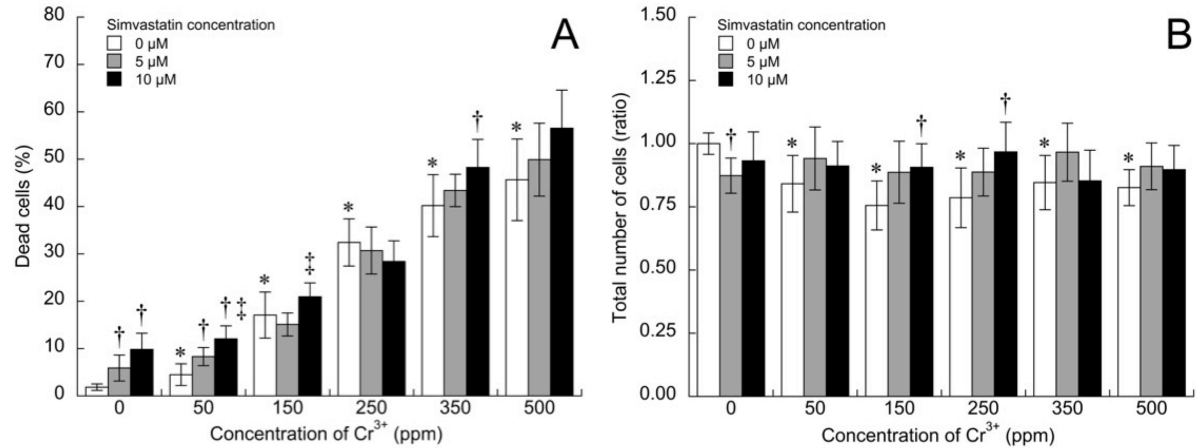

Figure 1: Mortality of J774A.1 macrophages after a 24-hour exposure to Cr^{3+} with and without simvastatin: (A) Percentage of dead cells; (B) Total number of cells (viable and dead) expressed as a ratio of the total number of cells in the absence of Cr^{3+} and simvastatin. An asterisk (*) indicates a significant difference ($p<0.05$) between the negative control without simvastatin and a given Cr^{3+} concentration. A dagger (†) indicates a significant difference ($p<0.05$) between a given Cr^{3+} concentration without simvastatin and the same Cr^{3+} concentration with simvastatin. A double dagger (‡) indicates a significant difference ($p<0.05$) between 5 and 10 μM simvastatin at a given Cr^{3+} concentration. Cells were incubated with the indicated concentrations of Cr^{3+} and simvastatin at 37 °C under constant rotation (8 rpm). Cells were counted by hemocytometry and dead cells were identified using the trypan blue dye-exclusion method. Data are presented as means ± S.E.M. of 7 independent experiments performed in triplicate.

2.5.2 Effects of Cr^{3+} on the morphology of J774A.1 macrophages with and without simvastatin

Photomicrographs of macrophages incubated in the absence and presence of 250 ppm Cr^{3+} (Figure 2A and 2B, respectively) show that Cr^{3+} at this concentration increased macrophage mortality, as indicated by the presence of cellular debris (Figure 2B), and induced vacuolization and cell swelling. Simvastatin at 10 μM effectively prevented Cr^{3+}-induced vacuolization and swelling (Figure 2D), while it had no detectable effects on the morphology of the macrophages in the absence of Cr^{3+} (Figure 2C). Interestingly, as exemplified in the upper right corner of Figure 2D, simvastatin also prevented the loss of cell division observed in the presence of 250 ppm Cr^{3+} without simvastatin.

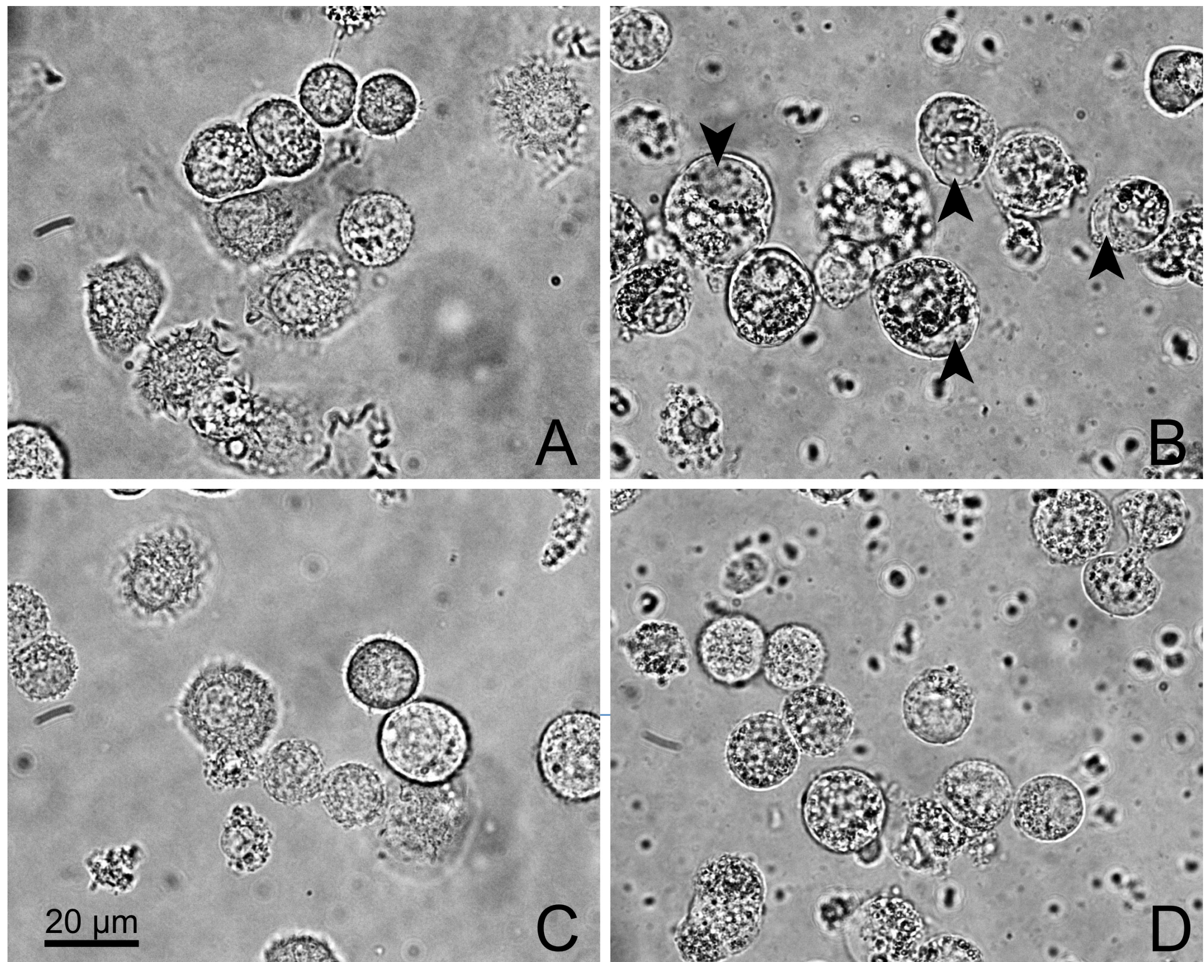

Figure 2: Morphology of J774A.1 macrophages after a 24-hour incubation with: (A) no Cr^{3+} and no simvastatin; (B) 250 ppm Cr^{3+} without simvastatin; (C) no Cr^{3+} with 10 μM simvastatin; and (D) 250 ppm Cr^{3+} with 10 μM simvastatin. Vacuoles are indicated by arrowheads. Cell suspensions were transferred into poly-D-lysine-coated glass-bottom culture dishes for photomicrography.

2.5.3 Effects of Cr^{3+} on TNF–α release by J774A.1 macrophages with and without simvastatin

ELISAs revealed a Cr^{3+}-induced increase in TNF–α release of 112% to 160% with 150 ppm to 350 ppm Cr^{3+} ($p<0.002$), relative to the negative control (macrophages with no Cr^{3+}) (Figure 3). In contrast, a decrease was observed with 500 ppm Cr^{3+} ($p<0.001$), likely reflecting the high toxicity of Cr^{3+} at this elevated concentration.

Simvastatin at 5 μM induced a 40% decrease in TNF–α release in the negative control ($p<0.001$), and a 39%, 112%, and 118% decrease with 50 ppm, 150 ppm, and 350 ppm Cr^{3+}, respectively ($p<0.002$) (Figure 3). The effect of simvastatin was not significant with 250 ppm Cr^{3+}, possibly due to the large S.E.M. at this concentration. Increasing the concentration of simvastatin from 5 μM to 10 μM did not further decrease TNF–α release with any chromium (III) (Cr^{3+}) concentrations. Importantly, the difference between TNF–α release in the negative control (macrophages with no Cr^{3+}) and the highest TNF–α release was 160% without simvastatin but only 131% and 76% with 5 and 10 μM simvastatin, respectively, demonstrating that simvastatin decreased Cr^{3+}-induced release of TNF–α.

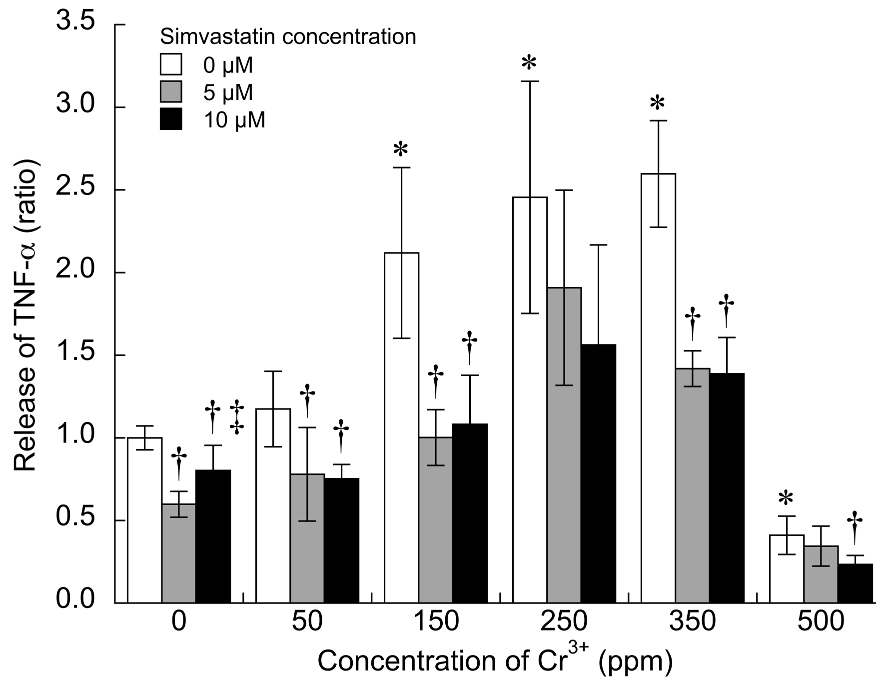

Figure 3: Tumor necrosis factor–α (TNF–α) release by J774A.1 macrophages after a 24-hour exposure to Cr^{3+} with and without simvastatin. TNF–α release is expressed as a ratio of the release in the absence of Cr^{3+} and simvastatin. An asterisk (*) indicates a significant difference ($p<0.05$) between the negative control without simvastatin and a given Cr^{3+} concentration without simvastatin. A dagger (†) indicates a significant difference ($p<0.05$) between a given Cr^{3+} concentration without simvastatin and the same Cr^{3+} concentration with simvastatin. A double dagger (‡) indicates a significant difference ($p<0.05$) between 5 and 10 µM simvastatin at a given Cr^{3+} concentration. Cells were incubated with the indicated concentrations of Cr^{3+} and simvastatin at 37 °C under constant rotation (8 rpm). TNF–α release was measured by ELISA. Data are presented as means ± S.E.M. of 4 independent experiments performed in triplicate.

2.5.4 Effects of Cr^{3+} on CC chemokine release by J774A.1 macrophages with and without simvastatin

2.5.4.1 MCP–1

Incubation of the macrophages with Cr^{3+} induced a low but significant increase in MCP–1 of 27% and 20% with 50 ppm and 150 ppm Cr^{3+}, respectively ($p<0.004$) (Figure 4A). However, a significant decrease of 15% and 77% was observed with 350 ppm and 500 ppm Cr^{3+} ($p<0.02$), likely reflecting the high toxicity of Cr^{3+} at these elevated concentrations, especially 500 ppm.

Simvastatin at 5 μM decreased the release of MCP–1 by 75% in the negative control (macrophages with no Cr^{3+}) ($p<0.001$) and by 14% to 88% with all Cr^{3+} concentrations analyzed ($p<0.001$) (Figure 4A). Increasing the concentration of simvastatin from 5 μM to 10 μM further decreased the release of MCP–1 in all conditions analyzed, by 3% to 25% ($p<0.001$).

2.5.4.2 **MIP–1α**

Results showed a Cr^{3+}-induced increase in MIP–1α release ranging from 46% to 100% with 50 ppm to 350 ppm Cr^{3+} ($p<0.001$), relative to the negative control (macrophages with no Cr^{3+}) (Figure 4B). In contrast, a significant decrease of 69% was observed with 500 ppm Cr^{3+} ($p<0.001$), likely reflecting the high toxicity of Cr^{3+} at this elevated concentration.

Similarly to what was observed with TNF–α and MCP–1 release, simvastatin at 5 μM decreased the release of MIP–1α by 66% in the negative control ($p<0.001$), and by 8% to 130% with all Cr^{3+} concentrations analyzed ($p<0.005$) (Figure 4B). Increasing the simvastatin concentration from 5 μM to 10 μM further decreased the release by 9% in the negative control ($p=0.011$) and by 9% to 31% with all Cr^{3+} concentrations analyzed ($p<0.001$). Importantly, the difference between MIP–1α release in the negative control and the highest MIP–1α release was 100% without simvastatin but only 47% and 30% with 5 μM and 10 μM simvastatin, respectively, demonstrating that simvastatin decreased Cr^{3+}-induced release of MIP–1α.

2.5.4.3 **MIP–1β**

In contrast to what was observed with MIP–1α release, 50 ppm Cr^{3+} did not significantly increase MIP–1β release in macrophages, and Cr^{3+} concentrations of 250 ppm to 500 ppm decreased MIP–1β release by 32% to 84% ($p<0.001$) (Figure 4C).

Simvastatin at 5 μM decreased the release of MIP−1β by 8% to 81% in all conditions analyzed ($p<0.02$), relative to the release in the same conditions without simvastatin (Figure 4C). Increasing the concentration of simvastatin from 5 μM to 10 μM further decreased the release by 4% to 26% ($p<0.03$), with the maximum effect observed in the negative control (macrophages with no Cr^{3+}).

2.5.4.4 RANTES

A significant increase in RANTES release was observed in macrophages incubated with 50 ppm and 150 ppm Cr^{3+} (18% and 63% relative to the negative control; $p=0.040$ and $p<0.001$, respectively) (Figure 4D). In contrast, a significant decrease was observed with 350 ppm and 500 ppm Cr^{3+} (39% and 76%, respectively; $p<0.001$) likely reflecting the high toxicity of the metal ion at these elevated concentrations.

Simvastatin at 5 μM decreased the release of RANTES by 58% in the negative control (macrophages with no Cr^{3+}) ($p<0.001$), and by 15% to 65% with all Cr^{3+} concentrations analyzed ($p<0.03$) (Figure 4D). Increasing the concentration of simvastatin from 5 μM to 10 μM further decreased the release by 2% to 49% with all Cr^{3+} concentrations ($p<0.005$), except 500 ppm. Importantly, the difference between RANTES release in the negative control (macrophages with no Cr^{3+}) and the highest RANTES release was 63% without simvastatin but only 55% and 18% with 5 μM and 10 μM simvastatin, respectively, demonstrating that simvastatin decreased Cr^{3+}-induced release of RANTES.

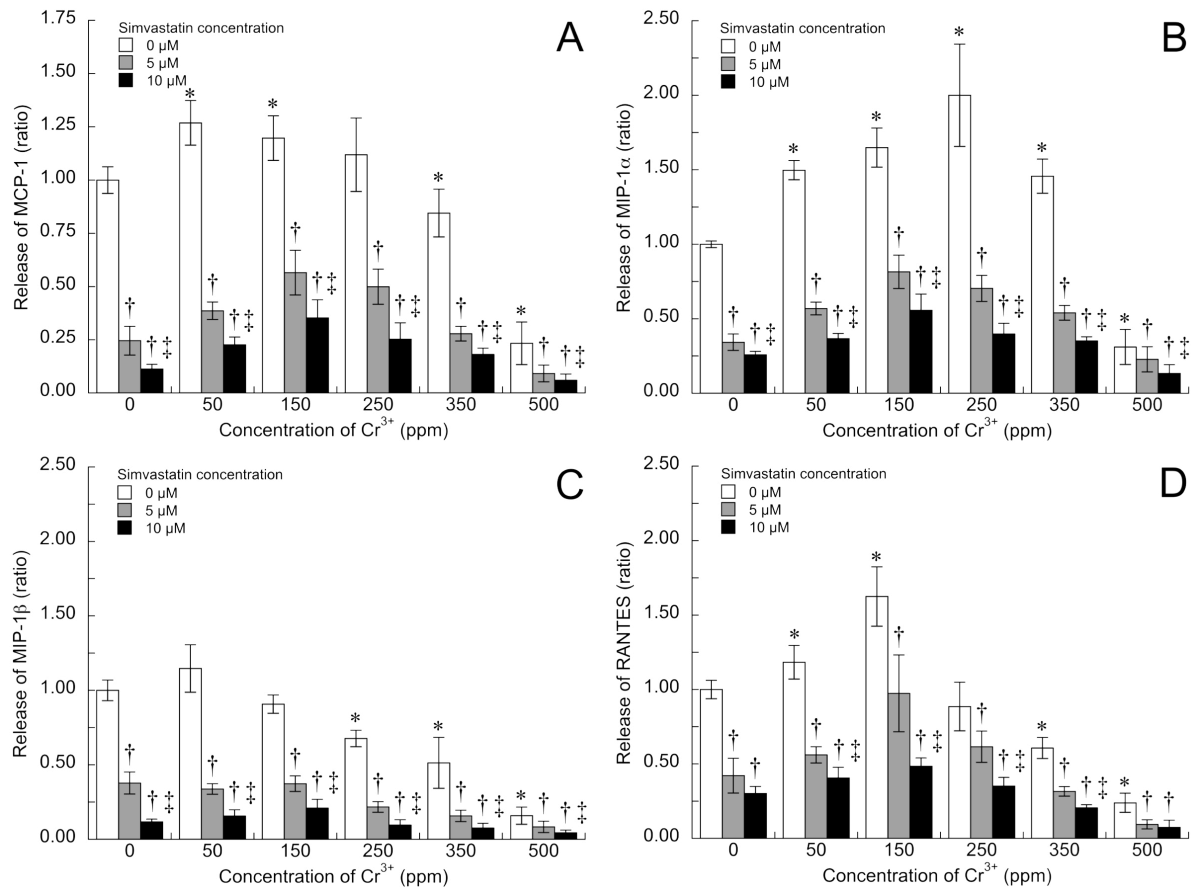

Figure 4: Chemokine release by J774A.1 macrophages after a 24-hour exposure to Cr^{3+} with and without simvastatin. (A) Monocyte chemotactic protein–1 (MCP–1); (B) Macrophage inflammatory protein–1α (MIP–1α); (C) Macrophage inflammatory protein–1β (MIP–1β); (D) Regulated on activation, normal T cell expressed and secreted (RANTES). Release is expressed as a ratio of the release in the absence of Cr^{3+} and simvastatin. An asterisk (*) indicates a significant difference ($p<0.05$) between the negative control without simvastatin and a given Cr^{3+} concentration without simvastatin. A dagger (†) indicates a significant difference ($p<0.05$) between a given Cr^{3+} concentration without simvastatin and the same Cr^{3+} concentration with simvastatin. A double dagger (‡) indicates a significant difference ($p<0.05$) between 5 and 10 μM simvastatin at a given Cr^{3+} concentration. Cells were incubated with the indicated concentrations of Cr^{3+} and simvastatin at 37 °C under constant rotation (8 rpm). Chemokine release was measured by ELISA. Data are presented as means ± S.E.M. of 3 independent experiments performed in triplicate.

2.6 DISCUSSION

The present study analyzed cell mortality, cell morphology, as well as the release of TNF–α and CC chemokines following macrophage exposure to Cr^{3+} with and without simvastatin.

This study focused on the effects of chromium ions because stainless steel alloys and CoCrMo alloys, two alloys used extensively in orthopaedic applications, contain large proportions of chromium. *In vivo*, these alloys are subjected to wear and corrosion that leads to significant metal ion release. While cobalt ions are also of interest, being released from CoCrMo alloys, they have been studied more extensively because of their greater toxicity and consequent clinical concerns.[27,28,44] Chromium ions exist in two main oxidation states: trivalent (Cr^{3+}) and hexavalent (Cr^{6+}). This study focused on Cr^{3+} because of the reactivity and consequent instability of Cr^{6+}.[45] The selection of relatively high Cr^{3+} concentrations was based on previous *in vitro* studies using the same culture model,[2,23,24] and because ion levels in periprosthetic tissues are likely higher than those in body fluids where they get diluted and are eventually partially eliminated.[2] In addition, the stimulation of non-prestimulated cells *in vitro* requires higher concentrations of an exogenous stimulating agent than the stimulation of cells *in vivo* since the latter are simultaneously subjected to multiple stimulating factors.[24]

Macrophages were used in this study because they are an important player in the immune system and the most prominent cell type in periprosthetic tissues.[46] They can also act as antigen-presenting cells in type-IV hypersensitivity reactions, which are lymphocyte-mediated.[47] More specifically, the J774A.1 mouse monocyte/macrophage cell line was selected because it has been demonstrated to release similar inflammatory cytokines and exhibit a similar morphology to macrophages in periprosthetic tissues at the bone-cement interface.[48] It is also a cell line commonly used in *in vitro* studies of the inflammatory response to orthopaedic wear products including bone cement, polyethylene, ceramic, and metal particles, as well as metal ions.[2,23,24,40,42,49–57]

Mortality results showed that Cr^{3+} induced J774A.1 macrophage death in a dose-dependent manner, in agreement with previous studies using the same cell line.[2,23,24] The maximum percentage of dead cells in the present study was similar to previously reported results with the same Cr^{3+} concentration (46% dead cells compared to 41% reported by Catelas et al.[2]). It should however be noted that the percentage of dead cells can be either over- or under-estimated because of the difficulty in distinguishing dark trypan blue-stained cells from cells darkened by the adherence of green chromium ions to the cell membrane. Finally, Cr^{3+}-induced cell death, particularly at high concentrations of the metal ion, can be both rapid and violent causing the cells to burst thereby producing large cellular debris that can be misidentified as trypan blue-stained cells. Total cell counts showed a 15% to 25% decrease in the overall cell number with all Cr^{3+} concentrations analyzed, compared to the negative control (macrophages with no Cr^{3+}). This difference may reflect a Cr^{3+}-induced cell mortality that was undetectable by dye-

exclusion analysis because of cell lysis. Alternatively, the difference may reflect an inhibition of cell proliferation by the ions. In either case, the results reflect an additional form of Cr^{3+} cytotoxicity.

Measurements of TNF–α release showed a dose-dependent response to Cr^{3+}, similar to the one previously reported by Catelas et al. with the same cell line.[2] The maximum release was however higher in the present study, possibly because of differences in the components of the culture medium and serum as well as culture conditions (rotation vs. static). Both studies demonstrated that stimulation with 500 ppm Cr^{3+} resulted in a significant decrease in TNF–α release. This may be due to the high toxicity of Cr^{3+} at elevated concentrations inducing rapid cell death, and/or to non-specific interactions cell-Cr^{3+} interactions interfering with the ability of the macrophages to produce TNF–α.[2]

Previous work by Nakashima et al. demonstrated that titanium and poly(methyl methacrylate) (PMMA) wear particles upregulate the ability of monocytes/macrophages to produce MCP–1 and MIP–1α in a time- and dose-dependent manner.[32] The analysis of CC chemokine release in the present study demonstrated that Cr^{3+}, up to 250 ppm, induced a significant dose-dependent increase in MIP–1α release (up to 200% of the negative control). As previously mentioned, MIP–1α is known to stimulate the production of IL–1, IL–6, and TNF–α, as well as regulate the migration of monocytes/macrophages, B cells, cytotoxic T cells, NK cells, and eosinophils. MIP–1α may therefore play a significant role in the inflammatory response observed in periprosthetic tissues containing metal wear products. A significant increase in the release of RANTES (up to 63%) and a low but significant increase in the production of MCP–1 (up to 27%) were also observed with the lower Cr^{3+} concentrations (50 ppm and 150 ppm). Although these increases may be attributed to macrophage activation resulting from constant rotation of the cells, it is also possible that larger Cr^{3+}-induced increases would be observed with prolonged incubation. A 24-hour incubation period was chosen to avoid high levels of metal ion-induced mortality. It is also a commonly-used incubation period for *in vitro* cell culture models with implant wear products.[2,23,32,42,50,53,54,58–62]

Interestingly, while 50 ppm to 350 ppm of Cr^{3+} increased MIP–1α release, a significant decrease in MIP–1β was observed with 250 ppm and 350 ppm Cr^{3+}, reaching concentrations lower than those in the negative controls. This may be due to increased cell mortality with increasing Cr^{3+} concentration, but it may also reflect a downregulation of MIP–1β in the presence of Cr^{3+}, and/or a time-dependent relationship between inter- and intra-cellular signaling molecules. As previously mentioned, Nakashima et al.[32] showed an increase in MCP–1 and MIP–1α release in macrophages exposed to titanium and PMMA particles, but no increase in RANTES. Although the cell culture system was different from that used in the present study, comparison of the results suggests that different wear products induce the upregulation of different CC chemokines, possibly reflecting differences in the interactions of the cells with these products and/or in the cell activation mechanisms. Finally, a large decrease in the release of all CC chemokines was observed with high concentrations of Cr^{3+} (especially

500 ppm). As previously mentioned, this decrease may be due to the high toxicity of Cr^{3+} at elevated concentrations inducing rapid cell death, and/or non-specific cell-Cr^{3+} interactions interfering with the ability to produce chemokines. Overall, the results of the present study suggest that while high concentrations of Cr^{3+} may be too toxic to stimulate CC chemokine release, lower concentrations of Cr^{3+} are capable of inducing selective inflammatory CC chemokine release, with a predominance of MIP–1α, at the time and ion concentrations analyzed.

Simvastatin (5 μM and 10 μM; concentrations selected based on previous *in vitro* studies[37,63–65]) significantly increased the percentage of dead (trypan blue-stained) cells in the negative control (macrophages with no Cr^{3+}). Also, the total cell numbers were slightly but significantly decreased by 5 μM simvastatin in the negative control (13%). However, simvastatin had a tendency to increase the total number of cells in the presence of Cr^{3+}, although a significant increase was observed only at 10 μM simvastatin and with 150 ppm and 250 ppm Cr^{3+} (15% and 18%, respectively). Although these changes remain small and may be attributed to limitations of the dye-exclusion and hemocytometry techniques, as previously discussed, it may also suggest a modest reduction of Cr^{3+}-induced cell mortality by simvastatin. Longer incubations would be required to further investigate this potential effect. Higher concentrations of simvastatin could also be considered, but the increased mortality observed with 10 μM simvastatin in the negative control suggests that higher concentrations may be contraindicated. Interestingly, light microscopy showed that simvastatin at 10 μM helped to preserve the morphology of the macrophages with 250 ppm Cr^{3+} by preventing the formation of large vacuoles and cell swelling. Simvastatin also prevented the Cr^{3+}-induced loss of cell division with 250 ppm Cr^{3+}. This observation is in agreement with cell counts showing that simvastatin had a tendency to increase the total number of cells in the presence of Cr^{3+} (Figure 1B), and more specifically increased the number of viable cells (from $55 \pm 11\%$ to $72 \pm 11\%$ with 250 ppm Cr^{3+}; $p<0.02$) (data not shown).

Simvastatin significantly decreased the release of TNF–α, and all chemokines analyzed, in all experimental conditions (i.e., with and without Cr^{3+}). While the specific pathways involved in the upregulation of pro-inflammatory mediators by Cr^{3+} are not well understood, the modulation of this response by simvastatin may involve the geranylgeranyl pyrophosphate pathway.[66] This pathway has also been proposed to be responsible for the beneficial effects of statins on osteoblasts[67,68] and osteoclasts.[69] With the exception of TNF–α, the effects of simvastatin on chemokine release were dose-dependent, inducing significant decreases relative to the corresponding conditions without simvastatin. Although the decrease of chemokine release was significantly greater with 10 μM simvastatin than with 5 μM, future studies aiming to further analyze the effects of simvastatin concentration on the macrophage response should also consider the potential cytotoxic effects of higher drug concentrations, as demonstrated by a low but significant increase (8%) in the percentages of dead cells in the negative control (macrophages with no Cr^{3+}) with 10 μM simvastatin.

Surprisingly, a high release of TNF–α and all chemokines was observed in the negative control without simvastatin, explaining why simvastatin decreased cytokine release below that of the negative control without simvastatin. The elevated baseline release can largely be attributed to the constant rotation of the cells in culture, as demonstrated by a supplementary experiment in which macrophages with no Cr^{3+} and no simvastatin were incubated in parallel with and without rotation. Results from this experiment showed that rotation increased concentrations of MCP–1, MIP–1α, and RANTES by two to four fold ($p<0.001$; data not shown). While incubations were performed under rotation to ensure constant resuspension of the cells that would otherwise adhere to the cell culture tubes, rotation may have activated the macrophages, leading to the release of TNF–α and chemokines. Therefore, this release of TNF–α and chemokines (that was not induced by Cr^{3+}) may have partially hidden the effects of simvastatin (at both 5 and 10 μM) on the Cr^{3+}-induced TNF–α and chemokine release. However, the difference between the negative control and the highest TNF–α, MIP–1α, and RANTES release with simvastatin was significantly lower than the corresponding difference without simvastatin, thereby demonstrating that the statin can decrease Cr^{3+}-induced TNF–α and chemokine release. This demonstrates the potential of simvastatin to decrease the Cr^{3+}-induced release of major inflammatory mediators by macrophages, and thereby the potential of this drug to reduce metal ion-induced inflammatory response. Other interesting properties of simvastatin relevant to orthopaedic applications, and specifically periprosthetic osteolysis, are its demonstrated ability to inhibit osteoclast-mediated bone resorption by decreasing osteoclast function[63] and to stimulate bone formation by enhancing osteoblast maturation and function.[70] *In vivo*, simvastatin has been shown to improve osseointegration of orthopaedic implants in animal trials[71,72] and to decrease osteolysis induced by polyethylene particles in mouse calvaria[73,74] and rabbit models.[75] *In vitro*, studies have also demonstrated the applicability of simvastatin treatments for the attenuation of titanium wear particle-induced IL–6 expression and production.[68]

Overall, this study showed that while high concentrations of Cr^{3+} may be too toxic to stimulate TNF–α and CC chemokine release, lower concentrations of Cr^{3+} are capable of inducing TNF–α and selective inflammatory CC chemokine release with a predominance of MIP–1α, at the time and Cr^{3+} concentrations analyzed. The presence of simvastatin prevented Cr^{3+}-induced vacuolization and cell swelling, and reduced the release of TNF–α and all chemokines (MCP–1, MIP–1α, MIP–1β, and RANTES) with all Cr^{3+} concentrations analyzed. This demonstrates that simvastatin may be a suitable candidate to reduce metal ion-induced inflammatory response leading to periprosthetic osteolysis and other adverse tissue reactions.

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2.8 REFERENCES

[1] P. E. Beaulé, I. Catelas, and J. B. Medley. *Current controversies, Bearing surface: metal-on-metal*, chap. 62, 468–477 (Elsevier, 2009), first edit edn.

[2] I. Catelas, A. Petit, D. J. Zukor, J. Antoniou, and O. L. Huk. TNF-alpha secretion and macrophage mortality induced by cobalt and chromium ions in vitro-qualitative analysis of apoptosis. *Biomaterials* **24**, 383–91 (2003).

[3] I. Catelas and J. J. Jacobs. Biologic activity of wear particles. *Instr. Course Lect.* **59**, 3–16 (2010).

[4] P. F. Doorn, J. M. Mirra, P. A. Campbell, and H. C. Amstutz. Tissue reaction to metal on metal total hip prostheses. *Clin. Orthop. Relat. Res.* **329 Suppl**, S187–205 (1996).

[5] L. D. Dorr, Z. Wan, D. B. Longjohn, B. Dubois, and R. Murken. Total hip arthroplasty with use of the Metasul metal-on-metal articulation. four to seven-year results. *J. Bone Joint Surg. Am.* **82**, 789–98 (2000).

[6] T. P. Schmalzried, P. C. Peters, B. T. Maurer, C. R. Bragdon, and W. H. Harris. Long-duration metal-on-metal total hip arthroplasties with low wear of the articulating surfaces. *J. Arthroplasty* **11**, 322–31 (1996).

[7] Y. Kwon, A. V. Lombardi, J. J. Jacobs, T. K. Fehring, C. G. Lewis, and M. E. Cabanela. Risk stratification algorithm for management of patients with metal-on-metal hip arthroplasty: consensus statement of the American Association of Hip and Knee Surgeons, the American Academy of Orthopaedic Surgeons, and the Hip Society. *J. Bone Joint Surg. Am.* **96**, e4 (2014).

[8] J. Antoniou, D. J. Zukor, F. Mwale, W. Minarik, A. Petit, and O. L. Huk. Metal ion levels in the blood of patients after hip resurfacing: a comparison between twenty-eight and thirty-six-millimeter-head metal-on-metal prostheses. *J. Bone Joint Surg. Am.* **90 Suppl 3**, 142–8 (2008).

[9] A. G. Cobb and T. P. Schmalzreid. The clinical significance of metal ion release from cobalt-chromium metal-on-metal hip joint arthroplasty. *Proc. Inst. Mech. Eng., H J. eng. med.* **220**, 385–98 (2006).

[10] H. S. Dobbs and M. Minski. Metal ion release after total hip replacement. *Biomaterials* **1**, 193–198 (1980).

[11] N. J. Hallab and J. J. Jacobs. Biologic effects of implant debris. *Bull. NYU Hosp. Jt. Dis.* **67**, 182–8 (2009).

[12] N. J. Hallab, M. Caicedo, K. McAllister, A. Skipor, H. Amstutz, and J. J. Jacobs. Asymptomatic prospective and retrospective cohorts with metal-on-metal hip arthroplasty indicate acquired lymphocyte reactivity varies with metal ion levels on a group basis. *J. Orthop. Res.* **31**, 173–82 (2013).

[13] A. Hartmann, F. Hannemann, J. Lützner, A. Seidler, H. Drexler, K.-P. Günther, and J. Schmitt. Metal ion concentrations in body fluids after implantation of hip replacements with metal-on-metal bearing–systematic review of clinical and epidemiological studies. *PLoS One* **8**, 1–25 (2013).

[14] A. K. Skipor, P. A. Campbell, L. M. Patterson, H. C. Anstutz, T. P. Schmalzried, and J. J. Jacobs. Serum and urine metal levels in patients with metal-on-metal surface arthroplasty. *J. Mater. Sci. Mater. Med.* **13**, 1227–34 (2002).

[15] R. B. Cook, B. J. R. F. Bolland, J. A. Wharton, S. Tilley, J. M. Latham, and R. J. K. Wood. Pseudotumour formation due to tribocorrosion at the taper interface of large diameter metal on polymer modular total hip replacements. *J. Arthroplasty* **28**, 1430–6 (2013).

[16] H. Cooper, C. Della Valle, R. Berger, M. Tetreault, W. Paprosky, S. Sporer, and J. Jacobs. Corrosion at the head-neck taper as a cause for adverse local tissue reactions after total hip arthroplasty. *J. Bone Joint Surg. Am.* **94**, 1655–61 (2012).

[17] H. J. Cooper, R. M. Urban, R. L. Wixson, R. M. Meneghini, and J. J. Jacobs. Adverse local tissue reaction arising from corrosion at the femoral neck-body junction in a dual-taper stem with a cobalt-chromium modular neck. *J. Bone Joint Surg. Am.* **95**, 865–72 (2013).

[18] I. P. S. Gill, J. Webb, K. Sloan, and R. J. Beaver. Corrosion at the neck-stem junction as a cause of metal ion release and pseudotumour formation. *J. Bone Joint Surg. Br.* **94**, 895–900 (2012).

[19] G. B. Higgs, J. A. Hanzlik, D. W. MacDonald, J. L. Gilbert, C. M. Rimnac, and S. M. Kurtz. Is increased modularity associated with increased fretting and corrosion damage in metal-on-metal total hip arthroplasty devices?: a retrieval study. *J. Arthroplasty* **28**, 2–6 (2013).

[20] H. Krishnan, S. P. Krishnan, G. Blunn, J. A. Skinner, and A. J. Hart. Modular neck femoral stems. *Bone. Joint. J.* **95-B**, 1011–21 (2013).

[21] R. Pivec, R. M. Meneghini, W. J. Hozack, G. H. Westrich, and M. A. Mont. Modular taper junction corrosion and failure: how to approach a recalled total hip arthroplasty implant. *J. Arthroplasty* **29**, 1–6 (2014).

[22] Y. Kwon, S. J. Ostlere, P. McLardy-Smith, N. A. Athanasou, H. S. Gill, and D. W. Murray. Asymptomatic pseudotumors after metal-on-metal hip resurfacing arthroplasty: prevalence and metal ion study. *J. Arthroplasty* **26**, 511–8 (2011).

[23] I. Catelas, A. Petit, D. J. Zukor, and O. L. Huk. Cytotoxic and apoptotic effects of cobalt and chromium ions on J774 macrophages - Implication of caspase-3 in the apoptotic pathway. *J. Mater. Sci.: Mater. Med.* **12**, 949–53 (2001).

[24] I. Catelas, A. Petit, H. Vali, C. Fragiskatos, R. Meilleur, D. J. Zukor, J. Antoniou, and O. L. Huk. Quantitative analysis of macrophage apoptosis vs. necrosis induced by cobalt and chromium ions in vitro. *Biomaterials* **26**, 2441–53 (2005).

[25] D. Granchi, E. Verri, G. Ciapetti, L. Savarino, E. Cenni, A. Gori, and A. Pizzoferrato. Effects of chromium extract on cytokine release by mononuclear cells. *Biomaterials* **19**, 283–91 (1998).

[26] J. Wang, B. Wicklund, R. Gustilo, and D. Tsukayama. Titanium, chromium and cobalt ions modulate the release of bone-associated cytokines by human monocytes/macrophages in vitro. *Biomaterials* **17**, 2233–40 (1996).

[27] B. M. Devitt, J. M. Queally, M. Vioreanu, J. S. Butler, D. Murray, P. P. Doran, and J. M. O'Byrne. Cobalt ions induce chemokine secretion in a variety of systemic cell lines. *Acta Orthop.* **81**, 756–64 (2010).

[28] J. M. Queally, B. M. Devitt, J. S. Butler, A. P. Malizia, D. Murray, P. P. Doran, and J. M. O'Byrne. Cobalt ions induce chemokine secretion in primary human osteoblasts. *J. Orthop. Res.* **27**, 855–64 (2009).

[29] S. B. Goodman and T. Ma. Cellular chemotaxis induced by wear particles from joint replacements. *Biomaterials* **31**, 5045–50 (2010).

[30] R. Chiu and S. B. Goodman. *Biological response of osteoblasts and osteoprogenitors to orthopaedic wear debris*, chap. 8, 183–222 (InTech, 2012).

[31] T. Lin, Y. Tamaki, J. Pajarinen, H. A. Waters, D. K. Woo, Z. Yao, and S. B. Goodman. Chronic inflammation in biomaterial-induced periprosthetic osteolysis: NF-κB as a therapeutic target. *Acta Biomater.* **10**, 1–10 (2014).

[32] Y. Nakashima, D. H. Sun, M. Trindade, L. E. Chun, Y. Song, S. B. Goodman, D. J. Schurman, W. J. Maloney, and R. L. Smith. Induction of macrophage C-C chemokine expression by titanium alloy and bone cement particles. *J. Bone Joint Surg. Br.* **81**, 155–62 (1999).

[33] K. Conlon, A. Lloyd, U. Chattopadhyay, N. Lukacs, S. Kunkel, T. Schall, D. Taub, C. Morimoto, J. Osborne, J. Oppenheim, H. Young, D. Kelvin, and J. Ortaldo. CD8+ and CD45RA+ human peripheral blood lymphocytes are potent sources of macrophage inflammatory protein 1 alpha, interleukin-8 and RANTES. *Eur. J. Immunol.* **25**, 751–6 (1995).

[34] E. M. Schwarz. What potential biologic treatments are available for osteolysis? *J. Am. Acad. Orthop. Surg.* **16**, S72–S75 (2008).

[35] A. Lübbeke, G. Garavaglia, K. J. Rothman, A. Bonvin, C. Roussos, H. Miozzari, and P. Hoffmeyer. Statins may reduce femoral osteolysis in patients with total hip arthroplasty. *J. Orthop. Res.* **31**, 814–820 (2013).

[36] T. M. Thillemann, A. B. Pedersen, F. Mehnert, S. P. Johnsen, and K. Søballe. The risk of revision after primary total hip arthroplasty among statin users: a nationwide population-based nested case-control study. *J. Bone Joint Surg. Am.* **92**, 1063–72 (2010).

[37] B. P. Leung, N. Sattar, A. Crilly, M. Prach, D. W. McCarey, H. Payne, R. Madhok, C. Campbell, J. A. Gracie, F. Y. Liew, and I. B. McInnes. A novel anti-inflammatory role for simvastatin in inflammatory arthritis. *J. Immunol.* **170**, 1524–1530 (2003).

[38] G. Weitz-Schmidt. Statins as anti-inflammatory agents. *Trends Pharmacol. Sci.* **23**, 482–6 (2002).

[39] M. M. Sadeghi, M. Collinge, R. Pardi, and J. R. Bender. Simvastatin modulates cytokine-mediated endothelial cell adhesion molecule induction: involvement of an inhibitory G protein. *J. Immunol.* **165**, 2712–8 (2000).

[40] A. Prabhu, C. E. Shelburne, and D. F. Gibbons. Cellular proliferation and cytokine responses of murine macrophage cell line J774A.1 to polymethylmethacrylate and cobalt-chrome alloy particles. *J. Biomed. Mater. Res.* **42**, 655–63 (1998).

[41] M. Taira, T. Kagiya, H. Harada, M. Sasaki, S. Kimura, T. Narushima, T. Nezu, and Y. Araki. Microscopic observations and inflammatory cytokine productions of human macrophage phagocytising submicron titanium particles. *J. Mater. Sci.: Mater. Med.* **21**, 267–75 (2010).

[42] R. VanOs, L. L. Lildhar, E. A. Lehoux, P. E. Beaulé, and I. Catelas. In vitro macrophage response to nanometer-size chromium oxide particles. *J. Biomed. Mater. Res. B Appl. Biomater.* **102**, 149–59 (2014).

[43] E. Soltysinska, B. Bentzen, M. Barthmes, H. Hattel, A. Thrush, M. Harper, K. Qvortrup, F. Larsen, T. Schiffer, J. Losa-Reyna, J. Straubinger, A. Kniess, M. Thomsen, A. Brüggemann, S. Fenske, M. Biel, P. Ruth, C. Wahl-Schott, R. Boushel, S. Olesen, and R. Lukowski. KCNMA1 encoded cardiac BK channels afford protection against ischemia-reperfusion injury. *PloS one* **9**, e103402 (2014).

[44] A. J. Hart, P. D. Quinn, F. Lali, B. Sampson, J. A. Skinner, J. J. Powell, J. Nolan, K. Tucker, S. Donell, A. Flanagan, and J. F. W. Mosselmans. Cobalt from metal-on-metal hip replacements may be the clinically relevant active agent responsible for periprosthetic tissue reactions. *Acta Biomater.* **8**, 3865–73 (2012).

[45] A. Sargeant and T. Goswami. Hip implants - paper VI - ion concentrations. *Mater. Des.* **28**, 155–71 (2007).

[46] P. A. Campbell, M. Wang, H. C. Amstutz, and S. B. Goodman. Positive cytokine production in failed metal-on-metal total hip replacements. *Acta Orthop. Scand.* **73**, 506–12 (2002).

[47] N. J. Hallab, K. Mikecz, and J. J. Jacobs. A triple assay technique for the evaluation of metal-induced, delayed-type hypersensitivity responses in patients with or receiving total joint arthroplasty. *J. Biomed. Mater. Res.* **53**, 480–9 (2000).

[48] S. M. Horowitz, S. B. Doty, J. M. Lane, and A. H. Burstein. Studies of the mechanism by which the mechanical failure of polymethylmethacrylate leads to bone resorption. *J. Bone Joint Surg. Am.* **75**, 802–13 (1993).

[49] D. Bagchi, M. Bagchi, and S. J. Stohs. Chromium (VI)-induced oxidative stress, apoptotic cell death and modulation of p53 tumor suppressor gene. *Mol. Cell. Biochem.* **222**, 149–58 (2001).

[50] I. Catelas, O. L. Huk, A. Petit, D. J. Zukor, R. Marchand, and L. Yahia. Flow cytometric analysis of macrophage response to ceramic and polyethylene particles: effects of size, concentration, and composition. *J. Biomed. Mater. Res.* **41**, 600–7 (1998).

[51] P. L. Palmbos, M. J. Sytsma, D. H. DeHeer, and J. D. Bonnema. Macrophage exposure to particulate titanium induces phosphorylation of the protein tyrosine kinase lyn and the phospholipases Cγ-1 and Cγ-2. *J. Orthop. Res.* **20**, 483–9 (2002).

[52] A. Rodrigo, G. Vallés, L. Saldaña, M. Rodríguez, M. Martínez, L. Munuera, and N. Vilaboa. Alumina particles influence the interactions of cocultured osteoblasts and macrophages. *J. Orthop. Res.* **24**, 46–54 (2006).

[53] I. Catelas, A. Petit, D. J. Zukor, R. Marchand, L. Yahia, and O. L. Huk. Induction of macrophage apoptosis by ceramic and polyethylene particles in vitro. *Biomaterials* **20**, 625–30 (1999).

[54] I. Catelas, A. Petit, R. Marchand, D. J. Zukor, L. H. Yahia, and O. L. Huk. Cytotoxicity and macrophage cytokine release induced by ceramic and polyethylene particles in vitro. *J. Bone Joint Surg. Br.* **81**, 516–21 (1999).

[55] S. M. Horowitz and M. A. Purdon. Mechanisms of cellular recruitment in aseptic loosening of prosthetic joint implants. *Calcif. Tissue Int.* **57**, 301–5 (1995).

[56] O. L. Huk, D. J. Zukor, J. Antoniou, and A. Petit. Effect of pamidronate on the stimulation of macrophage TNF-alpha release by ultra-high-molecular-weight polyethylene particles: a role for apoptosis. *J. Orthop. Res.* **21**, 81–7 (2003).

[57] S. S. Jakobsen, A. Larsen, M. Stoltenberg, J. M. Bruun, and K. Soballe. Effects of as-cast and wrought cobalt-chrome-molybdenum and titanium-aluminium-vanadium alloys on cytokine gene expression and protein secretion in J774A.1 macrophages. *Eur. Cell. Mater.* **14**, 45–54 (2007).

[58] A. Tsaousi, E. Jones, and C. P. Case. The in vitro genotoxicity of orthopaedic ceramic (Al2O3) and metal (CoCr alloy) particles. *Mutat. Res.* **697**, 1–9 (2010).

[59] C. Fleury, A. Petit, F. Mwale, J. Antoniou, D. J. Zukor, M. Tabrizian, and O. L. Huk. Effect of cobalt and chromium ions on human MG-63 osteoblasts in vitro: morphology, cytotoxicity, and oxidative stress. *Biomaterials* **27**, 3351–60 (2006).

[60] M. A. Germain, A. Hatton, S. Williams, J. B. Matthews, M. H. Stone, J. Fisher, and E. Ingham. Comparison of the cytotoxicity of clinically relevant cobalt-chromium and alumina ceramic wear particles in vitro. *Biomaterials* **24**, 469–79 (2003).

[61] A. M. Kaufman, C. I. Alabre, H. E. Rubash, and A. S. Shanbhag. Human macrophage response to UHMWPE, TiAlV, CoCr, and alumina particles: analysis of multiple cytokines using protein arrays. *J. Biomed. Mater. Res. A* **84**, 464–74 (2008).

[62] L. Luo, A. Petit, J. Antoniou, D. J. Zukor, O. L. Huk, R. C. W. Liu, F. M. Winnik, and F. Mwale. Effect of cobalt and chromium ions on MMP-1, TIMP-1, and TNF-alpha gene expression in human U937 macrophages: a role for tyrosine kinases. *Biomaterials* **26**, 5587–93 (2005).

[63] A. Hughes, M. J. Rogers, A. I. Idris, and J. C. Crockett. A comparison between the effects of hydrophobic and hydrophilic statins on osteoclast function in vitro and ovariectomy-induced bone loss in vivo. *Calcif. Tissue Int.* **81**, 403–13 (2007).

[64] T. T. Tuomisto, H. Lumivuori, E. Kansanen, S.-K. Häkkinen, M. P. Turunen, J. V. van Thienen, A. J. Horrevoets, A.-L. Levonen, and S. Ylä-Herttuala. Simvastatin has an anti-inflammatory effect on macrophages via upregulation of an atheroprotective transcription factor, Kruppel-like factor 2. *Cardiovasc. Res.* **78**, 175–84 (2008).

[65] A. A. Zeki, P. Thai, N. J. Kenyon, and R. Wu. Differential effects of simvastatin on IL-13-induced cytokine gene expression in primary mouse tracheal epithelial cells. *Respir. Res.* **13**, 1–9 (2012).

[66] N. R. Veillard, V. Braunersreuther, C. Arnaud, F. Burger, G. Pelli, S. Steffens, and F. Mach. Simvastatin modulates chemokine and chemokine receptor expression by geranylgeranyl isoprenoid pathway in human endothelial cells and macrophages. *Atherosclerosis* **188**, 51–8 (2006).

[67] T. Maeda, A. Matsunuma, I. Kurahashi, T. Yanagawa, H. Yoshida, and N. Horiuchi. Induction of osteoblast differentiation indices by statins in MC3T3-E1 cells. *J. Cell. Biochem.* **92**, 458–71 (2004).

[68] G. Vallés, C. Pérez, A. Boré, F. Martín-Saavedra, L. Saldaña, and N. Vilaboa. Simvastatin prevents the induction of interleukin-6 gene expression by titanium particles in human osteoblastic cells. *Acta Biomater.* **9**, 4916–25 (2013).

[69] J. Woo, H. Nakagawa, A. M. Krecic, K. Nagai, A. D. Hamilton, S. M. Sebti, and P. H. Stern. Inhibitory effects of mevastatin and a geranylgeranyl transferase i inhibitor (GGTI-2166) on mononuclear osteoclast formation induced by receptor activator of NFκB ligand (RANKL) or tumor necrosis factor-α (TNF-α). *Biochem. Pharmacol.* **69**, 87–95 (2005).

[70] G. Mundy. Inflammatory mediators and the destruction of bone. *J. Periodontal Res.* **26**, 213–7 (1991).

[71] Y. Ayukawa, Y. Ogino, Y. Moriyama, I. Atsuta, Y. Jinno, M. Kihara, Y. Tsukiyama, and K. Koyano. Simvastatin enhances bone formation around titanium implants in rat tibiae. *J. Oral Rehabil.* **37**, 123–30 (2010).

[72] K. Başarir, B. Erdemli, A. Can, E. Erdemli, and T. Zeyrek. Osseointegration in arthroplasty: can simvastatin promote bone response to implants? *Int. Orthop.* **33**, 855–9 (2009).

[73] F. von Knoch, C. Wedemeyer, A. Heckelei, G. Saxler, G. Hilken, J. Brankamp, T. Sterner, S. Landgraeber, F. Henschke, F. Löer, and M. von Knoch. Promotion of bone formation by simvastatin in polyethylene particle-induced osteolysis. *Biomaterials* **26**, 5783–9 (2005).

[74] F. von Knoch, A. Heckelei, C. Wedemeyer, G. Saxler, G. Hilken, F. Henschke, F. Löer, and M. von Knoch. The effect of simvastatin on polyethylene particle-induced osteolysis. *Biomaterials* **26**, 3549–55 (2005).

[75] T. Tanigo, R. Takaoka, and Y. Tabata. Sustained release of water-insoluble simvastatin from biodegradable hydrogel augments bone regeneration. *J. Controlled Release* **143**, 201–6 (2010).

Part IV

Thesis Discussion

Conclusions From the Thesis

If you thought that science was certain - well, that is just an error on your part.

Richard P. Feynman

The two manuscripts included in this thesis describe two complementary *in vitro* studies using a murine model and metal ions derived from chloride salts. The first study tested the effects of metal ions on both chemokine release by macrophages and lymphocyte migration, thereby fulfilling the first two objectives of the thesis. The second study tested the effects of simvastatin on the modulation of chemokine release, thereby fulfilling the third, and final, objective of the thesis.

These studies have shown that Co^{2+} and Cr^{3+}, metal ions released from CoCrMo alloys used in orthopaedic applications, induced the release of TNF–α and CC chemokines that was ion-specific and dose-dependent. Specifically, lower concentrations of Co^{2+} and Cr^{3+} primarily induced an upregulation in the release of TNF–α and MIP–1α. High concentrations, on the other hand, appeared to be too toxic to stimulate the release of cytokines. Results also suggested that Co^{2+} and Cr^{3+} may be capable of directly stimulating the migration of T cells but not that of B cells. All together, these results suggest that Co^{2+} and Cr^{3+} have the potential to create a micro-environment that would favour a T cell-mediated response (*i.e.*, type-IV hypersensitivity reaction) *in vivo*. Lastly, results showed that simvastatin prevented Cr^{3+}-induced vacuolization and cell swelling, as well as reduced the release of TNF–α and all chemokines (MCP–1, MIP–1α, MIP–1β, and RANTES) after 24h incubation with all Cr^{3+} concentrations analyzed, suggesting its potential as a candidate to modulate the Cr^{3+}-induced inflammatory response.

Clinical Importance of Findings

Science never solves a problem without creating ten more.

George Bernard Shaw

One of the reasons why the use of MM hip implants was revisited during the last two decades is the lower volumetric wear of these implants compared to conventional MPE implants and the expected lower inflammatory response. There are, however, clinical concerns regarding the risks of adverse tissue reactions to metal wear and corrosion products, and more specifically to metal ions.

Results of the studies presented in this thesis demonstrated that Co^{2+} and Cr^{3+} could induce the release of specific pro-inflammatory mediators, especially TNF–α and MIP–1α. Of particular interest, when comparing the current studies with previous *in vitro* work reported in the literature, is the selective nature of the upregulated chemokines by Co^{2+} and Cr^{3+}. This implies that there may be specific molecular targets for pharmaceutical modulation of inflammation induced by different wear or corrosion products from various implants.

The chemokine study presented in this thesis focused on several important CC chemokines because of their specificity to macrophage and lymphocyte migration. If the cocktail of chemokines in cultures of macrophages exposed to metal ions induced T cell chemotaxis, results would give some insights into the mechanisms of ion-mediated hypersensitivity. Interestingly, results showed T cell migration in culture supernatants from macrophages exposed to Co^{2+} and Cr^{3+}, suggesting that the chemokines present in the culture supernatants induced chemotaxis. However, a similar level of T cell migration was found in equilibrated culture medium (as opposed to culture supernatants) containing Co^{2+} and Cr^{3+}. This indicates that these ions alone, resuspended in culture medium containing FBS, may be capable of stimulating T cell migration, which would create a micro-environment that would favour a T-cell mediated response *in vivo*. Overall, the results of this thesis provide some insights into the mechanisms by which metal ions may induce a T cell-mediated response in periprosthetic tissues of patients with metal implants.

Considering the large number of patients with a hip implant, some may benefit from a pharmaceutical agent capable of modulating ion-induced inflammation that can potentially lead to further adverse tissue reactions. Results of this thesis showed that simvastatin significantly decreased ion-induced vacuolization and lowered the release of TNF–α and CC chemokines. This demonstrates that simvastatin

may be a suitable candidate to reduce metal ion-induced inflammation leading to periprosthetic osteolysis and other adverse tissue reactions in patients with metal implants.

Part IV — Chapter 3

Technical Considerations and Future Work

It is difficult to say what is impossible, for the dream of yesterday is the hope of today and the reality of tomorrow.

Robert H. Goddard

While the results of this thesis and their potential clinical impact are promising, some technical limitations may have interfered with the interpretation of the results and should be considered in future studies.

3.1 EXPERIMENTAL DESIGN CONSIDERATIONS

3.1.1 Ion cytotoxicity on J774A.1 cells

Adjustments to the experimental protocols could include a better control of the cell preparation steps. In the studies described in this thesis, macrophages were harvested and allowed to recover in 50-mL polypropylene tubes for 3–4 hours under cell culture conditions with periodic mixing. However, membrane resilience to trypan blue was the only parameter used to define the length of the recovery period and may not fully reflect the health of the cells. Additionally, although the rate of cell respiration did not appear to consume all the oxygen contained in the 5-mL polystyrene tubes used for the experiments, as explained in manuscript two, it is possible that when the tubes were not incubated in rotation, the partial pressure of O_2 in the medium may have been lower at the bottom of the tubes, implying greater resistance to oxygen diffusion and equilibration. A hypoxia control test could therefore be considered prior to future studies using the same set-up. A senescence control might also be helpful to determine if the medium column height and initial cell concentration could also affect the cell response. The height of the medium column would be easier to optimize if experiments were performed in multi-well culture plates. Experimental designs using multi-well culture plates would also be easier to scale, if needed. The trade-off, however, would be the use of a larger quantity of reagents.

Despite the fact that macrophages adhere locally to the site of injury or inflammation under normal *in vivo* conditions, experiments investigating the modulation of ion-induced inflammation using simvastatin were performed in rotation, which enabled the macrophages to remain in suspension. The rotating condition was chosen to be able to better mimic, and thereby be more comparable to previ-

ous studies using wear particles, especially polyethylene particles which float, stick to the walls of the culture tube, and have overall poor bioavailability [1, 2]. It also allowed direct cytotoxicity assessment by Trypan blue and flow cytometry, without having to detach and resuspend the otherwise strongly adherent cells. Interestingly, although rotation induced an increase in cytokine release (likely because of cell activation), the overall increases in the presence of Co^{2+} and Cr^{3+} relative to the negative control (cells with no ions) remained comparable, especially for TNF–α and MIP–1α.

Future studies could include prolonged incubation times, along with lower ion concentrations. In this case, the use of non-proliferating cells (*e.g.*, primary cells) should be investigated in order to avoid cell proliferation over time, creating confounding factors in the analysis. Extended incubations would however require supplementation or complete changes of the culture medium, which would change the culture conditions (*i.e.*, levels of statins or metal ions, cytokines released in the supernatants, etc.). Despite being proliferating (transformed) murine cells, the J774A.1 mouse monocyte/macrophage cell line was selected for the present work because: (1) it has been shown to exhibit a similar morphology to macrophages in periprosthetic tissues at the bone-cement interface [3]; (2) it is a cell line commonly used in *in vitro* studies of orthopaedic wear products including bone cement, polyethylene, ceramic, and metal particles, as well as metal ions [1, 2, 4–15], therefore allowing a direct comparison of results with the literature; (3) it gives flexible access to large numbers of cells. If primary cells were to be used in future studies, a few potential concerns should be considered, including the potential variability between donors and the difficulty to have regular access to large numbers of cells required for each experiment.

Finally, consideration could also be given to the use of ions obtained from clinically relevant wear products (as opposed to chloride salts), as previously described [16]. Extracting soluble products may, however, prove to be challenging.

3.1.2 Lymphocyte migration

While possible to obtain, the maintenance of lymphocyte cell lines are considerably more involved and costly than that of the J774A.1 monocyte/macrophage cell line. Therefore, for the migration experiments, lymphocytes were isolated from the spleen of C57BL/6 mice, a common murine lymphocyte model.

The initial experimental design of lymphocyte migration experiments only included culture supernatants from J774A.1 cells exposed to Co^{2+} and Cr^{3+} and culture supernatants from cells alone as a negative control. However, over the course of the study, it was observed that the levels of migration in the culture supernatants from cells exposed to the ions were lower than that in the supernatant control, at least at the ion concentrations analyzed. Additional controls consisting of equilibrated culture medium

supplemented with Co^{2+} or Cr^{3+} (as opposed to cell culture supernatants following macrophage exposure to Co^{2+} or Cr^{3+}) were then added in the last experiment. Similarly, the addition of a B cell marker was only included in the last experiment. Therefore, the migration experiments should be repeated to gain statistical confidence.

Additionally, a control consisting of serum-free culture medium supplemented with Co^{2+} or Cr^{3+} could be considered in order to better isolate the effects of the ions alone on cell migration. Endotoxin contents of the cobalt and chromium ion solutions should also be evaluated. Finally, the level of lymphocyte pre-sensitization to metals as well as the identification of specific T cell subtypes within the migrated cell population (*e.g.*, Th, Tc) could be considered.

3.1.3 Effects of simvastatin on macrophage response to metal ions

During experiments investigating the modulation of ion-induced inflammation using simvastatin, cells were exposed to both the drug and the metal ions simultaneously. Future work could consider exposing the cells to the drug prior to the ions, or, alternatively, after the cells have been exposed to the ions. These different experimental approaches would give additional insights into the potential preventive vs. curative effects of the drug.

3.2 *ELISA QUANTIFICATION*

Cytokine and chemokine release from J774A.1 cells quantified by ELISA may have been underestimated because of potential ion-protein binding that may have led to organometallic proteins presenting a different conformation or binding sites not recognizable by the ELISA. Thus, this possible interference should be investigated.

3.3 *SIMVASTATIN INVESTIGATIONS*

3.3.1 Effects of simvastatin on macrophage response to Co^{2+}

The second manuscript included in this thesis describes the effects of simvastatin on the modulation of J774A.1 cell response to Cr^{3+} under rotation. Future studies should consider analyzing the effects of simvastatin on the modulation of cell response to Co^{2+}. Preliminary experiments have been performed under rotation. However, the results of chemokine release induced by Co^{2+} in the absence of simvastatin were inconsistent. Therefore, troubleshooting would be required to complete these experiments.

3.3.2 Simvastatin and lymphocyte migration

Future studies could include testing the effects of simvastatin (and possibly other statins) on lymphocyte migration. Interestingly Ulivieri *et al.*, [17] demonstrated that simvastatin can inhibit migration of lymphocytes towards strong chemoattractants such as SDF–1α. This study suggests that simvastatin may have the potential to modulate lymphocyte migration in periprosthetic tissues.

References

[1] I. Catelas, O. L. Huk, A. Petit, D. J. Zukor, R. Marchand, and L. Yahia, "Flow cytometric analysis of macrophage response to ceramic and polyethylene particles: effects of size, concentration, and composition," *J. Biomed. Mater. Res.*, vol. 41, no. 4, pp. 600–7, Sep. 1998.

[2] I. Catelas, A. Petit, D. J. Zukor, R. Marchand, L. Yahia, and O. L. Huk, "Induction of macrophage apoptosis by ceramic and polyethylene particles in vitro," *Biomaterials*, vol. 20, no. 7, pp. 625–30, Apr. 1999.

[3] S. M. Horowitz, S. B. Doty, J. M. Lane, and A. H. Burstein, "Studies of the mechanism by which the mechanical failure of polymethylmethacrylate leads to bone resorption," *J. Bone Joint Surg. Am.*, vol. 75, no. 6, pp. 802–13, Jun. 1993.

[4] D. Bagchi, M. Bagchi, and S. J. Stohs, "Chromium (VI)-induced oxidative stress, apoptotic cell death and modulation of p53 tumor suppressor gene," *Mol. Cell. Biochem.*, vol. 222, no. 1-2, pp. 149–58, Jun. 2001.

[5] P. L. Palmbos, M. J. Sytsma, D. H. DeHeer, and J. D. Bonnema, "Macrophage exposure to particulate titanium induces phosphorylation of the protein tyrosine kinase lyn and the phospholipases Cγ-1 and Cγ-2," *J. Orthop. Res.*, vol. 20, no. 3, pp. 483–9, May 2002.

[6] A. Prabhu, C. E. Shelburne, and D. F. Gibbons, "Cellular proliferation and cytokine responses of murine macrophage cell line J774A.1 to polymethylmethacrylate and cobalt-chrome alloy particles," *J. Biomed. Mater. Res.*, vol. 42, no. 4, pp. 655–63, Dec. 1998.

[7] A. Rodrigo, G. Vallés, L. Saldaña, M. Rodríguez, M. Martínez, L. Munuera, and N. Vilaboa, "Alumina particles influence the interactions of cocultured osteoblasts and macrophages," *J. Orthop. Res.*, vol. 24, no. 1, pp. 46–54, Jan. 2006.

[8] R. VanOs, L. L. Lildhar, E. A. Lehoux, P. E. Beaulé, and I. Catelas, "In vitro macrophage response to nanometer-size chromium oxide particles," *J. Biomed. Mater. Res. B Appl. Biomater.*, vol. 102, no. 1, pp. 149–59, Aug. 2014.

[9] I. Catelas, A. Petit, R. Marchand, D. J. Zukor, L. H. Yahia, and O. L. Huk, "Cytotoxicity and macrophage cytokine release induced by ceramic and polyethylene particles in vitro," *J. Bone Joint Surg. Br.*, vol. 81, no. 3, pp. 516–21, May 1999.

[10] I. Catelas, A. Petit, D. J. Zukor, and O. L. Huk, "Cytotoxic and apoptotic effects of cobalt and chromium ions on J774 macrophages - Implication of caspase-3 in the apoptotic pathway," *J. Mater. Sci.: Mater. Med.*, vol. 12, no. 10-12, pp. 949–53, 2001.

[11] I. Catelas, A. Petit, D. J. Zukor, J. Antoniou, and O. L. Huk, "TNF-alpha secretion and macrophage mortality induced by cobalt and chromium ions in vitro-qualitative analysis of apoptosis," *Biomaterials*, vol. 24, no. 3, pp. 383–91, Feb. 2003.

[12] I. Catelas, A. Petit, H. Vali, C. Fragiskatos, R. Meilleur, D. J. Zukor, J. Antoniou, and O. L. Huk, "Quantitative analysis of macrophage apoptosis vs. necrosis induced by cobalt and chromium ions in vitro," *Biomaterials*, vol. 26, no. 15, pp. 2441–53, May 2005.

[13] S. M. Horowitz and M. A. Purdon, "Mechanisms of cellular recruitment in aseptic loosening of prosthetic joint implants," *Calcif. Tissue Int.*, vol. 57, no. 4, pp. 301–5, Oct. 1995.

[14] O. L. Huk, D. J. Zukor, J. Antoniou, and A. Petit, "Effect of pamidronate on the stimulation of macrophage TNF-alpha release by ultra-high-molecular-weight polyethylene particles: a role for apoptosis," *J. Orthop. Res.*, vol. 21, no. 1, pp. 81–7, Jan. 2003.

[15] S. S. Jakobsen, A. Larsen, M. Stoltenberg, J. M. Bruun, and K. Soballe, "Effects of as-cast and wrought cobalt-chrome-molybdenum and titanium-aluminium-vanadium alloys on cytokine gene expression and protein secretion in J774A.1 macrophages," *Eur. Cell. Mater.*, vol. 14, pp. 45–54, Sep. 2007.

[16] D. Granchi, E. Verri, G. Ciapetti, L. Savarino, E. Cenni, A. Gori, and A. Pizzoferrato, "Effects of chromium extract on cytokine release by mononuclear cells," *Biomaterials*, vol. 19, no. 1-3, pp. 283–91, Jan-Feb 1998.

[17] C. Ulivieri, D. Fanigliulo, D. Benati, F. L. Pasini, and C. T. Baldari, "Simvastatin impairs humoral and cell-mediated immunity in mice by inhibiting lymphocyte homing, T-cell activation and antigen cross-presentation," *Eur. J. Immunol.*, vol. 38, no. 10, pp. 2832–44, Oct. 2008.