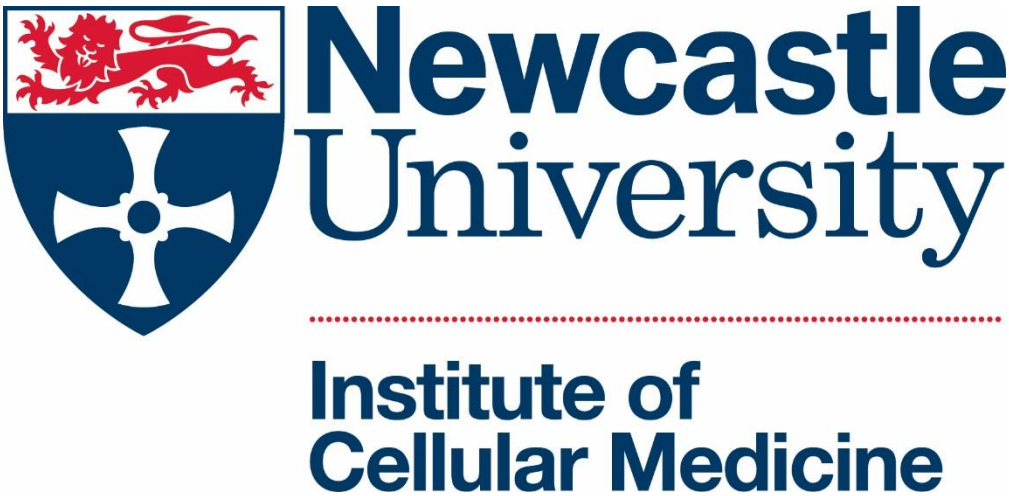

Cellular recognition of metal ions and mechanisms of reactivity

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June 2016

Thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy

Abstract

Hip replacement is the main therapeutic intervention for end-stage osteoarthritis (OA). Metal-on-metal (MoM) hip implants were introduced to provide a durable alternative to ceramic and polyethylene devices, particularly in a younger patient population. However they are associated with the development of adverse reactions to metal debris (ARMD) which includes osteolysis, soft tissues necrosis, and inflammatory pseudotumours.

MoM implants are usually fabricated from a cobalt-chrome alloy. Cobalt activates human Toll-like receptor 4 (TLR4), an innate immune receptor also activated by bacterial lipopolysaccharide (LPS). This study set out to investigate the inflammatory consequences of cobalt-mediated TLR4 activation through a series of *in vitro* assays developed throughout this work.

In human MonoMac 6 macrophages cobalt was found to increase secretion and expression of pro-inflammatory cytokines including IL-8, IL-6 and CCL20. Using IL-8 as a marker of TLR4 activation a small molecule TLR4 antagonist, CLI-095, was shown to inhibit these effects, indicating that they are TLR4-dependent. Similar responses were observed in endothelial cells and osteoblasts. A monoclonal anti-TLR4 neutralising antibody inhibited cobalt-mediated IL-8 expression and secretion, while a polyclonal anti-TLR4 neutralising antibody did not.

Further investigation showed that cobalt-mediated TLR4 activation increases expression of intercellular adhesion molecule 1 (*ICAM1*) and its soluble form sICAM-1. It also promotes primary monocyte and neutrophil migration. A TLR4 mutation did not prevent inflammatory responses to cobalt, although further assay optimisation is required. Co-stimulation of MonoMac 6 cells with cobalt and LPS or nickel caused downregulation of *IL6*, *CCL2* and *IL8* expression. Finally, unlike cobalt, chromium and strontium ions did not activate TLR4 and did not induce IL-6 or IL-8 secretion in macrophages.

In summary, this study has shown that TLR4 activation by cobalt ions from MoM hip implants results in increased cellular inflammatory responses. The use of TLR4 inhibitors in this study suggests that TLR4 is a potential therapeutic target in ARMD. Overall, the TLR4 signalling pathway is an interesting avenue for further investigation into factors underlying MoM implant failure.

Declaration

I declare that no portion of the work compiled in this thesis has been submitted in support of any other degree or qualification at Newcastle University or any other university or institute of learning. The work has been carried out by myself unless otherwise stated. All sources of information have been acknowledged accordingly by means of reference.

For Mum, Dad and Peter

With love and thanks

Acknowledgements

I am very grateful to my supervisors, Dr Alison Tyson-Capper, Professor John Kirby, Professor David Deehan and Mr Jim Holland for supporting this project from the very beginning and providing me with invaluable guidance throughout my PhD. I would also like to thank them for the opportunities they have given me over the last 4 years and for encouraging me to make the most of my research both in and out of the lab. This project was funded by the Newcastle NIHR Biomedical Research Centre and DePuy Synthes Ltd and I am grateful for their support.

I would like to thank Dr John Taylor (Newcastle University) and Professor Joanne Tipper (Leeds University) for the time they took to critique my thesis and for an enjoyable viva experience.

Many people have been very generous with their time throughout my studies. I would particularly like to thank Dr Jem Palmer for his help with MonoMac 6 cell culture and for allowing me to conduct countless ELISAs in his lab. I am very grateful to Dr Hannah Gautrey for providing sound advice and common sense when mine was (frequently) absent, and for always being willing to help me out. I would like to acknowledge Sami Anjum and Amy Mawdesley for their hard work during their MRes projects and for allowing me to use their data in this thesis. Thank you to all members of the Tyson-Capper lab past and present who have been great to work with and have patiently sat through many TLR4 talks. Thanks are also due to everyone in the Kirby/Ali lab for welcoming me into their lab and meetings.

A very big thank you goes to Ann, David and Anne-Marie Lilley for welcoming me to their family, and for kindly allowing me to write most of this thesis from the comfort of their sofa. I would particularly like to thank David for his thoughts on the introduction.

To Mum and Dad, I am more grateful than I can say for all the support you have given me over the years in everything I do. I hope that you enjoy reading this from cover to cover!

My final thank you is to Peter for bearing the brunt of final year angst, supplying words of encouragement (‘is it not done yet?’) and providing positive vibes when mine were lacking. I promise I’m going to be a real adult now.

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Abbreviations

ALVAL	Aseptic lymphocyte-dominated vasculitis-associated lesion
ANOVA	Analysis of variance
ARMD	Adverse reaction to metal debris
CCL2	Chemokine (C-C motif) ligand 2
CCL20	Chemokine (C-C motif) ligand 20
CD14	Cluster of differentiation 14
cDNA	Complementary deoxyribonucleic acid
$CoCl_2\,6H_2O$	Cobalt chloride hexahydrate
$CrCl_3\,6H_2O$	Chromium chloride hexahydrate
CXCL10	Chemokine (C-X-C motif) ligand 10
DAMP	Damage-associated molecular pattern
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
HBSS	Hanks' Balanced Salt Solution
HEK	Human embryonic kidney (cells)
HIF1α	Hypoxia-inducible factor 1α
HMEC-1	Human microvascular endothelial cells
HMGB1	High mobility group box protein 1
HSPA1A	Heat shock 70kDa protein 1A
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
IL-1α	Interleukin-1 alpha
IL-6	Interleukin-6
IL-8	Interleukin-8
IRAKM/IRAK3	Interleukin-1 receptor-associated kinase M/3
ITGA4	Integrin alpha 4 (CD49d)
ITGAL	Integrin alpha L (CD11a)
LFA-1	Lymphocyte function-associated antigen-1
LPS	Lipopolysaccharide
MAb2-hTLR4	Monoclonal anti-TLR4 neutralising antibody
MD2/LY96	Myeloid differentiation protein 2/lymphocyte antigen 96
MCG	Multi-nucleated giant cell
MoM	Metal-on-metal
MoP	Metal-on-polyethylene

mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation response protein 88
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
$NiCl_2\,6H_2O$	Nickel chloride hexahydrate
OA	Osteoarthritis
OPG	Osteoprotegerin
PAb-hTLR4	Polyclonal anti-TLR4 neutralising antibody
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PRR	Pattern recognition receptor
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
SEAP	Secreted alkaline phosphatase
sICAM-1	Soluble intercellular adhesion molecule-1
SNP	Single nucleotide polymorphism
$SrCl_2\,6H_2O$	Strontium chloride hexahydrate
THR	Total hip replacement
TICAM1/TRIF	TIR-domain-containing adaptor molecule 1/TIR-domain-containing adaptor-inducing interferon-β
TICAM2/TRAM	TIR-domain-containing adaptor molecule 2/TRIF-related adaptor molecule
TLR4	Toll-like receptor 4
TRAP	Tartrate-resistant acid phosphatase
UHMWPE	Ultra-high molecular weight polyethylene
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very late antigen-4

Chapter 1. Introduction

1.1. Total hip replacement

Total hip replacement (THR) is a major therapeutic intervention for those with end-stage joint diseases such as osteoarthritis and rheumatoid arthritis. In these conditions the cartilage that forms the articulating surface of the hip joint is worn away, increasing friction and causing pain. The modern THR was devised by Sir John Charnley in 1962 and involves removal of the femoral head and acetabular component of the affected hip and replacement with a prosthetic joint implant. The structure of a modern THR is shown in **Figure 1.1**. In the majority of cases THR restores hip function and alleviates the pain caused by damaged joints (Ng et al., 2007), making it one of the most successful surgical interventions of recent times.

1.1.1. THR biomaterials

Charnley's first THR consisted of a metal femoral stem and a polyethylene (plastic) acetabular component, cemented into the bone using an acrylic cement (Charnley, 1964, Knight et al., 2011). Most modern day THRs have a metal femoral stem and trunnion (**Figure 1.1**) while the bearing surfaces are made from metal, ceramic or polyethylene; these can be used in combination, for example a metal femoral head with a polyethylene liner (termed metal-on-polyethylene, MoP). The same materials can be used for both bearing surfaces, such as the metal-on-metal THR shown in **Figure 1.1**. The material composition of THRs is constantly being modified to reduce wear rates, provide improved biocompatibility, and increase implant longevity. These changes are also increasingly required to meet the needs of a patient population with a rapidly altering demographic.

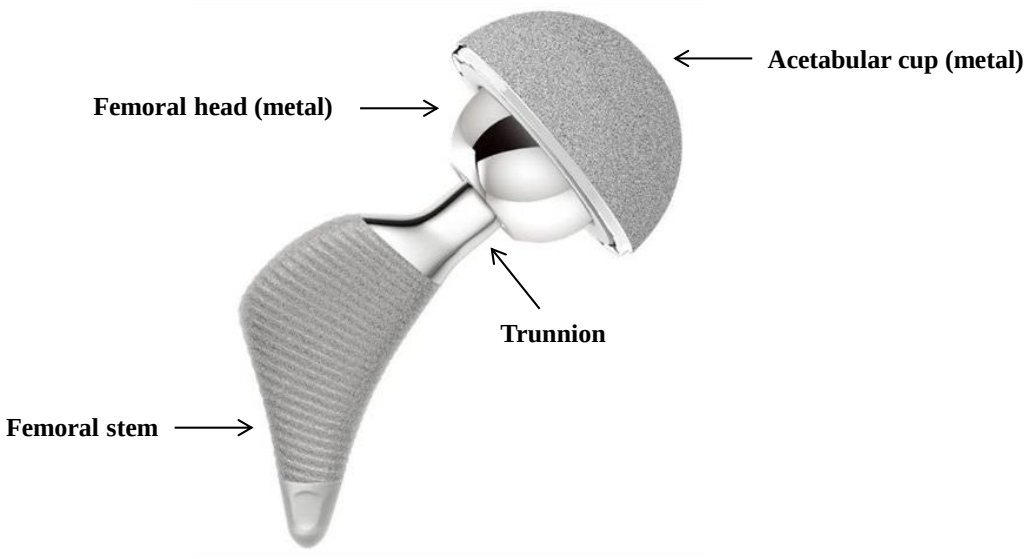


Figure 1.1: Total hip replacement structure
Structure of a metal-on-metal total hip replacement. Image obtained and adapted from www.medicalplasticsnews.com.

1.1.2. THR patient demographic

Osteoarthritis (OA) is usually the result of joint 'wear and tear' and is the leading indication for THR (Powers-Freeling, 2015). OA can result from the damage caused by constant joint use over a lifetime, as well as increased stress placed on a joint if an individual is overweight. As such, OA development is closely correlated with age and obesity (Messier et al., 2005, Sowers and Karvonen-Gutierrez, 2010). The UK has an ageing population and an ever-increasing problem with obesity meaning that the number of OA cases is on the rise (Conaghan et al., 2014). Higher obesity rates coupled with improved methods of OA detection have resulted in rising rates of hip OA; 8% of people over the age of 45 have hip OA (Chidambaram and Cobb, 2009, Jordan et al., 2009). To combat obesity and the effects of ageing, an active lifestyle is encouraged and this also contributes to the joint damage that can result in OA.

Increased numbers of OA cases, as well as those of other joint conditions, have caused the number of THRs performed worldwide annually to grow rapidly. In 2003 there were 14,424 reported primary THR surgeries in England and Wales and by 2014 this had reached 88,125 (Powers-Freeling, 2015) **(Figure 1.2)**. As each THR procedure costs upwards of £6000 they place a considerable financial burden on the NHS (Hamilton et al., 2009). In the USA the number of hip replacements per year is predicted to reach 572,000 by 2030 (Kurtz et al., 2007) and similar increases are forecast across Europe (Singh, 2011). In addition younger patients increasingly require THR surgery with 50% of all hip replacements predicted to be in people under 65 by 2030 (Kurtz et al., 2009).

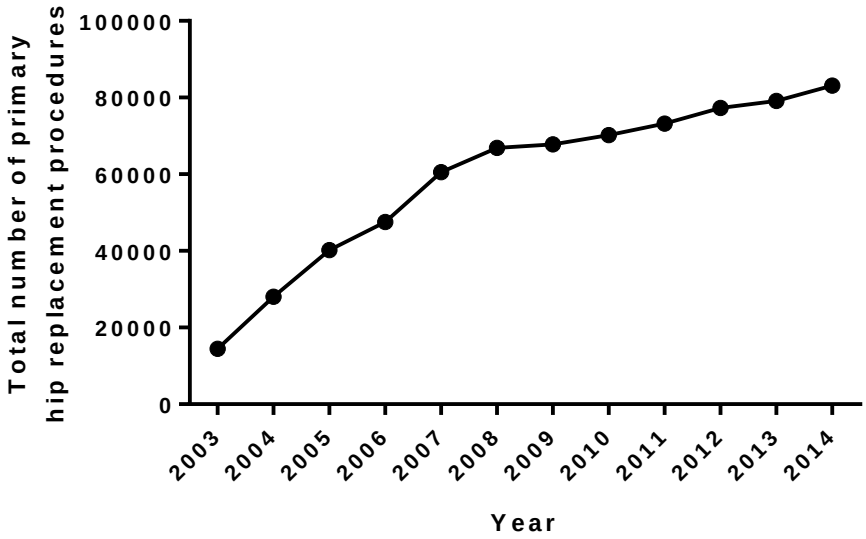


Figure 1.2: Total number of primary hip replacement procedures in England and Wales from 2003-2014.
Figure adapted from data in the National Joint Registry 2015 (Powers-Freeling, 2015).

THR is very successful at restoring joint mobility and improving patient quality of life (O'Boyle et al., 1992). However the changing THR patient demographic has revealed problems with conventional ceramic-on-polyethylene and metal-on-polyethylene hip implants. Younger THR patients (i.e. under 50 years old) are more likely to experience THR failure (Girard et al., 2011) and require revision surgery in which the failed hip implant is replaced with a new device. The revision procedure is more complex than a primary THR procedure because of reduced bone stock but remains the only way of resolving a failed joint replacement. Higher THR failure rates among a younger population also means that they may require multiple surgeries in their lifetime (Berry et al., 2002); this issue is compounded by the UK's ageing population and therefore the number of revisions performed each year is rising rapidly (Powers-Freeling, 2015). There is a higher rate of complications for revisions in younger individuals (Girard et al., 2011). Consequently the need has arisen for hip implants that will be hard-wearing and long-lasting with the aim of reducing the risk of revision. This has led to the development of metal-on-metal (MoM) hip implants.

1.2. Metal-on-metal hip replacement

In a MoM THR both of the bearing surfaces (i.e. femoral head and acetabular cup liner) are fabricated from metals including cobalt, chromium and molybdenum. Titanium can also be used in the trunnion and femoral stem of MoM implants The first generation of MoM implants (McKee and Watson-Farrar, 1966) was used from the 1960s to the 1980s

with mixed results. Although successes were reported (Djerf and Wahlström, 1986) the hip implants ultimately showed unacceptably high levels of failure(August et al., 1986). This was largely due to osteolysis (breakdown of the bone surrounding the implant) which reduced the stability of the implant. In the absence of infection this is termed aseptic loosening. Following improvements in design and manufacture, second generation MoM hip replacements were introduced in the 1990s. In these newer implants the bearing surfaces are usually made from a cobalt-chrome-molybdenum (CoCrMo) alloy and they were considered to have decreased wear rates compared to metal-on-polyethylene and ceramic-on-polyethylene devices. Second generation MoM implants also allowed the reintroduction of MoM hip resurfacing in which a shorter femoral stem is used meaning that more bone is preserved **(Figure 1.3A)**. Resurfacings were more commonly used in younger patients and were reported to have similar success rates to conventional THRs **(Figure 1.3B)** (Mont et al., 2009).

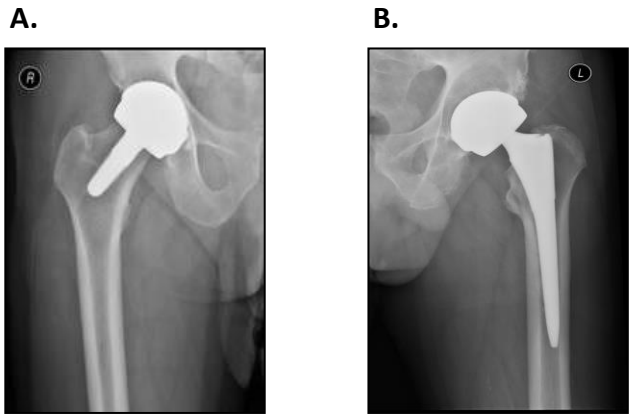


Figure 1.3: Metal-on-metal hip resurfacing versus hip replacement
A. Hip resurfacing in which a shorter femoral stem is used for greater bone preservation. **B.** Conventional total hip replacement. Images were provided by Mr Jim Holland (Consultant orthopaedic surgeon, Freeman Hospital, Newcastle upon Tyne).

Initial reports on these new MoM hip implants were positive (Long, 2005) and showed little indication of the component loosening or osteolysis that led to the failure of first generation MoM implants (Dorr et al., 2000). As a result their use increased dramatically, particularly among the younger patient population. However studies soon began to report high failure rates for MoM implants compared to ceramic-on-polyethylene and metal-on-polyethylene devices. Some types of implant seemed at greater risk of failure than others; the DePuy Articulating Surface Replacement (ASR) has a 7-year failure rate of 48.8% for the total hip replacement and 25% for the hip resurfacing (Langton et al., 2011a). In contrast, a 10-year survival rate of 92% is reported for the Birmingham Hip Resurfacing (Holland et al., 2012, Jameson et al., 2012).

1.3. Adverse reactions to metal debris (ARMD)

The major cause of MoM implant failure is the development of adverse reactions to metal debris (ARMD) (Langton et al., 2011b). ARMD is an umbrella term given to conditions associated with MoM implants including soft tissues necrosis, osteolysis, metallosis, aseptic implant loosening, and the development of benign growths known as pseudotumours (Daniel et al., 2012). It has also been termed adverse local tissue reaction (ALTR) but for the purposes of this study ARMD will be used. ARMD can present as pain or swelling in the joint and groin area, or the feeling that the hip is 'giving way'. Predicting and diagnosing ARMD is made more challenging because it is estimated that up to 50% of MoM patients could have asymptomatic ARMD (e.g. where a pseudotumour is detected by an MRI scan (Kwon et al., 2011, Wynn-Jones et al., 2011)). MoM failure as a result of adverse reactions normally requires revision to a different type of implant (e.g. metal-on-polyethylene) and is responsible for the high MoM THR and resurfacing revision rates shown in **Figure 1.4A**. MoM patients have a 12-13% risk of revision 7 years post-surgery while for other bearing types the incidence is just 2-3% within the same timeframe (Powers-Freeling, 2015). The revision procedure usually reduces metal ion levels in the body and resolves ARMD (Lainiala et al., 2015) which strongly suggests a link between metal release from the hip implant and the development of ARMD.

The impact of concerns surrounding MoM implants, ARMD development, and the increased revision risk can be clearly seen in **Figure 1.4B**. The use of primary MoM hip replacements peaked in 2007 and has since declined dramatically, now representing <1% of all hip replacements (Powers-Freeling, 2015). However understanding ARMD remains important because there are still an estimated 1 million patients with MoM hips worldwide (AAOS, 2012) and ARMD can develop at any time. Furthermore, MoM hip joints are still being implanted and metals are present in existing and new medical devices such as knee replacements and dental implants. A greater understanding of their biological effects could therefore benefit many patients with a broad range of conditions.

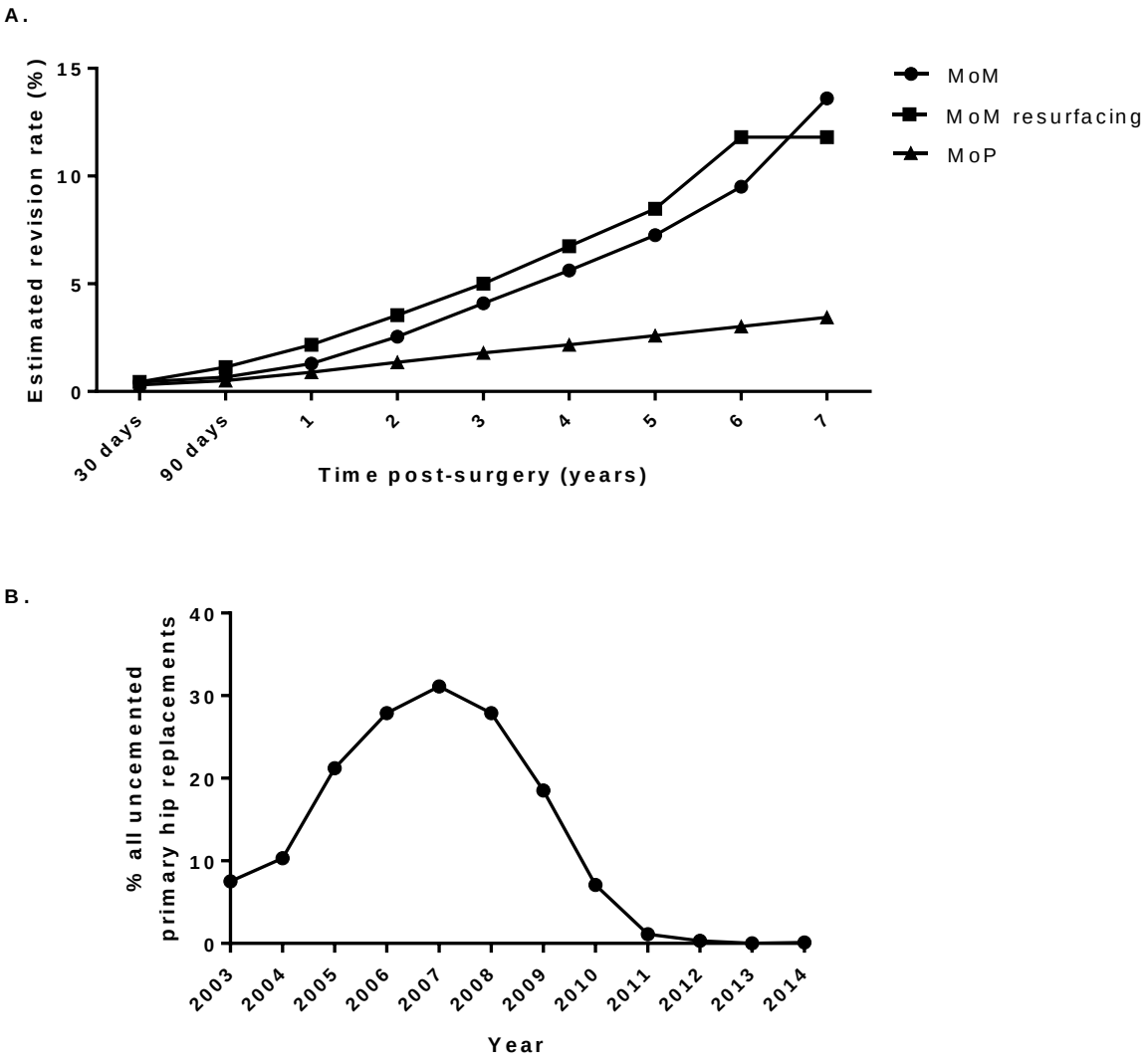


Figure 1.4: MoM hip revision rates and use
A. Estimated revision rates for metal-on-metal hip replacement (MoM), metal-on-metal hip resurfacing (MoM resurfacing) and metal-on-polyethylene hip replacement (MoP). **B.** Use of MoM hip replacements from 2003-2014 shown as a percentage of all uncemented primary hip replacements. Data for figures was obtained from the National Joint Registry Annual Report 2015, available online at www.njrcentre.org.uk.

1.3.1. Metal debris

Release of wear debris occurs in all hip replacements because of the large range of motion and constant surface articulation. Although MoM implants were designed to have low wear rates compared to their counterpart ceramic-on-polyethylene and metal-on-polyethylene devices, many have actually been shown to have increased wear rates and consequently more debris is released than was previously anticipated (De Smet et al., 2008, Kwon et al., 2011, Bosker et al., 2012). In most hip replacements the main debris release comes from the two articulating bearing surfaces and in the case of MoM implants this results in cobalt and chromium being released as debris or ions. Larger debris can also be corroded by the biological environment, resulting in the generation of metal ions. In addition to bearing surface articulation, corrosion at the femoral head-neck junction or trunnion can occur (see **Figure 1.1** for THR structure). Trunnions are usually manufactured from a titanium-aluminium or CoCr alloy (Porter et al., 2014) and wear at this junction can result in metal debris and ion release. Trunnion wear is a significant clinical problem because metal trunnions are used in metal-on-polyethylene and ceramic-on-polyethylene implants as well as MoM, and have been associated with adverse reactions in MoP devices (Whitehouse et al., 2015). Consequently, although the majority of ARMD cases develop in MoM implant patients, patients with other types of hip implant can also be affected.

1.3.2. Cobalt as a biomaterial

The main focus of this study is on cobalt ions as they are often found at higher levels around MoM implants than chromium or molybdenum (Whitehouse et al., 2015), and also have a stronger association with adverse responses (Ninomiya et al., 2013). Cobalt is present in the body in trace amounts (<0.3µg/L) as an important human micronutrient that forms a component of vitamin B12 (Yamada, 2013). Vitamin B12 is required for DNA synthesis, amino acid metabolism (Boss, 1985) and nervous system function (Reynolds, 2006, Kandula et al., 2014). Environmental exposure to cobalt comes from industrial sources such as in dust formed from tungsten carbide synthesis, or medical implants including MoM hip replacements, coronary artery stents, and spinal hardware used in scoliosis treatment.

Even before problems surrounding MoM implants emerged, the potential toxic or harmful effects of cobalt have been studied because of exposure of industry workers to the metal via inhalation or skin contact (Bucher et al., 1999). Cobalt toxicity, also referred to as

cobaltism, can lead to neurological symptoms including dizziness, blindness and impaired cognitive function, as well as cardiomyopathy (Stephen, 2012). Cobaltism is a rare condition associated with high systemic levels of cobalt ions (>60μg/L (Tower, 2010)) and is not usually linked to MoM hip implants, although a small number of cases have been reported (Machado et al., 2012, Stephen, 2012, Bradberry et al., 2014). In MoM hip replacement patients, cobalt concentrations are at their highest around the joint as debris and ions are released during implant articulation. Cobalt cannot be broken down metabolically but it can travel systemically and has been detected in the blood, urine and hair of MoM hip patients (Rodríguez De La Flor et al., 2013).

Following the reports of high MoM failure rates and debris release, as well as local and systemic adverse effects, safe upper limits were proposed for blood metal ion concentrations, above which clinical investigation is recommended. The safe levels vary from study to study; Van Der Straeten *et al* reported 95% specificity in using 4μg/L (unilateral) or 5μg/L (bilateral) cobalt as a predictor of poor MoM implant function (Van Der Straeten et al., 2013). The Medicines and Healthcare Products Regulatory Agency (MHRA) set the upper cobalt limit slightly higher at 7 parts per billion or 119nmol/L (MHRA, 2015). However patients with metal concentrations within these limits may still develop adverse reactions.

1.3.3. Patient variability in ARMD development

It is evident from previous studies that excessive wear of MoM implants is associated with ARMD development, although the mechanisms that result in these effects remain unknown. However the situation is more complicated than a direct correlation between implant wear, metal ion release, and progression to ARMD. Firstly, not every patient with a MoM implant develops an adverse reaction, showing that they can be beneficial and work effectively in the 'right' patient. Secondly, there is a proportion of patients that do not show any association between metal ion concentrations and risk of ARMD development. Langton *et al* found that tissue damage observed in patients undergoing revision for MoM failure was not related to increased wear or metal ion concentrations (Langton et al., 2011b), and similar results have been reported in more recent studies (Bayley et al., 2014, Campbell et al., 2014, Ebramzadeh et al., 2014, Krishnan et al., 2015, Hjorth et al., 2016).

Identifying patients who will respond well to a MoM implant is extremely difficult due to the multifactorial nature of ARMD which appears to involve both mechanical (e.g.

implant misalignment and increased wear) and biological factors. The biological factors can vary both in terms of the exact presentation of the reaction (i.e. a pseudotumour may be a solid fibrotic mass or a fluid-filled cyst-like growth (Pandit et al., 2008)) and in severity; some patients may be asymptomatic and a pseudotumour is only detected by an MRI scan while others experience severe pain and considerable loss of mobility (Kwon et al., 2011).

Additional patient variability is introduced by gender. Sixty percent of patients receiving a primary THR in 2014 were female (Powers-Freeling, 2015) and ARMD is also more common in women than men (Smith et al., 2012). This bias is particularly prevalent following hip resurfacing and applies to aseptic loosening, osteolysis and overall risk of revision, as well as to rarer complications such as dislocation (Glyn-Jones et al., 2009, Haughom et al., 2015). Studies have suggested that the gender disparity is due to the smaller implant size used in female patients, or an increased likelihood of metal ion sensitisation in women through wearing nickel-containing jewellery. However these factors cannot explain every ARMD case. It has also been noted that while being female does increase a patient's risk of MoM implant failure, there must be underlying causative factors that lead to this outcome, rather than gender alone (Amstutz and Le Duff, 2015), supporting a role for additional biological factors in ARMD. There is also a correlation between age, gender and risk of revision, with young female patients most at risk of MoM implant failure and subsequent revision **(Table 1.1)** (Powers-Freeling, 2015). No data was available for females aged 75+ and this is probably because resurfacings are used in younger individuals.

The lack of association between ARMD and metal ion levels, coupled with the high degree of patient variability, point to complex biological factors involved in adverse reactions. Consequently it is essential to improve the understanding of the biology underlying ARMD to reduce its incidence and improve the longevity of hip replacements.

Risk of revision at 10 years (%)		
MoM hip replacement		
Age (years)	**Males**	**Females**
<55	19.48	28.37
55-64	17.72	24.04
65-74	16.04	23.37
75+	9.83	12.31
MoM hip resurfacing		
<55	8.86	21.63
55-64	8.21	19.54
65-74	9.09	16.32
75+	5.24	-
MoP hip replacement		
<55	6.35	5.33
55-64	6.22	4.91
65-74	4.79	3.40
75+	3.94	3.53

Table 1.1: Risk of revision 10 years post-THR by gender, age and implant material
Risk of revision 10 years after surgery is increased in younger women with MoM replacements and resurfacings compared to older women and men. MoP data is included for comparison (Powers-Freeling, 2015).

1.4. Immune response to wear debris

The focus of this study was to understand the cellular responses to metal ions from MoM joint replacements. However there is a large body of work on the inflammatory response to wear debris from other types of hip implant that can inform the specific study of reactions to metal ions. In the following sections 'wear debris' will be used in reference to studies of non-metal bearings (e.g. ultra-high molecular weight polyethylene (UHMWPE) and ceramic) while 'metal debris' or 'metal ions' will be used for MoM implants.

Ceramic-on-polyethylene and metal-on-polyethylene hip replacements generally have excellent survival rates compared to MoM implants (Kandala et al., 2015) but can still fail after many years of use. Given significant advances in biomedical engineering, the causes of failure are usually due to biological factors such as osteolysis and subsequent implant loosening. Studies have indicated that osteolysis is a result of persistent, low-grade inflammation caused by the presence of wear debris from the implant (Goodman et al., 2013a), and suggests a role for the immune system in hip implant failure. The other clinical signs of ARMD such as swelling, pain and soft tissue necrosis are all hallmarks of inflammation, further suggesting involvement of the immune system.

All patients undergoing THR surgery will develop an immune response as a natural and necessary reaction to surgical trauma and the presence of a foreign implant. In the majority of cases this initial 'running-in' phase is short-lived and the immune system soon adapts to the presence of the implant because they are designed to have excellent biocompatibility. In a minority of patients it appears that the immune system remains activated, resulting in the persistent production of inflammatory mediators, chronic inflammation, and ultimately implant failure. This is likely to be a result of exposure to wear debris, and in the case of ARMD to metal debris and ions. However the mechanisms that lead to the immune response to metals are unknown.

The innate immune system is at the forefront of the inflammatory response to pathogen invasion and as such is also the first component of the immune system to encounter wear debris from orthopaedic implants. Innate immune cells such as macrophages are present in the synovial fluid that lubricates the hip implant as well as in the synovial membrane, and they participate in pathogen and debris removal. Innate immunity appears to be the main mediator of wear particle-induced inflammation because mice without functioning adaptive immunity develop similar adverse responses to wear debris as those with a full immune system (Taki et al., 2005). Neutrophils and eosinophils are found at low levels in failed MoM peri-implant tissues but it is macrophages that dominate the innate immune cell infiltrate (Mahendra et al., 2009, Pajarinen et al., 2010, Dapunt et al., 2014, Perino et al., 2014). As such they are considered one of the main targets for wear debris and metal ions (Gu et al., 2012) and therefore there has been considerable research into their role in adverse reactions to orthopaedic implants.

1.4.1. Development and function of monocytes, macrophages and neutrophils

The majority of immune cells are derived from pluripotent haematopoietic stem cells (HSCs) in the bone marrow. HSCs have the potential to become any type of immune cell but begin to differentiate down two distinct lineages, the lymphoid or myeloid lineage. Lymphoid progenitor cells ultimately develop into B and T lymphocytes, or natural killer cells. Myeloid progenitor cells develop into crucial innate immune cells, including eosinophils, neutrophils, monocytes and basophils. As the present study is investigating innate immune responses, the focus of this section will be on the development of monocytes, macrophages and neutrophils.

HSCs destined for the monocyte lineage first develop into common myeloid progenitor cells, followed by monoblasts and pro-monocytes before becoming monocytes. This differentiation requires growth factors including macrophage colony-stimulating factor (M-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF). Following their differentiation in the bone marrow, monocytes enter the circulation where they have a surveillance function and can phagocytose any encountered pathogens. After several days in the circulation monocytes migrate into the tissues where they undergo further maturation to become macrophages. Myeloid progenitor cells can also develop into immature dendritic cells which ultimately become mature dendritic cells upon entering the tissues. It should be noted that as well as bone marrow-derived macrophages, other tissue resident macrophages arise directly in the tissues during embryonic development and do not develop from monocytes or enter the circulation. The exact macrophage phenotype depends on specific stimuli received in the tissues, including factors secreted or expressed on the surface of neighbouring cells and the extracellular matrix. Examples of tissue-specific macrophages are Kupffer cells (liver) and Langerhans cells (dendritic cells in the skin and mucosa). Monocytes can also be recruited directly to pathogen-affected tissues by inflammatory cytokines such as CCL2, and once at the site of inflammation they mature into macrophages.

The most widely described function of macrophages is phagocytosis (‘macrophage’ means ‘big eaters’ in Greek) but they are also capable of inflammatory cytokine and chemokine secretion, and antigen presentation to stimulate adaptive immune responses. In addition, many specialised tissue macrophages (e.g. Kupffer cells) also regulate tissue homeostasis, for example by clearing senescent or dead cells. They can also fuse together to form multi-nucleated giant cells (MGCs) which promote inflammation. MGCs have been identified in failed MoM peri-implant tissues (Carli et al., 2011) where they aid osteoclast formation (known as osteoclastogenesis) and this could in turn contribute to aseptic loosening. The function of macrophages in inflammatory responses is described in more detail later in this section.

Neutrophils arise from the same pluripotent HSCs as monocytes but develop down a different lineage, becoming myeloblasts, promyelocytes and then neutrophilic myelocytes. This process includes the development of intracellular granules that eventually characterise mature neutrophils. Like monocytes, neutrophils are also released into the circulation from the bone marrow but only circulate for 6-10h before they enter the tissues. Neutrophils perform several functions that are critical to pathogen eradication.

They phagocytose pathogens into intracellular vesicles where the ingested organism is degraded by enzymes; this process is described in more detail in the next paragraph. Degranulation is another feature of neutrophils and involves the release of factors previously stored in granules, including matrix metalloproteinases (collagenases and gelatinases), defensins, and lysozymes which target bacterial cell walls. Neutrophils also released nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and through a series of chemical reactions this results in the generation of hydrogen peroxide and then hypochlorous acid, which is highly toxic to bacteria. All of these factors function to provide an initial and effective response against pathogen invasion.

As mentioned previously, phagocytosis is a major function of both macrophages and neutrophils, and wear debris phagocytosis has been studied in-depth in the context of ARMD. Phagocytosis involves pathogen endocytosis, which occurs when a phagocyte uses its plasma membrane to surround and internalise a pathogen in an intracellular vesicle known as a phagosome. The phagosome then fuses with lysosomes to form a phagolysosome. Here the pathogen is degraded by a range of mechanisms including enzymes (NADPH oxidase, DNases, lipases and proteases) and antimicrobial peptides (defensins, lactoferricin). Pathogen internalisation also leads to further macrophage activation and increased release of pro-inflammatory cytokines and chemokines to coordinate subsequent immune responses. In some cases, digested components of the antigen may also be presented by the phagocyte to trigger adaptive immunity. Wear debris from joint replacements can be opsonised by human serum proteins, including complement proteins, albumin and alpha-1 antitrypsin, which promotes macrophage activation and particle phagocytosis (Sun et al., 2003). Sun *et al* found that the pattern of opsonising proteins was unique to each particle tested; for example, CoCr debris was opsonised by albumin and alpha-1 antitrypsin, while polyethylene debris could also be opsonised by apolipoprotein (Sun et al., 2003).

Phagocytosed debris has been observed in macrophages present in tissues surrounding aseptically-loosened hip implants (Lähdeoja et al., 2009). However wear debris presents a complex problem to phagocytes for several reasons. Firstly, implant wear results in the constant generation of debris to challenge the immune system and if the debris is difficult to break down it may result in persistent cell activation and inflammation. Secondly, Scharf *et al* showed that macrophages can generate ions from phagocytosed cobalt and chromium nanoparticles, which could perpetuate the inflammatory response (Scharf et al., 2014). Finally, in the case of MoM implants, cobalt ions have been shown to reduce

the phagocytic function of neutrophils (Rae, 1983) which could complicate the response to both the metal ions themselves and any infection that may develop around the joint.

1.4.2. Cytokine and chemokine release

As previously mentioned phagocytosis leads to cytokine and chemokine secretion by the phagocytic cell. However cytokines and chemokines are not just by-products of phagocytosis but can also be released via different mechanisms such as activation of innate immune receptors, and they have critical functions in inflammation. Cytokines are small (<30kDa) secreted molecules involved in cell signalling and communication. Chemokines (chemotactic cytokines) are a subclass of cytokines that coordinate cell migration or chemotaxis. Chemokines can be classified into structurally distinct groups based on the location of the first cysteine residues in their amino acid sequences. The main classes of chemokines described in this study belong to either the CC chemokines where there are two adjacent cysteine residues near the N-terminus (e.g. CCL2), or to the CXC chemokines where one amino acid separates the two cysteine residues (e.g. CXCL12).

Cytokines and chemokines are released when a cell encounters a pathogen such as a bacteria or virus. They coordinate cell migration, adhesion, proliferation, activation and maturation, and exert their effects by binding to specific receptors on their target cell. There is a high degree of redundancy among cytokines and chemokines with many having overlapping functions and binding to the same receptor. Although they mediate inflammatory responses their release is not limited to immune cells as endothelial cells, fibroblasts and numerous other cell types can also secrete cytokines and chemokines. In the context of adverse reactions to wear debris, persistent chemokine release results in chronic inflammation, fibrosis, and osteolysis (Fahey et al., 2014, Chu et al., 2015).

Macrophages secrete cytokines and chemokines but their activity can also be regulated by them, particularly in the context of polarisation and differentiation. Macrophage polarisation occurs when cells are driven towards a particular phenotype by external stimuli. Macrophages are often classified into two phenotypically distinct subsets, M1 (pro-inflammatory) and M2 (anti-inflammatory), although it has been suggested that there should be a more comprehensive classification based on pathogen recognition, cell activation, and interaction with other immune cells (Martinez and Gordon, 2014). As most wear particle studies describe M1 and M2 macrophages, the nomenclature will be used in this section. However as the classification needs updating to reflect the complexity

and diversity of macrophage activation and function, it will not be used extensively elsewhere in this thesis.

M1 macrophages are induced by lipopolysaccharide (LPS) and interferon gamma (IFNγ) and secrete high levels of pro-inflammatory cytokines and chemokines such as interleukin-12 (IL-12), interleukin-23 (IL-23), and chemokine (C-C motif) ligand 2 (CCL2) (Duque and Descoteaux, 2014). They are also responsible for driving Th1 (pro-inflammatory, microbicidal) inflammatory responses. In contrast, M2 macrophages develop upon interleukin-4 (IL-4) exposure and have an immunomodulatory phenotype, secreting anti-inflammatory interleukin-10 (IL-10) and promoting tissue repair functions such as fibrosis.

There is conflicting data on the role of macrophage polarisation in adverse reactions to wear debris. Jämsen *et al* found that the cytokine profile induced by wear debris does not induce a specific M1 or M2 macrophage profile, indicating the importance of cellular activation by the wear debris itself (Jämsen et al., 2014). However other studies have reported increased levels of M1 polarising macrophages in tissues surrounding aseptically loosened hip implants, suggesting that M1 macrophages may promote osteolysis and implant loosening (Gallo et al., 2013).

1.4.3. Osteolysis

The balance of osteoblast (bone-forming) and osteoclast (bone-resorbing) cells is crucial to maintaining bone homeostasis. These cells regulate the bone remodelling that is required for normal development but their balance can be disrupted, resulting in skeletal disorders including osteoporosis. The role of osteoblasts and osteoclasts is of interest to the present study because a drive towards osteoclastogenesis can promote the osteolysis that leads to aseptic implant loosening. Aseptic loosening is the leading indication for hip implant failure (regardless of bearing surface type), accounting for 46% of all revision procedures in 2009-2014 (Powers-Freeling, 2014), and is also one of the characteristics of ARMD. Loosening is the result of osteolysis, which is mediated by osteoclasts and macrophages. Consequently, considerable research has focused on the effects of wear debris on these cells (Ingham and Fisher, 2005, Nich et al., 2013) and their potential as therapeutic targets in the prevention of aseptic loosening (Chen et al., 2015).

Osteoclasts are essential in normal physiology, and resorb bone by secreting acid, matrix metalloproteinases (MMPs), and cathepsin K, a hydrolytic enzyme that degrades collagen. However in ARMD they appear to have a detrimental effect, resulting in

osteolysis and implant loosening. Like macrophages, osteoclasts are of haematopoietic stem cell origin and develop down the same lineage to become monocytes. Osteoclast precursor cells develop when monocytes are exposed to TNFα and IL-1. The differentiation of these precursor cells into osteoclasts is driven by interactions between receptor activator of nuclear factor kappa-B (RANK) on their surface, and its ligand RANKL which is expressed by osteoblasts on the bone surface (**Figure 1.5)**. Interaction between these two cell surface proteins initiates a signalling cascade in osteoclast precursor cells that promotes the upregulation of osteoclastogenic genes such as tartrate-resistant acid phosphatase (TRAP) and ultimately drives osteoclast formation. The precursor cells also fuse together to form multinuclear osteoclasts. Osteoprotegerin (OPG) can act as a soluble decoy molecule for osteoclastogenesis by binding to RANKL and preventing its interaction with RANK, which stops the precursor cell from developing into an osteoclast (**Figure 1.5**). Cobalt and chromium ions can have a direct effect on the RANK/RANKL/OPG axis by decreasing the ratio of OPG to RANKL and promoting osteolysis (Zijlstra et al., 2012). In addition they can also have an indirect effect by increasing the secretion of pro-inflammatory cytokines and chemokines which creates an environment favouring M1 macrophage formation and thus osteoclast development (Adamopoulos and Mellins, 2015).

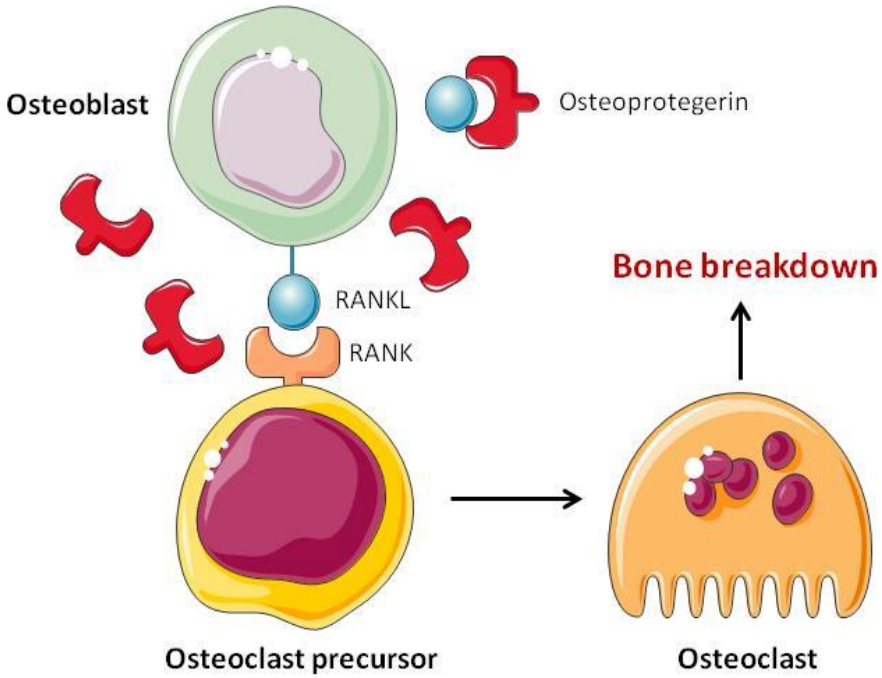


Figure 1.5: RANK/RANKL/OPG axis in osteoclast formation
RANK is expressed by osteoclast precursors and can bind RANKL which is expressed on osteoblasts and stromal cells. Engagement of the two molecules drives cell fusion, multinuclear osteoclast formation and subsequent osteolysis. Osteoprotegerin is a decoy molecule that can bind RANKL, preventing osteoclast differentiation.

It is widely recognised that cytokines and chemokines contribute to ARMD development and progression but the nature of their involvement is highly complex and not fully understood. Much of the current research into adverse reactions focuses on cytokines and chemokines in osteolysis as bone breakdown causes aseptic loosening of the implant. The role of inflammatory cytokines in osteolysis is complex and the same cytokine can have multiple and even opposing functions in osteoclastogenesis. This is particularly the case for IL-6 which can reportedly promote osteoclastogenesis by RANKL-dependent (Hashizume et al., 2008) and independent (Kudo et al., 2003) mechanisms. However other studies have indicated that the IL-6 signalling pathway has a protective effect on bone by downregulating NFκB signalling, decreasing the expression of osteoclastogenic genes, and thereby preventing osteoclast development (Yoshitake et al., 2008). Other cytokines including tumour necrosis factor α (TNFα) can also stimulate osteoclast formation by increasing cellular RANKL expression (Zhang et al., 2001, Kitaura et al., 2004) while IL-8 can drive osteoclastogenesis via RANKL-independent mechanisms (Bendre et al., 2003, Kamalakar et al., 2014).

In addition to increased activity of osteoclasts, osteolysis can also develop through reduced activity of bone-forming osteoblasts. Wear debris increases inflammatory cytokine and matrix metalloproteinase-1 (MMP-1) secretion by osteoblasts and at the same time decreases their ability to synthesise collagen (Lochner et al., 2011); it is particularly interesting to note that the most dramatic effects were observed with CoCrMo debris compared to titanium and stainless steel. Furthermore, osteoblasts exposed to cobalt ions secrete IL-8 and CCL2, and display compromised calcium deposition ability compared to untreated controls (Queally et al., 2009). Taken together this information highlights the importance of cytokines and chemokines in adverse reactions to wear debris.

1.4.4. Adaptive immune response in ARMD

As well as innate immunity, the adaptive immune system is also implicated in ARMD as immunohistochemical analysis has shown that MoM peri-implant tissues are infiltrated by lymphocytes (Davies et al., 2005, Natu et al., 2012). This is termed aseptic lymphocyte-dominated vasculitis-associated lesion, or ALVAL (Watters et al., 2010). The lymphocyte-dominated nature of ALVAL led researchers to suggest that adverse reactions to metal ions are due to Type IV hypersensitivity (Catelas et al., 2015). Type IV hypersensitivity or delayed hypersensitivity is a cell-mediated response driven by T

helper 1 (Th1) cells. Metal ions can act as haptens and are presented by dendritic cells to T lymphocytes causing them to assume a Th1 (pro-inflammatory) phenotype (Thierse et al., 2005). The activity of Th1 cells stimulates further macrophage proliferation and inflammatory responses including fibroblast development. In view of the apparent hypersensitivity response, patch testing for metal allergies has been suggested as a method of predicting implant failure and has been reported as an effective method in some cases (Granchi et al., 2012). However the role of Type IV hypersensitivity as the sole cause of MoM implant failure has been contradicted by several studies. Kwon *et al* used a lymphocyte proliferation assay to investigate the cellular response in patients with pseudotumours following MoM arthroplasty (Kwon et al., 2010). They found that there was no difference in lymphocyte proliferation in response to cobalt, chromium and nickel between patients with or without pseudotumours (Kwon et al., 2010). Furthermore, metal allergies are not associated with increased risk of revision surgery (Thyssen et al., 2009). Although this does not rule out metal hypersensitivity as a cause of ARMD, it suggests that there are other reactions taking place in response to MoM implants and this is supported by ARMD cases where macrophages dominate the inflammatory infiltrate.

In summary, the immune system clearly plays a significant role in the development of adverse reactions to wear debris, including metal ions. Cytokines and chemokines released by immune cells mediate adverse effects including osteoclast formation and osteolysis. However the mechanisms that lead to the initiation of these responses are unclear.

1.5. Metal ions and the Toll-like receptor family

1.5.1. Nickel and TLR4

Nickel is widely used in everyday items such as jewellery and coins, and generates the metal ions most commonly associated with hypersensitivity. More than 8% of North American and European populations has a nickel allergy (Thyssen et al., 2007) and in young females the prevalence can reach nearly 20% (Carøe et al., 2011). The major clinical manifestation of this allergy is skin hypersensitivity and resulting allergic contact dermatitis.

In 2010 Schmidt *et al* investigated the mechanisms by which nickel can cause contact hypersensitivity reactions. It was already understood that nickel hypersensitivity is the result of antigen presentation to T lymphocytes by dendritic cells which creates a population of T cells sensitised to the metal ion. Upon re-exposure the T cells react against

nickel but a second inflammatory stimulus is required for a complete immune response and it was not clear what this stimulus might be. Nickel ions upregulate NFκB activity and increase release of pro-inflammatory cytokines (Goebeler et al., 1995). Schmidt *et al* recognised that the first step in pathogen-mediated pro-inflammatory cytokine secretion is usually activation of a series of cell surface and intracellular receptors known as pattern recognition receptors (PRRs) which recognise pathogens. These include receptor families such as the Toll-like receptors (TLRs) and nucleotide oligomerisation domain (NOD)-like receptors (NLRs). The authors hypothesised that nickel ions can activate one or more of these PRRs leading to an immune response (Schmidt et al., 2010).

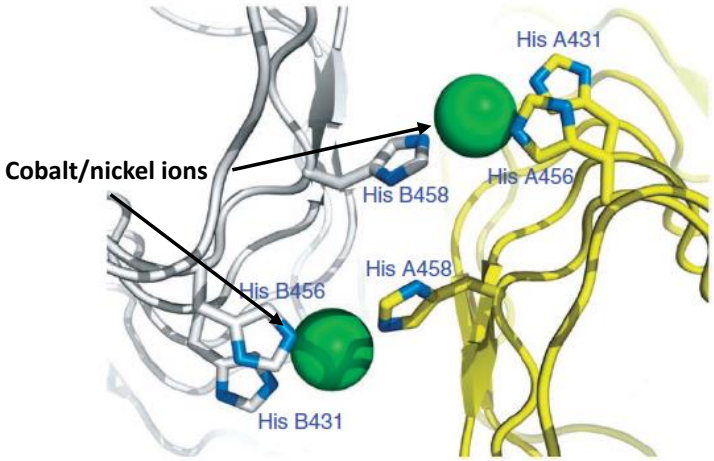
The first challenge for Schmidt *et al* was to identify which receptor family might be implicated in the inflammatory response to nickel. PRRs induce diverse intracellular signalling cascades and therefore specific proteins involved in these cascades were investigated. Myeloid differentiation primary response gene 88 (MyD88) is an adaptor protein essential for signalling via the TLRs. Knockdown of MyD88 in endothelial cells was found to abrogate nickel-induced cytokine secretion, suggesting that the TLRs are involved in the response (Schmidt et al., 2010).

TLR4, a member of the TLR family, is activated by bacterial lipopolysaccharide (LPS). The study authors observed similarities between the LPS and nickel-induced cytokine responses, including increased IL-8 secretion. TLR4 was transfected into human embryonic kidney (HEK) 293 cells which do not express any endogenous TLRs and the cells were then challenged with nickel ions. HEK293/TLR4 cells significantly increased IL-8 production in response to nickel ions compared to untransfected controls, and showed that nickel can activate TLR4. The involvement of other TLRs was eliminated because their transfection into HEK293 cells did not elicit inflammatory responses to nickel ions (Schmidt et al., 2010).

The same study (Schmidt et al., 2010) found that although murine TLR4 is activated by LPS, it is unresponsive to nickel ions. Expression of human TLR4 by a murine model conferred nickel sensitivity, showing that the effect is species-specific. The species difference arises from a so-called ‘histidine pocket’ consisting of 3 histidine residues at positions 431, 456 and 458 in the human TLR4 sequence **(Figure 1.6)**. TLR4 homodimer formation brings these six residues into close proximity, creating a binding site for nickel ions. Although H431 is conserved, murine TLR4 lacks H456 and H458 and as a result it is not activated by nickel ions (Schmidt et al., 2010).

The study concluded that TLR4 activation by nickel could play a crucial role in the development of hypersensitivity to the metal ion, but that the observed receptor activation may be an inflammatory mechanism occurring independently of hypersensitivity. Furthermore, TLR4 was proposed as a potential therapeutic target for allergic contact dermatitis because nickel does not share the LPS binding site. Finally, the authors cautioned the use of animal models in the study of inflammatory responses to chemical agents because of the species differences in nickel activation of TLR4 (Schmidt et al., 2010).

A.



B.

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O00206 TLR4_HUMAN    421 LEQLEHLDFQHSNLKQMSEFSVFLSLRNLIYLDISHTHTRVAFNGIFNGLSSLEVLKMAG  480
L0CL36 L0CL36_MOUSE  419 LEELQHLDFQHSTLKRVTEFSAFLSLEKLLYLDISYTNTKIDFDGIFLGLTSLNTLKMAG  478
```

Figure 1.6: Human and murine TLR4 sequences
A. Proposed model of TLR4 homodimer showing the histidine residues (H431, H456 and H458) that bind nickel and cobalt ions for receptor activation. The model is based on the crystal structure of TLR4 and is adapted from Schmidt *et al* (Schmidt et al., 2010) **B.** Human and mouse TLR4 sequences were aligned using the UniProt online database. H431 is shown in the first box and H456 and H458 are shown in the second box. Murine TLR4 lacks H456 and H458 and is therefore unresponsive to nickel (Schmidt et al., 2010).

Nickel is present in most MoM joints but because of its close association with hypersensitivity reactions it is only used in trace amounts and is therefore unlikely to be responsible for ARMD. Instead the composition of most MoM implants is around 60-70% cobalt-chromium. Cobalt is a less common allergen than nickel, affecting around 3-5% of the population (Carøe et al., 2011, Diepgen et al., 2016) and accounting for 4% of all contact dermatitis cases (Athavale et al., 2007). However nickel and cobalt are adjacent transition metals in the periodic table **(Figure 1.7)** which means that they share a number of similar properties, including ionic radius and charge density. Both metals are known to bind histidine as nickel and cobalt columns can be used in the laboratory to isolate histidine-tagged proteins. Given that histidine binding is a critical step in nickel-mediated TLR4 activation, it was hypothesised that cobalt ions could also activate human TLR4.

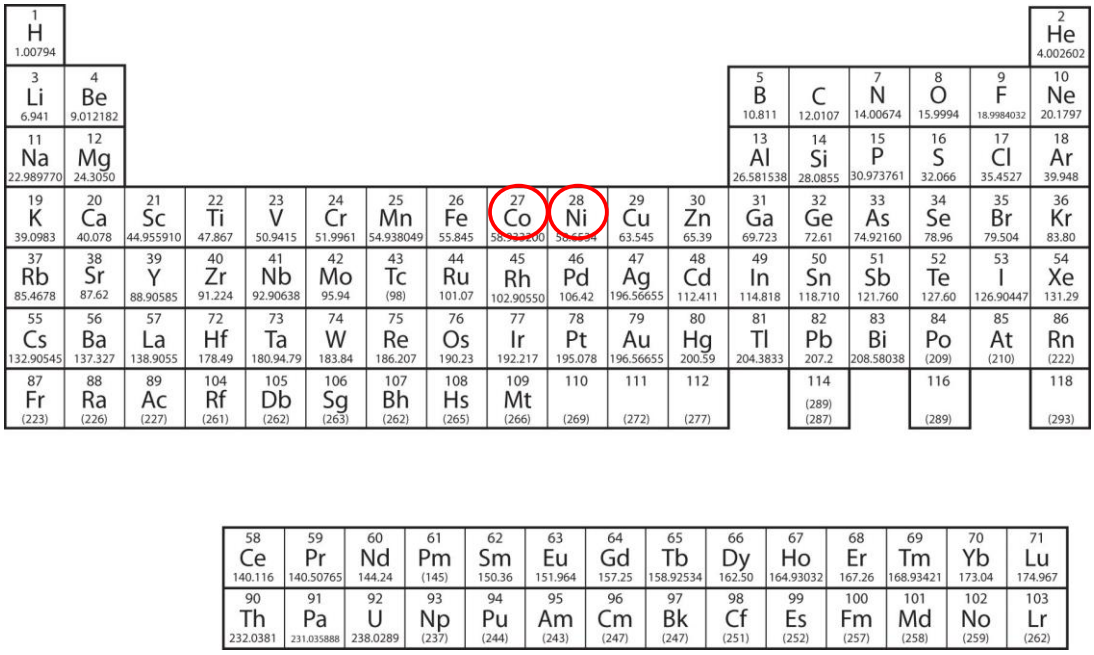

Figure 1.7: Periodic table of elements
Cobalt and nickel (red circles) are adjacent transition metals within the periodic table and share a number of similar properties including the ability to bind to histidine residues.

1.5.2. Cobalt and TLR4

A preliminary study (MRes project) to the work described in this thesis used a TLR4 reporter cell assay to assess the effect of cobalt ions on human and murine TLR4 activation. The reporter cells contained a secreted alkaline phosphatase (SEAP) reporter gene induced by NFκB activation. When TLR4 is activated, NFκB activity increases and SEAP is released. SEAP secretion can then be quantified by a colorimetric assay.

An initial positive control experiment demonstrated human and murine TLR4 responsiveness to LPS (**Figure 1.8A&B**) (Tyson-Capper et al., 2013a). Nickel ions also activated human TLR4 (**Figure 1.8C**) but not murine TLR4 (**Figure 1.8D**), confirming the findings of previous work (Schmidt et al., 2010). The addition of cobalt ions to the assay caused a significant increase in SEAP secretion by human TLR4-expressing reporter cells showing that cobalt can activate human TLR4 (**Figure 1.8E**). However murine TLR4-expressing cells did not produce SEAP following cobalt stimulation, indicating that murine TLR4 does not respond to cobalt ions (**Figure 1.8F**). These findings were supported by the work of Raghavan *et al* and Potnis *et al*, and showed for the first time that cobalt ions from MoM implants can have immunological activity through direct activation of human TLR4 (Raghavan et al., 2012, Potnis et al., 2013).

The study described above used reporter cell assays and consequently the focus was on TLR4 activation using SEAP as a marker. This provides insight into the potential of a ligand to activate a receptor but does not allow investigation into responses such as inflammatory cytokine secretion, and therefore the effect of cobalt-mediated TLR4 activation on these factors remains unclear.

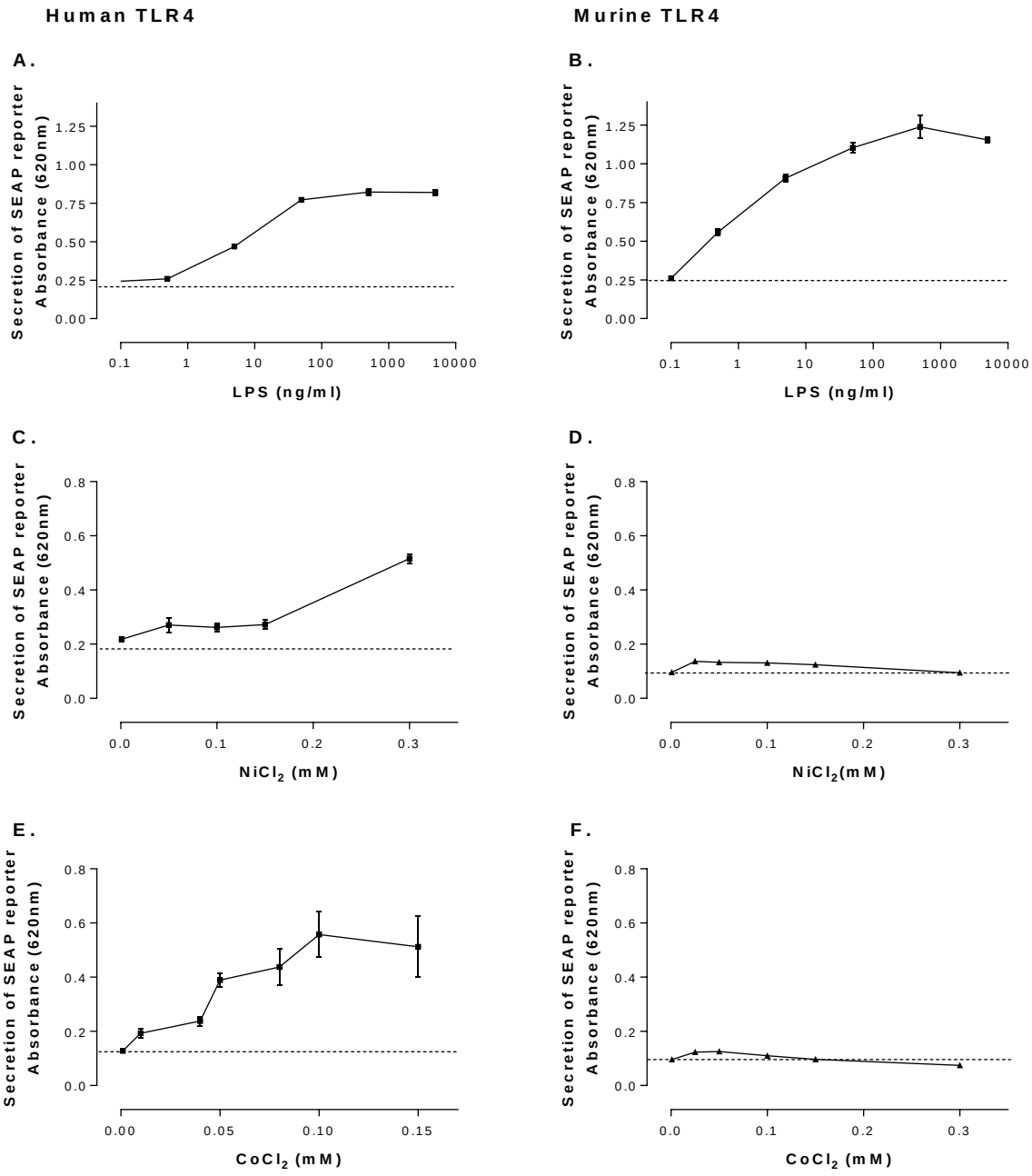

Figure 1.8: Human and murine TLR4 activation by LPS, $NiCl_2$ and $CoCl_2$
Human and murine TLR4 reporter cells were used to determine TLR4 activation by a range of LPS, $NiCl_2$ or $CoCl_2$ concentrations. Human TLR4 was activated by **A.** LPS, **C.** $NiCl_2$ and **E.** $CoCl_2$. Murine TLR4 was activated by **B.** LPS, but not by **D.** $NiCl_2$ or **F.** $CoCl_2$. Figure adapted from previously published work (Tyson-Capper et al., 2013a).

1.6. Toll-like receptors

TLRs are an evolutionarily conserved family of PRRs and were first discovered in *Drosophila melanogaster* (Anderson et al., 1985, Gay and Keith, 1991). They are essential in defence against pathogen invasion and are responsible for initiating immune responses when a cell encounters a foreign organism. To date 10 human TLRs have been identified. Of these, TLR1, 2, 4, 5, 6 and 10 are on the cell surface while TLR3, 7, 8 and 9 are intracellular. TLRs recognise pathogen-associated molecular patterns, which are unique to individual pathogens; for example TLR3 is activated by double-stranded RNA (Alexopoulou et al., 2001) while TLR9 recognises CpG nucleotides which are prevalent in bacterial DNA (Hemmi et al., 2000). Between them the TLRs recognise many diverse ligands, although compared to the antigen recognition capacity of the adaptive immune system this is a relatively small number. There is some overlap between ligands recognised by the TLRs, with TLR1 and TLR2 both recognising peptidoglycan and lipopeptides. The full list of human TLRs and their ligands is shown in **Table 1.2**.

The ligand repertoire of TLRs is not limited to PAMPs as they can also recognise damage-associated molecular patterns (DAMPs, also known as 'alarmins'). DAMPs are self-derived molecules that are released by damaged or stressed cells, often when they are undergoing necrosis. Among the major DAMPs known to be TLR ligands is high mobility group box protein 1 (HMGB1), which is a nuclear protein released by necrotic but not apoptotic cells. HMGB1 can activate TLR2, TLR4 and TLR9. A second TLR-stimulating DAMP is serum amyloid A, which is primarily expressed by hepatocytes and activates TLR2 and TLR4. A comprehensive list of TLR-activating DAMPs is shown in **Table 1.2**.

Activation of TLRs by DAMPs is usually beneficial to the host because it drives TLR-mediated innate and adaptive immune signalling (described later in this section) in a similar way to PAMPs; this is known as 'sterile inflammation' because immune responses occur in the absence of a pathogen. For example, DAMP activation of TLRs on innate immune cells can recruit and stimulate dendritic cells, resulting in increased antigen presentation to the adaptive immune system. They also promote repair mechanisms such as fibrosis, which is important for redressing homeostasis after an inflammatory response. However, their ability to promote self-recognition also means that DAMPs are implicated in the development of autoimmune conditions such as rheumatoid arthritis (Goh and Midwood, 2012). Consequently, DAMPs and TLRs have been proposed as therapeutic targets in the prevention of autoimmune diseases (Midwood and Piccinini, 2010).

For effective pathogen recognition most TLRs undergo dimerisation; homodimerisation is more common but heterodimers can also form, particularly in the case of TLR2 which can heterodimerise with TLR1 and TLR6. Heterodimerisation is thought to expand the repertoire of PAMPs recognised by the TLRs (Farhat et al., 2008). Dimerisation is also important for bringing the intracellular TIR domains of the receptors into closer proximity, creating a binding site for adaptor proteins such as MyD88. Following receptor dimerisation a downstream intracellular signalling cascade is initiated which activates transcription factors including NFκB, AP-1 and IRF3. These factors regulate transcription of pro-inflammatory cytokines and chemokines such as IL-8 and tumour necrosis factor alpha (TNFα). There is also upregulation of co-stimulatory molecules like intercellular adhesion molecule-1 (ICAM-1) to assist in the cell-cell interactions required for adaptive immune responses. TLR activation also results in increased phagocytosis and antigen presentation to the adaptive immune system. The exact nature of the cellular immune response depends on which TLR is activated, and the downstream adaptor proteins that are recruited; the result is an inflammatory response that is appropriate to the activating PAMP or DAMP. For example, TLR3 and TLR4 activation can drive a Type I interferon response, but TLR2 and TLR5 cannot do so (Toshchakov et al., 2002). This study will focus on TLR4 and the inflammatory consequences of its activation.

Toll-like receptor	PAMPs (source)	DAMPs	References
1	Peptidoglycan, lipopeptide (Gram positive bacteria)	B-defensin 3	(Takeuchi et al., 2002)
2	Peptidoglycan, lipopeptide (Gram positive bacteria)	Heat shock proteins, HMGB1, biglycan, antiphospholipid antibodies, hyaluronic acid fragments, serum amyloid A	(Aliprantis et al., 1999)
3	Double-stranded RNA (viruses)	mRNA	(Alexopoulou et al., 2001)
4	Lipopolysaccharide (Gram negative bacteria)	HMGB1, heat shock proteins, fibronectin, hyaluronan fragments, heparan sulphate, fibrinogen, lung surfactant protein A, antiphospholipid antibodies, serum amyloid A	(Poltorak et al., 1998)
5	Flagella (bacteria)	None identified	(Hayashi et al., 2001)
6	Lipopeptide (bacteria and fungi)	None identified	(Takeuchi et al., 2001)
7	Single-stranded RNA (viruses)	Antiphospholipid antibodies, ssRNA	(Hemmi et al., 2002, Lund et al., 2004)
8	Single-stranded RNA (viruses)	Antiphospholipid antibodies, ssRNA	(Heil et al., 2004)
9	CpG nucleotides (bacteria and viruses)	Hypomethylated CpG-DNA, HMGB1	(Bauer et al., 2001)
10	Unknown	None identified	(Chuang and Ulevitch, 2001)

Table 1.2: Human Toll-like receptors and their ligands
The table shows the 10 human TLRs and the pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) that activate them. DAMPs section of table was adapted from (Midwood and Piccinini, 2010). The table is not exhaustive but describes the major DAMPs identified for each TLR. To-date no endogenous ligands for TLR5, TLR6 and TLR10 have been identified.

1.6.1. Toll-like receptor 4

Toll-like receptor 4 (TLR4) is a type I transmembrane receptor with a molecular weight of 95kDa. It is expressed on numerous cell types including monocytes, macrophages, dendritic cells and endothelial cells. Human TLR4 was first discovered by Medzhitov *et al* in 1997 (Medzhitov et al., 1997) and subsequently identified as the receptor for bacterial lipopolysaccharide (LPS) from Gram negative bacteria (Poltorak et al., 1998).

1.6.2. LPS activation of TLR4

TLR4 cannot initiate an immune response against LPS without accessory proteins that aid in the transfer of LPS to the receptor. LPS first binds to LPS-binding protein (LBP) which forms a complex with cluster of differentiation 14 (CD14), a protein usually expressed in either soluble or membrane-bound form alongside TLR4. This complex then transfers LPS to myeloid differentiation protein 2 (MD2), which is complexed with TLR4. MD2/TLR4 binding of LPS induces receptor homodimerisation. MD2 is essential to the LPS-mediated response as MD2-negative cells are unable to respond to LPS (Zhang et al., 2009). A TLR4/MD2 dimer and its adaptor proteins are shown in **Figure 1.9**.

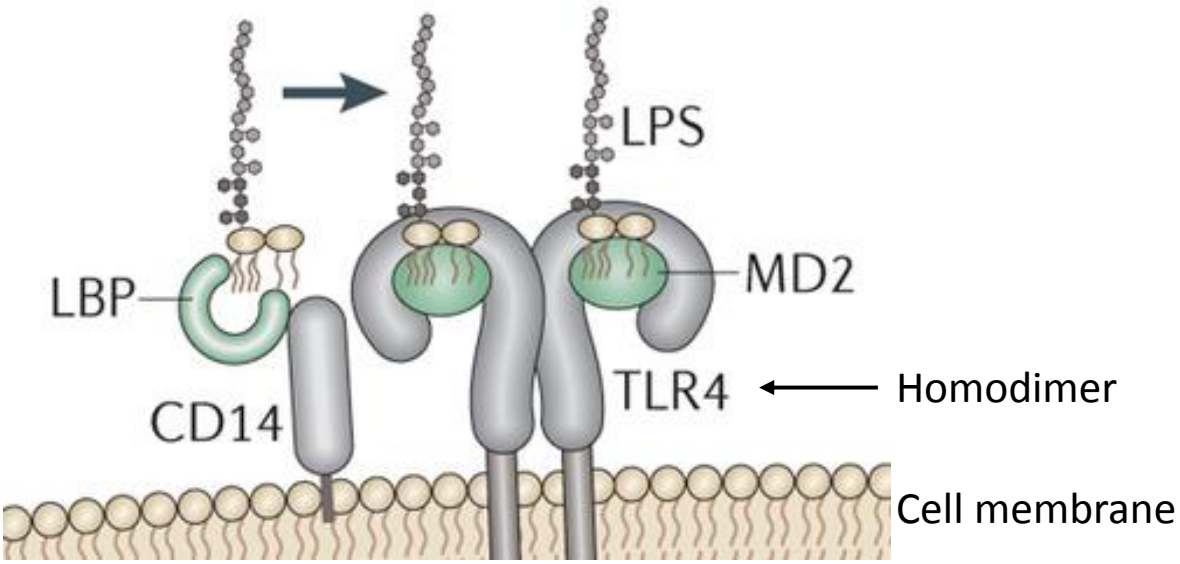

Figure 1.9: TLR4/MD2 dimer and its adaptor proteins
TLR4 is a cell surface receptor and requires adaptor proteins LBP, MD2 and CD14 for activation by LPS. LPS is initially bound by LBP, which complexes with CD14 and then transfers LPS to MD2, which is present in a heterodimer with TLR4. This causes TLR4/MD2 complex homodimerisation and results in the recruitment of intracellular adaptor proteins (e.g. MyD88, TRIF) and subsequent initiation of downstream signalling cascades (described in later figures). Image adapted from (Needham and Trent, 2013)

TLR4 contains Toll IL-1 resistance (TIR) domains which, following activation andhomodimerisation, recruit other adaptor proteins with TIR domains. These include MyD88, TIRAP, TIR domain-containing adaptor-inducing interferon-β (TRIF, also known as TIR domain-containing adaptor molecule 1 or TICAM1) and TRIF-related adaptor molecule (TRAM or TICAM2) (O'Neill et al., 2013). The recruitment of adaptor proteins activates downstream signalling pathways via interleukin-1 receptor-associated kinase (IRAK) and TNF receptor-associated factor (TRAF) molecules, and transcription factors including NFκB. This leads to increased transcription of pro-inflammatory cytokines and chemokines such as interleukin-6 (IL-6) and interleukin-8 (IL-8). Whilst TLR4 activation is an essential first step in the immune response to LPS, it is these effector molecules that go on to promote an inflammatory response against the pathogen. An outline of the TLR4 signalling pathway is shown in **Figure 1.10**.

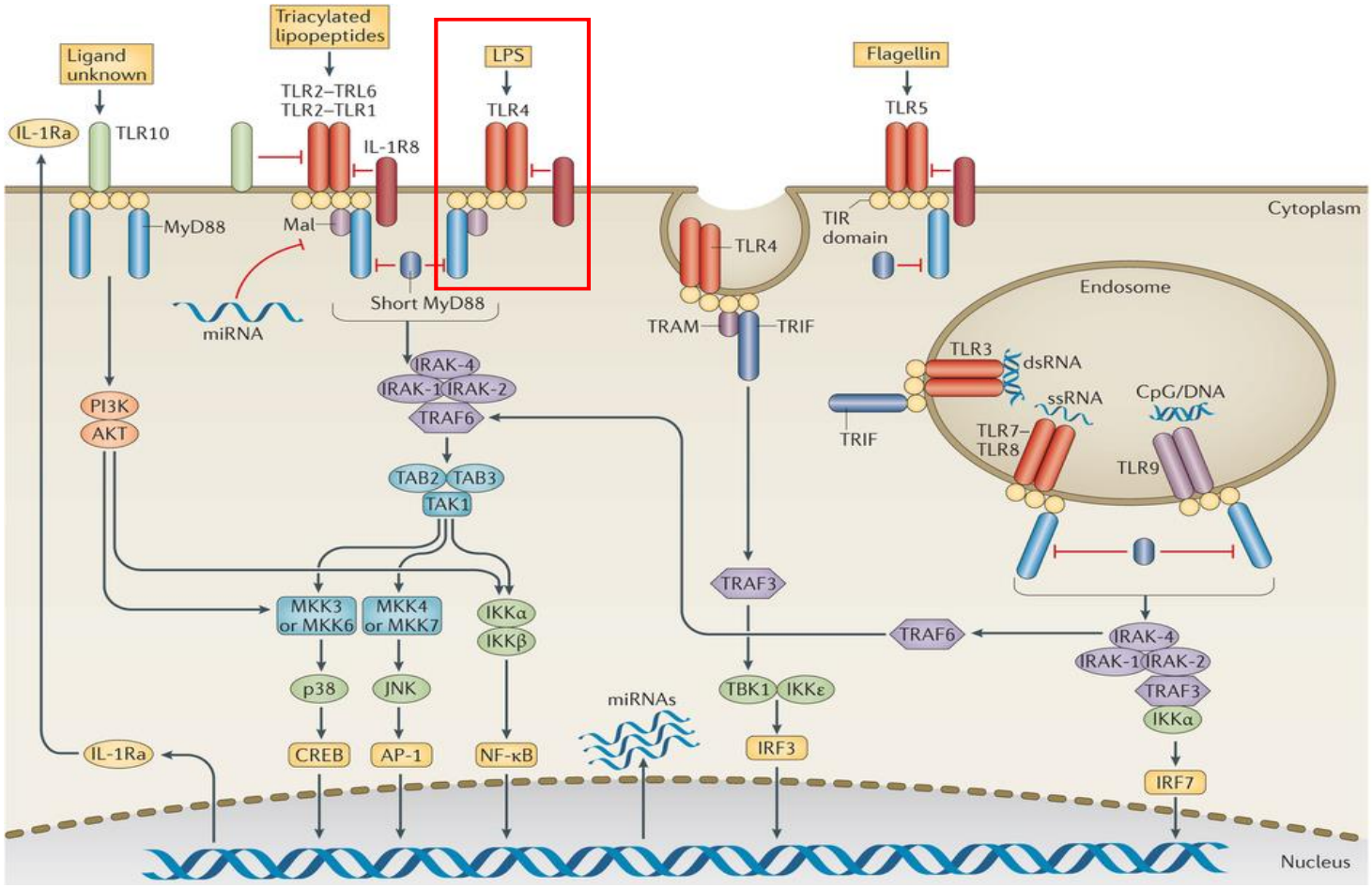

Figure 1.10: TLR4 signalling pathway

Figure shows an outline of human TLR signalling. TLRs can be expressed on the cell surface (TLRs 1, 2, 4, 5, 6 and 10) or in intracellular endosomes (TLRs 3, 7, 8 and 9). There is considerable overlap in the downstream signalling pathways induced by the different TLRs. TLR4 (red box) is activated by LPS, resulting in receptor homodimerisation and recruitment of adaptor proteins such as MyD88 and TRIF. This initiates a downstream signalling cascade involving IRAK protein complexes and activation of MAP kinases, among the many other factors shown in the figure. The outcome of TLR4 signalling is increased activity of transcription factors including NFκB, AP-1 and IRF3; this is dependent on adaptor proteins, for example the MyD88 pathway tends to activate NFκB signalling. Transcription factor activity results in increased expression of pro-inflammatory cytokines and chemokines. Image adapted from (Joosten et al., 2016).

LPS was the first TLR4 ligand to be identified and as such remains the most widely studied. However, as discussed earlier, TLR4 is activated by other factors including high-mobility group box protein 1 (HMGB1) (Yu et al., 2006), hyaluronic acid fragments (Termeer et al., 2002), and heat shock protein 60 (HSP60) (Cohen-Sfady et al., 2005). There is considerable debate over whether or not these DAMPs are 'true' TLR4 ligands or if their effects are enhanced by endotoxin contamination (Erridge, 2010). However it is clear that TLR4 can respond to a range of stimuli that includes pathogens and host-derived factors, as well as ligands not classified as PAMPs or DAMPs, such as nickel and cobalt ions.

1.6.3. Cobalt activation of TLR4

Unlike LPS, which requires transfer across several adaptor proteins to access and activate TLR4, cobalt and nickel ions bind directly to the receptor. Raghavan *et al* observed that cobalt activation of TLR4 requires histidine residues at positions 456 and 458 in the TLR4 sequence, which is the same as those that facilitate nickel binding (Raghavan et al., 2012). Murine TLR4 lacks these histidine moieties and consequently cobalt cannot activate the murine receptor. Mutation of H456 and H458 significantly reduces the inflammatory response to cobalt ions but does not affect the LPS response (Raghavan et al., 2012)**,** suggesting that there are differences between LPS- and cobalt-mediated TLR4 activation.

The same study (Raghavan et al., 2012) aimed to elucidate the role of MD2, a TLR4 co-receptor, in cobalt activation of TLR4. It was found that both LPS and cobalt ions cause receptor homodimerisation. However while MD2 is essential for homodimerisation following LPS stimulation, it is not required following cobalt stimulation. In contrast, a more recent study showed that cobalt cannot activate TLR4 in the absence of MD2 (Oblak et al., 2015). Raghavan *et al* found that mutating N433, an asparagine residue present in the TLR4 dimerisation interface, prevented TLR4 homodimerisation in response to cobalt and LPS and subsequent inflammatory cytokine release (Raghavan et al., 2012), indicating the importance of this process in TLR4 activation by cobalt.

There is limited information on the downstream signalling pathways that may be induced following cobalt activation of TLR4. Silencing of the MyD88 and IRAK1 genes inhibits inflammatory responses to cobalt ions in THP-1 macrophages (Rachmawati et al., 2013) but little is known about the cytokine and chemokine profile produced, or the functional effects that this may have.

1.7. Inflammatory effects of LPS-mediated TLR4 activation

The following sections describe the inflammatory effects of LPS-mediated TLR4 activation. These are important to the present study because they provide insight into the potential immunological consequences of cobalt activation of TLR4.

1.7.1. Cytokine and chemokine secretion

LPS activation of the TLR4 signalling pathway causes increased secretion of pro-inflammatory cytokines and chemokines including IL-8 (Yoshimura et al., 1987), IL-6 (Song et al., 2007) and CCL2 (Guijarro-Muñoz et al., 2014). The cytokine profile release following LPS activation of TLR4 depends on which transcription factors are activated. NFκB and AP-1 activation tends to drive secretion of cytokines and chemokines like IL-6, IL-8 and TNFα, while IRF3 activation promotes type I interferon release, which is often associated with anti-viral responses. The cytokines and chemokine released by LPS-mediated TLR4 activation have a broad range of immunological functions that combine to produce an effective inflammatory response. For example, TNFα increases vascular permeability to allow immune cells and complement access to the affected tissues. IL-8 recruits neutrophils and basophils to the site of inflammation, while IL-12 can activate natural killer cells which are cytotoxic for pathogens. It also promotes differentiation of $CD4^+$ T cells into Th1 cells, showing a clear link between TLR4 activation and stimulation of adaptive immunity **(Section 1.7.4)**.

As with any aspect of the inflammatory response, TLR4 signalling must be tightly regulated. Very high doses of LPS result in overwhelming cytokine and chemokine secretion (sometimes referred to as a 'cytokine storm'), and increased vascular permeability; these are the events that occur in septic shock. One of the most well-studied negative regulators of TLR4 is IRAKM, which functions by preventing the formation of the IRAK1-TRAF6 complex (**Figure 1.10**) that is required for downstream signalling. Suppressor of cytokine signalling 1 (SOCS1) is a second inhibitor of TLR4 signalling and is thought to act by binding IRAK1 and preventing IRAK complex formation (Naka and Fujimoto, 2010). Most endogenous negative regulators of TLR4 are also increased by LPS stimulation of the receptor in order to prevent the overwhelming cytokine response; mice negative for such factors (e.g. SOCS1) develop severe sepsis (Nakagawa et al., 2002).

1.7.2. Phagocytosis

Activation of TLR4 by LPS also promotes phagocytosis. The role of TLRs in phagocytosis was first identified by Blander and Medzhitov in 2004; they identified that TLR4-/- mice had a reduced ability to phagocytose *E. coli* compared to wildtype control mice (Blander and

Medzhitov, 2004). Since their discovery, other studies have reported similar central roles for TLR4 in phagocytosis, although the exact pathways involved are not entirely clear. Chen *et al* reported that TLR4-MyD88 interactions are essential for phagocytosis while others have described activation of a MyD88-independent pathway that promotes phagocytosis (Kong and Ge, 2008). Given the apparent number of factors critical to TLR4-dependent phagocytosis reported in different studies, it is feasible that the exact signalling pathway induced is specific to the pathogen encountered. However the eventual outcome is increased phagocytic capabilities, particularly in the case of macrophages; this is due to elevated expression of scavenger receptors and increased complement production which drives phagocytosis (Doyle et al., 2004). The function of macrophages as phagocytes is described in more detail in **section 1.4.1**.

1.7.3. Leukocyte extravasation

The presence of a pathogen in the tissues requires movement of circulating leukocytes from the blood, across the endothelial barrier and into the affected tissues where they can coordinate an inflammatory response. This process is known as extravasation and is a multi-step process involving local and systemic cytokine and chemokine signals from immune cells and endothelial cells that promote leukocyte adhesion (**Section 1.7.3.1**) and migration **(Section 1.7.3.2)**. TLRs are critical in these processes because they identify the specific pathogen and mediate the secretion of a unique cytokine and chemokine profile required for an appropriate immune response to the pathogen. In particular, LPS activation of TLR4 can promote adhesion molecule expression, both directly and indirectly, through increased expression of cytokines and chemokines such as TNFα (**Section 1.7.3.1**).

1.7.3.1. Adhesion

Circulating immune cells such as monocytes and neutrophils must adhere to the endothelial cell layer in order for them to move from the blood into the surrounding tissues. The process requires interaction between adhesion molecules expressed on both cell types. As blood flows rapidly through vessels at a high shear rate, the first step in cell adhesion is a low-level ('rolling') adhesion that slows the passage of circulating leukocytes. This involves engagement of carbohydrate structures such as Sialyl Lewis^X expressed on monocytes and neutrophils with selectins (e.g. E-selectin) on the endothelial cell surface (**Table 1.3**). The interaction allows cells to roll more slowly along the endothelial cell layer and progress to firm adhesion via engagement of different groups of adhesion molecules.

Endothelial cell factor	Constitutively expressed?	Ligand on monocytes/neutrophils	Function
Selectins, e.g. E-selectin	Yes	Sialyl Lewis^X	Rolling adhesion
ICAM-1	Yes	LFA-1 (CD18/CD11a), Mac-1 (CD18/CD11b)	Firm adhesion
VCAM-1	No	VLA-4 (CD49d, CD29)	Firm adhesion

Table 1.3: Factors involved in endothelial cell-monocyte/neutrophil adhesion
Table adapted from Panés and Granger (Panés and Granger, 1998)

Most endothelial cells constitutively express two forms of intercellular adhesion molecule (ICAM) for firm adhesion; these are ICAM-1 and ICAM-2. Soluble forms of these factors (e.g. soluble ICAM-1 or sICAM-1) also exist. Upon cell stimulation, ICAM-1 expression is increased and an additional adhesion molecule, vascular adhesion molecule-1 (VCAM-1), is induced (Panés and Granger, 1998). The integrin family are capable of rapid conformational changes, which allows them to switch rapidly between binding and release of their ligands; this is important for binding the cell and then allowing it to migrate through the endothelial cell layer.

The ligands for ICAM-1 are the integrins CD18/CD11a (lymphocyte function-associated antigen-1, LFA-1) and CD18/CD11b (macrophage-1 antigen, Mac-1) while VCAM-1 binds very late antigen-4 (VLA-4) (**Table 1.3**). LFA-1 is expressed constitutively, mostly by neutrophils, whilst Mac-1 is mainly stored in granules. Upon cell stimulation, Mac-1 can be rapidly trafficked to the cell surface and at the same time the selectins involved in the initial low adherence step are shed by the endothelial cell, allowing rapid transition from rolling to firm adhesion. The cell adhesion process is summarised in **Figure 1.11**.

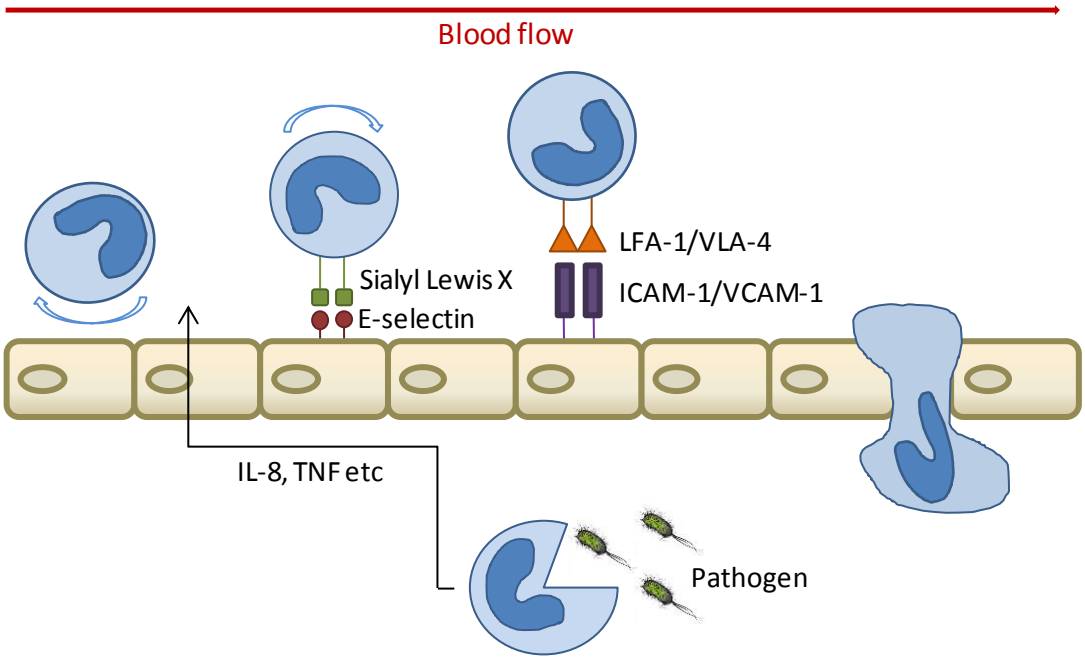

Figure 1.11: Monocyte adhesion and migration
Monocyte infiltration of tissues (extravasation) requires adhesion molecules and chemotactic stimuli. Rolling adhesion occurs through interaction between molecules including Sialyl Lewis^X (monocyte) and selectins (endothelial cell). This is followed by firm adhesion mediated by LFA-1 or VLA-4 (monocyte) and ICAM-1 and VCAM-1 (endothelial cell). Adhesion allows monocyte extravasation and maturation into a macrophage following exposure to a pathogen in the tissues. Macrophages secrete chemokines in response to the pathogen that recruit more inflammatory cells and facilitate their migration through the endothelial cell layer. Circulating monocytes are shown in the diagram but the process is the same for neutrophils, which are usually the first immune cells to enter affected tissues.
.

Adhesion molecule expression is tightly regulated under non-pathogenic conditions to prevent unwanted inflammation. In the presence of a pathogen expression is increased and this is largely due to the regulation of adhesion molecules by cytokines including IL-6, IFNγ and TNFα (Zhang et al., 2011), which induce endothelial inflammation including increased expression of adhesion molecules. LPS can also directly affect ICAM-1 expression; breast cancer cells challenged with LPS increase ICAM-1 expression via a TLR4 and MyD88-dependent pathway (Park and Kim, 2015) and a similar mechanism has been demonstrated in endothelial cells (Sawa et al., 2008). TLR4 inhibition can prevent these changes, suggesting that they are mediated by receptor activation and upregulation of the transcription factors NFκB and AP-1 (Sawa et al., 2008, Hung et al., 2010).

The effect of TLR4 ligands on adhesion molecules is not limited to ICAM-1 and VCAM-1 as LFA-1 and VLA-4 can also be regulated through activation of TLR4 (Liu et al., 2015). Engagement of ICAM-1 on endothelial cells with LFA-1 on neutrophils is a crucial aspect of neutrophil recruitment in the inflammatory response to LPS (Basit et al., 2006) and interaction between the two molecules also promotes neutrophil phagocytosis and the oxidative burst (Schnitzler et al., 1999). VLA-4 binds to VCAM-1 and plays a similar role to LFA-1; it is required for neutrophil recruitment following endotoxin challenge (Burns et al., 2001), showing its importance in TLR4-mediated inflammatory responses.

In addition to its role in innate immune cell binding, LFA-1 is expressed on T lymphocytes and in this case its binding to ICAM-1 on an antigen-presenting cell is a key step in T cell activation. Consequently LFA-1 is important in a broad range of pro-inflammatory responses by mediating cellular interactions and, as such, has been proposed as a therapeutic target in autoimmune conditions such as psoriasis because of its role in coordinating T cell-mediated inflammation (Cather et al., 2003).

1.7.3.2. Migration

Following cell adhesion the next step is migration of the leukocyte through the endothelial cell barrier and into the tissue. This occurs in response to a local chemokine gradient generated by tissue-resident cells (such as macrophages) upon pathogen recognition. The process also requires homophilic interactions between platelet endothelial cell adhesion molecule-1 (PECAM-1) on the leukocyte and the endothelial cell, which allows the leukocyte to pass through the endothelial cell barrier. The type of leukocyte recruited to the tissues is dependent on the secreted chemokine profile and receptor expression on the target cells, e.g. IL-8 acts via chemokine (C-X-C motif) receptors 1 and 2 (CXCR1 and CXCR2) to promote neutrophil

migration. Chemokine receptors are G protein-coupled receptors and when activated they initiate cytoskeletal changes in the cell that drive migration. They also promote conformational changes in the adhesion molecules described earlier, which allows for more effective leukocyte-endothelial binding. Once the newly recruited cells arrive at the site of inflammation they can promote an immune response. For example, monocyte extravasation results in cell maturation into a macrophage which can carry out functions such as phagocytosis. When migrated cells encounter the pathogen they also begin to secrete chemokines that recruit more inflammatory cells to the site which ultimately leads to eradication of the pathogen.

1.7.4. Role of TLR4 inand adaptive immune responses

As well as its crucial role in innate immunity, TLR4 signalling can also promote adaptive immunity. TLR4 is expressed on dendritic cells, which provide a bridge between the innate and adaptive immune systems. Upon LPS challenge and TLR4 activation, immature DCs begin to mature by upregulating their expression of MHC molecules for antigen presentation and co-stimulatory molecules for cell-cell interactions; these include cluster of differentiation 40 (CD40), CD80 and CD86, and are essential for initiating a Th1-dependent adaptive immune response (Fang et al., 2011). The result of LPS-driven DC maturation is migration to the lymph nodes where they present antigen for priming naïve T cells. Interestingly, a recent paper has shown that LPS activation of TLR4 can cause a transient decrease in phago-lysosomal fusion (and thereby phagocytosis). This was accompanied by a temporary increase in antigen cross-presentation to cytotoxic $CD8^+$ cells (Alloatti et al., 2015), suggesting that TLR4 is involved in improving the antigen presenting ability of DCs.

Expression of TLR4 by T and B lymphocytes confers their responsiveness to LPS and other TLR4 ligands (Cohen-Sfady et al., 2005, Zanin-Zhorov et al., 2007). Although T cells do not usually display increased cytokine secretion following TLR4 activation (Reynolds et al., 2012), LPS activation of TLR4 on T cells increases cell adhesion (Zanin-Zhorov et al., 2007) and promotes clonal expansion (Gandhapudi et al., 2013). In TLR4-mediated inflammatory responses, TLR4 and MyD88 inhibition significantly generation of antigen-specific T cells (Uto et al., 2011). TLR4-deficient mice also have a reduced $CD4^+$ T cell population in response to *Salmonella* infection compared to wildtype controls. However other studies have found that LPS activation of TLR4 promotes immunomodulatory IL-10 secretion by regulatory T cells (Higgins et al., 2003, Den Haan et al., 2007), which may be a mechanism of controlling inflammation.

Activation of TLR4 on B lymphocytes results in cytokine secretion (IL-6, IL-10 (Barr et al., 2007)), cell maturation (Hayashi et al., 2010), increased antigen presentation and antibody production. However, unlike for T lymphocytes, there appears to be species differences in B cell expression of TLR4 (for example murine TLR4 expression is constitutive but this is not the case in humans) (Bekeredjian-Ding and Jego, 2009). Consequently the exact effects of TLR4 activation on human B cells remain to be elucidated.

The primary focus of the present study will be on the role of TLR4 as an innate immune receptor but it is important to note that the receptor and its signalling pathway are also crucial in adaptive immune system activation. Furthermore, activation can occur via either direct (i.e. LPS activation of TLR4 on T and B lymphocytes) or indirect (i.e. stimulation of DC maturation for increased antigen presentation) mechanisms, highlighting the central role of TLR4 in immunity.

1.8. Toll-like receptors in hip arthroplasty

TLR4 expression has been demonstrated in tissues surrounding aseptically-loosened joint replacements (Takagi et al., 2007, Pajarinen et al., 2010). The majority of this expression is attributed to monocytes and macrophages infiltrating the peri-implant tissues, as well as macrophage clustering in response to wear debris, known as foreign body macrophage giant cells. Small numbers of TLR4-expressing neutrophils are also present (Lähdeoja et al., 2009) and together these are indicative of an innate immune response. TLRs are expressed in vascular endothelial and synovial cells surrounding aseptically-loosened implants and TLR4 expression is particularly high; this appears to be a response to the presence of the hip replacement as expression levels are increased in tissues from aseptically-loosened implants compared to in osteoarthritic tissues (Lähdeoja et al., 2009). These studies suggest that the cells and tissues around MoM implants can be responsive to TLR4 PAMPs and DAMPs, including cobalt ions. However little is known about how this may be linked to the inflammation observed in ARMD.

1.9. ARMD therapy

The only current treatment for ARMD is revision to an implant made from an alternative material (e.g. metal-on-polyethylene). Although this is an effective intervention it is also costly and more complex than primary hip replacement surgery. The development of a treatment option that could prevent or reduce ARMD without the need for surgery is consequently an attractive area for research. Current research into ARMD therapies focuses on modifying the existing inflammatory response, for example by polarising the macrophage population towards the M2 (anti-inflammatory or immunomodulatory) phenotype through IL-4 treatment (Pajarinen et al., 2015). Another option is introducing mutant and therefore dysfunctional

chemokines such as a mutant form of CCL2 known as 7ND, which dominantly inhibits activity of the wildtype form. In a murine model of wear particle-induced osteolysis, 7ND significantly decreased bone breakdown through downregulation of osteoclast formation (Jiang et al., 2015). Other targets include NFκB as it plays a key role in inducing inflammatory cytokine secretion in response to wear debris (Lin et al., 2014, Sato et al., 2015).

Understanding the cause of ARMD is the greatest obstacle in the development of novel therapies but challenges are also presented by the method of delivery (i.e. targeting the joint in isolation) and nature of the treatment itself (e.g. use of an antibody or other immune-modulating agent). Several methods of therapy delivery have been suggested, including coating hip implants with the therapeutic agent so that they are released as a product of the wear process (Goodman et al., 2013b). An insight into the molecular mechanisms underlying ARMD could aid in the development and delivery of preventative therapies.

1.10. Hypothesis

In summary of the current situation, cobalt-containing MoM implants fail at high rates because of adverse tissue reactions including osteolysis, pain, soft tissue necrosis, and pseudotumour development. These reactions are inflammatory and mediated by cytokines and chemokines, but the mechanisms that initiate them are unclear. Cobalt is known to activate human TLR4, an innate immune receptor that also recognises LPS from Gram negative bacteria, usually resulting in a pro-inflammatory response. This study therefore hypothesises that cobalt activation of human TLR4 leads to cellular inflammatory responses that may be linked to ARMD. The aim of the study is to investigate inflammatory effects induced by cobalt ions using a series of *in vitro* cell culture assays, and to assess the role of TLR4 in these responses.

1.11. Objectives

A. To investigate the overall effect of cobalt ions on cellular immune responses

Quantitative real-time polymerase chain reaction (qRT-PCR) arrays and proteome profiling arrays were performed to assess gene expression and protein secretion changes following stimulation of MonoMac 6 cells with cobalt ions.

B. To determine the role of TLR4 in inflammatory responses to cobalt ions

qRT-PCR and enzyme-linked immunosorbent assays (ELISA) were conducted to investigate more closely potential markers of TLR4 activation by cobalt. A small molecule TLR4 antagonist and two different anti-TLR4 neutralising antibodies were studied for their potential to inhibit the cellular inflammatory response induced by cobalt.

C. To investigate the effect of cobalt ions on immune cell adhesion and migration

Inflammatory cytokine secretion by cobalt-stimulated endothelial cells was assessed by ELISA with the aim of selecting an appropriate *in vitro* cell culture model. The effect of cobalt ions on membrane-bound adhesion molecule expression in human macrophages and endothelial cells was assessed by qRT-PCR, and soluble adhesion molecule secretion was quantified by ELISA. Inflammatory cell migration following cobalt stimulation was investigated using transwell chemotaxis assays.

D. To investigate factors that may impact upon patient variability in adverse reactions to metal ions

Stable cell lines expressing wildtype or mutant TLR4 were generated and investigated for their response to cobalt stimulation using qRT-PCR and ELISA assays. Co-stimulation assays were conducted using LPS or nickel ions to determine their effect on the cellular response to cobalt. The effect of chromium and strontium ions on TLR4 activation was also investigated.

Chapter 2. Materials and Methods

2.1. General laboratory practice

All experiments were carried out in accordance with the university standards for safe working with chemical substances in laboratories, which comply with the Control of Substances Hazardous to Health (COSHH) regulations. All laboratory work was conducted in compliance with the university safety policy.

2.2. Cell culture

Cell culture was carried out in compliance with regulations for the containment of Class II pathogens. All cells were maintained in 25 or 75cm^2 flasks in a humidified incubator at 37ºC with 5% CO_2.

2.2.1. Cell lines

MonoMac 6

MonoMac 6 cells are a human monocytic cell line derived from acute monocytic leukaemia. They are known to express TLR4 (part of MRes project) and respond to LPS by secreting IL-8 (Dr Jem Palmer, Institute of Cellular Medicine, Newcastle University personal communication). MonoMac 6 cells were cultured in RPMI-1640 medium (Sigma-Aldrich, Gillingham, UK) supplemented with 10% v/v foetal bovine serum (FBS), 2mM L-glutamine, 50U/ml penicillin and 50µg/ml streptomycin (all Sigma-Aldrich).

THP-1

THP-1 monocytes are derived from acute pro-monocytic leukaemia and were cultured in RPMI-1640 medium supplemented with 10% v/v FBS, 2mM L-glutamine, 50U/ml penicillin and 50µg/ml streptomycin.

U2OS

U2OS cells are a human osteoblast cell line derived from osteosarcoma. They were cultured in Dulbecco's Modified Eagle's Medium (DMEM) 1g/L glucose supplemented with 10% v/v FBS, 2mM L-glutamine, 50U/ml penicillin and 50µg/ml streptomycin.

J774

J774 murine macrophages are from BALB/c mice and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (1g/L) glucose supplemented with 10% v/v FBS, 2mM L-glutamine, 50U/ml penicillin, 50µg/ml streptomycin, 1mM non-essential amino acids and 1mM sodium pyruvate (both Lonza, Basel, Switzerland).

HUVEC

Human umbilical vein endothelial cells (HUVEC) from pooled donors (Promocell, Heidelberg, Germany) were cultured in Endothelial Cell Growth Medium with Supplement Mix containing FBS and additional required nutrients (all Promocell). 50U/ml penicillin and 50µg/ml streptomycin was also added.

HMEC-1

Human microvascular endothelial cells (HMEC-1) are derived from dermal foreskin. Cells were cultured in MCDB131 medium supplemented with 10% v/v FBS, 50U/ml penicillin, 50µg/ml streptomycin, 10ng/ml epidermal growth factor (EGF) and 1µg/ml hydrocortisone (all Sigma-Aldrich).

2.2.2. Cell maintenance and passage

All cell culture experiments were performed under aseptic conditions in a Class II laminar flow microbiological safety cabinet. Cells were maintained in the media described in **section 2.2.1** and passaged at 70-80% confluency. Passage methods were dependent on the cell line and cell characteristics affecting passage methods are detailed below.

Suspension cells	Adherent cells
MonoMac 6	U2OS
THP-1	HMEC-1
J774	HUVEC

Table 2.1: Cell line characteristics

Suspension cells were centrifuged at 179g for 5 minutes, supernatant was discarded and pelleted cells were resuspended in fresh complete media. Adherent cells (except HUVEC) were rinsed with phosphate-buffered saline (PBS) (Sigma Aldrich) following removal of supernatant. Cells were then trypsinised in 2mM trypsin-EDTA (Sigma-Aldrich) at 37ºC, centrifuged at 179g for 5 minutes and resuspended in complete media. HUVEC were passaged using a DetachKit (Promocell); the kit contained HEPES-buffered saline solution (HEPES-BSS) for washing, trypsin–EDTA and a trypsin neutralisation solution. All reagents were used according to the manufacturer's protocol.

2.2.3. Cryopreservation of cells

Cell cryopreservation was routinely performed for stock maintenance. Cells were frozen in cryovials at approximately 1 million per ml in FBS with 10% v/v dimethyl sulphoxide (DMSO) (Sigma-Aldrich) at -80ºC and then moved to liquid nitrogen for long-term storage. To thaw stocks, cryovials were rapidly warmed in a 37ºC water bath and the contents transferred to a

vial of pre-warmed PBS. Cells were then centrifuged at 179g for 5 minutes to remove DMSO and seeded in pre-warmed culture medium.

2.2.4. Mycoplasma testing

All cell lines were routinely tested for mycoplasma contamination using a MycoAlert mycoplasma detection kit (Lonza, Slough, UK). The assay lyses any mycoplasma present in cell culture supernatant, releasing enzymes that react with the MycoAlert substrate and convert adenosine diphosphate (ADP) to adenosine triphosphate (ATP). The ratio of ATP before and after the addition of substrate is then calculated. A ratio of <0.8 is considered mycoplasma-free while >1.2 shows mycoplasma contamination.

2.3. Cell stimulation

2.3.1. Positive and negative controls

LPS is a known TLR4 ligand and therefore TLR4-specific LPS (from *E.coli* serotype J5, Alexis Biochemicals, California, USA) was used as a positive control throughout this study. This LPS does not contain TLR2 agonists, nor any protein or DNA contaminants known to activate TLRs. LPS was diluted in complete culture medium appropriate for each cell line and used at a range of concentrations up to 1000ng/ml. Untreated cells were incubated in complete media as a negative control.

2.3.2. Metal ions

Nickel chloride hexahydrate ($NiCl_2\,6H_2O$) and cobalt chloride hexahydrate ($CoCl_2\,6H_2O$) (both Sigma-Aldrich) were diluted in complete culture medium appropriate for each cell line. Complete media was selected as the metals ions precipitated in PBS and blank cell culture medium (previously optimised during MRes project). Metal salts were selected because nickel salts have previously been used to demonstrate nickel activation of human TLR4 (Schmidt et al., 2010).

2.3.2.1. Metal ion concentrations

Concentrations of $NiCl_2$ and $CoCl_2$ used for *in vitro* cell stimulation in previous studies vary considerably. The concentrations used in this study were selected based on research in similar cell lines and refined following consideration of metal ion levels surrounding failed MoM joints observed in clinical studies (De Smet et al., 2008, Davda et al., 2011). This will be discussed in more detail in **section 4.4**.

2.4. TLR4 inhibition

2.4.1. CLI-095

CLI-095 (Invivogen, San Diego, USA) is a small molecule TLR4 antagonist. It is also known as TAK-242 or resatorvid and has been shown to block LPS-mediated TLR4 signalling by binding to Cys747 in the intracellular domain of TLR4 (Takashima et al., 2009). This prevents recruitment of adaptor proteins such as MyD88 and TRIF to the activated receptor (Matsunaga et al., 2011) and inhibits subsequent downstream signalling **(Error! Reference source not found.A)**. CLI-095 was reconstituted in DMSO and diluted in complete cell culture medium according to the manufacturer's protocol. Cells were pre-incubated with CLI-095 for 6h prior to stimulation with a TLR4 ligand.

2.4.2. PAb-hTLR4

PAb-hTLR4 (Invivogen) is a polyclonal rat IgG antibody against human TLR4. It is reported to inhibit TLR4-mediated signalling through receptor neutralisation and has been shown to be effective in MonoMac 6 cells (Ekaney et al., 2014) **(Error! Reference source not found.B)**.

2.4.3. MAb2-hTLR4

MAb2-hTLR4 (Invivogen) is a monoclonal mouse IgG1 antibody (clone 3C3) against human TLR4. The exact antibody binding site is unknown (**Figure 2.1C**) but like PAb-hTLR4 it is reported to neutralise human TLR4 activation and prevent subsequent intracellular signalling (Scarpa et al., 2015).

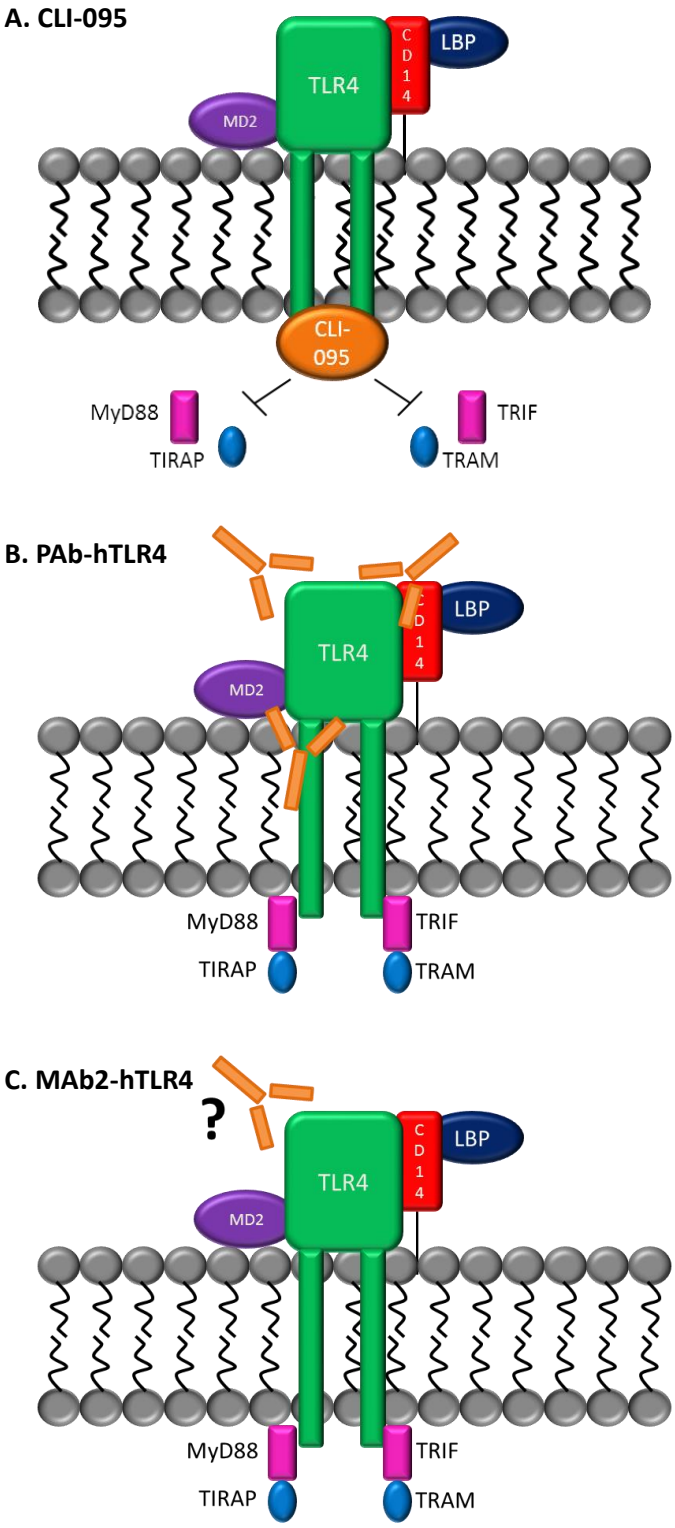

Figure 2.1: Mechanism of action of TLR4 inhibitors
A. CLI-095 binds to Cys747 in the intracellular domain of TLR4, preventing the recruitment of adaptor proteins including MyD88, TIRAP, TRIF and TRAM which are required for downstream signalling. **B. PAb-hTLR4** is a polyclonal anti-TLR4 neutralising antibody and therefore binds multiple epitopes. **C. MAb2-hTLR4** is a monoclonal anti-TLR4 neutralising antibody. Its exact binding site is unknown.

2.5. Cytotoxicity

Cytotoxicity was assessed by trypan blue staining. 10µl trypan blue dye (Logos Biosystems, Anyang-City, South Korea) was mixed with 10µl cell suspension and the number of live and dead cells was counted using a Luna II automated cell counter (Logos Biosystems). The cell counter determines cell viability by detecting trypan blue uptake; cells with disrupted membranes and reduced viability cannot exclude the dye and appear blue, while healthy cells can exclude the dye and appear white/clear. However during the optimisation of this assay it was noted that the automated counts being generated were not accurate as healthy cells were falsely detected as nonviable. An example of this is shown in **Figure 2.2**; cells circled in red are classed as unviable (tagged image) but analysis of the raw image indicates that the cells are clear of trypan blue and therefore viable**.** To combat this issue the same staining process was used but the raw images collected using the Luna II cell counter were counted manually.

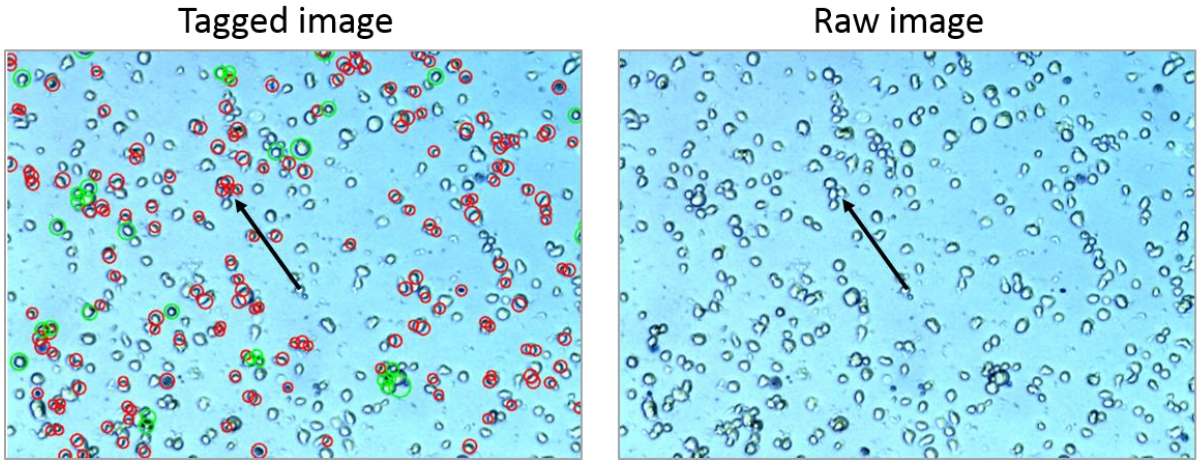

Figure 2.2: Cytotoxicity assay optimisation
Unstimulated MonoMac 6 cells were stained with trypan blue dye and counted using a Luna II automated cell counter. In some experiments it was noted that during the counting process viable cells were mistakenly being classified as nonviable (circled in red in tagged image but appear viable in raw image). Green circles indicate viable cells.

2.6. Gene expression analysis

2.6.1. RNA extraction

Total RNA was extracted from cells using an RNeasy Mini kit (Qiagen, Venlo, Netherlands) following the manufacturer's protocol. Briefly, cells were collected in a 1.5ml microcentrifuge tube and lysed in Buffer RLT containing 1% v/v β-mercaptoethanol to inhibit RNase activity. The lysed solution was further homogenised using a needle and syringe before the addition of 70% v/v ethanol and transfer to the spin column assembly. A series of washing and centrifugation steps were performed followed by incubation of the spin column membrane with DNase I for 15 minutes. Several further washing and centrifugation steps were performed

before elution of the purified RNA in 50µl nuclease-free water. RNA quantity and quality was measured by Nanodrop ND-1000 spectrophotometer at wavelengths of 260nm and 280nm. A 260/280 ratio of between 1.8 and 2.2 indicates good quality RNA. Isolated RNA was stored at -80^{o}C if required.

Integrity of isolated RNA was confirmed by gel electrophoresis on a 1.2% w/v agarose gel. Two distinct bands should be seen representing the 28S and 18S ribosomal RNA subunits indicating intact RNA **(Figure 2.3)**.

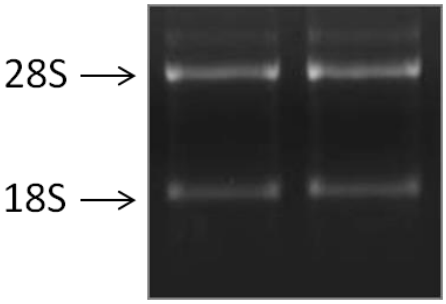

Figure 2.3: RNA gel electrophoresis
Isolated RNA was separated by electrophoresis on a 1.2% w/v agarose gel. The 28S and 18S bands are the two ribosomal subunits and show intact RNA. The two samples shown are for reference only.

2.6.2. Reverse transcription

cDNA was synthesised from total RNA using either Superscript III reverse transcriptase or a Maxima First Strand cDNA Synthesis kit (both ThermoFisher Scientific, Waltham, USA). The Superscript III method uses oligodT primers while the Maxima method includes oligodT primers and random hexamers, and this has been shown to produce increased amounts of cDNA from the same starting amount of RNA (Dr Alison Tyson-Capper, personal communication).

2.6.2.1. Superscript III reverse transcriptase

Each Superscript III reverse transcription reaction contained 1µg total RNA, 10µl 5X First Strand Buffer, 2µl 10mM dNTP mix, 1µl oligo(dT)$_{20}$ (50µM), 1µl DTT (0.1M), 1µl RNase OUT (40 units/µl) (all ThermoFisher Scientific) and nuclease-free H_2O to a final volume of 49µl. As the final step 1µl Superscript III reverse transcriptase was added to each reaction and stirred with a pipette tip. The reverse transcription reactions were incubated in a G-Storm thermal cycler for 50 minutes at 50^{o}C followed by 5 minutes at 85^{o}C. This method was optimised by previous members of the laboratory.

2.6.2.2. Maxima First Strand cDNA synthesis

Each Maxima First Strand cDNA Synthesis Reaction contained 1µg total RNA, 4µl 5X Reaction Mix, 2µl Maxima Enzyme Mix (all ThermoFisher Scientific) and nuclease-free H_2O to a final volume of 20µl. Reactions were incubated in a G-Storm thermal cycler for 10 minutes at 25ºC, 15 minutes at 50ºC and finally 5 minutes at 85ºC. Synthesised cDNA was stored on ice for immediate use or at -20ºC for long-term storage. If required cDNA was diluted in H_2O before further assays were conducted.

2.6.3. Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted using TaqMan gene expression probes **(Table 2.2)**. The TaqMan-based method was selected for its high specificity compared to SYBR Green **(Figure 2.4)** and, aside from the arrays described in chapter 3, all qRT-PCR conducted in this study used TaqMan assays. Each reaction contained 1µl 20X TaqMan gene expression assay (containing primer pair and gene-specific probe) (ThermoFisher Scientific), 10µl 2X TaqMan Universal MasterMix II (ThermoFisher Scientific), 4µl diluted cDNA template and 5µl nuclease-free water. No template controls (NTC) were included for each primer and contained nuclease-free H_2O instead of cDNA. Unless otherwise stated gene expression was normalised to *GAPDH* as a housekeeping gene.

20µl reaction was added in triplicate to each well of a 96-well reaction plate. qRT-PCR was performed using a StepOnePlus real-time PCR thermal cycler (Applied Biosystems, Massachusetts, USA). Reactions were incubated at 50ºC for 2 minutes and 95ºC for 10 minutes followed by 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute.

Target gene	Abbreviation	Species	RefSeq	Assay ID	Exon boundary
Beta actin	*ACTB*	Human	NM_001101.3	Hs01060665_g1	2-3
Beta actin	*Actb*	Mouse	-	Mm00607939_s1	6-6
Chemokine (C-C motif) ligand 2	*CCL2*	Human	NM_002982.3	Hs00234140_m1	1-2
Chemokine (C-C motif) ligand 20	*CCL20*	Human	NM_001130046.1	Hs00355476_m1	2-3
Chemokine (C-C motif) ligand 25	*CCL25*	Human	NM_001201359.1	Hs00608373_m1	5-6
Chemokine (C-X-C motif) ligand 1	*Cxcl1*	Mouse	NM_008176.3	Mm04207460_m1	3-4
Chemokine (C-X-C motif) ligand 10	*CXCL10*	Human	NM_001565.3	Hs01124251_g1	2-3
Glyceraldehyde 3-phosphate dehydrogenase	*GAPDH*	Human	NM_002046.4	Hs99999905_m1	3-3
Hypoxia-inducible factor 1α	*HIF1A*	Human	NM_001243084.1	Hs00153153_m1	4-5
High mobility group box protein 1	*HMGB1*	Human	NM_002128.4	Hs01590761_g1	1-2
Heat shock protein A1A	*HSPA1A*	Human	NM_005345.5	Hs00359163_s1	1-1
Intracellular adhesion molecule-1	*ICAM1*	Human	NM_000201.2	Hs00164932_m1	2-3
Interleukin-1α	*IL1A*	Human	NM_000575.3	Hs00174092_m1	6-7
Interleukin-6	*IL6*	Human	NM_000600.3	Hs00985639_m1	2-3
Interleukin-8	*IL8*	Human	NM_000584.3	Hs00174103_m1	1-2
Interleukin-1 receptor-associated kinase 2	*IRAK2*	Human	NM_001570.3	Hs00176394_m1	11-12
Integrin alpha 4	*ITGA4 (CD49D)*	Human	NM_000885.4	Hs00168433_m1	2-3
Integrin alpha L	*ITGAL (CD11a)*	Human	NM_001114380.1	Hs00158218_m1	13-14
Vascular adhesion molecule-1	*VCAM1*	Human	NM_001078.3	Hs00365480_g1	1-2

Table 2.2: TaqMan gene expression assays
TaqMan gene expression assays were purchased from ThermoFisher Scientific. Table shows the target genes investigated in this study, the relevant assay IDs (identification numbers used by the manufacturer) and the reference sequences used for primer and probe design (taken from the NCBI RefSeq database). Most TaqMan probes bind across exon boundaries because this ensures that the assay only detects genes that have been correctly spliced.

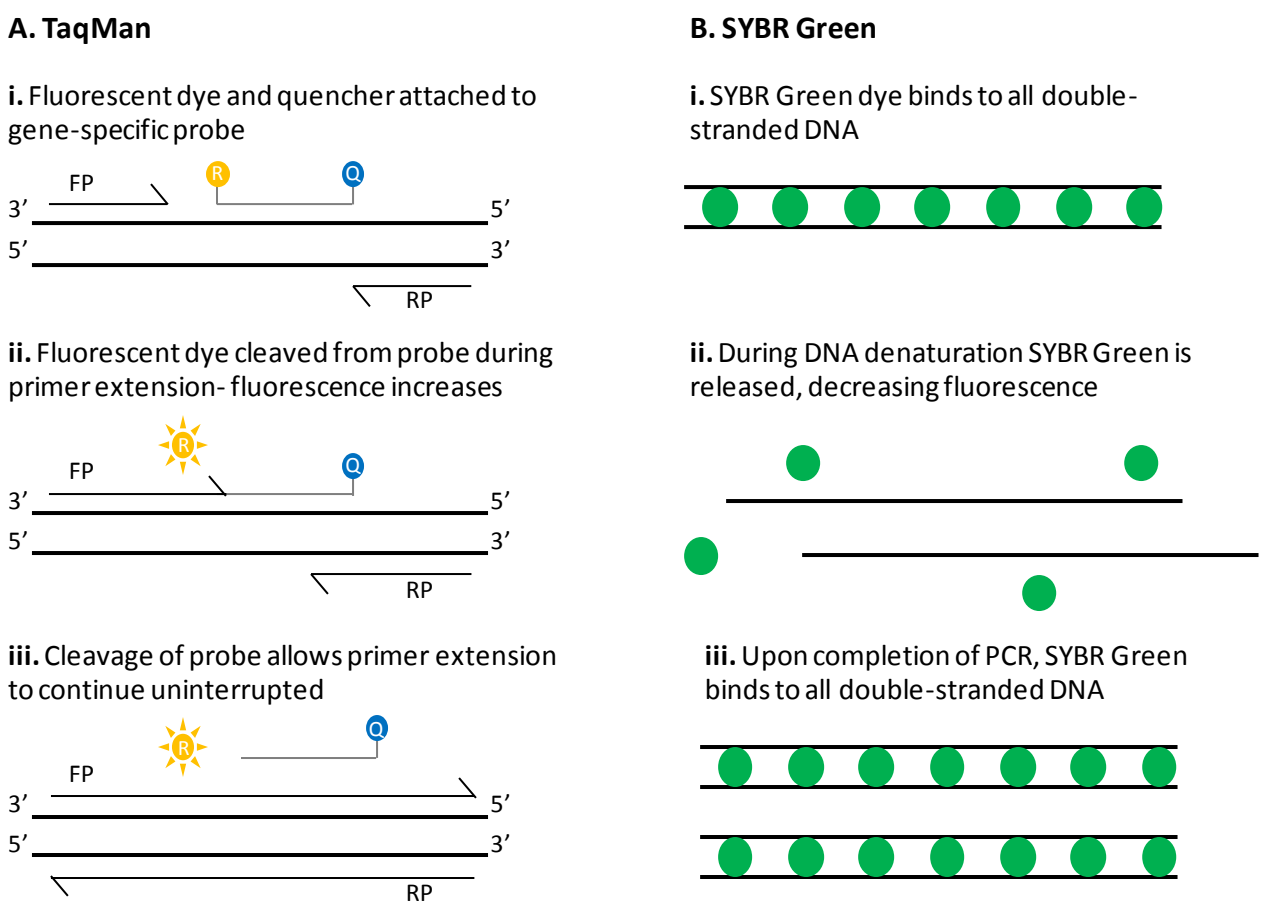

Figure 2.4: TaqMan versus SYBR Green chemistry in qRT-PCR
A. TaqMan gene expression assays contain forward (FP) and reverse (RP) primers and a gene-specific probe. **i.** The probe has a fluorescent reporter dye (R) and quencher (Q) attached and binds to the target gene. **ii.** During primer extension DNA polymerase cleaves the probe, releasing the reporter dye from the quencher and increasing fluorescence. **iii.** Cleavage of the probe by DNA polymerase allows the primer to continue extension without affecting binding. **B. i.** SYBR Green dye binds to all double-stranded DNA. **ii.** As DNA is denatured during qRT-PCR, SYBR Green is released and fluorescence is decreased. **iii.** When qRT-PCR is complete the dye binds to all double-stranded DNA. Figure adapted from https://www.thermofisher.com/uk/en/home/life-science/pcr/real-time-pcr/qpcr-education/taqman-assays-vs-sybr-green-dye-for-qpcr.html

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2.7. Protein analysis

2.7.1. Enzyme-linked immunosorbent assay

Supernatant from stimulated cells was collected in a 1.5ml microcentrifuge tube and centrifuged at 179g for 5 minutes to pellet cells. The supernatant was then stored at -80^{o}C prior to further assays.

Enzyme-linked immunosorbent assays (ELISA) were used to detect cellular cytokine secretion. IL-6, IL-8, CXCL10 and sICAM-1 ELISA kits (Peprotech, London, UK) were used according to the manufacturer's protocol with minor modifications for the assay development step, as optimised by Dr Jem Palmer. Unless otherwise stated all steps were followed by aspiration of the plate and 2 wash cycles using an automated plate washer (ThermoFisher Scientific) and wash buffer (1X PBS and 0.01% v/v Tween).

An Immulon 4HBX 96-well plate (ThermoFisher Scientific) was coated with capture antibody at 4^{o}C overnight. The plate was blocked for 1h at room temperature (RT) using 5% w/v bovine serum albumin (BSA) in PBS. The block buffer was diluted 1:10 in wash buffer to give the reagent diluent. Samples were diluted in reagent diluent as appropriate and a seven-point standard curve was generated before standards and samples were added to the plate in triplicate and incubated for 3h at 4^{o}C. Reagent diluent was included to give a blank reading for normalisation. The detection antibody was added to the plate and incubated at RT for 2h. Instead of using the avidin provided in the kit, streptavidin-HRP (horseradish peroxidise) conjugate (ThermoFisher Scientific) was diluted 1:5000 in reagent diluent before addition to the plate and incubation for 30 minutes at RT. The detection solution was composed of 200µl o-phenylenediamine dihydrochloride (OPD), 5µl hydrogen peroxide and 12.5 ml citrate buffer and incubated at RT until colour had fully developed. No aspiration and wash step was performed at this point. The reaction was stopped with 2M sulphuric acid (H_2SO_4). Absorbance was read at 490nm on a BioTek Synergy HT plate reader (BioTek, Vermont, USA). The Peprotech ELISA protocol is summarised in **Table 2.3** and reagent concentrations are shown in **Table 2.4**.

Step	Diluent	Volume (µl)	Time (h)	Temperature (ºC)
Capture antibody	PBS	100	Overnight	4
Block buffer (5% w/v BSA)	PBS	150	1	RT
Samples and standards	Reagent diluent	100	3h	4
Detection antibody	Reagent diluent	100	2	RT
Streptavidin-HRP conjugate	Reagent diluent	100	0.5	RT
OPD detection solution	N/A	100	N/A	RT

Table 2.3: Peprotech ELISA protocol
All volumes are per well of a 96 well plate. 'RT' indicates room temperature. The detection solution in the final step was incubated for approximately 3 minutes or until colour had fully developed.

CCL20 secretion was quantified by DuoSet ELISA (R&D Systems, Minneapolis, USA) according to the manufacturer's protocol. The protocol was similar to the Peprotech ELISA procedure with some minor differences. The capture antibody coating step was performed at RT and the plate was blocked in 1% w/v BSA in PBS. The plate was developed using a substrate solution with tetramethylbenzidine (R&D Systems) and absorbance was read at 450nm. Reagent concentrations for the CCL20 ELISA are shown in **Table 2.4**.

	IL-6	IL-8	sICAM-1	CXCL10	CCL20
Capture antibody (µg/ml)	1	0.5	1	0.5	2
BSA in block buffer (%)	5	5	5	5	1
Maximum standard (pg/ml)	2000	1000	3000	1000	1000
Detection antibody (µg/ml)	0.5	0.5	0.5	0.25	0.02

Table 2.4: ELISA reagent concentrations

2.7.2. ELISA data analysis

Blank (reagent diluent) values were subtracted from all other standards and samples. A standard curve was generated **(Figure 2.5**) and used to calculate the sample concentrations. Values were multiplied by the appropriate dilution factor.

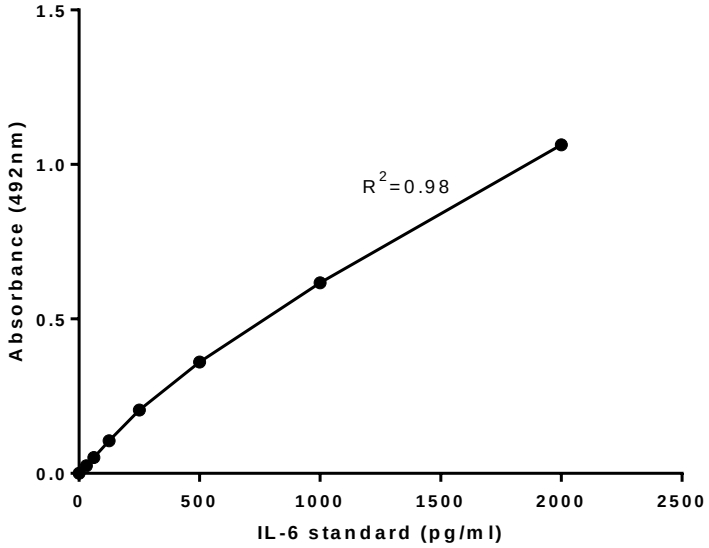


Figure 2.5: Representative IL-6 ELISA standard curve

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, USA). All error bars show the standard error of the mean (SEM) unless otherwise stated. The analysis method is described for each individual experiment. Statistical significance is shown as follows *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$.

2.9. Graphs and images

All graphs were generated using GraphPad Prism 6.0. Unless otherwise stated diagrams were created in Microsoft Powerpoint 2013 using Servier Medical Art images, available online at http://www.servier.com/Powerpoint-image-bank.

Chapter 3. Medium-throughput study to investigate the effect of cobalt ions

3.1. Introduction

Cobalt ions activate human TLR4 but it is not known how they regulate other TLR4 signalling factors including MyD88, TRIF, IRAK1 and IRAK3 (IRAKM). It is important to improve our understanding in this regard because these factors influence the inflammatory outcome of TLR4 activation. Consequently, one of the initial aims of the study was to determine the effect of cobalt ions on a broad range of TLR4 signalling factors, inflammatory cytokines, chemokines, and their receptors, with the subsequent goal of identifying a marker of cobalt-mediated TLR4 activation that can be used to further define the immunological effects of cobalt ions.

3.1.1. MonoMac 6 cells

A human cell line was required for this study because, as described in **section 1.5.1**, murine TLR4 is not activated by cobalt ions. Further sequence analysis of this region of TLR4 revealed that the histidine pocket is only present in humans and other primates, precluding the use of conventional small animal models (**Figure 3.1**). Although humanised mouse models represent a realistic option for future research, one of the aims of the present study was to develop an *in vitro* model to test the effects of cobalt and other metal ions, and therefore a human cell line was considered appropriate. Macrophages are highly relevant to ARMD as they often dominate the inflammatory infiltrate of MoM peri-implant tissues (Natu et al., 2012) and their presence in synovial fluid makes them primary responders to wear debris. MonoMac 6 cells were chosen for this study because they are responsive to LPS through their expression of TLR4 (Lee and Sullivan, 2001).

```
SP|O00206|TLR4_HUMAN RNLIYLDISHTHTRVAFNGIFNGLSSLEVLKMAGNSFQENFLPDIFTELRNLTFLDLSQC 506
SP|Q9QUK6|TLR4_MOUSE EKLLYLDISYTNTKIDFDGIFLGLTSLNTLKMAGNSFKDNTLSNVFANTTNLTFLDLSKC 504
SP|Q496Z2|TRIL_RAT   ----------+----------------------------------HLPRLGLLSLSGN 263
SP|Q9TSP2|TLR4_PAPAN RNLIYLDISHTHTTVAFNGIFDGLLSLKVLKMAGNSFQENFLPDIFTDLKNLTFLDLSQC 506
SP|Q9WV82|TLR4_CRIGR EKLLYLDISYTNTKIDFNGIFFGLTSLNTLKMAGNSFKDNILSNVFTNTTNLTFLDISKC 504
SP|Q9TTN0|TLR4_PANPA RNLIYLDISHTHTRVAFNGIFNGLSSLEVLKMAGNSFQENFLPDIFTELRNLTFLDLSQC 506
SP|Q68Y56|TLR4_PIG   RNLHYLDISYTNIHVVFRGIFAGLVSLQTLKMAGNSFQNNLLPDVFTDLTNLILLDLSKC 506
```

Figure 3.1: Presence of TLR4 histidine pocket in different species
The histidine pocket (red box) present at positions 456 and 458 in the human TLR4 sequence is also present in other primates such as the baboon ('PAPAN') and chimpanzee ('PANPA'). However it is not found in the mouse, rat or Chinese hamster ('CRIGR') TLR4 sequences, nor in larger animals including the pig. Analysis was performed using the UniProt alignment tool available online at http://www.uniprot.org/align/A201601082M3UUO3L42.

3.2. Objectives

- To investigate the effect of cobalt ions on expression of a range of inflammatory cytokine and TLR signalling genes using qRT-PCR arrays
- To validate any notable findings from the arrays using TaqMan-based gene expression assays for individual target genes
- To investigate the effect of cobalt ions on secretion of pro-inflammatory cytokines using Proteome Profiler (dot blot) arrays

3.3. Specific materials and methods

3.3.1. RT^2 Profiler qRT-PCR arrays

MonoMac 6 cells were seeded at $0.2x10^6$ cells per well in a 6-well plate and treated with 0.75mM $CoCl_2$ or left untreated for 4h. The $CoCl_2$ concentration was selected based on previous *in vitro* studies on cobalt and TLR4 (Raghavan et al., 2012), as well as the MRes project preceding this work, and will be discussed in more detail in chapter 4. RNA was isolated using an RNeasy Mini Kit (Qiagen, Venlo, Netherlands) as described in **section 2.6.1**. cDNA was reverse transcribed from 1µg RNA using an RT^2 First Strand Kit (Qiagen) following the manufacturer's protocol, which included a genomic DNA elimination step. cDNA was then used for SYBR Green-based RT^2 Profiler PCR arrays (Qiagen). The arrays used were Inflammatory Cytokines and Receptors (PAHS-011C) and Toll-like Receptor Signalling Pathway (PAHS-018Z), and were performed according to manufacturer's protocol. Briefly, 102µl cDNA was mixed with 1350µl 2x RT^2 SYBR Green Mastermix (Qiagen) and 1248µl RNase-free H_2O before 25µl of the reaction was added to each well of the array plate. Each array included a series of housekeeping gene controls and human genomic DNA contamination controls. qRT-PCR was conducted using a StepOnePlus thermal cycler and the reactions were heated to 95ºC for 10 minutes before 40 cycles of 15 seconds at 95ºC and 1 minute at 60ºC. Three independent $CoCl_2$ stimulation experiments were performed meaning that six arrays (three each for stimulated and unstimulated) were conducted in total.

3.3.2. qRT-PCR array data analysis

Data obtained from the qRT-PCR arrays was analysed using Qiagen's RT^2 Profiler PCR Array Data Analysis program Version 3.5 (available online at http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php) and the Excel analysis spreadsheet (available via the same website). All three pairs of arrays were analysed together. p values of <0.05 were considered significant, together with a fold change in gene expression of greater than 2.

Any genes in which the average Ct value for both treated and untreated samples was >35 were excluded from the analysis as very low Ct values can affect results. In addition, genes in which one sample (e.g. unstimulated) had an average Ct value of >35 were noted and this is taken into consideration in the analysis.

3.3.3. Proteome Profiler human cytokine array

Proteome Profiler Human Cytokine Arrays (R&D Systems, Minneapolis, USA) detect inflammatory cytokines in cell culture supernatant using a principle similar to an ELISA.

Capture antibodies are bound to a nitrocellulose membrane and after incubation with cell culture supernatant a cocktail of biotinylated detection antibodies is added. The signal is detected using streptavidin-HRP and chemiluminescence meaning that it is proportional to the amount of cytokine present. Arrays were conducted according to the manufacturer's protocol using conditioned media (supernatant) from MonoMac 6 cells stimulated with 0.75mM $CoCl_2$, 1000ng/ml LPS or left unstimulated for 24h. This longer timepoint was chosen to allow for protein expression changes compared to the shorter 4h stimulation used for gene expression.

3.4. Results

3.4.1. Effect of cobalt ions on MonoMac 6 cell viability

Cobalt is known to be cytotoxic in both its ionic and particulate forms (Kwon et al., 2009). Before the qRT-PCR arrays were conducted it was important to determine whether or not $CoCl_2$ caused cytotoxicity in MonoMac 6 cells as this could influence the assay outcome. MonoMac 6 cells were stimulated for 4h with 0.75mM $CoCl_2$ and a cytotoxicity assay was conducted **(Figure 3.2A&B)** using trypan blue staining. Images were taken using a Luna II automated cell counter and counted manually for the reasons detailed in **section 2.5**. Viability in untreated MonoMac 6 cells was normalised to 100%. $CoCl_2$ stimulation caused cell viability to decrease to 94% ($p<0.0059$) **(Figure 3.2C)** showing that $CoCl_2$ does cause a degree of cytotoxicity in MonoMac 6 cells.

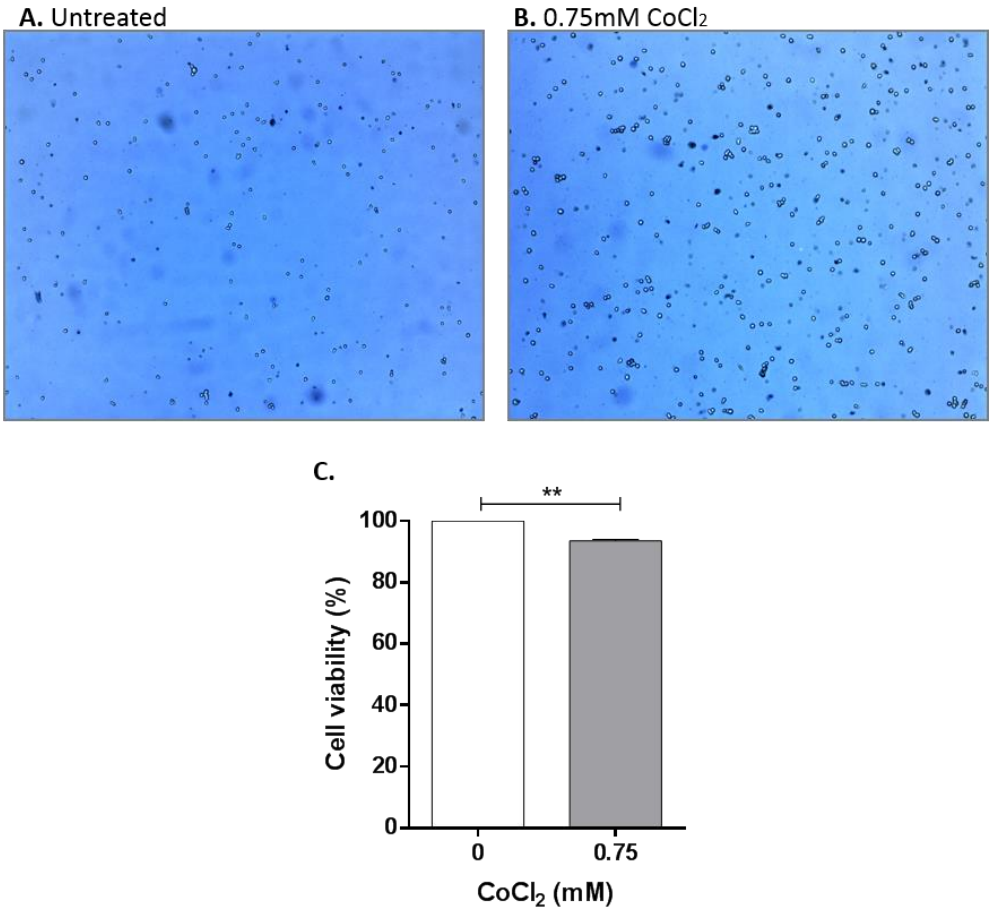


Figure 3.2: Effect of $CoCl_2$ on MonoMac 6 cell viability
MonoMac 6 cells were stimulated with 0.75mM $CoCl_2$ for 4h and cytotoxicity was assessed by trypan blue staining. Images were taken on a Luna II automated cell counter and counted manually. **A.** Luna cell counter image of unstimulated cells. **B.** Luna cell counter image of $CoCl_2$-stimulated cells. **C.** Percentage cell viability of MonoMac 6 cells following $CoCl_2$ stimulation. Data was normalised to 100% viability in untreated cells. Graph shows combined results from two independent experiments. Statistical significance was calculated by unpaired Student's *t* test.

3.4.2. Effect of cobalt ions on *TLR4* expression

Many studies report that TLR4 ligands such as LPS exert their effects in part through upregulation of TLR4 expression (Bosisio et al., 2002, Yokota et al., 2010, Guzzo et al., 2012). *TLR4* expression was investigated to determine whether or not this might be the case for cobalt ions, which could contribute to any observed changes. Analysis by qRT-PCR using TaqMan gene expression assays revealed that 4h stimulation of MonoMac 6 cells with 0.75mM $CoCl_2$ did not cause any changes in *TLR4* expression compared to the unstimulated control (p=0.1018) **(Figure 3.3)**.

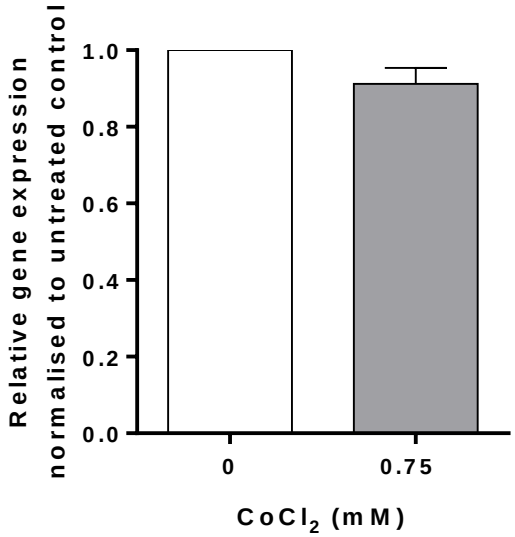


Figure 3.3: Effect of $CoCl_2$ on TLR4 expression
MonoMac 6 cells were stimulated with 0.75mM $CoCl_2$ for 4h. RNA was collected and cDNA synthesised by reverse transcription. *TLR4* expression was analysed by qRT-PCR (TaqMan gene expression assay). Data is representative of three independent experiments and statistical significance was calculated using a Student's *t* test (p=0.1018).

3.4.3. Effect of cobalt ions on TLR signalling genes

qRT-PCR arrays were used to determine the effect of cobalt ions on genes related to TLR signalling, and inflammatory cytokine and receptor genes. The arrays were specifically selected to give an overview of the inflammatory response to cobalt in relation to TLR signalling as cobalt ions activate human TLR4.

MonoMac 6 cells were stimulated with 0.75mM $CoCl_2$ for 4h before RNA was isolated and cDNA synthesised from 1µg RNA by reverse transcription. TLR Signalling qRT-PCR arrays (PAHS-018Z) were then performed. Genes with a fold upregulation greater than 2 are shown in **Table 3.1** while those showing a fold downregulation greater than 2 are detailed in **Table 3.2**. Overall 23 genes were upregulated by $CoCl_2$ treatment and 23 were downregulated. An initial overview of the affected genes shows the widespread effects of cobalt as it regulated the

expression of genes encoding PRRs, signalling factors, transcription factors, adaptor proteins, and cytokines. The full gene list along with detected fold changes and p values is shown in **Appendix A**.

There was no change in *TLR4* expression which supports the results of the TaqMan-based analysis in **Figure 3.3**. However, expression of other TLRs (*TLR1, TLR3, TLR5, TLR6, TLR8* and *TLR9*) was downregulated following cobalt stimulation, most notably *TLR1* which decreased more than 30-fold. Downregulation of *TLR6* reached statistical significance (p=0.0251).

TLR4 and other TLRs recruit adaptor proteins to their intracellular domains to initiate downstream signalling pathways. The MyD88-dependent and TICAM1 (TRIF)-dependent pathways can both be activated when LPS binds TLR4 (Kawai et al., 2001, Weighardt et al., 2004). The arrays showed a significant increase in *TICAM1* expression (p=0.0393) following cobalt stimulation and *TICAM2* (TRAM) was also upregulated. In contrast *MYD88* expression was decreased 3-fold by cobalt ions (**Table 3.2**), although other components of the MyD88-dependent pathway were upregulated, including interleukin receptor-associated kinase 2 (*IRAK2)* (p=0.0133).

The expression of genes encoding transcription factors was also regulated by cobalt ions, particularly members of the NFκB family. *NFKB1* was upregulated 7-fold and an inhibitor of NFκB, *NFKBIA*, was similarly increased **(Table 3.1)**. However signalling factors involved in activation of other transcription factors were also upregulated, including Jun proto-oncogene (*JUN)* (p=0.0159) which is part of the JNK/p38 pathway and results in AP-1 transcription factor activation.

The gene showing the largest fold upregulation in the TLR signalling arrays was *HSPA1A* which encodes heat shock 70kDa protein 1A (an Hsp70 family member); its expression was upregulated more than 200-fold by $CoCl_2$ **(Table 3.1)**. Two pro-inflammatory cytokine genes, interleukin-8 (*IL8*) and interleukin-1A (*IL1A*) were significantly upregulated ($p<0.001$ and p=0.0255 respectively). A second gene encoding heat shock 60kDa protein, *HSPD1*, was also upregulated, although this was not statistically significant. In addition to the very large expression changes detected for these genes, smaller fold changes were observed for other cytokines and chemokines, including interleukin-1B (*IL1B*), chemokine (C-X-C motif) ligand 10 (*CXCL10*) and colony stimulating factor 2 (*CSF2*). In contrast other pro-inflammatory cytokines were downregulated, including interferon gamma (*IFNG*) and interleukin-2 (*IL2*).

CCL2 showed the largest downregulation (over 400-fold) of all genes on the TLR signalling arrays (**Table 3.2**).

Gene	RefSeq	Gene name	Fold change
HSPA1A	**NM_005345**	**Heat shock 70kDa protein 1A**	**202.39**
IL8	**NM_000584**	**Interleukin 8**	**40.91**
HSPD1	NM_002156	Heat shock 60kDa protein 1	28.04
IL1A	**NM_000575**	**Interleukin 1 alpha**	**17.11**
NFKBIA	**NM_020529**	**Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha**	**7.81**
NFKB1	**NM_003998**	**Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1**	**7.62**
UBE2N	NM_003348	Ubiquitin-conjugating enzyme E2N	5.03
TICAM2	NM_021649	Toll-like receptor adaptor molecule 2	4.92
JUN	**NM_002228**	**Jun proto-oncogene**	**4.47**
PELI1	**NM_020651**	**Pellino homologue 1**	**4.42**
IL1B	NM_000576	Interleukin 1 beta	4.32
TICAM1	**NM_182919**	**Toll-like receptor adaptor molecule 1**	**3.68**
TIRAP	NM_001039661	Toll-interleukin 1 domain-containing adaptor protein	3.47
CXCL10	NM_001565	Chemokine (C-X-C motif) ligand 10	3.28
TLR10	NM_030956	Toll-like receptor 10	3.2
IRAK2	**NM_001570**	**Interleukin-1 receptor-associated kinase 2**	**3.17**
CSF2	NM_000758	Colony stimulating factor 2 (granulocyte-macrophage)	2.42
CHUK	NM_001278	Conserved helix-loop-helix ubiquitous kinase	2.35
REL	**NM_002908**	**V-rel reticuloendotheliosis viral oncogene homologue (avian)**	**2.32**
TBK1	NM_013254	TANK-binding kinase 1	2.28
RIPK2	NM_003821	Receptor-interacting serine-threonine kinase 2	2.25
CASP8	NM_001228	Caspase 8, apoptosis-related cysteine peptidase	2.06
CLEC4E	NM_014358	C-type lectin domain family 4, member E	2.01

Table 3.1: Upregulated TLR signalling genes following cobalt stimulation
MonoMac 6 cells were stimulated with 0.75mM $CoCl_2$ for 4h before RNA was isolated and cDNA synthesised by reverse transcription. Gene expression was assessed by TLR Signalling qRT-PCR array (PAHS-018Z). Table shows genes that were upregulated more than 2-fold. Significant ($p<0.05$) changes are shown in bold. Analysis was performed using Qiagen's online array analysis software **(see section 3.3.2)** using three pairs of arrays (stimulated versus unstimulated). The full array analysis results are shown in **Appendix A**.

Gene	RefSeq	Gene name	Fold change
CCL2	NM_002982	Chemokine (C-C motif) ligand 2	-410.75
TLR1	NM_003263	Toll-like receptor 1	-33.91
IL2	NM_000586	Interleukin 2	-32.56
TLR8	NM_138636	Toll-like receptor 8	-12.88
IFNG	NM_000619	Interferon gamma	-10.96
CD180	NM_005582	CD180 molecule	-9.00
CD14	**NM_000591**	**Cluster of differentiation 14**	**-8.76**
IL12A	NM_000882	Interleukin 12A	-5.46
FADD	**NM_003824**	**Fas (TNFRSF6)-associated via death domain**	**-3.70**
TLR3	NM_003265	Toll-like receptor 3	-3.45
MYD88	NM_002468	Myeloid differentiation primary response gene (88)	-3.36
TLR5	NM_003268	Toll-like receptor 5	-3.23
IFNB1	NM_002176	Interferon beta 1	-3.21
NFKBIL1	NM_005007	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1	-3.18
HRAS	NM_005343	V-Ha-ras Harvey rat sarcoma viral oncogene homologue	-3.05
TAB1	**NM_006116**	**TGF-beta activated kinase 1**	**-3.01**
TLR6	**NM_006068**	**Toll-like receptor 6**	**-2.75**
ELK1	NM_005229	ELK1, member of ETS oncogene family	-2.67
TLR9	NM_017442	Toll-like receptor 9	-2.41
SIGIRR	NM_021805	Single immunoglobulin and toll-interleukin 1 receptor (TIR) domain	-2.39
CD86	NM_006889	CD86 molecule	-2.22
IRAK4	**NM_016123**	**Interleukin-1 receptor-associated kinase 4**	**-2.18**
MAP2K4	NM_003010	Mitogen-activated protein kinase kinase 4	-2.02

Table 3.2: Downregulated TLR signalling genes following cobalt stimulation
MonoMac 6 cells were stimulated with 0.75mM $CoCl_2$ for 4h before RNA was collected, cDNA synthesised by reverse transcription and gene expression assessed by TLR Signalling qRT-PCR arrays (PAHS-018Z). Table shows genes that were downregulated more than 2-fold. Significant ($p<0.05$) changes are shown in bold. Analysis was performed using Qiagen's online array analysis software **(see section 3.3.2)** using three pairs of arrays (stimulated versus unstimulated). The full array analysis results are shown in **Appendix A**.

3.4.4. Effect of cobalt ions on inflammatory cytokine genes

Using the same method as described earlier (4h stimulation of MonoMac 6 cells with 0.75mM $CoCl_2$), cobalt-mediated gene expression changes were analysed using Inflammatory Cytokine and Receptor qRT-PCR arrays (PAHS-011C). As seen in the TLR arrays, cobalt regulated expression of a broad ranges of genes including chemokines, cytokines, and their receptors. Fourteen genes were upregulated more than 2-fold **(Table 3.3)** while 32 were downregulated by the same factor **(Table 3.4)**. The full gene list along with detected fold changes and p values is shown in **Appendix B**.

Upregulated expression of 8 chemokines was observed (Table 3.3**)**. Two of these changes were found to be significant, the 35-fold upregulation of chemokine (C-C motif) ligand 20 (*CCL20*) (p=0.0020) and the 14.53-fold upregulation in *IL8* expression (p=0.0236). Other upregulated chemokine genes included chemokine (C-X-C motif) ligand 2, 3 and 14 (*CXCL2, CXCL3* and *CXCL14*) as well as chemokine (C-C motif) ligand 3, 4 and 25 (*CCL3, CCL4* and *CCL25*) but none of the changes reached statistical significance. Downregulation of 9 chemokine genes was noted; *CCL7, CCL2, CCL8, CCL13, CCL11, CXCL12, CXCL13 CXCL6* and *CCL23* were all downregulated by more than 2-fold but none were statistically significant.

Interleukin-36 gamma *(IL36G),* lymphotoxin alpha *(LTA)* and interleukin-1 beta (*IL1B)* expression was upregulated following cobalt stimulation (**Table 3.3**). However as with chemokine expression there was not a uniform upregulation of cytokine expression, with downregulation observed in expression of interleukin-36 alpha (*IL36A*), interleukin-10 (*IL10)* and interleukin-5 (*IL5)* (**Table 3.4**).

Only one cytokine or chemokine receptor, interleukin-1 receptor 1 (*IL1R1*), was upregulated following cobalt treatment (**Table 3.3**). Several receptor genes were downregulated, including chemokine (C-C motif) receptor 2 (*CCR2),* chemokine (C-X-C motif) receptor 1 and 2 (*CXCR1* and *CXCR2*), interleukin-10 receptor beta *(IL10RB)* and interleukin-13 receptor alpha 1 (*IL13RA1).* Of these *IL10RB* was significantly decreased ($p<0.001$) by cobalt ions (**Table 3.4**).

Gene	RefSeq	Gene name	Fold change
CCL20	**NM_004591**	**Chemokine (C-C motif) ligand 20**	**34.96**
CXCL3	NM_002090	Chemokine (C-X-C motif) ligand 3	15.13
IL8	**NM_000584**	**Interleukin 8**	**14.53**
CXCL2	NM_002089	Chemokine (C-X-C motif) ligand 2	11.21
CCL4	NM_002984	Chemokine (C-C motif) ligand 4	10.27
CCL25	NM_005624	Chemokine (C-C motif) ligand 25	7.71
C3	NM_000064	Complement component 3	7.20
IL13	NM_002188	Interleukin 13	2.97
CXCL14	NM_004887	Chemokine (C-X-C motif) ligand 14	2.96
IL1R1	NM_000877	Interleukin 1 receptor type 1	2.76
IL36G	NM_019618	Interleukin 36 gamma	2.73
LTA	NM_000595	Lymphotoxin alpha	2.53
CCL3	NM_002983	Chemokine (C-C motif) ligand 3	2.21
IL1B	NM_000576	Interleukin 1 beta	2.11

Table 3.3: Upregulated inflammatory cytokine and receptor genes following cobalt stimulation

MonoMac 6 cells were stimulated with 0.75mM $CoCl_2$ for 4h before RNA was isolated and cDNA synthesised by reverse transcription. Gene expression changes were assessed by Inflammatory Cytokine and Receptor qRT-PCR arrays (PAHS-011C). Table shows all genes that were upregulated more than 2-fold. Significant changes ($p<0.05$) are shown in bold. Analysis was performed using Qiagen's Excel-based software using three pairs of arrays (stimulated versus unstimulated). Full results of the array analysis are shown in **Appendix B**.

Gene	RefSeq	Gene name	Fold change
CCR2	NM_001123396	Chemokine (C-C motif) receptor 2	-77.1
CD40LG	NM_000074	CD40 ligand	-11.78
LTB4R	NM_181657	Leukotriene B4 receptor	-11.04
CCR1	NM_001295	Chemokine (C-C motif) receptor 1	-9.43
CCL7	NM_006273	Chemokine (C-C motif) ligand 7	-9.11
CCL2	NM_002982	Chemokine (C-C motif) ligand 2	-6.81
IL36A	NM_014440	Interleukin 36 alpha	-6.25
LTB	NM_002341	Lymphotoxin beta	-5.91
IL10	NM_000572	Interleukin 10	-5.50
IL1F10	NM_173161	Interleukin 1 family member 10 (theta)	-5.47
CARD18	NM_021571	Caspase recruitment domain family member 18	-4.42
CCR6	NM_004367	Chemokine (C-C) motif receptor 6	-4.35
CCL8	NM_005623	Chemokine (C-C motif) ligand 8	-4.10
CCL13	NM_005408	Chemokine (C-C motif) ligand 13	-3.96
CCL11	NM_002986	Chemokine (C-C motif) ligand 11	-3.82
IL36B	NM_173178	Interleukin 36 beta	-3.60
TNF	NM_000594	Tumour necrosis factor	-3.25
CXCL12	NM_000609	Chemokine (C-X-C motif) ligand 12	-3.03
IL1RN	NM_000577	Interleukin 1 receptor antagonist	-2.86
IL5	NM_000879	Interleukin 5	-2.82
CXCL13	NM_006419	Chemokine (C-X-C motif) ligand 13	-2.66
AIMP1	**NM_004757**	**Aminoacyl tRNA synthetase**	**-2.58**
ABCF1	**NM_001090**	**ATP-binding cassette sub-family 4**	**-2.57**
TOLLIP	NM_019009	Toll interacting protein	-2.54
CXCL6	NM_002993	Chemokine (C-X-C) motif ligand 6	-2.53
CXCR2	NM_001557	Chemokine (C-X-C motif) receptor 2	-2.50
CCR5	NM_000579	Chemokine (C-C motif) receptor 5	-2.33
C5	NM_001735	Complement component 5	-2.12
IL10RB	**NM_000628**	**Interleukin 10 receptor beta**	**-2.10**
CXCR1	NM_000634	Chemokine (C-X-C motif) receptor 1	-2.10
CCL23	NM_005064	Chemokine (C-C motif) ligand 23	-2.05
IL13RA1	NM_001560	Interleukin 13 receptor alpha 1	-2.02

Table 3.4: Downregulated inflammatory cytokine and receptor genes following cobalt stimulation

MonoMac 6 cells were stimulated with 0.75mM $CoCl_2$ for 4h before RNA was isolated and cDNA synthesised by reverse transcription. Gene expression changes were assessed by Inflammatory Cytokine and Receptor qRT-PCR arrays (PAHS-011C). Table shows all genes that were downregulated more than 2-fold. Significant changes ($p<0.05$) are shown in bold. Analysis was performed using Qiagen's Excel-based software using three pairs of arrays (stimulated versus unstimulated). Full array analysis results are shown in **Appendix B**.

3.4.5. Validation of target genes by TaqMan-based qRT-PCR

The qRT-PCR arrays use SYBR Green dye for gene expression quantification. TaqMan gene expression assays contain a probe specific for the target gene, providing a higher degree of specificity than the SYBR Green method **(Figure 2.4)**. Selected results obtained from the qRT-PCR arrays were therefore validated using TaqMan gene expression assays for individual genes. The chosen target genes and their reasons for selection are detailed in **Table 3.5**. They included genes that were significantly up- or downregulated following cobalt stimulation, those that showed inter-assay variability, and others that gave different results than predicted based on previous studies.

Target gene	Array fold change	Reason for validation
IL8	40.91	Significantly upregulated in arrays
CCL20	34.96	Significantly upregulated in arrays
CCL25	7.71	Upregulated in arrays
CXCL10	3.28	Upregulated in TLR array, no change detected in cytokine array
IL1A	17.11	Significantly upregulated in TLR array, no change detected in cytokine array
IRAK2	3.17	Significantly upregulated in arrays, key TLR4 signalling factor
HMGB1	1.70	Known TLR4 ligand (Jong et al., 2006)
HSPA1A	202.39	Significantly upregulated in arrays, known TLR4 ligand (Klink et al., 2012)
CCL2	-410.75	Downregulated in arrays, literature suggests usually upregulated (Queally et al., 2009)

Table 3.5: Target genes selected for further validation
Table shows target gene expression fold changes detected by qRT-PCR arrays. *IL8* and *CCL2* were investigated on both sets of arrays; the fold changes shown in the table are the largest of the two detected.

cDNA was synthesised using the Superscript III kit (**section 2.6.2.1**) and the RNA collected for the qRT-PCR arrays. TaqMan-based qRT-PCR analysis confirmed upregulation of *IL8* ($p<0.001$), *CCL20* ($p<0.001$), *CCL25* ($p=0.0074$), *CXCL10* ($p=0.0114$), *IL1A* ($p<0.001$), *IRAK2* ($p<0.001$) and *HSPA1A* ($p<0.001$) by MonoMac 6 cells in response to $CoCl_2$ **(Figure 3.4A-G)**. There was no change in *HMGB1* expression ($p=0.9188$) (**Figure 3.4H)** and a significant downregulation of *CCL2* following cobalt stimulation ($p<0.001$) **(Figure 3.4I)**, both of which were consistent with the SYBR Green array results. The fold change values were different from those obtained using the arrays and this will be discussed later in the chapter.

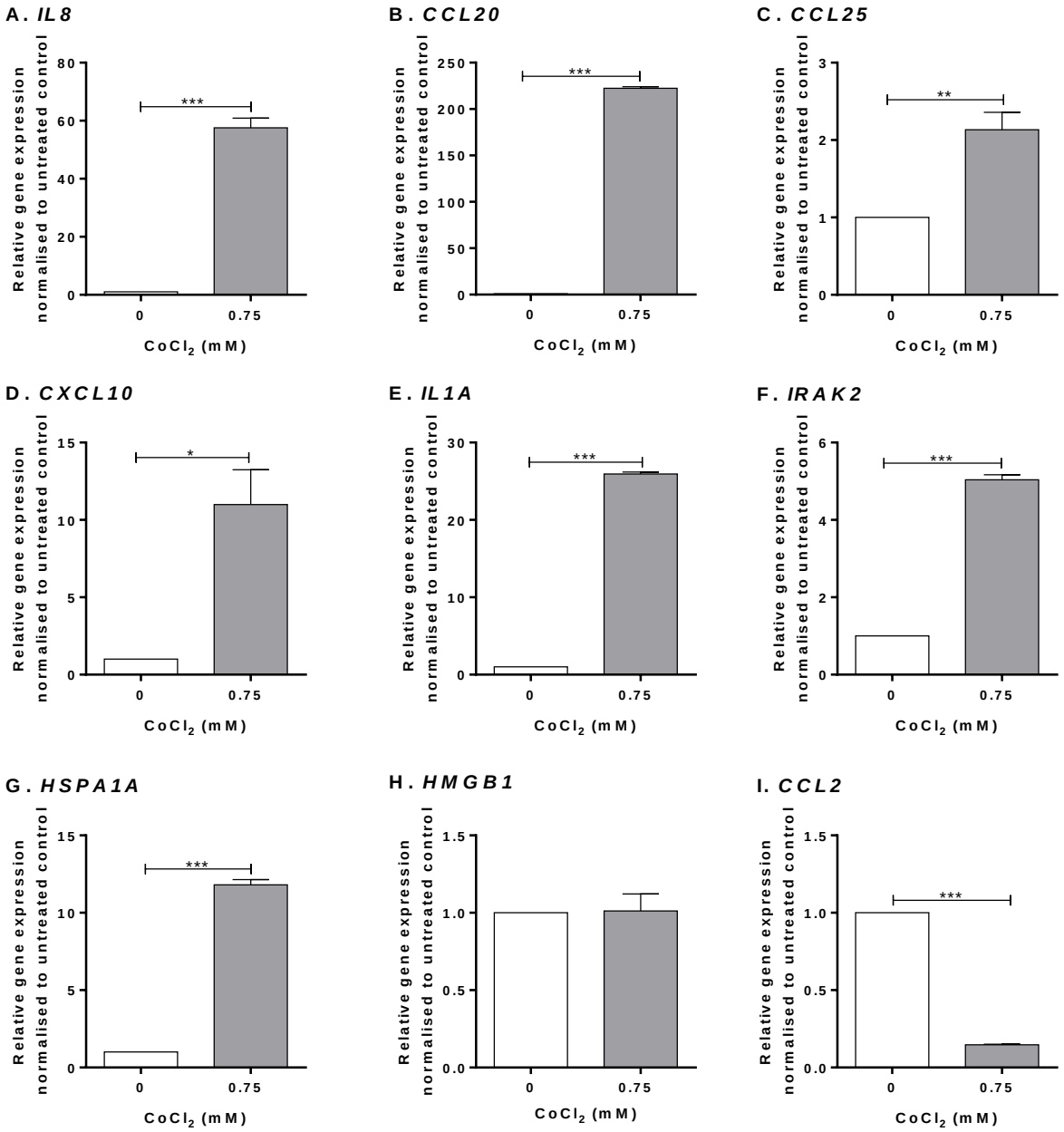


Figure 3.4: Validation of target gene expression by TaqMan-based qRT-PCR
MonoMac 6 cells were stimulated with 0.75mM $CoCl_2$ for 4h before RNA was isolated and cDNA synthesised by reverse transcription. qRT-PCR was performed using TaqMan probes specific for the target genes. All data is representative of at least two independent experiments and statistical significance was calculated by an unpaired Student's *t* test.

3.4.6. Effect of cobalt ions on inflammatory cytokine secretion

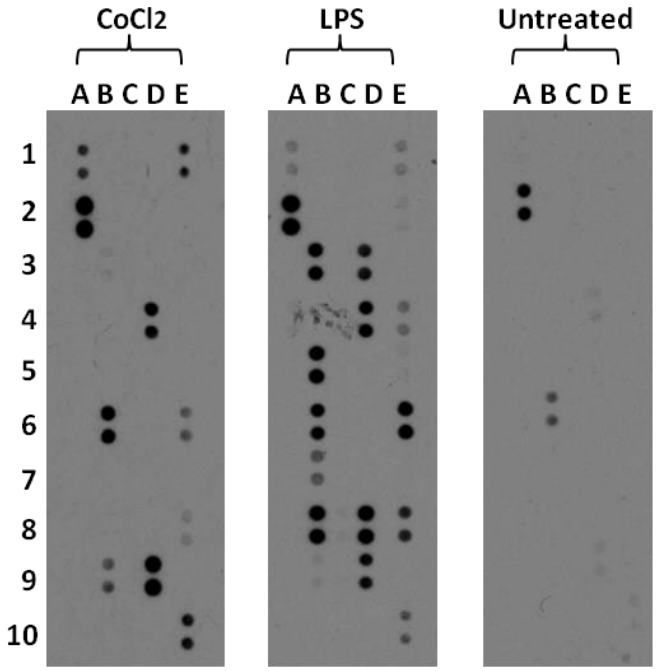
Human Cytokine Proteome Profiler (dot blot) arrays (R&D Systems) were used to investigate the effect of cobalt ions on cytokine secretion by MonoMac 6 cells. Cells were treated with 0.75mM $CoCl_2$, 1000ng/ml LPS or left untreated for 24h. Conditioned media was then collected and incubated with the array membranes as per the manufacturer's protocol. Relative dot intensity was calculated by densitometry using Photoshop as shown below:

(Mean intensity of sample dot x number of pixels)/
(Mean intensity of control dot x number of pixels)

Using this method the cytokine showing the largest change in secretion following $CoCl_2$ stimulation was IL-8, which was upregulated **(Figure 3.5A)**. Other notable $CoCl_2$-mediated increases were observed for interleukin-1 receptor antagonist (IL-1ra), macrophage migration inhibitory factor (MIF), serpin E1 and chemokine (C-X-C motif) ligand (CXCL1). Very small changes were observed in soluble intracellular adhesion molecule 1 (sICAM-1), CCL5 and CXCL10 secretion (**Figure 3.5A**).

Following LPS stimulation MonoMac 6 cells considerably increased secretion of CCL2, CCL4, CXCL1, CXCL10 and IL-1ra. Elevated secretion of IL-1β, IL-8, sICAM-1, MIF and CCL3 was also observed **(Figure 3.5)**. There were significant differences between the $CoCl_2$ and LPS-induced cytokine profiles, including in CXCL10 ($p<0.001$), CXCL1 ($p=0.0026$) and IL-1β ($p<0.001$) secretion (**Figure 3.6**). In all of these cases LPS caused a larger increase in cytokine secretion than $CoCl_2$. These differences will be discussed in more detail in **section 3.5**.

A.



B.

Coordinate	Cytokine	Coordinate	Cytokine	Coordinate	Cytokine
A1	Reference	**B9**	Serpin E1	**D7**	IL-5
A2	CCL5	**B10**	-	**D8**	IL-6
A3	CXCL12	**C1**	-	**D9**	IL-8
A4	TNFα	**C2**	IL-10	**D10**	-
A5	sTREM-1	**C3**	IL-12 p70	**E1**	Reference
A6	-	**C4**	IL-13	**E2**	C5/C5a
A7	-	**C5**	IL-16	**E3**	CD40 ligand
A8	-	**C6**	IL-17	**E4**	G-CSF
A9	-	**C7**	IL-17E	**E5**	GM-CSF
A10	Negative	**C8**	IL-23	**E6**	CXCL1
B1	-	**C9**	IL-27	**E7**	CCL1
B2	IL-32α	**C10**	-	**E8**	sICAM-1
B3	CXCL10	**D1**	-	**E9**	IFN-γ
B4	CXCL11	**D2**	IL-1α	**E10**	Reference
B5	CCL2	**D3**	IL-1β		
B6	MIF	**D4**	IL-1ra		
B7	CCL3	**D5**	IL-2		
B8	CCL4	**D6**	IL-4		

Figure 3.5: Proteome Profiler human cytokine arrays
MonoMac 6 cells were treated with 0.75mM $CoCl_2$, 1000ng/ml LPS or left untreated for 24h. Supernatant was collected and used for Proteome Profiler human cytokine arrays (R&D Systems). **A.** Dot blot showing changes in cytokine secretion following $CoCl_2$ and LPS stimulation. **B.** Coordinates of antibodies on dot blot arrays. Dashes indicate that there was no antibody present.

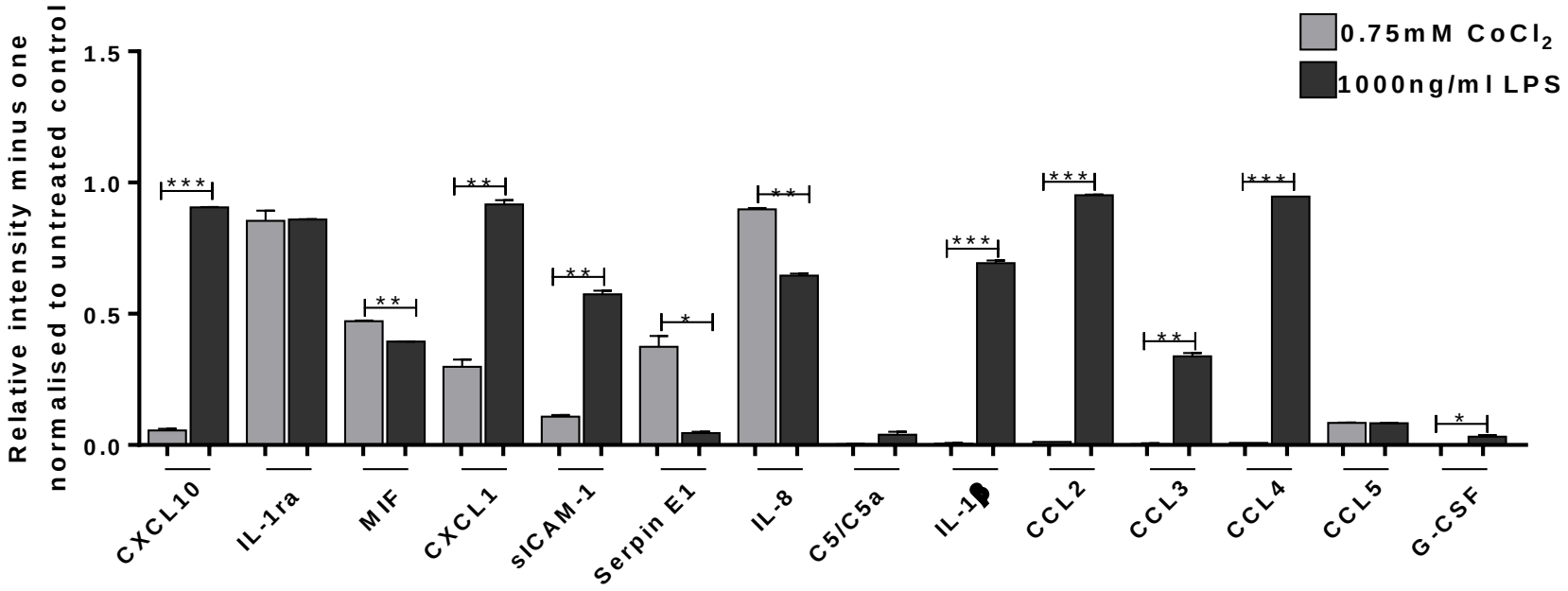


Figure 3.6: Densitometric analysis of Proteome Profiler arrays

Proteome Profiler human cytokine arrays were conducted using supernatant from MonoMac 6 cells stimulated with either 0.75mM $CoCl_2$ or 1000ng/ml LPS for 24h. Relative intensity of membrane dots was calculated and normalised to the untreated control (set to 0). Graph shows all upregulated cytokines and compares secretion of each cytokine in response to $CoCl_2$ (light bars) and LPS (dark bars). n=1. Statistical significance was calculated by unpaired Student's *t* test. p values: CXCL10 ($p<0.001$), IL-1ra ($p=0.9139$), MIF ($p=0.0014$), CXCL1 ($p=0.0026$), sICAM-1 ($p=0.0011$), Serpin E1 ($p=0.0158$), IL-8 ($p=0.0011$), C5/C5a ($p=0.0824$), IL-1β ($p<0.001$), CCL2 ($p<0.001$), CCL3 ($p=0.0015$), CCL4 ($p<0.001$), CCL5 ($p=0.3422$), G-CSF ($p=0.0321$).

3.5. Discussion

MonoMac 6 cells were chosen for this aspect of the study because as a cell line they should provide a consistent and reproducible response to stimuli. They have also previously been shown to express TLR4 and respond to LPS by increasing their secretion of cytokines including IL-8 (Dr Jem Palmer, Newcastle University, personal communication). Although primary blood mononuclear cells (PBMC) would be interesting to study, they introduce an element of variability (e.g. TLR4 expression levels) that was not desirable at this point in the investigation.

Initial observations of the qRT-PCR array analysis shows that cobalt ions have a broad effect on inflammatory genes, regulating cytokine, chemokine, and receptor expression, as well as that of PRRs and signalling factors from different pathways, including the MyD88-dependent and TRIF-dependent arms of TLR signalling. Most previous studies have focused on the outcome of inflammatory signalling (i.e. cytokine secretion or macrophage activation) rather than on the activated signalling pathways and how their components may be regulated. This section will discuss the results obtained from the qRT-PCR arrays in signalling pathway order from immune receptors (e.g. TLRs) through to cytokines, chemokines, and other inflammatory mediators.

Toll-like receptor expression

The TLR signalling arrays detected downregulation of *TLR1, TLR3, TLR5, TLR6*, *TLR8* and *TLR9* expression following cobalt treatment, while *TLR4* expression was unaffected. This is largely consistent with the results of tissue analysis by Takagi *et al* who observed decreased expression of all TLRs (except TLR4 and TLR9) in tissues surrounding aseptically-loosened hip implants compared to controls (Takagi et al., 2007). The same study showed that TLR4 expression around these joints is largely due to infiltrating macrophages and thus highlights the benefit of using MonoMac 6 cells as a model in this study. The absence of cobalt-mediated changes in *TLR4* expression is interesting because TLR4 is often upregulated following LPS stimulation, which enhances the inflammatory response (Guzzo et al., 2012). It suggests that other gene expression changes, such as upregulation of *IL8*, are a consequence of downstream signalling pathway activation and effector molecule expression rather than increased TLR4 expression and activation. Attempts were made during this study to investigate the effect of cobalt ions on TLR4 cell surface expression using flow cytometry. However optimisation of the TLR4 antibody proved challenging and given the timeframe of the project analysis by this method was not possible. Further investigation into TLR4 protein expression would therefore be required to fully validate the conclusions drawn in this section of the study.

TLR10 was the only *TLR* gene upregulated by $CoCl_2$. The reasons for this are unclear as the ligand for TLR10 has not yet been identified. It has been suggested that TLR10 recognises viral PAMPs (Lee et al., 2014) but a recent study has also indicated that it is able to dampen inflammatory cytokine secretion and therefore may be the only TLR to inhibit inflammation (Oosting et al., 2014). Given this conflicting information it is difficult to interpret cobalt-mediated *TLR10* upregulation.

Co-receptors, adaptor proteins and co-stimulatory molecules

LPS activation of TLR4 requires co-receptors LBP, CD14 and MD2 (also known as lymphocyte antigen 96 or LY96) but the effect of cobalt on these proteins is not yet clear. No change in *MD2* expression was detected after $CoCl_2$ stimulation but *CD14* expression was downregulated. CD14 only becomes involved in TLR4 pathogen recognition once LPS has bound to LBP and as cobalt binds directly to TLR4, the downregulation of *CD14* may indicate that it is not part of the signalling complex required for cobalt activation of the receptor.

Downregulation of *CD180* expression was detected by the arrays. CD180 forms a complex with myeloid differentiation protein 1 (MD1, also known as lymphocyte antigen 86 or LY86) and works with TLR4 to promote B cell proliferation and differentiation in response to LPS. *LY86* expression was unaffected by cobalt ions. Expression of another co-stimulatory molecule, *CD86*, was also downregulated, although to a lesser degree than *CD180* (2-fold compared to 9-fold). CD86 is expressed by antigen-presenting cells and engagement with its ligand CD80 stimulates T cell activation, as well as maturation of the antigen-presenting cell. Decreased expression of co-stimulatory molecules is surprising as previous studies have shown that they can be upregulated in response to TLR4 activation by LPS (Hoebe et al., 2003). Caicedo *et al* also reported increased CD80 and CD86 expression in cobalt-stimulated macrophages (Caicedo et al., 2007), although these changes were detected at 48h post-stimulation which may account for the differences between the studies. It must be acknowledged that the *in vitro* approach used in the present study does not model the cell-cell interactions that take place *in vivo* and a co-culture system (e.g. of endothelial cells with macrophages) would perhaps reveal different results; this method would better replicate the *in vivo* environment in which cell behaviour is influenced by factors (growth factors, cytokines, hormones etc) released by neighbouring cells, as well as by engagement with cell surface molecules. It may also be the case that at this very early timepoint cobalt does not induce effects that would result in activation of the adaptive immune system.

MyD88 and TRIF are the major intracellular adaptor proteins involved in LPS-mediated TLR4 activation (Kawai et al., 1999, Yamamoto et al., 2003). Their recruitment to the activated receptor complex results in diverse but overlapping stimulation of inflammatory signalling pathways. MyD88-dependent signalling leads to upregulation of the transcription factors NFκB and AP-1 while TRIF-dependent signalling promotes IRF-mediated transcription (**Figure 1.10**).

. As a result there are differences in the cytokine profile resulting from activation of each pathway; MyD88/NFκB signalling leads to increases in cytokines like IL-6 and IL-8 (He et al., 2013a, Morandini et al., 2013) while TRIF promotes release of type I interferons and CXCL10 (Weighardt et al., 2004).

Cobalt activation of TLR4 has previously been shown to activate the MyD88-dependent pathway (Potnis et al., 2013) so it was surprising to find a 3-fold downregulation of *MYD88* expression in this study. However Toll-interleukin 1 domain-containing adaptor protein (*TIRAP*) was upregulated; TIRAP binds to MyD88 to promote signalling pathway activation, indicating that downstream components of the MyD88 pathway can be activated by $CoCl_2$. *TRIF* (referred to in the arrays as Toll-like receptor adaptor molecule 1 or *TICAM1)* and *TRAM* (Toll-like receptor adaptor molecule 2 or *TICAM2*) were both upregulated, suggesting increased activation of TRIF-dependent signalling by $CoCl_2$. The clearest indication of MyD88 and TRIF pathway activation is given by the cytokine and chemokine profile generated in response to cobalt ions which will be discussed in more detail later in this section.

As well as upregulation of positive regulators of TLR4 signalling, downregulation of inhibitory single immunoglobulin and toll-interleukin 1 receptor domain (*SIGIRR*) was detected. *SIGIRR* has a TIR domain that can engage with the homologous domain in TLR4 and prevent recruitment of MyD88 and TRIF, thus inhibiting downstream TLR4 signalling. Its reduced expression may indicate that MyD88 and TRIF are free to bind TLR4 and mediate activation of intracellular signalling cascades.

Intermediate Toll-like receptor signalling factors

Intracellular signalling pathways determine the outcome of cell stimulation, and are themselves dependent on the activity of the adaptor proteins and co-receptors described above. The TLR4 signalling pathway is complex and there is considerable cross-talk between different pathways, as well as modulation or inhibition of the pathway by endogenous factors such as interleukin 1 receptor-associated kinase 3 (IRAK3, IRAKM).

The IRAK proteins are essential to the MyD88 pathway of TLR4 signalling. IRAK2, upregulated by $CoCl_2$, interacts with MyD88 and then with TRAF6 to mediate NFκB activation. A second IRAK, *IRAK4*, was slightly (2.18-fold) downregulated by $CoCl_2$; IRAK4 is recruited to MyD88 when TLR4 is activated. TGF-beta activated kinase 1 binding protein 1 (*TAB1*) which forms part of the TRAF6 protein complex in the MyD88-dependent pathway was also downregulated. In the clinical context of this study it is interesting to note that *TAB1* downregulation is associated with increased activity of matrix metalloproteases (MMPs) which degrade collagen (Ciechomska et al., 2014). $CoCl_2$ regulation of the TLR signalling pathway may therefore have broader effects aside from direct stimulation of cytokine secretion.

Pellino homologue 1 (*PELI1*) was upregulated 4-fold by cobalt ions while ubiquitin-conjugating enzyme E2N (*UBE2N*) expression was increased 5-fold. These two proteins may function together as Pellino-1 mediates the Lys-63 ubiquitination of IRAK1 which leads to NFκB activation while UBE2N is responsible for generating Lys-63 ubiquitin chains. A recent study has also shown that LPS stimulation upregulates Pellino-1 activity in MonoMac 6 cells, resulting in increased polyubiquitination of IRAK1, TBK1, and TAK1, and subsequent enhanced NFκB activity and IL-8 release (Murphy et al., 2015). The increased *PELI1* expression observed in the present study may contribute to potentiation of TLR4 activation and the increased *IL8* expression also observed.

The mitogen-activated protein (MAP) kinases control cell proliferation and differentation. Seven *MAPK* genes were investigated using the qRT-PCR arrays and of these only one, MAP kinase kinase 4 (*MAP2K4*), was affected by $CoCl_2$. As this was a fold downregulation of just 2.01 and there was no change in any of the other MAP kinase genes, it does not appear that MAP kinases play a significant role in the cellular response to cobalt at this early timepoint. It is important to acknowledge that no change in gene expression does not necessarily mean that there is no change in enzyme activity so inhibitors targeting MAP kinase function would be required to define their role in the response.

Transcription factors

The outcome of TLR signalling results in activation of one or more transcription factors including NFκB, AP-1 and interferon regulator factor 3 (IRF3). Array analysis showed that cobalt has a considerable impact on NFκB signalling. NFκB is present in most eukaryotic cells and is involved in the regulation of many inflammatory genes that express an NFκB binding site in their promoter region. NFκB itself is sequestered in the cytoplasm as part of a protein complex that prevents its activity. For NFκB-mediated gene transcription the inhibitor proteins

in the complex are phosphorylated and targeted for degradation. NFκB is then released and translocates to the nucleus to regulate gene transcription. Two of the kinases involved in the phosphorylation process were upregulated following cobalt stimulation; these were conserved helix-loop-helix ubiquitous kinase (*CHUK)*, also known as IκB kinase-α (IKK-α, *IKK1*) and TANK-binding kinase 1 (*TBK1)*. This supports the results of a previous study demonstrating that cobalt particles can induce degradation of inhibitors of κB (IκB) (Rachmawati et al., 2013), resulting in increased NFκB activity. In addition to upregulation of the NFκB-activating kinases there was a 7-fold increase in *NFKB1* expression, which encodes a subunit of NFκB, again suggesting promotion of NFκB-mediated transcription by cobalt ions. There was also downregulation of NFκB inhibitor-like 1 (*NFKBIL1)*. Although its function is not completely clear, NFκBIL1 is thought to inhibit inflammatory responses to LPS by preventing NFκB activation (Atzei et al., 2010) and consequently its downregulation following cobalt stimulation suggests a drive towards NFκB-mediated gene transcription.

It is important to note that in addition to the increased expression of promoters of NFκB signalling, NFκB inhibitor alpha (*NFKBIA*) was upregulated by a similar fold change to *NFKB1*. This could potentially inhibit activity of the transcription factor, thus having a modulatory effect on immune responses. NFκBIα has also recently been implicated in carbon nanoparticle-induced cell death (Periasamy et al., 2016) and may therefore be a sign of cobalt cytotoxicity, although only a small cytotoxic effect was observed following 4h $CoCl_2$ stimulation.

NFκB is a key regulator of immune responses, and chronic inflammatory diseases such as irritable bowel syndrome are associated with persistent NFκB-mediated inflammation (Asquith and Powrie, 2010). LPS induces the expression of NFκB-dependent genes such as IL-8 via a TLR4-dependent mechanism (Chow et al., 1999). NFκB activation through TLR4 requires the MyD88-dependent and TRIF-dependent pathways; MyD88-deficient mice display impaired cytokine production upon LPS exposure but although there is a time delay NFκB is still activated, which shows involvement of the TRIF pathway (Kawai et al., 1999). TRIF-deficient cells also show decreased LPS-mediated cytokine secretion but near normal NFκB activation because MyD88 compensates for the absence of TRIF (Yamamoto et al., 2003). However when both adaptor proteins are inhibited there is no NFκB activation following LPS challenge (Yamamoto et al., 2003). This information suggests that the NFκB-mediated cytokine secretion observed here could therefore be the result of activation by either arm of the TLR4 signalling pathway.

Although NFκB signalling dominated the results of the arrays, signalling to other transcription factors was affected by $CoCl_2$. Jun proto-oncogene (*JUN*) encodes c-Jun and complexes with other proteins to form the transcription factor AP-1. AP-1 regulates transcription of genes associated with bacterial and viral responses such as cytokines, and can also contribute to cell differentiation and apoptosis. Cobalt has previously been shown to induce apoptosis via activation of AP-1 at comparable concentrations (0.2-1mM) but this was at longer timepoints (36h) to those used for the arrays (Zou et al., 2001). Upregulation of *JUN* may contribute to AP-1 formation and subsequent apoptosis, which could in turn account for the cytotoxic effects of cobalt ions. These results show that the effects of cobalt are not limited to NFκB signalling.

Cytokines and secreted factors

The factor with the largest upregulation detected across all arrays was heat shock 70kDa protein 1A (*HSPA1A*) followed by heat shock 60kDa protein 1A (*HSPD1)*. Heat shock proteins (Hsps) are released as a response to cell stress and damage, and modulate inflammation by assisting in the refolding of proteins damaged during these processes. The increased expression of Hsps here is likely due to cobalt causing cytotoxicity, although as mentioned previously there was only a small decrease in cell viability in the presence of cobalt. Hsps and particularly *HSPA1A* will be discussed in more detail in chapter 4.

The pleiotropy of cytokines makes analysing the potential inflammatory outcome of their up- or downregulation more complex. For example IL-13 is released during Th2 responses to modulate and dampen inflammation but it also increases the activity of matrix metalloproteases which promotes collagen deposition and fibrosis (Kaviratne et al., 2004). In this section cytokines will be discussed based on their dominant or most well-described function but it is acknowledged that their effects can be multifactorial and depend considerably on other factors such as receptor expression and the activity of other cytokines.

The up- and downregulated cytokines detected in both sets of arrays were categorised as predominantly pro- or anti-inflammatory (**Table 3.6**). Of the five upregulated cytokines, four were pro-inflammatory (*IL1A, IL1B, CSF2* and *IL36G)*. For example *IL1A* encodes IL-1α which promotes inflammatory cell proliferation and increases activity of the transcription factors NFκB and AP-1 to drive further inflammatory cytokine secretion (Wolf et al., 2001). IL-1β, encoded by *IL1B*, is induced by NFκB-mediated transcription and is released when TLRs are activated by DAMPs (Eltom et al., 2014). However there was also increased expression of *IL13* which is a Th2-associated cytokine and can inhibit Th1 pro-inflammatory responses.

Downregulation of seven pro-inflammatory cytokines was observed (**Table 3.6**), including two interferons, *IFNG* (IFNγ) and *IFNB1* (IFNβ). IFNγ is associated with decreased receptor activator of NFκB (RANK) expression (Ji et al., 2009) and therefore confers protection against osteoclastogenesis and subsequent osteolysis (Xu et al., 2009). In this case IFNγ downregulation could affect the balance between osteoblast and osteoclast formation and drive osteoclastogenesis. IFNβ promotes release of additional inflammatory cytokines and chemokines, as well as apoptosis. There was also a downregulation in TNFα expression, which is surprising because TNFα is usually upregulated in response to TLR4 stimulation by LPS and is often released early in inflammatory responses. However it is possible that using the array method (i.e. only studying one timepoint) means that any upregulation in TNFα expression is missed as changes in TNFα expression are often very transient. Additional dose response curves would be required to more fully investigate the effect of cobalt ions on TNFα expression and secretion.

Interestingly, aside from *IL13*, the anti-inflammatory cytokines (*IL2, IL10* and *IL1F10*) regulated by $CoCl_2$ were all downregulated (**Table 3.6**). IL-2 contributes to lymphocyte proliferation, including regulatory T cells, and as such is considered anti-inflammatory or immunomodulatory. *IL2* expression may be decreased here because the early timepoint used is unlikely to involve the adaptive immune system. Consequently, any of the cobalt-mediated inflammatory responses observed in this and other studies may not only be the result of increased pro-inflammatory cytokine secretion but also a decrease in the expression and activity of immunomodulatory cytokines.

Pro-inflammatory	**Anti-inflammatory**
Upregulated cytokines	
IL1A *CSF2* *IL1B* *IL36G*	*IL13*
Downregulated cytokines	
IFNG *IL36B* *IL12A* *TNF* *IFNB1* *IL5* *IL36A*	*IL10* *IL2* *IL1F10 (IL38)*

Table 3.6: Regulation of pro- and anti-inflammatory cytokines by cobalt ions

Chemokine expression

The expression of many chemokines was altered in response to $CoCl_2$. *IL8* expression was significantly upregulated in both sets of arrays although with different fold changes (40-fold in the TLR signalling arrays and 14.5-fold in the cytokine arrays). IL-8 promotes neutrophil migration, and other neutrophil chemotactic cytokines were upregulated including *CCL20,*

CXCL3, CXCL2 and *CCL3* **(Table 3.7)**. This is interesting as neutrophils are not present at high levels in failed MoM peri-implant tissues (Lähdeoja et al., 2009). However they do have a very short half-life and if they are indeed being recruited by these chemokines neutrophils may initiate recruitment of other inflammatory cells through secretion of cytokines such as CXCL10 (chemotactic for lymphocytes).

The arrays also detected increased expression of several monocyte/macrophage chemokines. These were *CXCL10, CCL4, CCL25* and *CXCL14* (**Figure 3.7**). Macrophages are often found in large numbers in ARMD tissues and these chemokines may be responsible for their recruitment. The only lymphocyte-specific upregulated chemokines were *CXCL10* and *CCL20*. This could be because the adaptive immune response occurs over time and 4h post-$CoCl_2$ stimulation is too early to see any changes related to adaptive immunity.

Chemokine	Target cell		
	Monocyte/macrophage	Neutrophil	T lymphocyte
IL-8		++	
CXCL10	++		++
CCL20		+	++
CXCL3		++	
CXCL2		++	
CCL4	++		
CCL25	++		
CXCL14	++		
CCL3		++	

Table 3.7: Target cells for chemokines upregulated by $CoCl_2$
Table shows the immune cells attracted by particular chemokines. '++' indicates that a chemokine is strongly chemotactic for that particular cell, while '+' indicates that the effect is weaker, or less well-described in the literature.

Expression of several of the CCL chemokines was downregulated. However in some cases (e.g. *CCL11*) the change in expression was less than one Ct value but because their expression levels were low (Ct >30) this was amplified to give a large fold change. The exception to this is chemokine C-C motif ligand 2 (*CCL2*). *CCL2* expression was higher (Ct<30) than that of the other CCL chemokines, although there was still a large downregulation in expression following $CoCl_2$ stimulation. This is a very interesting observation as other studies have reported CCL2 upregulation by cobalt-stimulated cells (Queally et al., 2009). However it is conceivable that the observed effect is a result of cobalt-mediated hypoxia as similar hypoxic effects have been reported (Bosco et al., 2004). Downregulation of *CCL2* will be discussed in more detail later in this chapter and in chapter 6. .

Cytokine and chemokine receptor expression

Of all the cytokine and chemokine receptor genes found to have a fold change >2, only interleukin-1 receptor type 1 (*IL1R1*) was upregulated. The encoded protein, IL-1R1, is very closely related to TLR family and receptor activation leads to recruitment of MyD88, activation of the MyD88-dependent pathway, and increased NFκB-mediated gene transcription (Muzio et al., 1997). Unlike TLRs IL-1R1 is activated by the endogenous cytokines IL-1α, IL-1β and IL-1ra rather than PAMPs. Interestingly, *IL1A* expression was upregulated following cobalt stimulation, suggesting that this signalling pathway may be involved in the cellular response to cobalt ions.

Downregulation of several other cytokine receptors was observed; these were chemokine (C-C motif) receptor 1 (*CCR1*) and 2 (*CCR2*), leukotriene B4 receptor (*LTB4R*), chemokine (C-X-C) motif receptor 2 (*CXCR2*), interleukin-10 receptor beta (*IL10RB*) and interleukin-13 receptor alpha 1 (*IL13RA1*). The ligands for these receptors are shown in **Table 3.8**. *IL10RB* expression was significantly downregulated and is the receptor for members of the IL-10 family. Cytokines belonging to this family, including IL-9, IL-10 and IL-26, are primarily associated with inhibition of inflammatory responses and a drive towards a Th2 response. Downregulation of *IL10RB* following cobalt stimulation could indicate a decrease in the activity of immunomodulatory cytokines such as IL-10 and a Th1-dominated immune response.

Receptor gene	Ligands
IL1R1	IL-1α, IL-1β, IL-1ra
CCR2	CCL2, CCL7, CCL13
LTB4R	Leukotriene B4
CCR1	CCL3, RANTES (CCL5), CCL7
CXCR2	IL-8
CXCR1	IL-8, CXCL6
IL10RB	IL-10, IL-22, IL-26, IL-28, IL-29
IL13RA1	IL-13

Table 3.8: Cytokine and chemokine receptors and their ligands
Table shows cytokine and chemokine receptor genes affected by cobalt ions and their ligands. *IL1R1* was the only receptor found to be upregulated and expression of all others shown in the table was downregulated.

The downregulation of so many cytokine and chemokine receptors following cobalt stimulation at first seems to suggest that the effect of cytokines may be inhibited, which could be aimed at protecting the cell from an overwhelming inflammatory response. However the downregulation may also be a direct result of increased cytokine and chemokine release. This is particularly the

case for expression of the IL-8 receptors *CXCR1* and *CXCR2* which were both downregulated by cobalt treatment. IL-8 can dose-dependently downregulate CXCR1 and CXCR2 expression by neutrophils which inhibits further neutrophil migration and allows increased cell adhesion, thus promoting localised inflammation (Hu et al., 2011). CXCR1 and CXCR2 expression can also be downregulated as a consequence of TLR4 activation by pathogens like LPS (Alves-Filho et al., 2010). The impaired neutrophil migration causes inflammation at the neutrophil site and increased sepsis susceptibility (Rios-Santos et al., 2007). The change in *CXCR1* and *CXCR2* expression detected by the arrays may therefore be the result of increased IL-8 expression and secretion, cobalt activation of TLR4, or a combination of the two.

When assessing the impact of cobalt ions on inflammatory factors it is important to consider the redundancy of cytokines, chemokines, and their receptors, as well as cross-talk between pathways. Chemokines can activate multiple receptors and the receptors themselves often have multiple ligands. For example CCL4 binds to CCR1, CCR5 and CCR8. *CCR1* and *CCR5* expression was downregulated by cobalt ions whilst *CCR8* expression was unaffected, meaning that even in the absence of two of its receptors CCL4 could still induce inflammatory effects. There is also evidence of compensatory mechanisms and cross-talk within the chemokine system whereby knockdown of a chemokine receptor results in upregulation of chemokines with similar functions (Yanaba et al., 2004). Furthermore, factors such as CXCL4 have been shown to reduce chemokine receptor expression (Schwartzkopff et al., 2012) to modulate inflammation. Functional studies such as migration assays would provide insights into the overall effects of the cytokine, chemokine, and receptor changes revealed by the arrays.

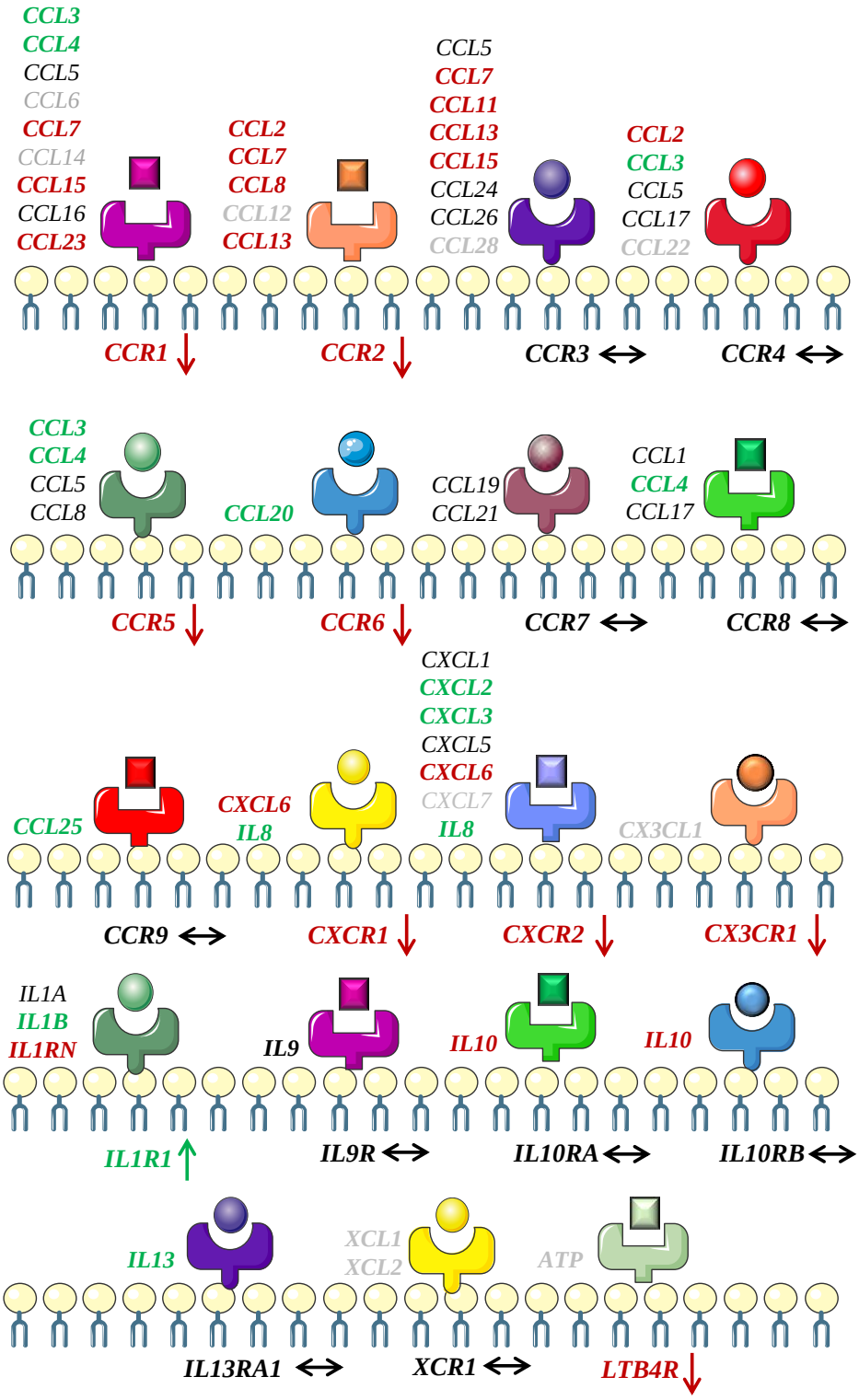


Figure 3.7: Cobalt-regulated cytokines, chemokines and receptors
Figure shows cytokines, chemokines, and their receptors as regulated by cobalt ions in the qRT-PCR arrays. Upregulated genes are shown in green and downregulated genes are shown in red. Those unaffected by cobalt (fold change<2) are shown in black. Genes in grey were not included in the arrays. Image created using information from Balkwill *et al* (Balkwill, 2004).

Apoptosis mediators

There is evidence that cobalt can cause cytotoxicity from preliminary experiments in this study and from the literature (Kwon et al., 2009). Consequently it was interesting to observe that Fas (TNFRSF6)-associated via death domain (*FADD*), a key receptor in apoptosis, was downregulated following cobalt stimulation, although there was a very small (2.06-fold) upregulation in expression of the FADD ligand caspase 8 apoptosis-related cysteine peptidase *(CASP8).* Aminoacyl tRNA synthetase (*AIMP1)* is a cytokine released under apoptotic conditions and was also downregulated by cobalt ions, which suggests that any cell death occurring may not be due to apoptosis. This supports a previous study showing that high cobalt concentrations can induce necrosis rather than apoptosis (Huk et al., 2004).

Taken together the data obtained from the arrays suggests that cobalt ions can activate inflammatory signalling pathways, particularly through increased NFκB activity. There are many similarities between $CoCl_2$ responses and LPS-induced responses reported in previous studies, such as increased expression of *IL8* and *CCL20*. However there are also some striking differences, including downregulation of *CCL2* and decreased expression of co-stimulatory molecules following cobalt treatment. These factors are often upregulated by LPS (Hoebe et al., 2003, Guijarro-Muñoz et al., 2014).

Array validation

Following analysis of the SYBR Green qRT-PCR arrays, nine genes were selected for further validation using qRT-PCR with TaqMan gene expression probes. TaqMan assays were used because they contain a gene-specific fluorescent probe alongside primers. This gives improved specificity in comparison to SYBR Green chemistry where a fluorescent dye binds to all double-stranded DNA and increases the likelihood of false positive results **(Figure 2.4)**.

TaqMan-based qRT-PCR analysis revealed gene expression changes comparable to those of the SYBR Green arrays in terms of the overall trend (i.e. upregulation, downregulation or no change) in gene expression. There were however some notable differences in the fold change values obtained for each individual gene. For example *CCL20* showed a 35-fold upregulation in the arrays but more than a 200-fold increase using the TaqMan probe method. These discrepancies may arise through differences in the chemistry of TaqMan and SYBR Green assays, differences in the analysis process, or be the result of *in vitro* assay variability.

The validated genes were selected for their relevance to the clinical context of this study. Their general function is discussed below and their potential significance in ARMD and MoM hip failure will be discussed in more detail in chapter 4.

IL8

IL8 was significantly upregulated by cobalt ions at gene level in both sets of arrays, as well as in the validation TaqMan-based qRT-PCR assays. The encoded IL-8 protein is a pro-inflammatory chemokine that targets neutrophils (Baggiolini et al., 1989) and recruits them to the site of inflammation through activation of the CXCR1 and CXCR2 receptors. The role of IL-8 in TLR4 signalling and ARMD will be discussed in full in later chapters.

CCL20

CCL20 (also known as macrophage inflammatory protein 3 alpha or MIP3α) acts on its target cell via the CCR6 receptor (Liao et al., 1999). CCR6 is expressed on T and B lymphocytes (Kondo et al., 2007, Wiede et al., 2013) and therefore CCL20 induces the migration of these cells (Liao et al., 2002, Cook et al., 2014). The elevated *CCL20* expression observed in this study supports the findings of Raghavan *et al* who demonstrated that cobalt increased *CCL20* expression by human endothelial cells (Raghavan et al., 2012).

CXCL10

CXCL10 is secreted via the TRIF-dependent arm of TLR4 signalling (Bandow et al., 2012). It recruits T lymphocytes (Stanford and Issekutz, 2003) and monocytes (Petrovic-Djergovic et al., 2015) to the site of inflammation. Upregulation of *CXCL10* along with that of MyD88-dependent chemokines such as *IL8* suggests that cobalt activates both the MyD88 and TRIF-dependent arms of the TLR4 signalling pathway. This hypothesis was recently supported by Oblak *et al* who showed that cobalt increases activation of the IFN-β (MyD88-dependent) and CXCL10 (TRIF-dependent) promoters (Oblak et al., 2015).

CCL25

CCL25 is chemotactic for macrophages and dendritic cells and acts via CCR9 (Youn et al., 1999). Although a very large fold upregulation in *CCL25* was detected using the qRT-PCR arrays, validation by TaqMan-based assays showed only a 2-fold change in gene expression.

IL1A

IL1A expression was upregulated following cobalt stimulation and its encoded protein IL-1α is a pro-inflammatory cytokine that is associated with cell damage and apoptosis. In the context of this study it is interesting to note that IL-1α promotes osteoclast formation and subsequent bone resorption (Lader and Flanagan, 1998). MoM implants are associated with higher rates of aseptic loosening compared to ceramic-on-polyethylene and metal-on-polyethylene implants and this is due to bone resorption (Abu-Amer et al., 2007).

HMGB1

HMGB1 is a DNA-binding protein that stabilises DNA structure. It is also involved in inflammation as it is released from necrotic and apoptotic cells (Scaffidi et al., 2002, Bell et al., 2006), as well as in response to PAMPs like LPS (El Gazzar, 2007). Despite the lack of change detected by the arrays *HMGB1* expression was validated because it can bind and activate TLR4 (Jong et al., 2006). It was important to consider that other TLR4 ligands may be secreted via an alternative pathway and then feedback to activate TLR4, perpetuating inflammation independently of cobalt. However there was no change in *HMGB1* expression suggesting that it is not having an inflammatory effect in this case.

HSPA1A

HSPA1A expression was also investigated as it was shown to be significantly upregulated following cobalt treatment in the TLR arrays. *HSPA1A* encodes Hsp70 which activates TLR4 (Klink et al., 2012). qRT-PCR using TaqMan probes confirmed upregulation of *HSPA1A* expression in the presence of cobalt, although there were considerable differences in the detected fold changes (202-fold in the arrays compared to 12-fold with the TaqMan method). The observed upregulation raises the question of whether Hsp70 may be contributing to cobalt-mediated inflammation by activating TLR4. However a 4h cobalt stimulation was used for the qRT-PCR arrays and this is a very short time for *HSPA1A* to be upregulated, translated into Hsp70, secreted from the cell, and then feedback to activate TLR4, causing the changes in other genes such as *IL8*. Hsp70 is purported to decrease IL-8 secretion (Malago et al., 2005) which suggests that it is not responsible for all of the gene expression changes detected by the qRT-PCR arrays.

CCL2

CCL2 secretion is known to occur upon LPS activation of TLR4 (Guijarro-Muñoz et al., 2014). Its release has also been observed in response to nickel particles (Glista-Baker et al., 2012), as well as following cobalt ion stimulation of osteoblasts and epithelial cells (Queally et al., 2009, Devitt et al., 2010). The $CoCl_2$-induced downregulation of *CCL2* is therefore one of the most surprising results from the qRT-PCR arrays. CCL2 protein secretion can be increased by IL-6 (Biswas et al., 1998, Ekhlassi et al., 2008) and as there was no change in IL-6 expression in either the qRT-PCR arrays or protein arrays, it may be that this contributes to the change in *CCL2*. Interestingly, other studies have shown that *CCL2* can be downregulated by using cobalt as a hypoxia mimetic (Negus et al., 1998, Safronova et al., 2003) which may also have influenced the observed response.

Proteome Profiler cytokine arrays

Cytokine secretion by $CoCl_2$ and LPS-stimulated MonoMac 6 cells was assessed by Proteome Profiler array. An initial visual assessment of the arrays shows clear differences not only between the $CoCl_2$ and untreated arrays, but also between $CoCl_2$ and LPS. The differing cytokine profiles suggest that cobalt ions and LPS do not activate identical signalling pathways. This may be due to the different mechanisms of receptor activation; cobalt binds directly to TLR4 while LPS requires transfer across adaptor molecules prior to TLR4 activation. It is not known how the binding of the ligands, particularly cobalt, may affect receptor structure or the recruitment and binding of downstream signalling factors like MyD88. Additionally, as LPS and cobalt are very different ligands it is impossible to directly compare the agonist concentrations. Consequently, although the effect on a particular cytokine may seem more potent with LPS (for example), it does not necessarily mean that LPS has a greater pro-inflammatory effect than $CoCl_2$.

The array analysis revealed several cytokines that were increased in response to LPS or $CoCl_2$ and others that were affected by only one ligand (**Figure 3.8)**. Serpin E1 was the only cytokine increased by $CoCl_2$ but not by LPS while CCL2, CCL3, CCL4 and IL-1β were increased following LPS stimulation but not $CoCl_2$.

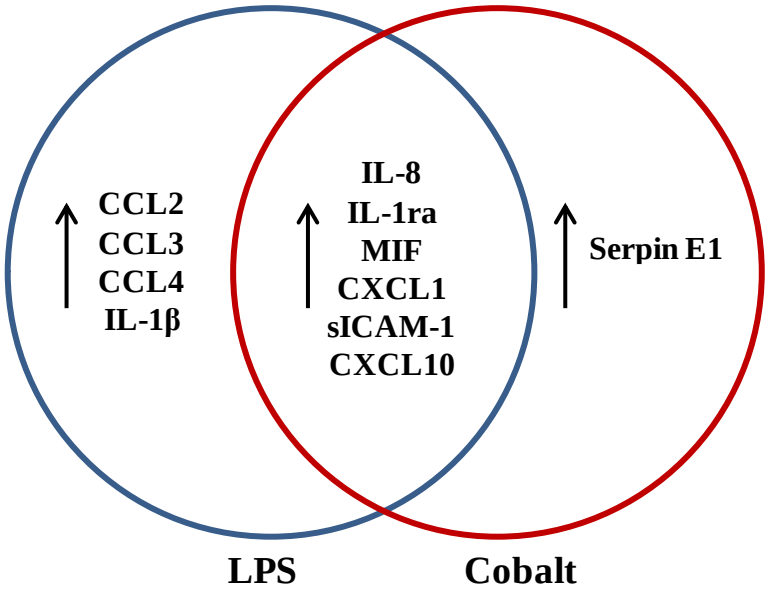


Figure 3.8: Differential regulation of cytokine secretion by LPS and $CoCl_2$
Figure shows upregulated cytokines from the cytokine profiler arrays and whether they were upregulated by LPS (blue), $CoCl_2$ (red) or both.

Cytokines and chemokines that were regulated by $CoCl_2$ in the Proteome Profiler arrays are discussed below:

IL-8

IL-8 is a pro-inflammatory chemokine that recruits neutrophils and mediates other inflammatory functions such as adhesion. It is discussed in more detail earlier in this chapter as well as in **section 4.4**.

IL-1ra

Interleukin-1 receptor antagonist (IL-1ra) binds to the interleukin-1 receptor (IL-1R1) and is able to block the action of IL-1 family members including IL-1α and IL-1β. Caicedo *et al* demonstrated that IL-1ra can prevent lymphocyte reactivity in patients who show nickel or cobalt hypersensitivity (Caicedo et al., 2010), which suggests that the observed upregulation in this study could have an immunomodulatory effect if similar effects occur *in vivo.*

MIF

Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine that inhibits the anti-inflammatory action of glucocorticoids on factors such as IL-6 and IL-8 (Calandra and Bucala, 1997), and as such is considered an immunoregulatory molecule. It is released by LPS-stimulated macrophages and induces further secretion of pro-inflammatory cytokines by macrophages and activated T cells. MIF inhibition can protect against an exaggerated immune response in bacterial sepsis (Calandra et al., 2000). This suggests that the increased MIF release following cobalt stimulation may promote further inflammation.

Serpin E1

Serine protease inhibitor E1 (serpin E1, also known as plasminogen activator inhibitor-1 or PAI-1) is an inhibitor of fibrolysis, the process of blood clot formation. Its secretion was increased in response to $CoCl_2$ stimulation but not in response to LPS. This is surprising as a previous study has shown that LPS can regulate serpin E1 expression through TLR4 activation (Ren et al., 2014). The same study showed that serpin E1 knockdown inhibits TLR4-mediated inflammatory responses including TNFα secretion. It is therefore possible that its upregulation in response to $CoCl_2$ may promote release of other cytokines.

CXCL1

Chemokine (C-X-C motif) Ligand 1 is chemotactic for neutrophils. CXCL1 has recently been implicated in osteoclast formation by binding to its receptor CXCR2. Activation of CXCR2 is associated with increased osteoclastogenesis (Hardaway et al., 2015), which is interesting

because several chemokines that bind CXCR2, including IL-8, are also upregulated by cobalt ions. It is therefore possible that a small upregulation of several different chemokines could still have an inflammatory effect because many act via the same receptor.

sICAM-1

Soluble intercellular adhesion molecule-1 (sICAM-1) is a secreted form of the membrane-bound adhesion molecule ICAM-1. The exact function of sICAM-1 is unclear but it has been linked to angiogenesis and tumour progression. sICAM-1 function and its potential significance in ARMD will be discussed in more detail in **section 5.5**.

CCL5

Chemokine (C-C motif) ligand 5, also known as regulated on activation, normal T cell expressed and secreted (RANTES), is chemotactic for monocytes and T cells. It is surprising that LPS had little effect on CCL5 release in this assay because previous studies have shown that LPS activation of TLR4 results in significantly increased CCL5 secretion (Fitzgerald et al., 2003). However a previous study has shown that $CoCl_2$ can downregulate both CCL5 and CCL2 release (Oh et al., 2014).

CXCL10

CXCL10 release was slightly increased following $CoCl_2$ stimulation, which correlates with the increased gene expression detected by qRT-PCR array. CXCL10 function is discussed earlier in this chapter and in **section 4.4**.

CCL2, CCL3 and CCL4

It is particularly interesting to observe that CCL2, CCL3 and CCL4 secretion was increased in LPS-stimulated MonoMac 6 cells but not in those treated with $CoCl_2$ **(Figure 3.8)**. The effect of $CoCl_2$ on CCL2 is consistent with the qRT-PCR array data. A possible mechanism underlying this inhibition could involve suppressor of cytokine signalling (SOCS) molecules as SOCS-3 has been shown to attenuate CCL chemokine secretion as a protective response (Qin et al., 2007, Qin et al., 2008). However other inflammatory factors (such as prostaglandins) can result in chemokine inhibition (Jing et al., 2004), and from the assays conducted in this study it is not clear which mechanisms are responsible.

3.5.1. Future work

Data presented here clearly shows that cobalt ions can regulate inflammatory responses in human MonoMac 6 macrophages. However the nature of the qRT-PCR arrays meant that only a limited number of timepoints and cobalt concentrations could be used. Further work could be

conducted to determine the effect of cobalt ions at different concentrations and also at longer timepoints (e.g. 12 or 24h). In addition, a number of genes were not expressed at high levels by MonoMac 6 cells and although there was some evidence of expression changes following cobalt treatment, their levels remained too low for informed analysis (examples include *CCL8* and *CXCL9*). The qRT-PCR arrays could therefore be performed on a second cell line to validate the results obtained in MonoMac 6 cells and assess the effect of cobalt on genes that were not highly expressed by these cells.

It is important to acknowledge that cytokines can be regulated via multiple signalling pathways. For example, CCL2 can be upregulated via JAK/STAT signalling (Lee et al., 2011) but NFκB may also be involved in the regulation of CCL2 expression as its promoter contains an NFκB binding element (Deng et al., 2013). siRNA knockdown could be performed to target JAK/STAT or NFκB signalling components, followed by quantification of CCL2 expression or secretion. This would provide greater insight into which signalling factors contribute to secretion and expression of individual cytokines.

The Proteome Profiler arrays provide an excellent starting point for studying the effects of cobalt ions on cellular cytokine secretion. As this was limited to investigating secreted inflammatory factors further work is required to validate the protein array work and assess expression of membrane-bound and intracellular signalling proteins following cobalt stimulation. Cell-based ELISA, Western immunoblotting, and flow cytometry would all be suitable techniques for analysis.

3.5.2. Conclusion

Overall the data obtained using the qRT-PCR and Proteome Profiler arrays indicates that cobalt ions can regulate expression of both pro- and anti-inflammatory cytokines and chemokines. For example, while pro-inflammatory *IL8* was upregulated, there was also downregulation of other inflammatory chemokines, most notably *CCL2*. This was consistent across both gene expression and protein arrays. Expression of TLR signalling factors was also altered by cobalt ions although *TLR4* itself was unaffected. While the NFκB pathway appeared to be upregulated overall, inhibitory *NFKBIA* expression was also upregulated and its effect on the subsequent cellular response is not clear. To determine the overall inflammatory effect of cobalt ions, functional studies are required and these will be discussed in later chapters.

Chapter 4. Role of the Toll-like receptor 4 signalling pathway in the inflammatory response to cobalt ions

4.1. Introduction

Chapter 3 describes an investigation into the cellular inflammatory response induced by cobalt ions in MonoMac 6 macrophages and shows that a wide range of signalling molecules and inflammatory factors are affected. However the overall aim of this study was to investigate the role of TLR4 in the immune response to cobalt ions, and subsequently a more focused approach was required. This necessitated selection of an appropriate marker of inflammatory responses to cobalt based on the qRT-PCR and Proteome Profiler arrays detailed in chapter 3. IL-8 was selected as a marker for cobalt-mediated inflammation because it was consistently and significantly upregulated by $CoCl_2$ at both the gene expression and protein levels. IL-8 is also secreted when LPS activates TLR4, meaning that TLR4-specific LPS could be used to provide an effective positive control for receptor activation. Finally, the NFκB signalling pathway appears to be regulated by cobalt ions (chapter 3) and IL-8 is an NFκB-regulated chemokine making it an appropriate marker for further study.

4.2. Objectives

- To define the response of MonoMac 6 macrophages to cobalt ions, using IL-8 as a marker of inflammation
- To investigate the potential of a small molecule TLR4 antagonist and anti-TLR4 neutralising antibodies in prevention of the inflammatory response to cobalt ions
- To further investigate cytotoxicity in response to cobalt ions
- To investigate other inflammatory factors regulated by cobalt activation of TLR4

4.3. Results

4.3.1. Development of an *in vitro* cell culture model for investigating cobalt-mediated inflammatory responses

The arrays detailed in chapter 3 offer insight into the inflammatory effects of cobalt but the nature of these assays meant that limited cobalt concentrations could be used. A series of additional assays was therefore utilised and optimised to further establish any dose-dependent effects of cobalt ions on IL-8 gene expression and secretion by MonoMac 6 cells.

MonoMac 6 cells were stimulated with 0.25-1mM $CoCl_2$ or 100ng/ml LPS for 24h (LPS concentration and timepoint previously optimised in MRes project) before RNA and supernatant were collected. *IL8* gene expression was quantified by qRT-PCR at this timepoint to confirm that the changes observed in the arrays were still in effect. Analysis using TaqMan probes in qRT-PCR revealed that *IL8* gene expression in MonoMac 6 cells is significantly upregulated following 24h treatment with $CoCl_2$ and LPS (all $p<0.001$) **(Figure 4.1A)**. A peak fold-change of approximately 70-fold occurred at 0.75mM $CoCl_2$. *GAPDH* expression was consistent across the different treatment conditions (**Appendix C**). IL-8 secretion was measured by ELISA and a dose-dependent response was observed with significant increases in chemokine release across all $CoCl_2$ concentrations (all $p<0.001$). Peak secretion of 7000pg/ml was reached at 1mM $CoCl_2$ **(Figure 4.1B)**. Both gene expression and protein secretion were very similar between cells stimulated with LPS and high $CoCl_2$ concentrations (0.75mM and 1mM). LPS therefore provided an effective positive control for further assays.

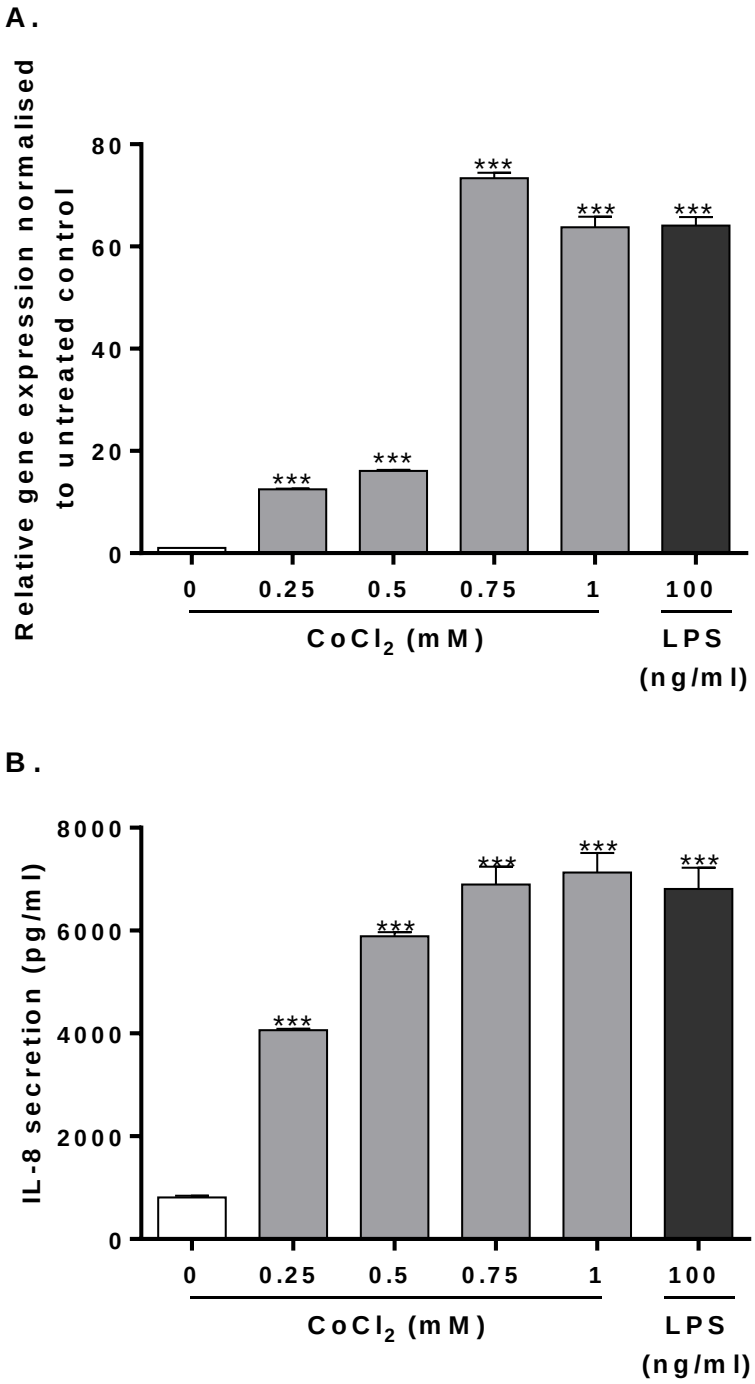


Figure 4.1: Effect of $CoCl_2$ on IL-8 gene expression and protein secretion
MonoMac 6 cells were stimulated with 0.25-1mM $CoCl_2$ or 100ng/ml LPS for 24h. **A.** RNA was collected, cDNA synthesised by reverse transcription, and qRT-PCR conducted to assess *IL8* expression. **B.** IL-8 secretion was measured by ELISA. Data is representative of at least three independent experiments. Statistical significance was calculated by one-way ANOVA with Dunnett's multiple comparisons test comparing treated samples to the untreated control.

4.3.2. Validation of IL-8 as a marker of inflammatory responses to cobalt

To determine that induction of IL-8 secretion by cobalt ions was not unique to MonoMac 6 cells, two other cell lines were investigated for their response to cobalt. They were THP-1, a monocyte cell line, and U2OS, an osteoblast cell line. Both cell lines were stimulated with 0.25-1mM $CoCl_2$ or 100ng/ml LPS for 24h before supernatant was collected and IL-8 secretion was measured by ELISA. THP-1 cells showed increased IL-8 release in response to $CoCl_2$, with peak $CoCl_2$-mediated secretion of 500pg/ml occurring at 0.75mM ($p<0.001$) (**Figure 4.2A)**. The positive control LPS caused the maximum IL-8 release of approximately 600pg/ml ($p<0.001$).

U2OS cells also increased IL-8 secretion following $CoCl_2$ and LPS stimulation, although the pattern was different to that of THP-1 cells. IL-8 levels increased from 0.5mM $CoCl_2$ ($p=0.0025$) and peaked at 650pg/ml with 1mM $CoCl_2$ treatment ($p<0.001$) (**Figure 4.2B**). LPS caused elevated IL-8 release ($p<0.001$) although at 400pg/ml this was less than the maximum $CoCl_2$-induced secretion.

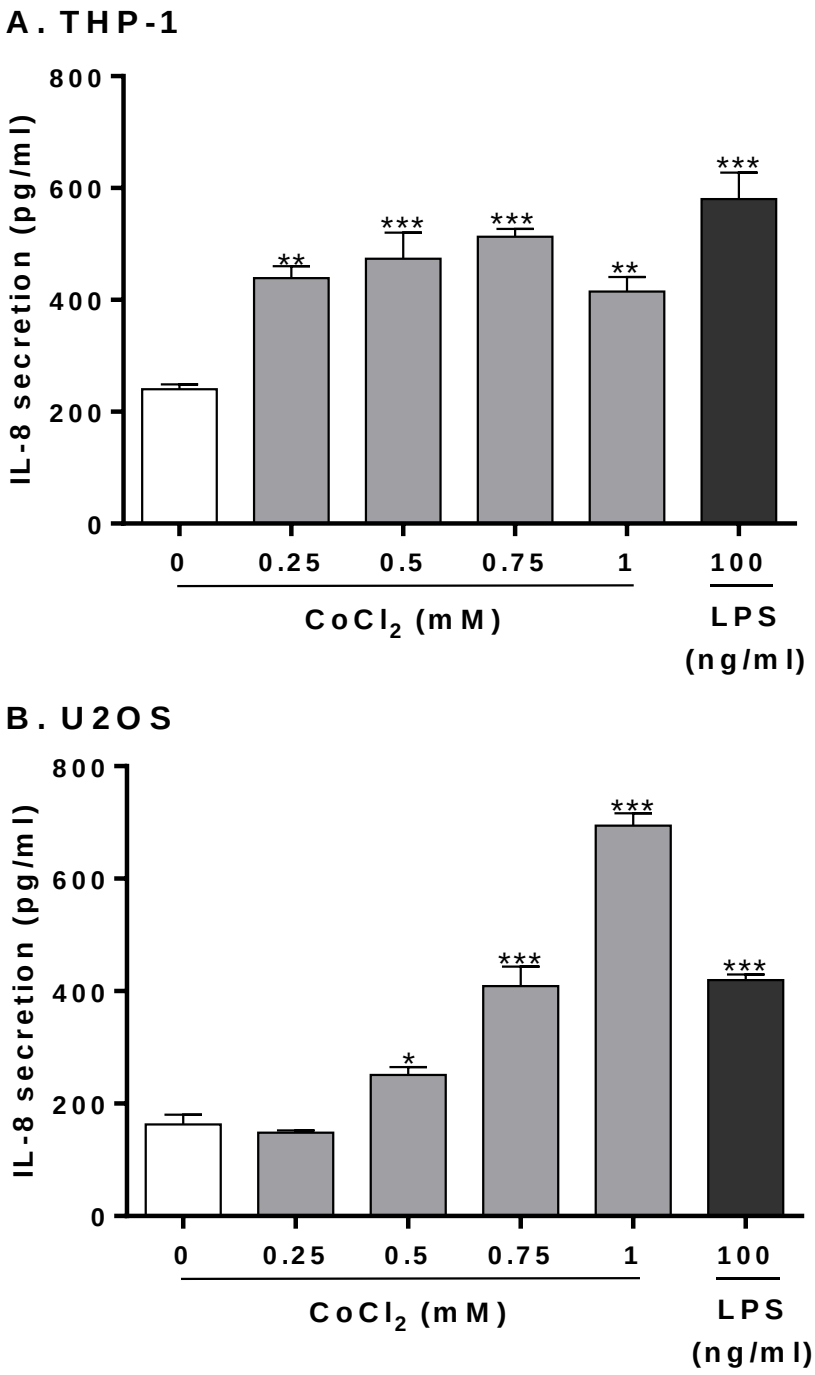


Figure 4.2: Effect of $CoCl_2$ on IL-8 secretion by THP-1 and U2OS cells
A. THP-1 and **B.** U2OS cells were stimulated with 0.25-1mM $CoCl_2$ or 100ng/ml LPS for 24h before supernatant was collected and IL-8 secretion quantified by ELISA. Data is representative of at least three independent experiments and statistical significance was calculated by one-way ANOVA with Dunnett's test for multiple comparisons comparing treated samples to the untreated control.

4.3.3. Selection of an optimal cobalt concentration

The results described so far in this chapter were used to select an optimum cobalt concentration for use in further assays investigating the role of TLR4. In all of the assays cobalt ions had a dose-dependent effect with the peak response usually occurring at 0.75mM or 1mM $CoCl_2$. The optimal concentration chosen for future use was 0.75mM $CoCl_2$ because it induced the maximum response in several assays (for example *IL8* expression) and is also closer to the clinically-relevant range than 1mM.

4.3.4. Effect of cobalt ions on MonoMac 6 cell viability

Cobalt ions can reduce cell viability (Kwon et al., 2009) and following selection of 0.75mM $CoCl_2$ as the optimal concentration in MonoMac 6 cells, cytotoxicity at this concentration was investigated. MonoMac 6 cells were stimulated with 0.75mM $CoCl_2$ for 24h and cell viability was assessed by trypan blue staining and counted manually using images obtained from a Luna II automated cell counter. Viability in the untreated cells was normalised to 100% while cobalt caused cytotoxicity, reducing viability to approximately 75% (**Figure 4.3**).

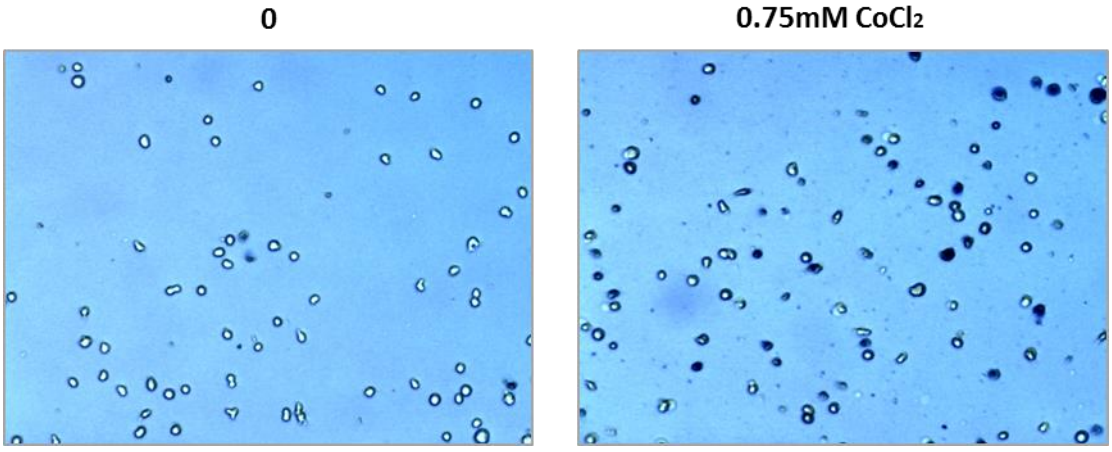


Figure 4.3: Effect of 24h $CoCl_2$ stimulation on MonoMac 6 cell viability
MonoMac 6 cells were stimulated with 0.75mM $CoCl_2$ or left untreated for 24h before cells were stained with trypan blue dye. Cell images were obtained using a Luna II automated cell counter and viability was assessed by manual counting due to the issues described in **section 2.5**. Viability in the untreated cells was normalised to 100% and $CoCl_2$ stimulation reduced cell viability to 75%. Images are representative of four independent experiments.

4.3.5. Effect of cobalt ions on murine *Cxcl1* expression

Previous research has shown that cobalt ions cannot activate murine TLR4 due to the absence of the histidine residues to which cobalt binds (Raghavan et al., 2012, Tyson-Capper et al., 2013a). To further validate this finding, J774 murine macrophages were assessed for *Cxcl1* (murine equivalent of *IL8*) expression in response to LPS and cobalt. J774 cells were treated for 4h with 1000ng/ml LPS or 0.75mM $CoCl_2$, RNA was collected and *Cxcl1* was quantified by qRT-PCR using *Actb* as a housekeeping gene. $CoCl_2$ induced a 4-fold upregulation ($p=0.0224$) in *Cxcl1* expression compared to a 550-fold change following LPS stimulation ($p<0.001$) **(Figure 4.4)**.

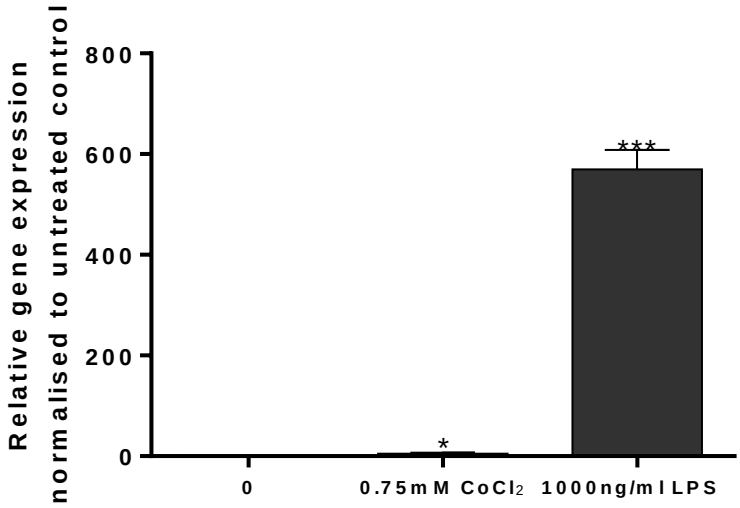


Figure 4.4: Cxcl1 expression in J774 macrophages
J774 murine macrophages were stimulated with 0.75mM $CoCl_2$ or 1000ng/ml LPS for 4h. RNA was isolated and cDNA synthesised by reverse transcription. *Cxcl1* expression was quantified by qRT-PCR using *Actb* as a housekeeping gene. Data is representative of three independent experiments and statistical significance was calculated by one-way ANOVA with Dunnett's test for multiple comparisons comparing treated samples to the untreated control.

4.3.6. Optimisation of TLR4 antagonist CLI-095

TLR4 activation is just one of many cellular processes that result in IL-8 secretion. It is therefore possible that the observed increases in IL-8 expression and secretion in response to $CoCl_2$ are a non-specific inflammatory response to metal ion exposure, rather than a result of TLR4 activation by cobalt ions. A small molecule TLR4 antagonist, CLI-095, was therefore used to elucidate the role of TLR4 in cobalt-mediated IL-8 release. CLI-095 binds to the intracellular domain of TLR4 to prevent recruitment of its adaptor proteins (e.g. MyD88, TRIF) and initiation of subsequent downstream signalling events. In previous studies CLI-095 has been shown to prevent LPS activation of TLR4 (Matsunaga et al., 2011). Consequently for this study an initial optimisation assay was performed using IL-8 as a marker to determine the inhibitory capacity of CLI-095. Following optimisation with $CoCl_2$ all subsequent assays included LPS as a positive control. MonoMac 6 cells were treated with 0-10µg/ml CLI-095 for 6h prior to stimulation with 0.75mM $CoCl_2$ for 24h and IL-8 secretion was measured by ELISA.

Maximal IL-8 secretion in response to 0.75mM $CoCl_2$ was approximately 4600pg/ml (**Figure 4.5**). Using 2300pg/ml as 50% inhibition, the half maximal inhibitory concentration (IC_{50}) was found to be approximately 2µg/ml. Near-complete inhibition of IL-8 secretion occurred at 10µg/ml CLI-095 and so this concentration was selected for further assays.

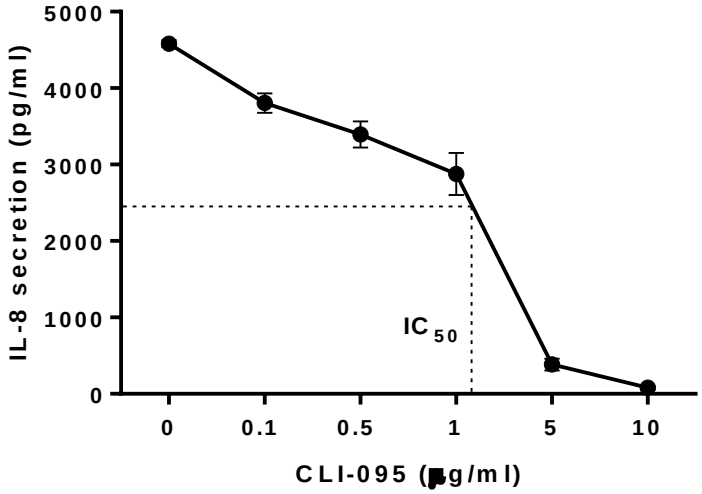


Figure 4.5: CLI-095 dose response curve
The optimal concentration of the TLR4 antagonist CLI-095 was determined using IL-8 as a marker of receptor activation by cobalt ions. MonoMac 6 cells were pre-incubated with 0-10µg/ml CLI-095 for 6h before 24h stimulation with 0.75mM $CoCl_2$. IL-8 secretion was measured by ELISA. The half maximal inhibitory concentration (IC_{50}) was calculated at 1.5µg/ml. Maximal inhibition occurred at 10µg/ml CLI-095. n=1.

4.3.7. Effect of cobalt ions on IL-8 and CXCL10 secretion

The Proteome Profiler arrays described in chapter 3 showed an increase in CXCL10 secretion following cobalt stimulation. Although small, this change is interesting because CXCL10 secretion can occur via activation of the TRIF-dependent pathway of TLR4 signalling as opposed to MyD88-dependent IL-8 release. To assess the role of TLR4 in the release of these cytokines MonoMac 6 cells were pre-treated with 10μg/ml CLI-095 for 6h before 24h stimulation with 0.75mM $CoCl_2$ or 100ng/ml LPS. IL-8 and CXCL10 levels were quantified by ELISA. As expected given previous results, IL-8 secretion was significantly increased to approximately 4000pg/ml by $CoCl_2$ and 7000pg/ml by LPS (both $p<0.001$). 10μg/ml CLI-095 abrogated release of IL-8 in response to both ligands ($p<0.001$), suggesting that it is TLR4-dependent (**Figure 4.6A**). CXCL10 secretion was increased by $CoCl_2$ and LPS (1000pg/ml CXCL10 for $CoCl_2$ and 1300pg/ml for LPS, both $p<0.001$) and significantly decreased in the presence of CLI-095 (both $p<0.001$) **(Figure 4.6B)**. CXCL10 secretion was also decreased by the antagonist in untreated cells ($p=0.0328$) and the reasons for this will be discussed later in the chapter.

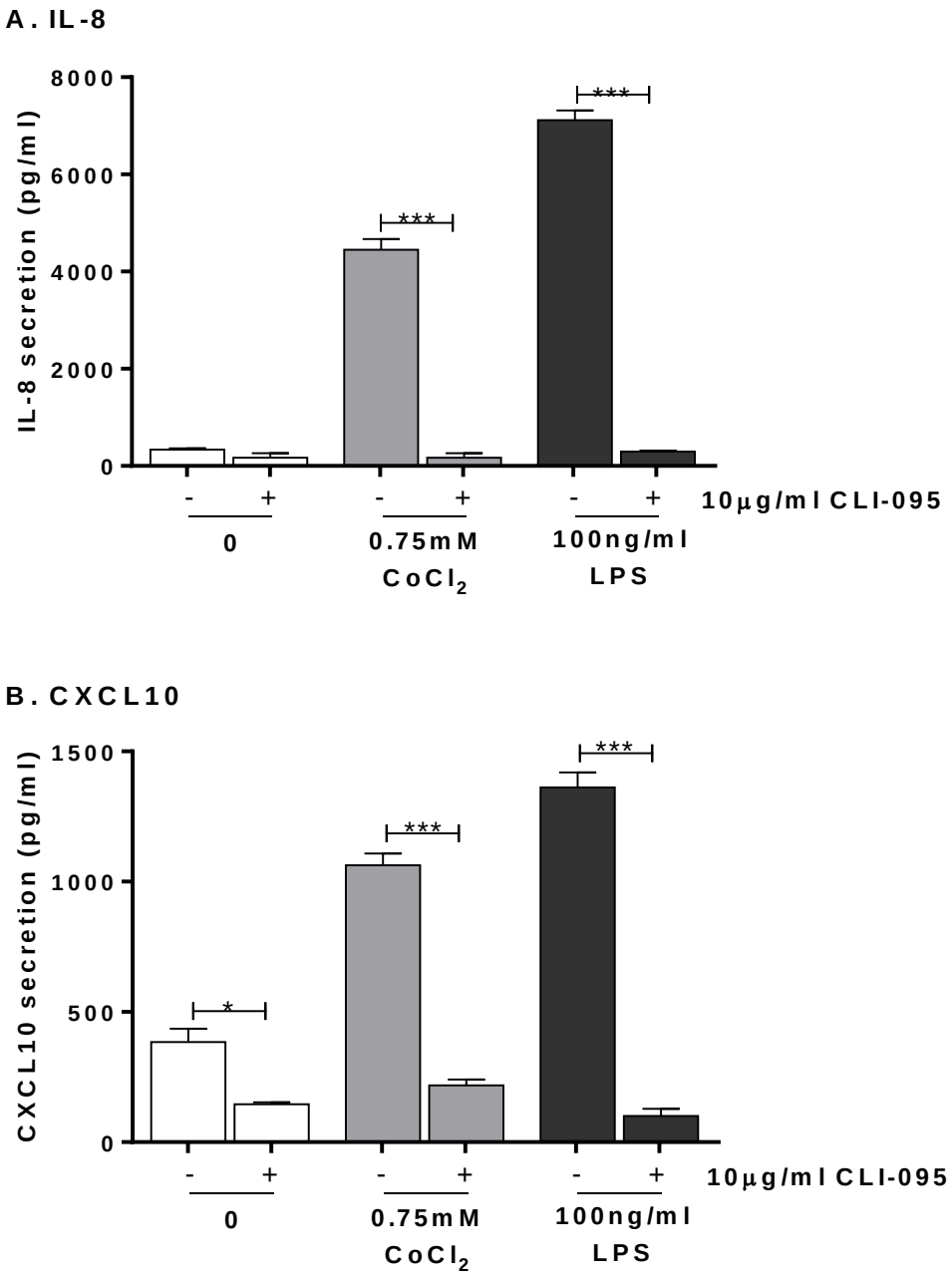


Figure 4.6: Effect of TLR4 inhibition on IL-8 and CXCL10 secretion
MonoMac 6 cells were pre-incubated with 10µg/ml CLI-095 for 6h prior to 24h stimulation with 0.75mM $CoCl_2$ or 100ng/ml LPS. **A.** IL-8 and **B.** CXCL10 secretion was measured by ELISA. Data is representative of three independent experiments and statistical significance was calculated by one-way ANOVA with Tukey's test for multiple comparisons comparing all samples to each other. This data appears in Lawrence *et al* (Lawrence et al., 2014).

4.3.8. Effect of CLI-095 on expression of inflammatory genes

To determine whether or not CLI-095 affected cobalt-mediated gene expression changes, *IL8* expression was used as a marker of TLR4 activation by cobalt ions. The same treatment method described in **section 4.3.7** was used and *IL8* expression was assessed by qRT-PCR. This revealed similar trends between gene and protein expression of IL-8; *IL8* expression was significantly upregulated by 24h stimulation with $CoCl_2$ and LPS (both $p<0.001$) and this was inhibited by CLI-095 (both $p<0.001$) (**Figure 4.7**). There was a slight decrease in *IL8* expression by untreated MonoMac 6 in the presence of CLI-095 but this was not statistically significant ($p=0.6950$).

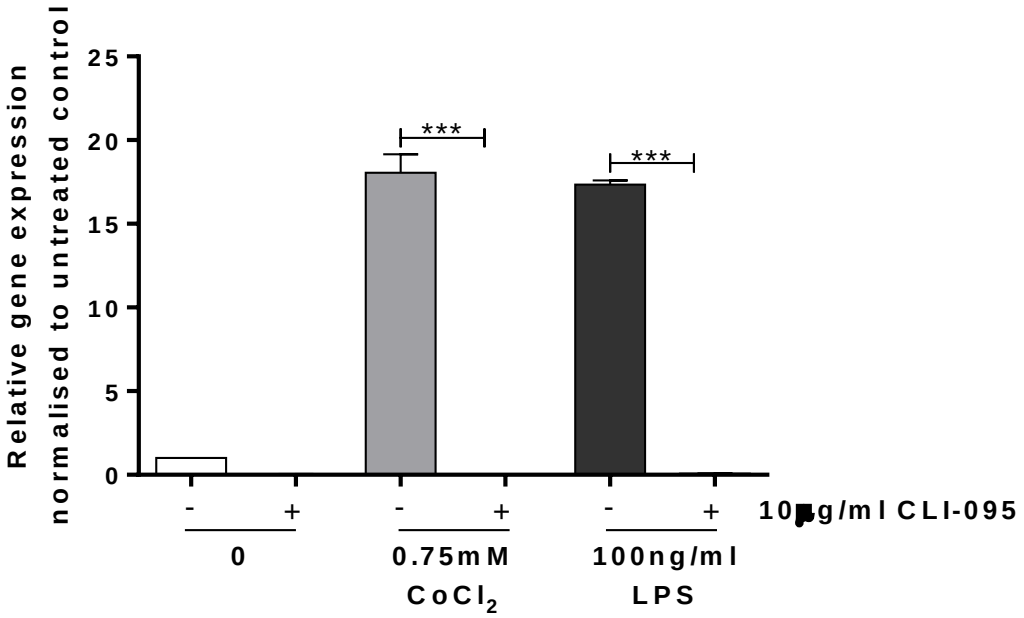


Figure 4.7: Effect of TLR4 inhibition on *IL8* expression
MonoMac 6 cells were pre-treated with 10µg/ml CLI-095 for 6h and then stimulated with 0.75mM $CoCl_2$ or100ng/ml LPS for 24h. RNA was isolated and cDNA synthesised by reverse transcription. *IL8* expression was assessed by qRT-PCR. Data is representative of three independent experiments and statistical significance was calculated by one-way ANOVA with Tukey's test for multiple comparisons comparing all samples to each other. This data appears in Lawrence *et al* (Lawrence et al., 2014).

4.3.9. Effect of CLI-095 on MonoMac 6 cell viability

As CXCL10 secretion and *IL8* expression were both decreased by 10µg/ml CLI-095 in untreated cells, a cytotoxicity assay was conducted to assess any potential toxic effects of the antagonist. Analysis by trypan blue staining revealed that >1µg/ml CLI-095 causes cytotoxicity in MonoMac 6 cells (**Appendix D**). As a result the optimal concentration was revised to 1µg/ml. As shown in **Figure 4.8** this concentration still effectively inhibited $CoCl_2$ and LPS-mediated IL-8 secretion, reducing IL-8 levels to approximately 2000pg/ml (both $p<0.001$). CLI-095 is reconstituted in DMSO and therefore an additional 1% v/v DMSO control was included to ensure that this did not have any effect on IL-8 secretion. There was no significant difference in IL-8 secretion by untreated cells and those challenged with DMSO ($p=0.6422$) **(Figure 4.8).**

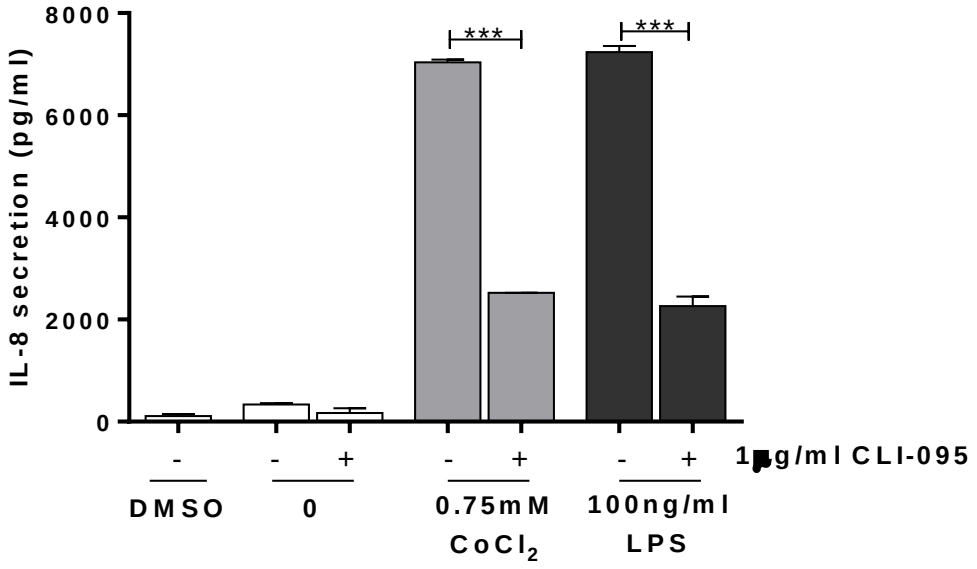


Figure 4.8: Effect of 1µg/ml CLI-095 on IL-8 secretion
MonoMac 6 cells were pre-treated with 1µg/ml CLI-095 for 6h before stimulation with 0.75mM $CoCl_2$ or 100ng/ml LPS for 24h. A 1% v/v DMSO control was included as CLI-095 is reconstituted in DMSO. Supernatant was collected and IL-8 secretion was measured by ELISA. Data is representative of three independent experiments and statistical significance was calculated by one-way ANOVA with Tukey's test for multiple comparisons comparing all samples to each other.

4.3.10. Effect of TLR4 inhibition on cobalt-mediated cytokine release

In addition to IL-8 and CXCL10 the secretion of two further inflammatory cytokines, CCL20 and IL-6, was investigated. CCL20 was selected because of the upregulation in gene expression shown by the qRT-PCR arrays and subsequent validation in chapter 3. CCL20 can also be regulated via activation of TLR4 by LPS (Guijarro-Muñoz et al., 2014). IL-6 was chosen because its secretion following cobalt stimulation is variable, with some studies reporting an increase in release (Queally et al., 2009) while others suggest that it is unaffected or even downregulated by cobalt ions (Posada et al., 2015).

MonoMac 6 cells were pre-treated with 1μg/ml CLI-095 for 6h followed by stimulation with 0.75mM $CoCl_2$ or 100ng/ml LPS for 24h. CCL20 and IL-6 levels were quantified by ELISA. CCL20 secretion was significantly increased to 300pg/ml ($p<0.001$) by $CoCl_2$ while LPS induced a concentration of approximately 400pg/ml ($p<0.001$) (**Figure 4.9A**). CLI-095 significantly inhibited CCL20 secretion in response to both ligands ($p<0.001$), reducing chemokine secretion to levels observed in untreated cells. LPS stimulation caused a significant upregulation in IL-6 secretion to 2000pg/ml ($p<0.001$) (**Figure 4.9B**) and in comparison $CoCl_2$-mediated IL-6 release was small at approximately 300pg/ml although it was still statistically significant ($p=0.0450$). CLI-095 pre-treatment inhibited IL-6 secretion in response to both $CoCl_2$ and LPS. This was statistically significant for LPS ($p<0.001$) but not for $CoCl_2$ ($p=0.0798$) (**Figure 4.9B**).

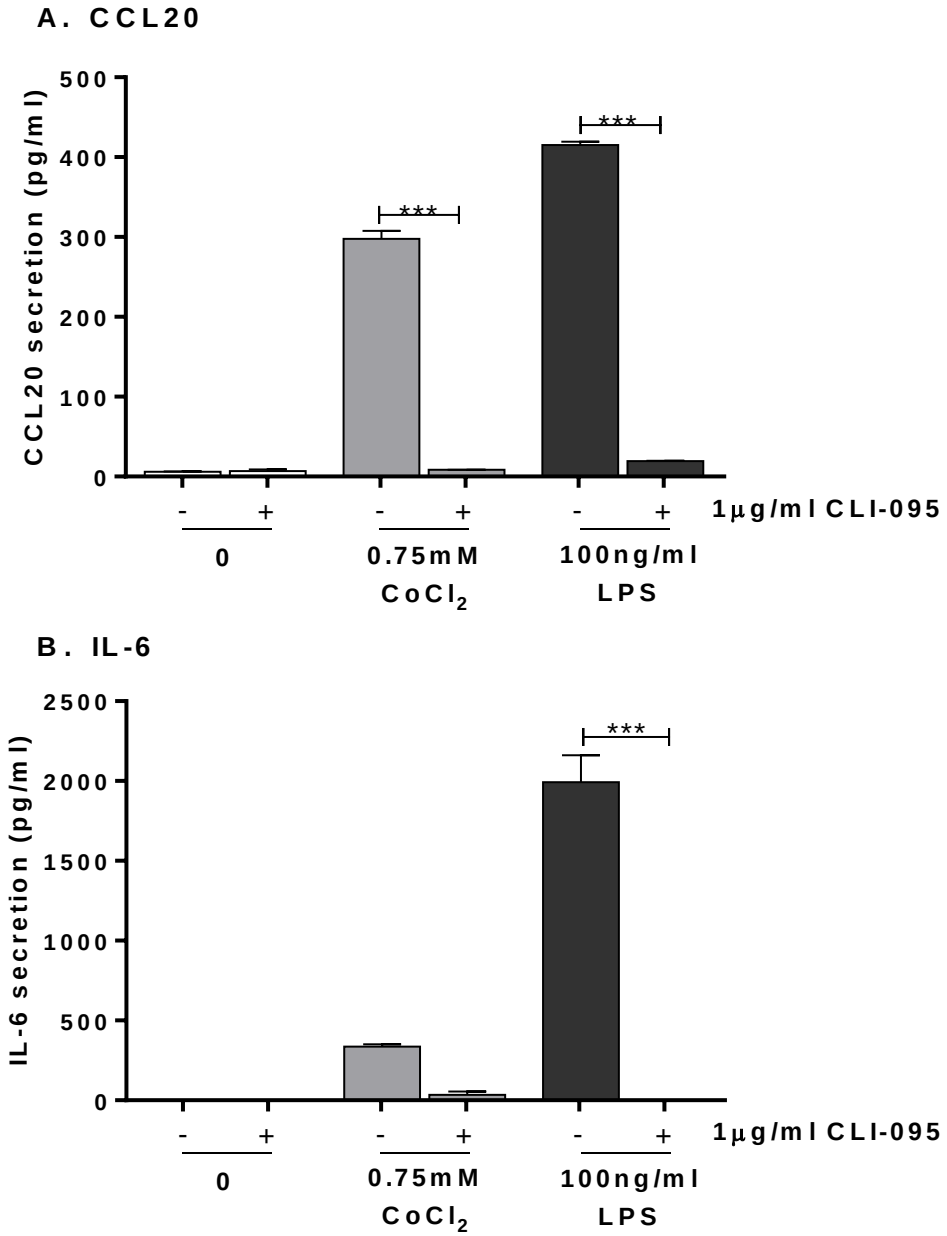


Figure 4.9: Effect of TLR4 inhibition on CCL20 and IL-6 secretion
MonoMac 6 cells were pre-treated with 1µg/ml CLI-095 for 6h before stimulation for 24h with 0.75mM $CoCl_2$ or 100ng/ml LPS. **A.** CCL20 and **B.** IL-6 secretion was measured by ELISA. Data is representative of three independent experiments and statistical significance was calculated by one-way ANOVA with Tukey's test for multiple comparisons comparing all samples to each other.

4.3.11. Effect of anti-TLR4 neutralising antibodies on the inflammatory response to cobalt ions

The data shown so far clearly demonstrates that cobalt regulates secretion and expression of cytokines and chemokines, and inhibition of TLR4 can prevent these responses. CLI-095 is a small molecule antagonist that requires intracellular access to successfully inhibit TLR4. Although its blockade of IL-8 and other inflammatory markers in response to cobalt demonstrates the potential of TLR4 as a therapeutic target in ARMD, CLI-095 itself is not a realistic therapeutic agent due to its mechanism of action. In contrast antibodies, particularly monoclonal antibodies, are constantly being developed for therapeutic use. This aspect of the study set out to investigate whether any commercially-available anti-TLR4 neutralising antibodies could prevent the inflammatory response to cobalt ions, again using IL-8 as a marker of cobalt-mediated TLR4 activation.

Unlike CLI-095 which has been used in previous studies to prevent LPS activation of TLR4, no studies could be found in which PAb-hTLR4 or MAb2-hTLR4 inhibit the effect of LPS, although the manufacturer reports LPS antagonism by both antibodies. Furthermore, the exact mechanism by which cobalt activates TLR4 is unclear and thus potential mechanisms of inhibition are also unclear. Consequently all neutralising antibody assays included LPS as a positive control for TLR4 activation.

4.3.12. Effect of PAb-hTLR4 on MonoMac 6 cell viability

An initial cytotoxicity assay was conducted in which MonoMac 6 cells were pre-treated with 5μg/ml PAb-hTLR4 (the highest concentration recommended by the manufacturer) for 16h. Cells were stained with trypan blue and cell viability assessed using a Luna II automated cell counter **(Figure 4.10)**. Viability was normalised to 100% in untreated cells and was 94% for those incubated with PAb-hTLR4.

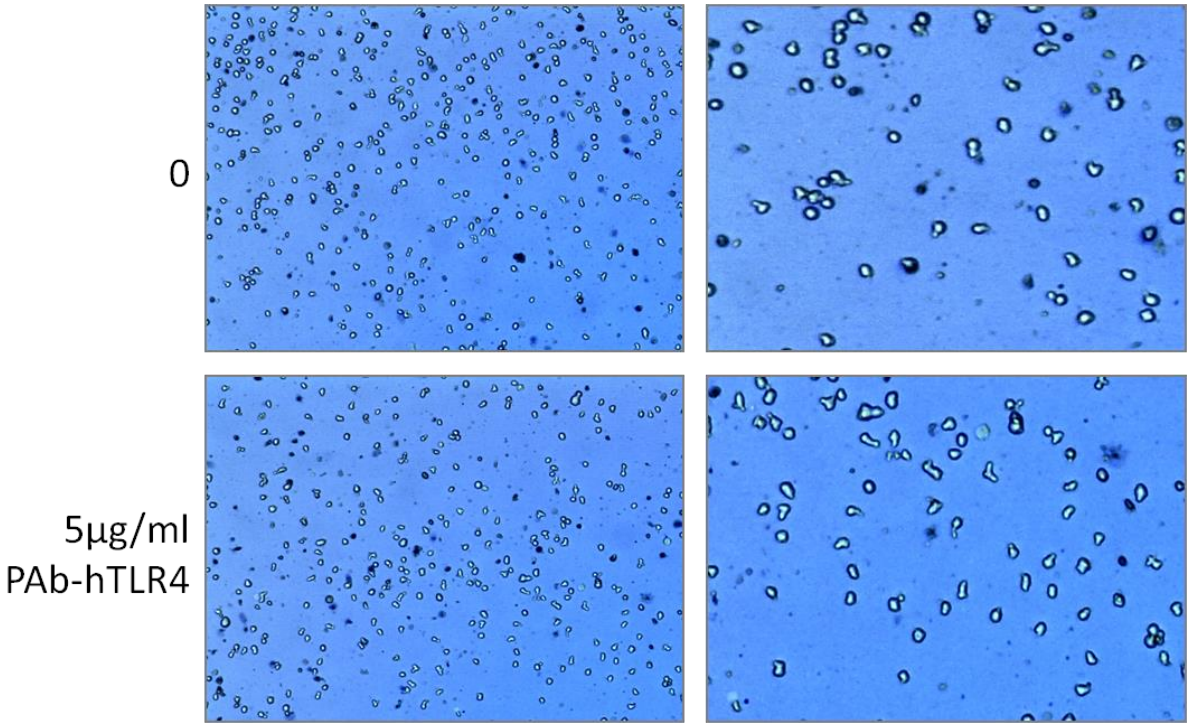


Figure 4.10: Effect of PAb-hTLR4 on MonoMac 6 cell viability.
MonoMac 6 cells were incubated with 5µg/ml PAb-hTLR4 for 16h and cell viability was measured using trypan blue staining and manual counting using images obtained from a Luna II automated cell counter. Viability was normalised to 100% in untreated cells and was 94% for those incubated with PAb-hTLR4. Images on the right are a magnified section of those on the left. Data is representative of two independent experiments. This experiment was conducted in collaboration with MRes student Amy Mawdesley and is published in Lawrence *et al* (Lawrence et al., 2016).

4.3.13. Effect of PAb-hTLR4 on cobalt-mediated IL-8 responses

The ability of PAb-hTLR4 to inhibit cobalt activation of TLR4 was assessed using the pre-treatment time (10 minutes) and ligand stimulation time (overnight or 16h) recommended by the antibody manufacturer. MonoMac 6 cells were incubated with 5μg/ml PAb-hTLR4 for 10 minutes before stimulation with 100ng/ml LPS or 0.75mM $CoCl_2$ for 16h. Changes in IL-8 gene expression and protein secretion were assessed by qRT-PCR and ELISA. PAb-hTLR4 did not inhibit *IL8* gene expression in response to cobalt ions and there was a slight increase in $CoCl_2$-mediated *IL8* expression in the presence of the antibody ($p=0.6516$) **(Figure 4.11A)**. However LPS-induced *IL8* expression was significantly decreased by PAb-hTLR4 ($p<0.001$). There was no significant difference in gene expression in untreated cells with and without the antibody ($p=0.6516$). IL-8 secretion showed a similar pattern to that of gene expression; PAb-hTLR4 did not have any effect on IL-8 secretion by cobalt-stimulated cells ($p>0.999$) **(Figure 4.11B)**. There was a small decrease in IL-8 release for LPS treatment in the presence of the inhibitor but this was not statistically significant ($p=0.4333$). Interestingly, IL-8 production by unstimulated cells increased with PAb-hTLR4 although this was not statistically significant ($p=0.0512$).

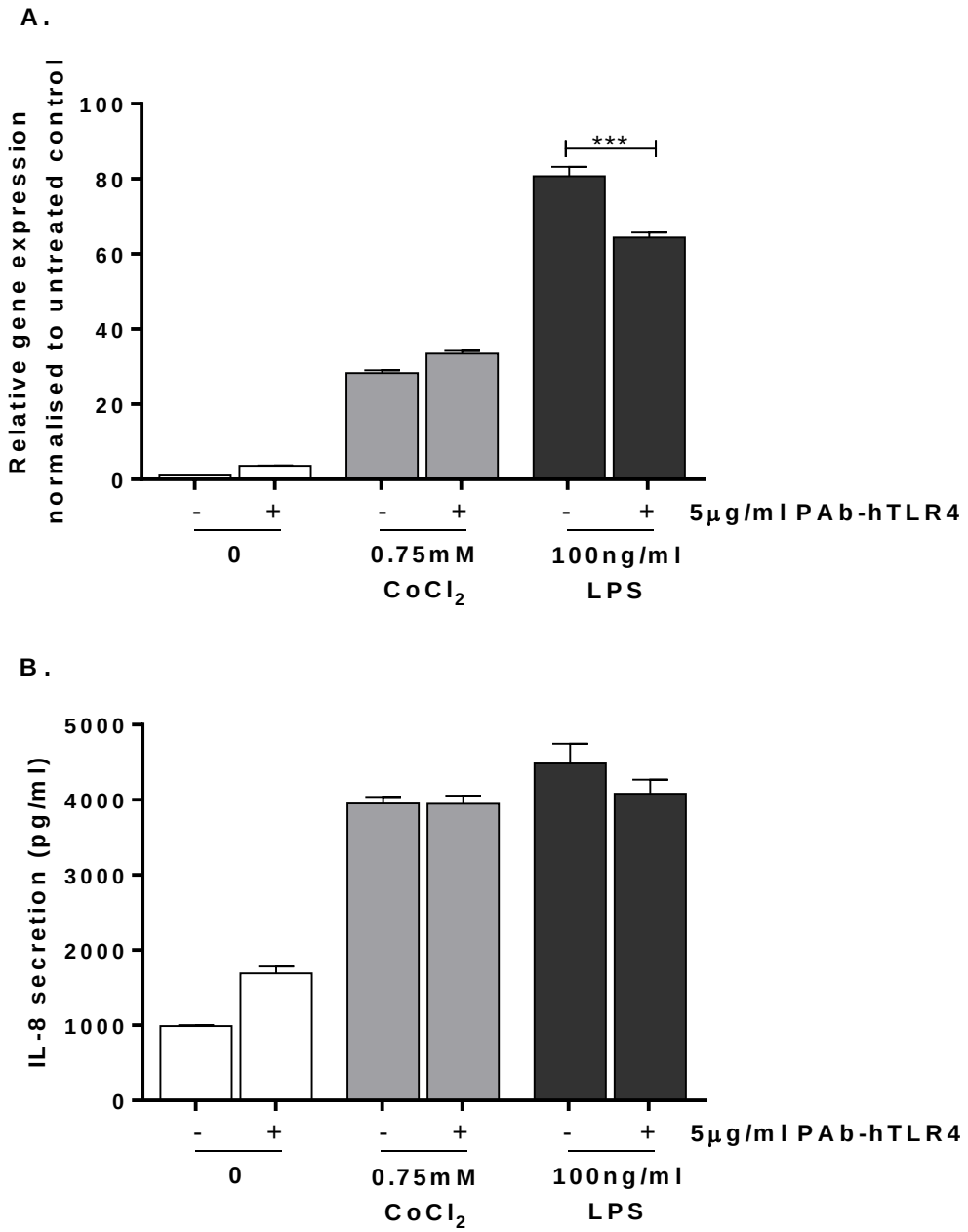


Figure 4.11: Effect of PAb-hTLR4 on IL-8 gene expression and protein secretion
MonoMac 6 cells were pre-treated with 5µg/ml PAb-hTLR4 for 10 minutes before 16h stimulation with 0.75mM $CoCl_2$ or 100ng/ml LPS. RNA was isolated and supernatant collected. **A.** cDNA was synthesised by reverse transcription and *IL8* expression measured by qRT-PCR. **B.** IL-8 secretion was quantified by ELISA. Data is representative of three independent experiments and statistical significance was calculated by one-way ANOVA with Tukey's test for multiple comparisons comparing all samples to each other. These experiments were conducted in collaboration with MRes student Amy Mawdesley and are published in Lawrence *et al* (Lawrence et al., 2016).

4.3.14. Optimisation of MAb2-hTLR4

MAb2-hTLR4 is also reported to inhibit activation of TLR4 (Scarpa et al., 2015). However as with PAb-hTLR4 this required optimisation in MonoMac 6 cells prior to determining the antibody's effect on cobalt-mediated activation. MonoMac 6 cells were incubated with 10µg/ml MAb2-hTLR4 for 16h and cytotoxicity was assessed by trypan blue staining and automated cell counting **(Figure 4.12)**. Viability was normalised to 100% for untreated cells. Cells treated with MAb2-hTLR4 were also found to be 100% viable, showing that the monoclonal antibody does not cause cytotoxicity (**Figure 4.12**).

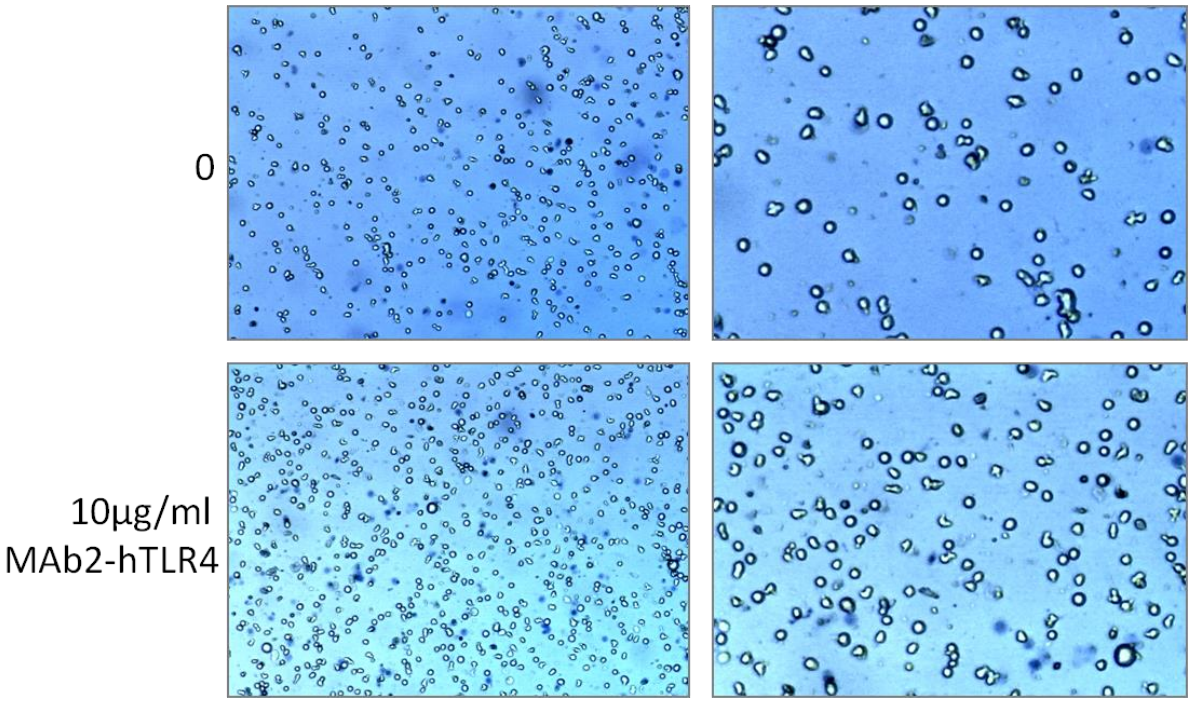


Figure 4.12: Effect of MAb2-hTLR4 on MonoMac 6 cell viability
MonoMac 6 cells were incubated with 10µg/ml MAb2-hTLR4 for 16h and cell viability was measured using trypan blue staining and an automated cell counter. Viability was 100% for untreated cells and 100% for those incubated with the antibody. This experiment was conducted in collaboration with MRes student Amy Mawdesley and is published in Lawrence *et al* (Lawrence et al., 2016).

The ability of MAb2-hTLR4 to inhibit LPS-induced *IL8* expression was investigated to determine the optimal antibody concentration for further assays. MonoMac 6 cells were pre-incubated with 0.1-10μg/ml MAb2-hTLR4 for 1h prior to 16h stimulation with 100ng/ml LPS. Untreated cells were incubated with 10μg/ml MAb2-hTLR4 as a negative control. RNA was isolated and cDNA synthesised by reverse transcription before *IL8* gene expression was quantified by qRT-PCR. There was a significant upregulation in *IL8* expression following LPS stimulation for 16h ($p<0.001$) **(Figure 4.13)**. Pre-incubation with 0.1μg/ml MAb2-hTLR4 did not cause a significant reduction in expression ($p=0.6925$) compared to the no antibody control (**Figure 4.13)**. However 1μg/ml MAb2-hTLR4 caused a significant decrease in LPS-mediated *IL8* expression ($p<0.001$) and this inhibitory effect was even greater following pre-incubation with 10μg/ml MAb2-hTLR4 ($p<0.001$). There was no difference in the response of untreated cells in the presence or absence of the antibody ($p>0.999$) **(Figure 4.13)**. 10μg/ml MAb2-hTLR4 was selected as the optimal concentration for inhibition of LPS-mediated TLR4 activation and this was included as a positive control in subsequent experiments.

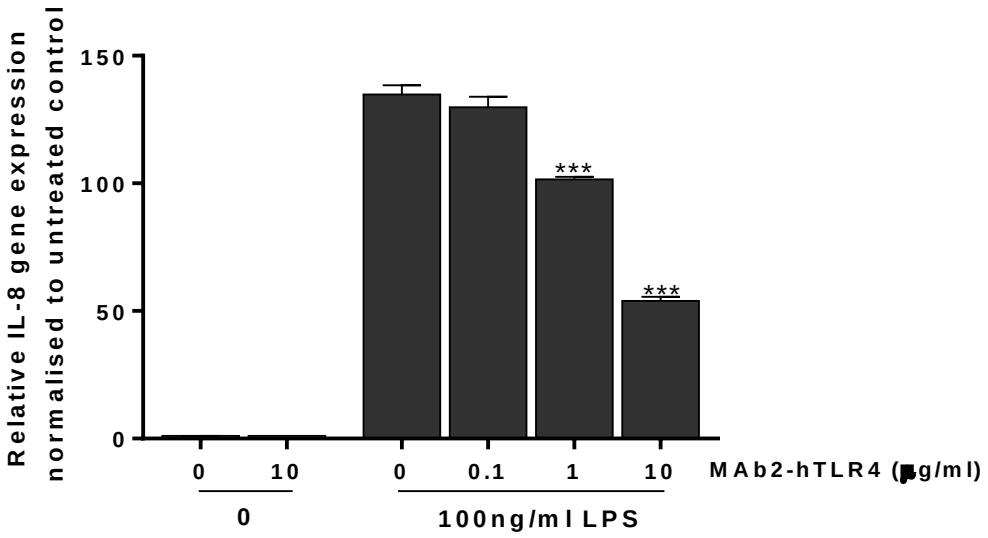


Figure 4.13: Optimisation of MAb2-hTLR4 in LPS-stimulated MonoMac 6 cells
MonoMac 6 cells were pre-treated with 0.1, 1 or 10μg/ml MAb2-hTLR4 for 1h before 16h stimulation with 100ng/ml LPS. An untreated control was also included, with and without the maximum dose of antibody. *IL8* gene expression was quantified by qRT-PCR. Data is representative of three independent experiments and statistical significance was calculated by one-way ANOVA with Tukey's test for multiple comparisons comparing all samples to each other. These experiments were conducted in collaboration with MRes student Amy Mawdesley.

4.3.15. Effect of MAb2-hTLR4 on cobalt-mediated IL-8 responses

Following optimisation of MAb2-hTLR4 with LPS, the ability of the antibody to inhibit cobalt activation of TLR4 was assessed. 0.1µg/ml MAb2-hTLR4 was excluded from further assays as it had no inhibitory effect on LPS, but 1µg/ml and 10µg/ml were both included to investigate their effect on $CoCl_2$. MonoMac 6 cells were pre-incubated with 1 or 10µg/ml MAb2-hTLR4 for 1h followed by 16h stimulation with 0.75mM $CoCl_2$. Positive control cells were pre-treated with 10µg/ml MAb2-hTLR4 and 100ng/ml LPS. RNA was isolated and cDNA synthesised for qRT-PCR while supernatant was collected for ELISA.

IL8 gene expression was upregulated following cobalt stimulation and this effect was significantly inhibited by 1µg/ml and 10µg/ml MAb2-hTLR4 (both $p<0.001$) **(Figure 4.14A)**. The most effective inhibition occurred with 10µg/ml MAb2-hTLR4, reducing the $CoCl_2$-induced *IL8* expression change from 20-fold to 5-fold. IL-8 ELISA analysis revealed the same pattern as that of gene expression; there was a significant reduction in $CoCl_2$-induced IL-8 secretion with both concentrations of MAb2-hTLR4 ($p=0.0375$ for 1µg/ml and $p<0.001$ for 10µg/ml) **(Figure 4.14B)**. Inhibition was dose-dependent, with the greatest effect observed at 10µg/ml MAb2-hTLR4.

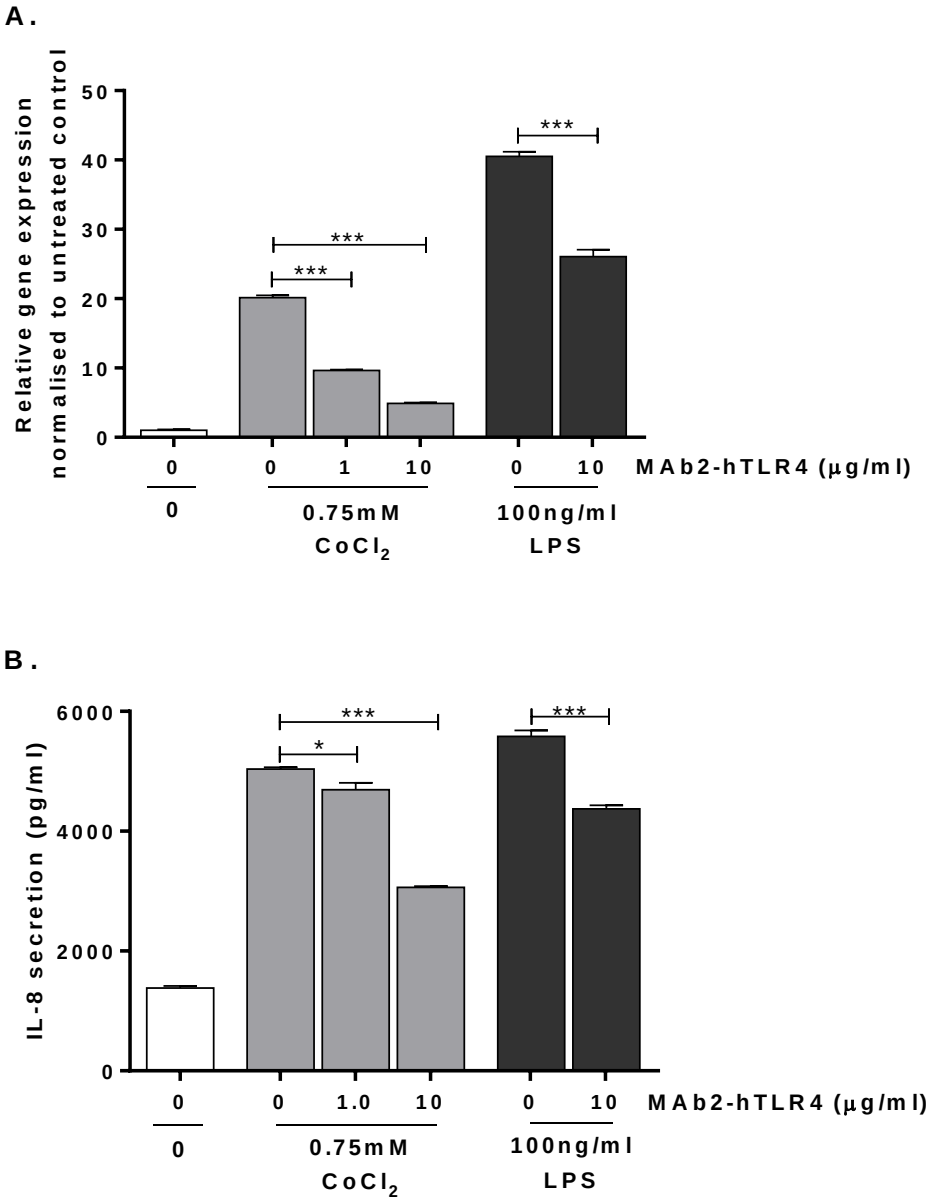


Figure 4.14: Effect of MAb2-hTLR4 on IL-8 gene expression and protein secretion
MonoMac 6 cells were pre-treated with a range of MAb2-hTLR4 concentrations for 1h and then stimulated with either 0.75mM $CoCl_2$ or 100ng/ml LPS for 16h. RNA and supernatant were collected for IL-8 analysis. **A.** cDNA was synthesised by reverse transcription and *IL8* expression was quantified by qRT-PCR. **B.** IL-8 secretion was measured by ELISA. Data is representative of three independent experiments and statistical significance was calculated by one-way ANOVA with Tukey's multiple comparisons test comparing all samples to each other. These experiments were conducted in collaboration with MRes student Amy Mawdesley and are published in Lawrence *et al* (Lawrence et al., 2016).

4.3.16. Effect of PAb-hTLR4 on inflammatory gene expression

The qRT-PCR arrays described in chapter 3 showed that as well as *IL8* gene expression changes, cobalt ions increase expression of other cytokines and chemokines such as *HSPA1A*, *IL1A* and *CCL20*. To determine whether or not the inability of PAb-hTLR4 to inhibit cobalt responses was specific to IL-8, the antibody's effect on these genes was assessed. As described previously, MonoMac 6 cells were pre-incubated with 5µg/ml PAb-hTLR4 for 10 minutes before 16h stimulation with 0.75mM $CoCl_2$ or 100ng/ml LPS. qRT-PCR was performed to determine whether or not PAb-hTLR4 and MAb2-hTLR4 could inhibit cobalt-mediated upregulation of *CCL20*, *IL1A* and *HSPA1A*.

CCL20 expression was significantly upregulated in response to both $CoCl_2$ and LPS (both $p<0.001$) (**Figure 4.15A**). The effect of LPS was much larger than that of $CoCl_2$ (40-fold compared to 10-fold). PAb-hTLR4 pre-treatment significantly decreased the change in *CCL20* expression induced by $CoCl_2$ and LPS ($p<0.001$). *IL1A* expression was also upregulated by $CoCl_2$ and LPS, although only the LPS response was statistically significant ($p=0.1021$ for $CoCl_2$ and $p<0.001$ for LPS) (**Figure 4.15B**). PAb-hTLR4 significantly inhibited LPS-mediated *IL1A* expression ($p=0.0044$) but had no effect on the response to $CoCl_2$ ($p=0.8246$). There was no change in *HSPA1A* expression in response to LPS ($p=0.9979$) **(Figure 4.15C)**. *HSPA1A* expression was increased 10-fold in $CoCl_2$-stimulated cells ($p<0.001$) but there was no change in response to LPS (**Figure 4.15C**). PAb-hTLR4 pre-treatment caused upregulation of *HSPA1A* expression in MonoMac 6 cells stimulated with $CoCl_2$ ($p<0.001$).

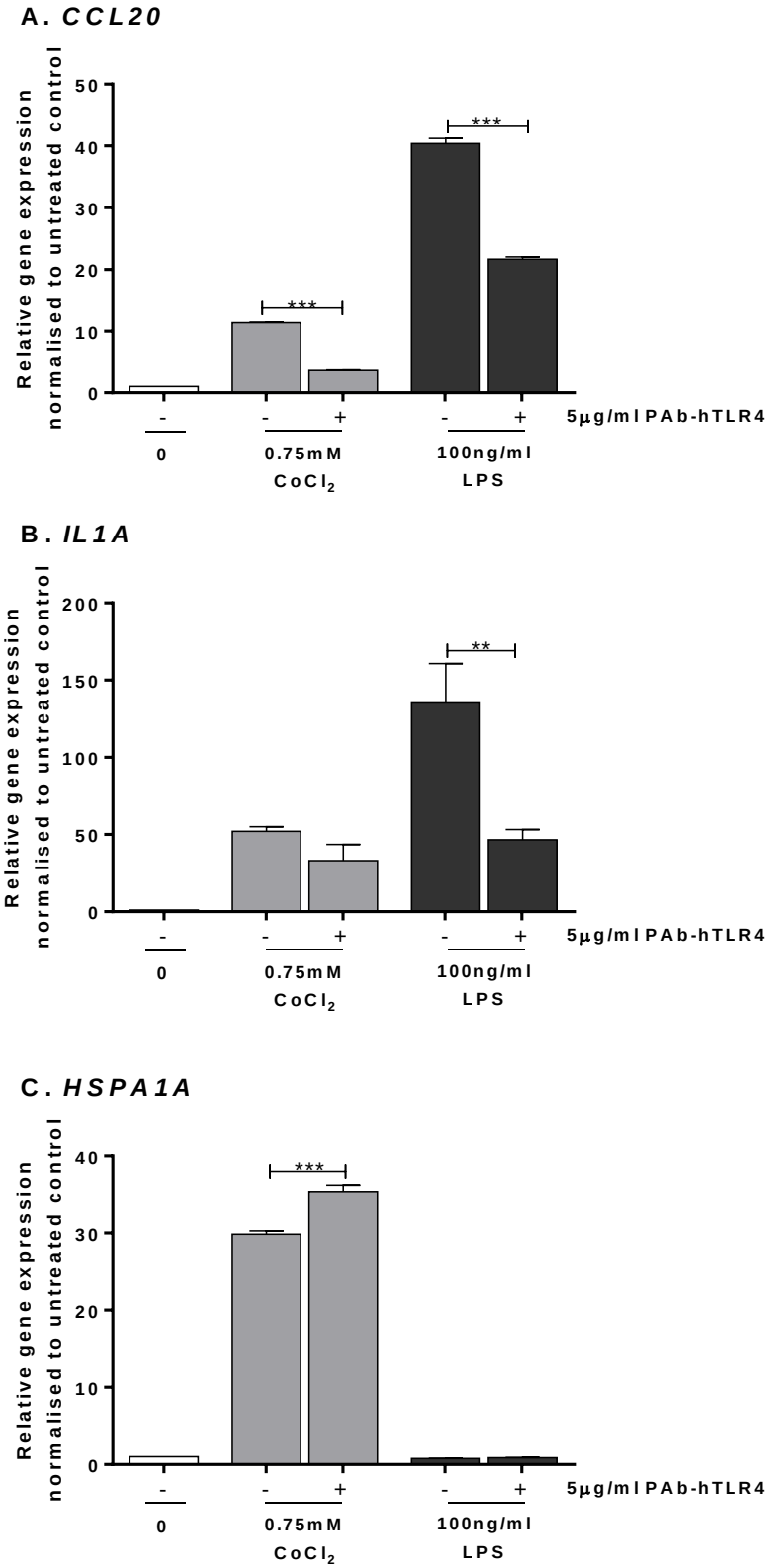


Figure 4.15: Effect of PAb-hTLR4 on inflammatory gene expression
MonoMac 6 cells were pre-incubated with 5µg/ml PAb-hTLR4 for 10 minutes before stimulation with 0.75mM $CoCl_2$ or 100ng/ml LPS for 16h. RNA was isolated and cDNA synthesised by reverse transcription. **A.** *CCL20* **B.** *IL1A* and **C.** *HSPA1A* expression was quantified by qRT-PCR. Data is representative of three independent experiments and statistical significance was calculated by one-way ANOVA with Tukey's test for multiple comparisons comparing all samples to each other. *This data appears in Lawrence* et al (Lawrence et al., 2016).

4.3.17. Effect of MAb2-hTLR4 on inflammatory gene expression

The effect of MAb2-hTLR4 on target genes *CCL20, IL1A* and *HSPA1A* was assessed. As previously, MonoMac 6 cells were pre-treated with 10μg/ml MAb2-hTLR4 for 1h before 16h stimulation with 0.75mM $CoCl_2$ or 100ng/ml LPS. Analysis by qRT-PCR revealed that LPS induces a very large 130-fold increase in *CCL20* expression **(Figure 4.16A)**, which was significantly reduced by MAb2-hTLR4 ($p<0.001$). The size of the fold change masks the change induced by cobalt and therefore **Figure 4.16B** shows the effect of cobalt alone. There was a 10-fold increase in *CCL20* expression following $CoCl_2$ stimulation and this effect was significantly inhibited by MAb2-hTLR4 ($p<0.001$). Similarly to *CCL20*, LPS and $CoCl_2$ caused upregulation of *IL1A* expression (both $p<0.001$), although the fold changes were more similar than for CCL20 **(Figure 4.16C)**. MAb2-hTLR4 abrogated this increase in the case of both ligands (both $p<0.001$); the observed inhibition was greater for LPS than for $CoCl_2$. Surprisingly, *HSPA1A* expression increased in the presence of the antibody in $CoCl_2$-stimulated cells. LPS did not increase *HSPA1A* expression in cells with or without MAb2-hTLR4 ($p<0.001$) **(Figure 4.16D)**.

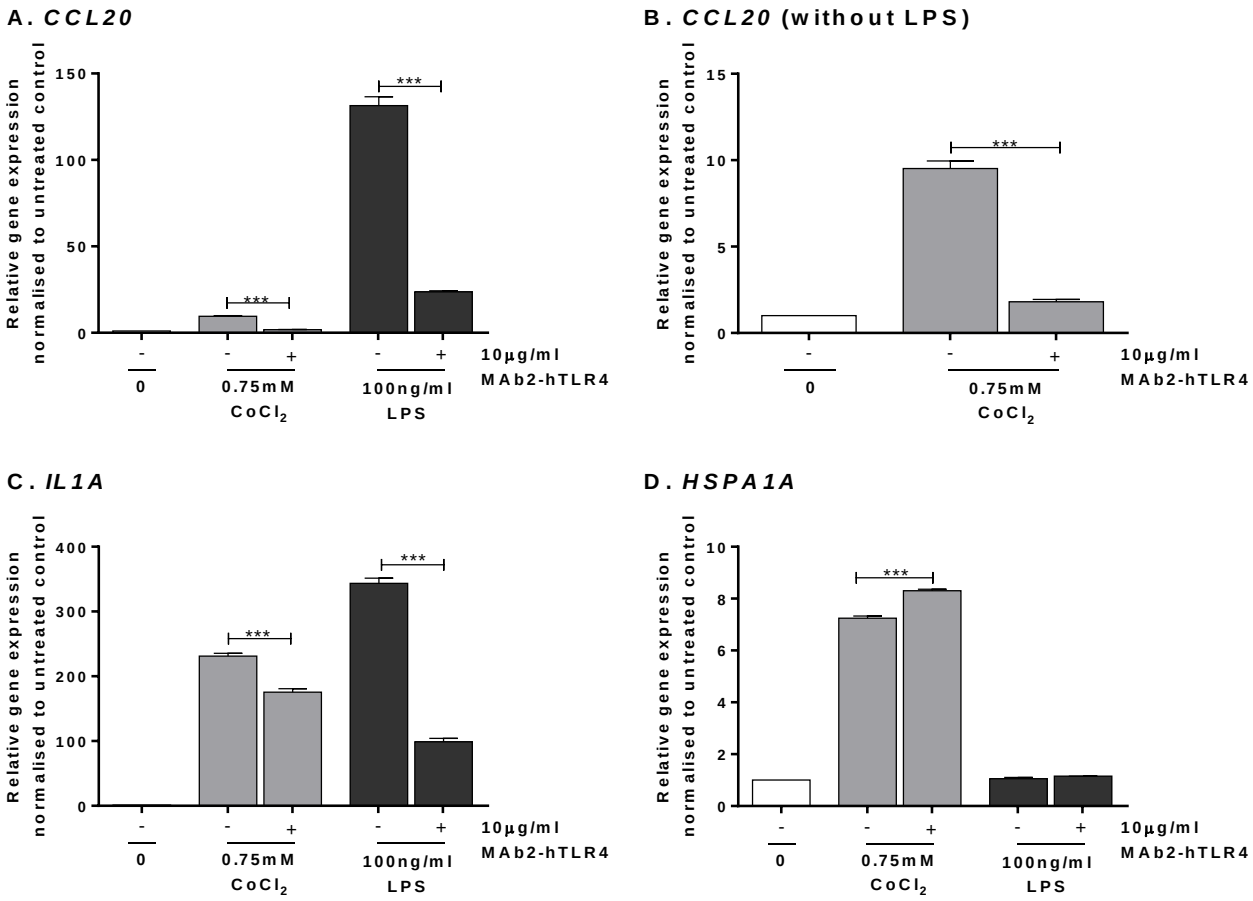


Figure 4.16: Effect of MAb2-hTLR4 on inflammatory gene expression
MonoMac 6 cells were pre-treated with 10µg/ml MAb2-hTLR4 for 1h before 16h stimulation with 0.75mM $CoCl_2$ or 100ng/ml LPS. RNA was isolated and cDNA synthesised by reverse transcription. qRT-PCR was performed to assess expression of **A&B.** *CCL20*, **C.** *IL1A*, and **D.** *HSPA1A.* Data is representative of three independent experiments and statistical significance was calculated by one-way ANOVA with Tukey's test for multiple comparisons comparing all samples to each other. *This data appears in Lawrence* et al (Lawrence et al., 2016).

4.4. Discussion

Selection of cobalt concentrations

Studies into the inflammatory effects of cobalt ions differ considerably in the concentrations used for cell stimulation. This is likely to be a reflection of the broad range of metal ion levels detected in patient samples used in clinical investigations, which can vary significantly even within the same study (Rodríguez De La Flor et al., 2013). In some cases this is due to different tissues being investigated, for example synovial fluid cobalt levels are generally higher than those in serum because wear debris is released directly into the synovial fluid and tissues (Lass et al., 2014). However cobalt concentrations can also vary greatly from patient to patient making it difficult to define a clinically-relevant concentration. For example Macnair *et al* measured serum cobalt concentrations and found a range of 0.7-60.6µg/L (MacNair et al., 2013) whilst Newton *et al* detected cobalt concentrations from 2-10,759nmol/L in urine (Newton et al., 2012). The range of concentrations selected for the present study were chosen following consideration of the first study investigating nickel activation of TLR4 which used 1.5mM $NiCl_2$ as the optimal concentration (Schmidt et al., 2010). This is a very high concentration when considering the results of most clinical studies and therefore 0.25-1mM $CoCl_2$ was selected as an appropriate range when considering clinical concentrations, together with previous *in vitro* studies using cobalt ions (Raghavan et al., 2012, Ninomiya et al., 2013).

Increased IL-8 secretion by MonoMac 6 cells was observed across all $CoCl_2$ concentrations, suggesting that cobalt ions can be inflammatory even at lower levels. There was also an upregulation in *IL8* gene expression by cobalt-stimulated MonoMac 6 cells, with peak expression occurring at 0.75mM $CoCl_2$. The effect on IL-8 release was validated in two other cell lines relevant to this study; THP-1 monocytes and U2OS osteoblasts. Both cell lines increased IL-8 secretion following $CoCl_2$ and LPS stimulation, showing the potential of cobalt to have widespread inflammatory effects. In all three cell lines studied 100ng/ml TLR4-specific LPS also caused increased IL-8 secretion (and gene expression in the case of MonoMac 6 cells), indicating that it is effective as a positive control for TLR4-dependent IL-8 secretion.

Total IL-8 levels released by stimulated THP-1 and U2OS cells were lower than those secreted by MonoMac 6 macrophages, and therefore MonoMac 6 cells were used as a model in further assays. MonoMac 6 cells are also a useful *in vitro* model for this clinical scenario because macrophages are resident in the synovial fluid that lubricates joint implants and are consequently some of the first cells to encounter metal debris and ions released from the bearing surfaces.

Given that maximal *IL8* gene expression occurred at 0.75mM $CoCl_2$ and there was little difference in the IL-8 release between 0.75mM and 1mM $CoCl_2$, 0.75mM was selected as the optimal cobalt concentration for further experiments. MonoMac 6, THP-1 and U2OS cells all released similar amounts of IL-8 when treated with 0.75mM $CoCl_2$ as when they were stimulated with 100ng/ml LPS which further supports the use of 0.75mM $CoCl_2$ as an optimal concentration, and LPS as a positive control.

Cytotoxicity of cobalt ions

Cobalt can cause cytotoxicity although previous studies have generated variable and conflicting results in this regard. Some have demonstrated no toxicity even with 1.5mM $CoCl_2$ (Raghavan et al., 2012) while others have found 0.1mM $CoCl_2$ to be toxic (Kanaji et al., 2014). It appears that cobalt-induced cytotoxicity is dependent on both cell type and the form of cobalt used (i.e. ions, nanoparticles, CoCrMo debris etc). To date no other studies have used MonoMac 6 cells to investigate the cellular response to cobalt ions. Consequently before any further assays were conducted the cytotoxic effect of $CoCl_2$ on MonoMac 6 cells was investigated. The optimal concentration of 0.75mM $CoCl_2$ was found to reduce cell viability from 100% (normalised value) in untreated cells to approximately 75% following 24h stimulation. Cytotoxicity is clearly a factor to consider in subsequent assays because it can influence cell responses but because 0.75mM $CoCl_2$ was optimised based on previous studies and *in vivo* levels, it was decided to proceed with this concentration.

Cytokine and chemokine expression and secretion

Murine *Cxcl1* expression was significantly increased by LPS stimulation whereas $CoCl_2$ caused only a very small change in expression. In MonoMac 6 cells IL-8 expression and secretion was comparable between $CoCl_2$ and LPS stimulation, suggesting that the effect in murine J774 cells is a TLR4-independent inflammatory response to $CoCl_2$. This result supports previous studies showing that cobalt ions do not activate murine Tlr4 (Raghavan et al., 2012, Tyson-Capper et al., 2013a) and highlights the importance of focusing on human TLR4 for further work.

This study clearly shows that cobalt can induce IL-8 release by MonoMac 6 macrophages and other cell lines. It was important to establish the role of TLR4 in the MonoMac 6 cell response to cobalt ions and this was done in the first instance using CLI-095, a small molecule TLR4 antagonist. CLI-095 has already been widely used in the prevention of inflammatory responses to LPS (Glushkova et al., 2013, Hussey et al., 2013) and therefore optimisation was performed using 0.75mM $CoCl_2$. This revealed that CLI-095 dose-dependently inhibits cobalt-mediated IL-8 secretion in MonoMac 6 cells and suggests a central role for TLR4 in the response. Similar

inhibitory effects were observed in *IL8* expression, indicating that cobalt-mediated gene expression changes are also TLR4-dependent. CLI-095 inhibited the majority of IL-8 secretion following cobalt stimulation and also prevented LPS-mediated responses, showing that LPS is a good positive control for both receptor activation and inhibition. The Proteome Profiler arrays described in chapter 3 showed a small increase in CXCL10 secretion by cobalt-stimulated MonoMac 6 cells. The use of CLI-095 demonstrates that TLR4 is essential to CXCL10 secretion in this case because pre-treatment with the inhibitor decreased CXCL10 release to that of untreated MonoMac 6 cells.

As CLI-095 prevents the recruitment of all adaptor proteins to activated TLR4 it is not possible to identify which of these is responsible for the observed responses. However the cytokines released may provide insight into the signalling pathways that are activated by cobalt ions. As described in **section 1.6.1**, TLR4 activation by LPS results in the recruitment of adaptor proteins including MyD88 and TRIF to the intracellular domain of the receptor. Each adaptor protein signals via a different pathway, resulting in activation of different transcription factors and the production of diverse inflammatory cytokines and chemokines. Secretion of IL-8 following TLR4 activation is thought to be MyD88-dependent as mutation of MyD88 abrogates the LPS-induced IL-8 response (He et al., 2013a). CXCL10 release can occur when the TRIF-dependent pathway is activated (Weighardt et al., 2004). TLR4-dependent secretion of both IL-8 and CXCL10 following cobalt stimulation therefore suggests that cobalt ions can activate both the MyD88 and TRIF-dependent arms of TLR4 signalling. Some caution is required here as CXCL10 can also be regulated by NFκB which in turn is modulated by MyD88 pathway activation. However a recent study has supported a role for the TRIF pathway in cobalt-mediated inflammatory responses, demonstrating CXCL10 promoter activity following cobalt stimulation (Oblak et al., 2015), and others have previously suggested involvement of the MyD88-dependent pathway (Potnis et al., 2013).

Untreated control cells with or without CLI-095 were included in all assays. This revealed a small but significant downregulation in CXCL10 secretion in the presence of the inhibitor. A similar effect was observed with IL-8 secretion and gene expression but this was not statistically significant. The effect may be due to release of endogenous TLR4 ligands such as HMGB1 by the cells, or a very low level of endotoxin contamination that activates TLR4. However the reduction in cytokine secretion may also be the result of cytotoxicity. The effect of the antagonist on cell viability was assessed because it is reconstituted in DMSO, a known cytotoxic agent. Treatment of MonoMac 6 cells with 10µg/ml CLI-095 reduced cell viability from 100% to approximately 80%. This is unlikely to be entirely responsible for the changes in

cobalt-mediated IL-8 and CXCL10 secretion because they are much larger changes than those observed in cell viability. However as cobalt can also cause toxicity the concentration of CLI-095 was revised to 1µg/ml, which was found to inhibit IL-8 secretion without affecting cell viability and is in line with concentrations used in previous studies (Glushkova et al., 2013).

The optimised concentration of CLI-095 was used to investigate the effect of cobalt ions on two additional inflammatory cytokines, CCL20 and IL-6. CCL20 was chosen for this aspect of the study because the qRT-PCR arrays showed that it was significantly upregulated by cobalt ions at gene level, and expression levels were high even at baseline, indicating that protein secretion would be detectable by ELISA. IL-6 was selected because there was no evidence of upregulation following cobalt stimulation in any of the arrays described in chapter 3 but there was an increase in response to LPS. There was a significant increase in CCL20 secretion by MonoMac 6 cells stimulated with $CoCl_2$ or LPS. Pre-treatment with CLI-095 significantly decreased this effect suggesting that chemokine release is a result of TLR4 activation by cobalt ions. IL-6 secretion was also significantly upregulated by LPS but there was only a very small increase in response to cobalt ions. These low levels of IL-6 may also have been outside the detection range of the Proteome Profiler arrays in chapter 3 which would account for the lack of changes in secretion. As with IL-8, CXCL10 and CCL20, IL-6 release was prevented by CLI-095 (although not statistically significant), showing that even this small change in cytokine secretion is dependent on TLR4 activation.

Cytokine and chemokine function: clinical significance in ARMD

The cytokines and chemokines released following cobalt activation of TLR4 could potentially contribute to adverse reactions to metal debris through their immunological functions. IL-8 is a pro-inflammatory chemokine secreted in response to cellular stress and damage, pathogen phagocytosis and PRR activation. It is particularly chemotactic for neutrophils (Baggiolini et al., 1989) via activation of CXCR1 and CXCR2 receptors on target cells. IL-8 is also an angiogenic factor, promoting blood vessel formation through activation of CXCR2 (Heidemann et al., 2003). In a study by Ma *et al* using an endothelial cell and osteoblast co-culture system, TLR4 activation by LPS caused significant upregulation of IL-6 and IL-8 secretion, along with pro-angiogenic factors ICAM-1 and E-selectin (Ma et al., 2015). These changes were associated with increased microvessel formation (Ma et al., 2015). This is of importance to the present study because one of the leading indications for MoM implant failure is inflammatory pseudotumour growth, a process that requires vascularisation. It is therefore possible that cobalt/TLR4-mediated IL-8 secretion could promote angiogenesis and aid pseudotumour formation.

Another characteristic of ARMD is aseptic loosening of the joint implant caused by peri-prosthetic osteolysis. Osteolysis is driven at least in part by inflammatory cytokines that promote the formation of osteoclasts (bone-resorbing cells) and prevent osteoblast activity. Bone metastasis in breast cancer requires osteolysis, and serum IL-8 levels correlate with the degree of metastasis in breast cancer patients (Bendre et al., 2002, Kamalakar et al., 2014). Furthermore, IL-8 stimulated the differentiation of peripheral blood mononuclear cells into osteoclasts and an anti-IL-8 antibody was able to prevent tumour osteolysis in an *in vivo* murine model (Kamalakar et al., 2014). Both IL-8 and CCL20 have been shown to have an indirect effect on osteoclast formation by increasing osteoblast secretion of IL-6 which in turn promotes osteoclastogenesis (Pathak et al., 2015). IL-8 levels in peri-implant tissues also correlate with incidences of aseptic loosening (Lassus et al., 2000, Tanaka et al., 2005). Effects such as these are associated with CXCR1 and CXCR2 expression levels on IL-8 target cells (Bendre et al., 2003) and cause variability from patient to patient. IL-8 therefore appears to play a key role in osteolysis and it could be speculated that if MoM patient cells secrete IL-8 in response to cobalt, an IL-8-mediated osteolytic response could develop, with the degree of osteolysis determined by CXCR1 and CXCR2 expression levels.

Patients with ARMD (and specifically ALVAL) have elevated CXCL10 levels in their synovial fluid compared to those with a metal-on-polyethylene implant (Kolatat et al., 2015). Dapunt *et al* also showed the presence of CXCL10 in tissues from aseptically loosened MoM implants and this was associated with a small population of T lymphocytes (Dapunt et al., 2014). It is conceivable that TLR4-dependent CXCL10 secretion contributes to the lymphocyte infiltration around MoM implants, providing a link between innate and adaptive immune responses. CXCL10 can be produced by bone and in a murine model it recruits cancer cells that promote bone metastasis (Lee et al., 2012). The same study found that CXCL10 acts by increasing RANKL expression without altering OPG, which drives osteoclastogenesis (Lee et al., 2012). The effect was inhibited by *Tlr4* knockdown indicating that the receptor is essential to the response. This suggests that TLR4 activation (e.g. by cobalt ions) may contribute to CXCL10-induced osteoclastogenesis and osteolysis.

CCL20 is a lymphocyte chemokine and promotes pro-inflammatory responses including B lymphocyte recruitment (Meissner et al., 2003). CCL20 is of interest to the present study because ALVAL, one of the features of ARMD, is characterised by lymphocyte infiltration of peri-implant tissues (Watters et al., 2010). If resident tissues macrophages secrete CCL20 it may contribute to the cellular infiltrate around failed MoM hip replacements by recruiting lymphocytes. Interestingly, CCL20 has been implicated in osteolysis as expression of CCL20

and CCR6 is elevated in leukaemia patients with osteolytic lesions compared to those with no bone breakdown (Giuliani et al., 2008). As a result CCL20 has been suggested as a biomarker of osteolysis (Palmer et al., 2015).

The immunobiology of IL-6 is complex and there is often conflicting information regarding its potential inflammatory effects. For example Yoshitake *et al* showed inhibition of osteoclast formation by IL-6 (Yoshitake et al., 2008), while others have suggested that IL-6 promotes osteoclastogenesis (Kudo et al., 2003) and inhibition of the IL-6 receptor can prevent bone loss (Axmann et al., 2009). Of interest to this study is the ability of IL-6 to promote vascular endothelial growth factor (VEGF) expression; VEGF in turn leads to increased angiogenesis which aids tumour (and pseudotumour) formation (Huang et al., 2004). IL-6 is associated with ARMD as significantly higher concentrations were found in patients experiencing MoM implant failure compared to those with failed ceramic-on-polyethyleneimplants (Singh et al., 2015).

Anti-TLR4 neutralising antibodies

Using IL-8 expression and secretion as a marker of TLR4 activation, the ability of anti-TLR4 neutralising antibodies to prevent receptor activation by cobalt ions was assessed. A monoclonal anti-TLR4 neutralising antibody, MAb2-hTLR4, inhibited cobalt-mediated IL-8 expression and secretion as well as expression of *CCL20* and *IL1A*. In contrast a polyclonal anti-TLR4 neutralising antibody, PAb-hTLR4, failed to inhibit IL-8 secretion following either $CoCl_2$ or LPS stimulation, while gene expression was marginally inhibited in LPS-stimulated cells. The polyclonal antibody inhibited *CCL20* and *IL1A* expression following LPS treatment but in the case of cobalt it could only inhibit *CCL20*. With both MAb2-hTLR4 and PAb-hTLR4 cobalt-mediated *HSPA1A* expression was further increased in the presence of the antibody.

It is surprising that PAb-hTLR4 could not effectively inhibit cobalt-mediated inflammatory responses to the same extent as MAb2-hTLR4 because both antibodies are reported to inhibit TLR4 activation (Ekaney et al., 2014, Lima et al., 2015). The differences in efficacy are likely to arise through different antibody binding sites. As PAb-hTLR4 inhibited LPS activation better than $CoCl_2$ it may bind to sites including those involved in the transfer of LPS from LBP to TLR4 itself. Cobalt binds directly to the receptor and consequently LBP is not required for TLR4 activation. The use of neutralising antibodies in this study supports the current understanding that cobalt and LPS bind and activate TLR4 by different mechanisms (Raghavan et al., 2012).

Another possible explanation for the lack of IL-8 and *IL1A* inhibition by PAb-hTLR4 is the presence of other TLR4 ligands such as Hsp70, as shown in the qRT-PCR arrays in chapter 3. It may be that cobalt ions cause increased secretion of inflammatory factors capable of activating TLR4 which feed back and perpetuate receptor activation. If these ligands bind to a site not targeted by PAb-hTLR4 they may be capable of promoting cellular responses even in the presence of the neutralising antibody. A third explanation could be that the increases in IL-8 secretion following cobalt and LPS stimulation are too strong for the antibody to inhibit. Finally, as IL-1α is released during cell stress its secretion may not be entirely TLR4-dependent and may be the result of generalised inflammation.

HSPA1A expression was increased in response to cobalt ions and this could not be prevented with either of the anti-TLR4 neutralising antibodies, indicating that it is not a TLR4-dependent effect. This is supported by the lack of change in *HSPA1A* expression following LPS stimulation; in all other assays LPS has been an effective positive control for TLR4 activation and inhibition by antagonists. Previous studies have shown that LPS can induce Hsp70 (encoded by *HSPA1A*) but this appears to require synergy with other TLRs and their ligands, as well as specific physiological factors such as increased temperatures (Gupta et al., 2013, Tulapurkar et al., 2015). The elevated *HSPA1A* expression detected in the present study may well be a result of the cytotoxicity also described in this chapter as heat shock proteins are released during cell stress responses. *HSPA1A* expression increased further with both MAb2-hTLR4 and PAb-hTLR4 pre-incubation and it is possible that some of the TLR4-dependent cytokines released have a protective effect on the cells which is abolished by TLR4 inhibition. However additional assays would be required to validate this theory.

CLI-095, MAb2-hTLR4 and PAb-hTLR4 were all selected for use in this study because they have previously been shown to inhibit inflammatory responses to TLR4 ligands. In addition, TLR4 is an attractive therapeutic target because it is at the start of a cascade of intra- and intercellular signalling that may be implicated in ARMD. Its cell surface expression also makes it a more accessible target, as well as its involvement in a broad range of inflammatory response. Studies have highlighted the potential of targeting TLR4 in treatments for Gram negative septic shock, cardiovascular disease, and rheumatoid arthritis (Jia et al., 2014, Lima et al., 2015, Monnet et al., 2015). In the present study the inhibitor and antibodies' blockade of LPS-mediated TLR4 activation provided a good positive control when investigating their potential to inhibit cobalt activation of the receptor, but their therapeutic potential in ARMD requires careful consideration. For a TLR4 antibody to be a therapeutic option in the prevention of ARMD it must be capable of inhibiting cobalt activation of TLR4 without affecting a patient's

ability to respond to LPS via the receptor; this is essential for mounting an immune response against Gram negative bacteria, as well as other PAMPs and DAMPs. As the LPS and cobalt binding sites within TLR4 are different, an antibody that specifically targets the histidine pocket may prevent cobalt (and nickel) activation of TLR4 while still conferring LPS responsiveness. The antibodies used in this study partially prevented LPS activation of TLR4 and therefore would not be suitable for ARMD prevention as they would block inflammatory responses to Gram negative bacteria as well as cobalt ions. However they clearly show that anti-TLR4 neutralising antibodies have potential in ARMD therapeutics. There are a number of TLR4 antibodies in clinical trials for inflammatory diseases, including NovImmune's NI-0101, which has successfully completed Phase I for use in rheumatoid arthritis (Monnet et al., 2015) showing that TLR4 is a realistic clinical target despite its importance in the immune response to bacteria.

Overall it is clear that cobalt activation of TLR4 results in increased secretion and expression of pro-inflammatory cytokines and chemokines. These factors (e.g. IL-6 and IL-8) have all previously been implicated in immune responses such as osteolysis and fibrosis. Persistent cytokine release appears central to the development of ARMD as it creates a pro-inflammatory environment that favours osteoclastogenesis and soft tissue necrosis (Gallo et al., 2013). However the mechanisms that lead to the release of these cytokines is not well understood. Based on the results of this study it is conceivable that the initial stimulus is cobalt-mediated TLR4 activation.

Importance of TLR4 in inflammatory responses to cobalt

From the data presented in this chapter it is clear that TLR4 plays a significant role in the cellular response to cobalt ions as the TLR4 antagonist and anti-TLR4 neutralising antibodies were able to prevent cobalt-mediated inflammatory cytokine secretion and gene expression. This raises the question of whether the immunological properties of cobalt in this *in vitro* cell culture model are entirely dependent on TLR4 activation, or whether other TLR4-independent responses also develop. CLI-095 was able to reduce IL-8 secretion to nearly the same level as in untreated cells suggesting that TLR4 significantly contributes to the inflammatory response.

Many animal studies have been conducted to model the immunological response to wear debris and metal ions, and the majority have shown an inflammatory effect (Saini et al., 2010b, Akbar et al., 2012). Most of these studies use conventional small animal models such as mice and guinea pigs, which do not have the histidine pocket in TLR4 that is present in humans and primates (**Figure 3.1**). As an inflammatory response still develops, TLR4 activation cannot be fully responsible for the observed inflammation and therefore the reaction may be generalised

inflammation, or immune system activation via an as yet unidentified pathway. This also suggests that TLR4 activation alone is not responsible for the inflammatory responses in ARMD but may combine with other stimulatory signals (e.g. antigen presentation) to drive inflammation. The TLR4 signalling pathway itself is also regulated by endogenous and exogenous factors and they may influence the immunological outcome of receptor activation. Some of these factors will be discussed in more detail in chapter 6.

4.4.1. Future work

The data presented in chapter 3 clearly shows that cobalt ions affect the expression of inflammatory genes and TLR-related signalling factors. To further elucidate the role of TLR4 in these responses, the same arrays could be performed using samples from MonoMac 6 cells pre-treated with CLI-095 or the anti-TLR4 neutralising antibodies before cobalt stimulation. This would give an improved overview of which changes are regulated by TLR4, as well as the efficacy of the TLR4 antagonists.

An important aspect of further work in the study is to investigate the functional effects of the cytokines and chemokines secreted in response to cobalt ions. In addition it would be interesting to identify which factors may influence particular aspects of the inflammatory response (e.g. CCL2 is often responsible for macrophage recruitment). As well as providing greater insight into how they may influence ARMD, this would also allow a more targeted approach in the design of therapeutic agents for the inhibition of adverse reactions. As mentioned earlier, an antibody targeting cobalt-mediated responses would need to be carefully designed to ensure that the capacity of TLR4 to respond to LPS is preserved.

The identification and optimisation of IL-8 as a biomarker for TLR4 activation by cobalt ions will be beneficial in further studies, including investigations into the specific downstream signalling factors involved in the response to cobalt. For example, siRNA knockdown of factors like *IRAK2* could be performed, using IL-8 as a biomarker of the effect of cobalt ions. TLR4 can also become tolerant or sensitised to its ligands and IL-8 could be used to assess cell responsiveness in appropriate assays.

4.4.2. Conclusion

The primary aim of this chapter was to use IL-8 as a biomarker of inflammation to investigate the effect of cobalt ions on TLR4 activation and subsequent cellular responses. Treatment with a clinically-relevant range of cobalt concentrations was found to increase IL-8 gene expression and protein secretion by MonoMac 6 cells. This was a TLR4-dependent effect as it was inhibited by CLI-095, a small molecule TLR4 antagonist. Similar patterns were observed in

cobalt-stimulated THP-1 monocytes and U2OS osteoblasts, as well as for *CXCL10* expression, and CCL20 and IL-6 secretion by MonoMac 6 cells. A monoclonal anti-TLR4 neutralising antibody proved effective at inhibiting the majority of $CoCl_2$-induced cytokine expression with the exception of *HSPA1A*. A polyclonal anti-TLR4 antibody was less successful in this regard. LPS provided an effective positive control for TLR4 activation in all experiments.

From the data presented in this chapter it can be concluded that the TLR4 signalling pathway is central to the *in vitro* cellular inflammatory response to cobalt ions. It could therefore contribute to the inflammatory response seen in a proportion of MoM implant patients, perhaps as a co-stimulatory signal alongside other mechanisms of immune activation. As such, TLR4 and its downstream signalling pathway are interesting targets both for further research into inflammatory responses to cobalt ions and as potential therapeutic options in the prevention of ARMD.

Chapter 5. Effect of cobalt ions on immune cell chemotaxis and binding

5.1. Introduction

Data presented in chapter 3 and 4 demonstrates that cobalt ions regulate expression and secretion of inflammatory cytokines and chemokines through direct activation of TLR4. Cytokines and chemokines in turn modulate numerous immunological functions including cell migration, adhesion, invasion, and proliferation. The overall outcome of cytokine signalling is determined by the cytokine profile released in response to a specific stimulus. The cumulative effect of the cobalt-induced cytokine profile is not yet clear.

Histopathological studies have shown elevated immune cell infiltration of tissues around failed MoM hip implants. Whilst there has been considerable focus on ALVAL, the lymphocytic reaction frequently associated with MoM joint replacement failure, many studies have identified an innate immune cell infiltrate composed primarily of monocytes and macrophages but also containing low levels of neutrophils and eosinophils (Pandit et al., 2008, Mahendra et al., 2009). However the stimuli that lead to inflammatory cell recruitment around MoM implants are unknown.

Cell adhesion and migration are critical steps in any inflammatory response and are particularly important for leukocyte extravasation. This is the process by which immune cells adhere to the vascular endothelial layer and then pass through the layer into the surrounding tissues where they can mount an inflammatory response against a pathogen. Extravasation (also known as diapedesis) requires interaction between adhesion molecules expressed on the leukocyte and endothelial cell for effective cell adhesion. Chemokine release is also essential to induce migration of target cells down a carefully coordinated chemokine concentration gradient. In the resting state these processes are tightly regulated to prevent unwanted inflammation but pathogen challenge results in upregulation of adhesion molecule and chemokine expression to drive leukocyte extravasation.

5.1.1. Effect of metal ions and wear debris on cell adhesion

Expression of adhesion molecules including intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin by leukocytes such as macrophages promotes the cell-cell interaction required for effective cellular communication and coordination of inflammation (**section 1.7.3**). This includes involvement in processes like extravasation and vascularisation.

The expression of factors such as ICAM-1 and VCAM-1 is altered in tissues surrounding joint implants; for example ICAM-1 expression is increased in blood vessels near metal and UHMWPE implants (Al-Saffar et al., 1994). The effect of cobalt ions on cell adhesion was first studied in the 1990s by Goebeler *et al* who demonstrated that they upregulate ICAM-1 and VCAM-1 expression in human umbilical vein endothelial cells (HUVEC) (Goebeler et al., 1993). More recently Ninomiya *et al* confirmed these findings and showed that the increase in adhesion molecule expression correlates with increased secretion of IL-8 and CCL2 by endothelial cells exposed to cobalt ions. These were functional changes as cobalt also increased lymphocyte binding to endothelial cells (Ninomiya et al., 2013). Interestingly, chromium, the second component of the CoCr alloy, did not have any effect on adhesion molecule expression and nor did molybdenum in work conducted by Caicedo *et al* (Caicedo et al., 2007, Ninomiya et al., 2013). As well as ions, cobalt nanoparticles have recently been shown to increase ICAM-1 and VCAM-1 expression (Alinovi et al., 2015). Although it is clear from the studies described above that cobalt ions regulate adhesion molecule expression the role of TLR4 in the response is not known.

5.1.2. Effect of metal ions and wear debris on cell migration

One of the main stimuli for cell migration is the secretion of chemokines by cells exposed to a pathogen. Chemokines form a gradient and act via receptors expressed by their target cells (often leukocytes) to guide them to the site of inflammation, where they extravasate to access the tissues and carry out their effector functions for efficient pathogen eradication.

A joint replacement causes an acute inflammatory response when it is first implanted into the body. In most patients this quickly subsides as the implant becomes integrated into the bone and the tissues heal from the initial surgical trauma. In a small number of patients the early acute inflammation is compounded by the presence of wear debris. *In vitro* studies have indicated that titanium-aluminium and cobalt-chromium wear debris can stimulate cells to release pro-inflammatory chemokines including CCL2 (MCP-1) and CCL3 (MIP-1α) (Jones et al., 2007, Kaufman et al., 2008). These chemokines recruit innate immune cells to the site of inflammation. However, as in the case of cell adhesion, the role of cytokines is not clear.

5.2. Objectives

- To identify an appropriate endothelial cell line for the study of cobalt and TLR4-mediated inflammatory responses
- To investigate the role of cobalt and TLR4 in adhesion molecule expression by endothelial cells and macrophages
- To investigate the effect of cobalt activation of TLR4 on primary monocyte and neutrophil migration using a trans-well chemotaxis assay

5.3. Specific materials and methods

5.3.1. Peripheral blood mononuclear cell transwell chemotaxis assay

Human peripheral blood mononuclear cells (PBMC) were isolated from the whole blood of healthy volunteers using Lympholyte-H (Cedarlane, Ontario, Canada) according to the manufacturer's protocol. Isolated PBMC were resuspended in pre-warmed RPMI-1640 medium supplemented with 2mM L-glutamine and 10% v/v FBS.

Conditioned media was generated by stimulating MonoMac 6 cells with $CoCl_2$ or LPS as described in earlier chapters and centrifuging samples to remove cells. A 24-well companion plate (VWR International, Pennsylvania, USA) was blocked with 1% w/v BSA in media for 1h before 800µl conditioned media was added to each well. A cell culture insert with 3µm pores (VWR International) was placed in each well and 500,000 PBMC added to each filter. Cells were incubated at 37ºC and 5% CO_2 for 2 hours to allow migration. Excess media and cells were removed by pipetting and cleaning with a cotton bud. Filters were fixed overnight in ice-cold methanol.

Filters were stained with haematoxylin for 30 minutes and washed in Scott's tap water for 10 minutes followed by sequential rinsing in 50%, 75%, 90% and 100% v/v ethanol (2 minutes each). They were then air-dried for a minimum of 3h, excised, and mounted using DPX mountant. Monocytes adhered to the filter were counted by selecting five different visual fields at 40x magnification. The chemotaxis assay protocol is summarised in **Figure 5.1**.

5.3.2. Neutrophil transwell chemotaxis assay

Neutrophil chemotaxis was performed using neutrophils isolated from healthy volunteers by dextran sedimentation (Dextran T500, Pharmacosmos, Holbaek, Denmark) and centrifugation on Percoll gradients (GE Healthcare, Buckinghamshire, UK) as previously described (Dransfield et al., 1994). Neutrophils were resuspended in Hanks' balanced salt solution (HBSS) supplemented with 10% v/v FBS prior to the assay. Following this step, the chemotaxis assay was conducted as described for PBMC chemotaxis (**section 5.3.1)**

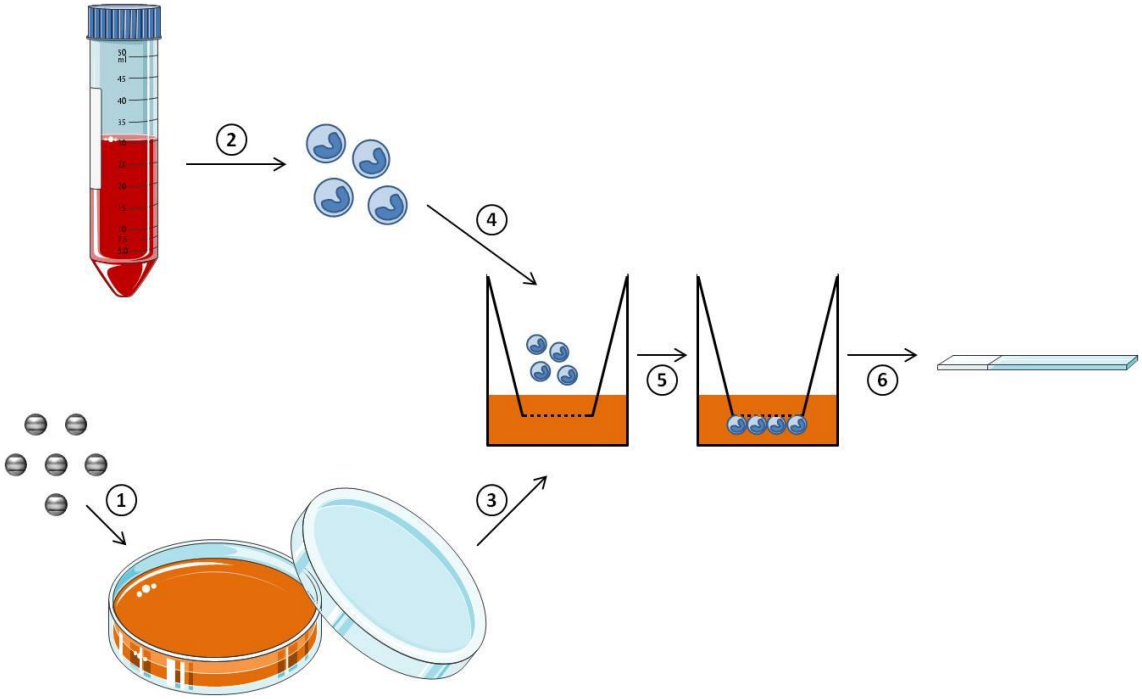


Figure 5.1: Chemotaxis assay protocol overview
1. MonoMac 6 cells in culture stimulated with cobalt ions. **2.** PBMC isolated from whole human blood. **3.** Conditioned media from cultured cells collected, centrifuged to remove cells and 800µl added to one well of a 24-well plate. **4.** Cell culture insert with 3µm filter pores placed in the well and 500,000 PBMC added to the top of the filter. **5.** PBMC incubated at 37ºC and 5% CO_2 for 2h to migrate. Migrated monocytes should adhere to the underside of the filter. **6.** Filters stained with haematoxylin, excised, mounted on slides with DPX mountant, and monocytes counted.

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5.4. Results

5.4.1. Effect of cobalt ions on human umbilical vein endothelial cells

Previous research into the effect of nickel and cobalt ions on adhesion molecule expression has centred on human umbilical vein endothelial cells (HUVEC). As a result pooled HUVEC (PromoCell, Heidelberg, Germany) were assessed for their suitability for this study. One of the main aims of this chapter was to investigate the effect of cobalt-mediated TLR4 activation on adhesion molecule expression. It was therefore important to determine that the endothelial cells were stimulated by cobalt ions and LPS through TLR4 activation. A preliminary experiment was conducted using the metal ion concentrations optimised for MonoMac 6 cells in chapter 4, and IL-8 as a marker of TLR4 activation. HUVEC were stimulated with 0.25-1mM $CoCl_2$ or 100ng/ml LPS for 24h before supernatant was collected and analysed by ELISA. There was no significant difference in IL-8 secretion between untreated cells and those treated with 0.25-0.75mM $CoCl_2$ **(Figure 5.2A)**. 1mM $CoCl_2$ caused a small but significant increase in IL-8 secretion from 550pg/ml to 650pg/ml ($p=0.0245$) while 100ng/ml LPS increased IL-8 levels to 700pg/ml ($p<0.001$) **(Figure 5.2A)**.

A previous study investigating the effect of cobalt on endothelial cells has demonstrated that high cobalt concentrations (up to 4mM) can induce IL-8 secretion by HUVEC (Ninomiya et al., 2013). Consequently HUVEC were treated with 1-4mM $CoCl_2$ to establish whether or not these higher concentrations would have an effect. IL-8 secretion was again measured by ELISA. There was a significant decrease in IL-8 release across all $CoCl_2$ concentrations compared to the untreated control ($p<0.001$ except 1mM where $p=0.0312$) **(Figure 5.2B)**. The same study (Ninomiya et al., 2013) also used longer timepoints for cobalt stimulation and therefore HUVEC were stimulated with 0.25-1mM $CoCl_2$ or 100ng/ml LPS for 48h after which IL-8 secretion was measured by ELISA. There was a significant reduction in IL-8 secretion with 0.25mM ($p<0.001$) and 1mM $CoCl_2$ ($p<0.001$) while LPS caused a small increase in IL-8 production ($p=0.0042$) **(Figure 5.2C)**. Finally, IL-6 secretion was investigated as it has been shown to be upregulated in LPS and nickel-stimulated HUVEC (Goebeler et al., 1995). Following treatment with 0.25-1mM $CoCl_2$ or 100ng/ml LPS for 24h, IL-6 secretion by HUVEC was assessed by ELISA. Although there was a significant increase in IL-6 release in LPS-treated cells ($p<0.001$), there was no increase in IL-6 production by HUVEC stimulated with $CoCl_2$ and a significant decrease was observed with 0.25mM ($p=0.0088$) and 0.5mM $CoCl_2$ ($p=0.0155$) **(Figure 5.2D)**.

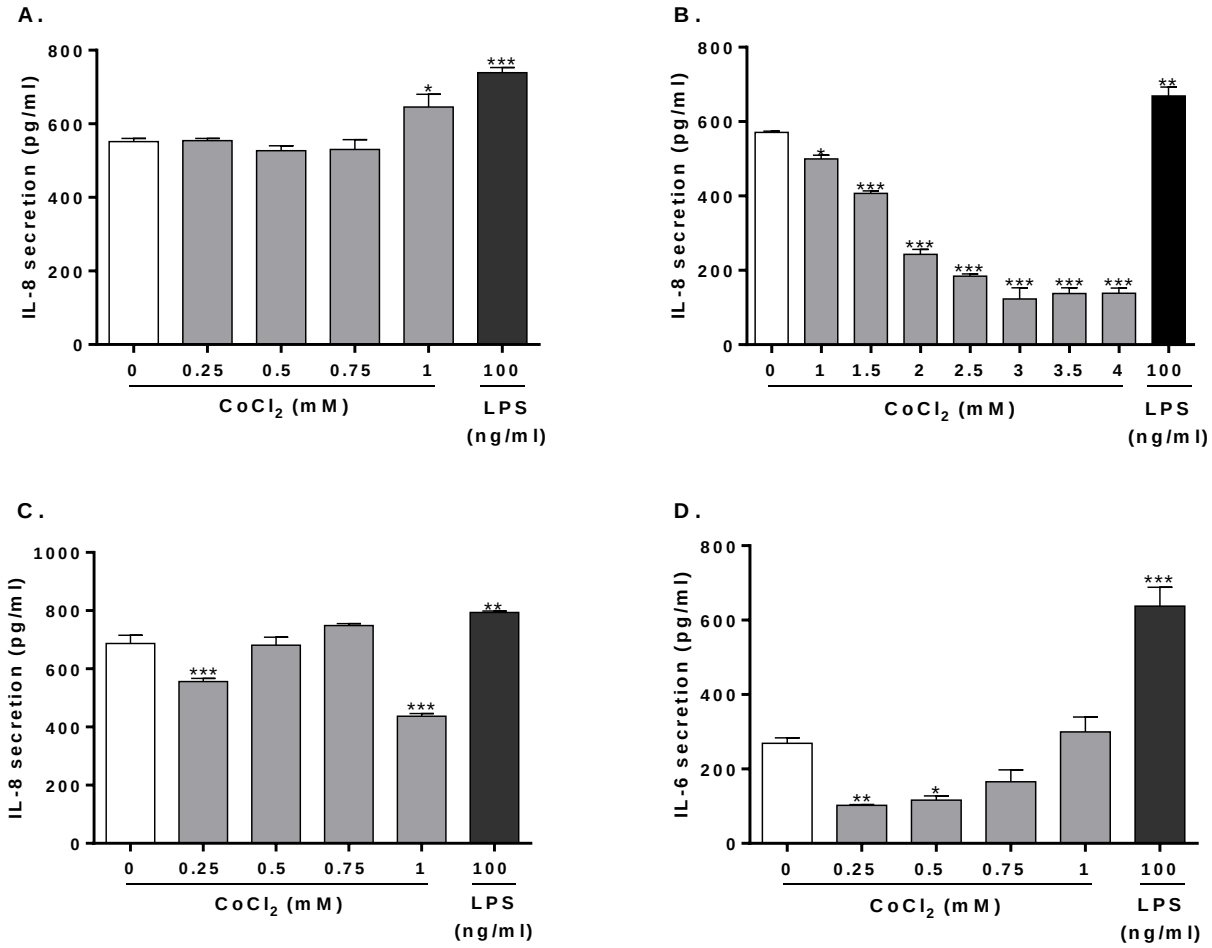

Figure 5.2: Effect of $CoCl_2$ on HUVEC

A. HUVEC were stimulated with 0.25-1mM $CoCl_2$ or 100ng/ml LPS for 24h and IL-8 secretion was quantified. Data is representative of two independent experiments. **B.** HUVEC were stimulated with 1-4mM $CoCl_2$ or 100ng/ml LPS and IL-8 secretion was quantified. Data is representative of two independent experiments. **C.** HUVEC were stimulated with 0.25-1mM $CoCl_2$ or 100ng/ml LPS for 48h and IL-8 secretion was quantified. n=1. **D.** HUVEC were stimulated with 0.25-1mM $CoCl_2$ or 100ng/ml LPS for 24h and IL-6 secretion was quantified. n=1. All cytokine quantification was performed by ELISA. Statistical significance was calculated by one-way ANOVA with Dunnett's multiple comparisons test comparing treated samples to the untreated control.

5.4.2. Effect of cobalt ions on human microvascular endothelial cells

Given the lack of cytokine secretion by $CoCl_2$ and LPS-stimulated HUVEC, a second endothelial cell line, HMEC-1, was assessed for its inflammatory response. As the HUVEC response to $CoCl_2$ indicated cytotoxicity (i.e. decreased cytokine secretion by treated cells compared to controls), a cytotoxicity assay was conducted on HMEC-1 prior to any further assays. HMEC-1 were incubated with 0.75mM $CoCl_2$ or left untreated for 24h before a cytotoxicity assay was performed using trypan blue staining and a Luna II automated cell counter. In contrast to the cytotoxicity observed in MonoMac 6 cells, there was no significant difference in HMEC-1 viability between $CoCl_2$-stimulated and unstimulated cells (100% viability normalised to untreated cells).

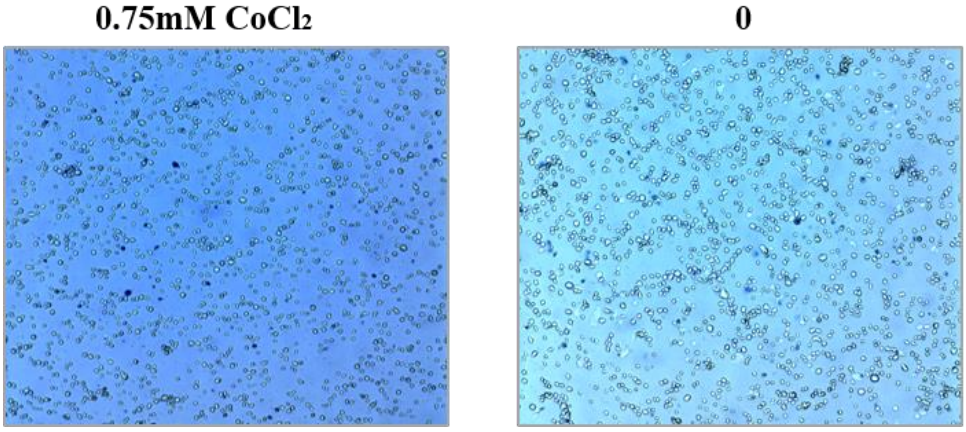

Figure 5.3: Effect of $CoCl_2$ on HMEC-1 viability
HMEC-1 cells were stimulated with 0.75mM $CoCl_2$ or untreated for 24h before a cytotoxicity assay was conducted using trypan blue staining. Images were obtained on a Luna II automated cell counter and counted manually. n=1. Cell viability was normalised to 100% in control cells, and was found to be 100% in $CoCl_2$-stimulated cells.

HMEC-1 were stimulated with 0.25-1mM $CoCl_2$ or 100ng/ml LPS for 24h. As well as being consistent with the concentrations used in MonoMac 6 cells, this $CoCl_2$ dose range is comparable with other studies using cobalt in endothelial cells (Kim et al., 2006). Following cell treatment supernatant was collected and IL-8 secretion quantified by ELISA. In contrast to HUVEC, HMEC-1 displayed a significant upregulation in IL-8 production across all $CoCl_2$ concentrations, as well as with LPS stimulation **(Figure 5.4A)**. Peak cobalt-mediated IL-8 secretion of approximately 1200pg/ml occurred at 1mM $CoCl_2$ ($p<0.001$) with LPS causing marginally higher secretion ($p<0.001$) (Figure 5.4A).

Cobalt-mediated IL-8 secretion by MonoMac 6 cells is TLR4-dependent, as shown in chapter 4. To further validate this data the small molecule TLR4 antagonist CLI-095 was used to

investigate HMEC-1 responses. In a preliminary assay HMEC-1 were pre-treated with a range of CLI-095 concentrations from 0.01-1µg/ml, followed by 24h stimulation with 0.75mM $CoCl_2$. IL-8 secretion was quantified by ELISA. The antagonist was found to be inhibitory across all of the concentrations used (**Appendix F**). 1µg/ml CLI-095 was selected as the optimal concentration as it caused the largest inhibition of IL-8 secretion and is consistent with previous work conducted in MonoMac 6 cells. To confirm this initial result HMEC-1 were pre-treated with 1µg/ml CLI-095 for 6h and then stimulated with 0.75mM $CoCl_2$ or 100ng/ml LPS for 24h. IL-8 secretion was measured by ELISA. In the presence of CLI-095 there was a significant reduction in IL-8 secretion in response to $CoCl_2$ ($p<0.001$) and the positive control LPS ($p<0.001$) compared to when no inhibitor was used **(Figure 5.4B)**. There was no significant difference in untreated samples with and without CLI-095 ($p=0.4454$).

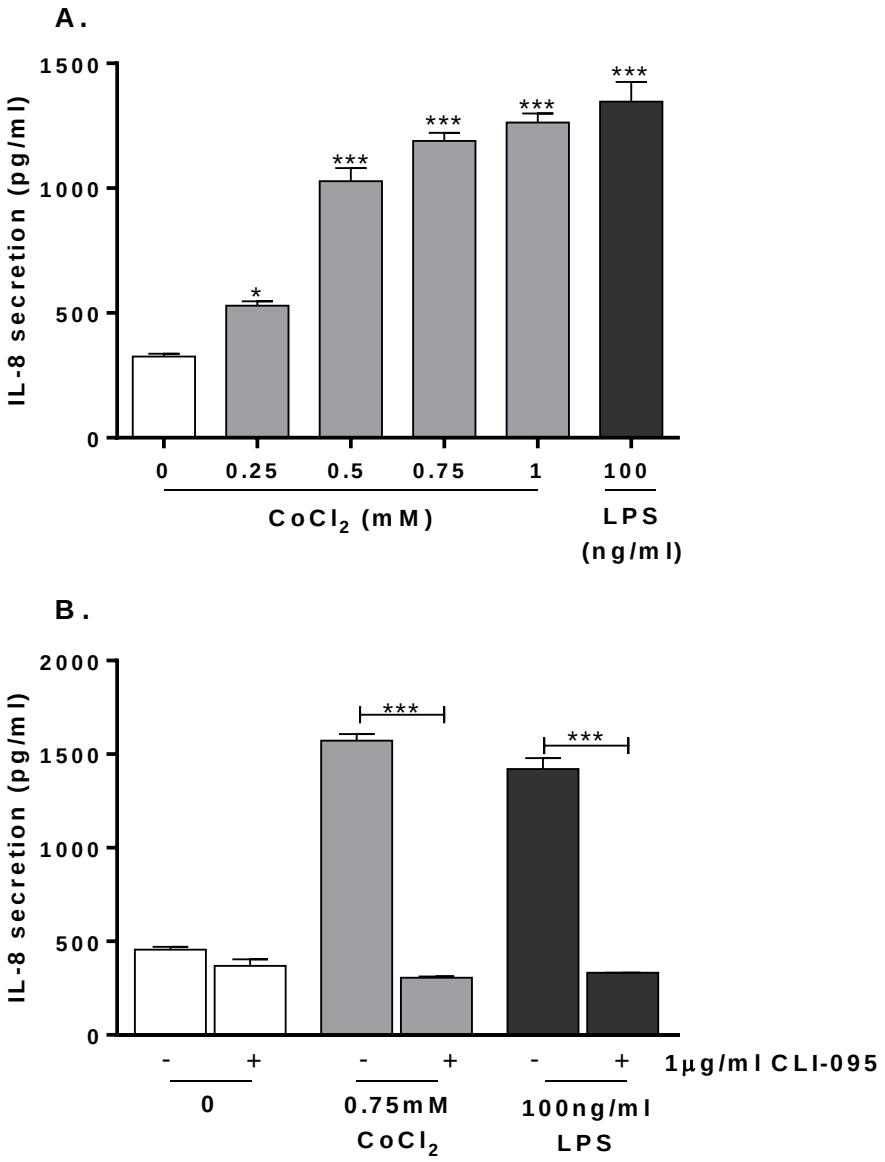

Figure 5.4: Effect of $CoCl_2$ and TLR4 inhibition on IL-8 secretion by HMEC-1
A. HMEC-1 were stimulated with 0.25-1mM $CoCl_2$ or 100ng/ml LPS for 24h before supernatant was collected and analysed by IL-8 ELISA. Statistical significance was calculated by one-way ANOVA with Dunnett's test for multiple comparisons comparing all treated samples to the untreated contol. **B.** HMEC-1 were pre-treated with 1µg/ml CLI-095 for 6h prior to 24h stimulation with 0.75mM $CoCl_2$ or 100ng/ml LPS. IL-8 production was measured by ELISA. Statistical significance was calculated by one-way ANOVA with Tukey's test for multiple comparisons comparing all samples to each other. All data is representative of three independent experiments. These experiments were conducted in collaboration with MRes students Sami Anjum and Amy Mawdesley.

Previous studies have demonstrated that cobalt ions induce secretion of IL-6 in different cell lines including keratinocytes and endothelial cells (Schmalz et al., 1998, Yang et al., 2015). However, as shown in **Figure 5.2D**, there was no increase in IL-6 release by HUVEC treated with $CoCl_2$. IL-6 secretion by $CoCl_2$-stimulated HMEC-1 cells was measured to establish whether or not this is a cell line-specific effect. HMEC-1 cells were stimulated with 0.25-1mM $CoCl_2$ or 100ng/ml LPS for 24h before IL-6 levels were measured by ELISA. IL-6 secretion was significantly increased by $CoCl_2$ across all concentrations ($p<0.001$ except for 0.25mM where $p=0.0247$) and by LPS ($p<0.001$) **(Figure 5.5A)**. Peak IL-6 secretion reached approximately 1500pg/ml and was induced by 0.75mM $CoCl_2$.

To assess the role of TLR4 in IL-6 release by $CoCl_2$-treated HMEC-1, cells were pre-treated with 1µg/ml CLI-095 for 6h followed by 24h treatment with 0.75mM $CoCl_2$ or 100ng/ml LPS. CLI-095 significantly decreased IL-6 secretion in response to both $CoCl_2$ and LPS to approximately 400pg/ml (both $p<0.001$). This is similar to IL-6 levels in untreated cells **(Figure 5.5B)**.

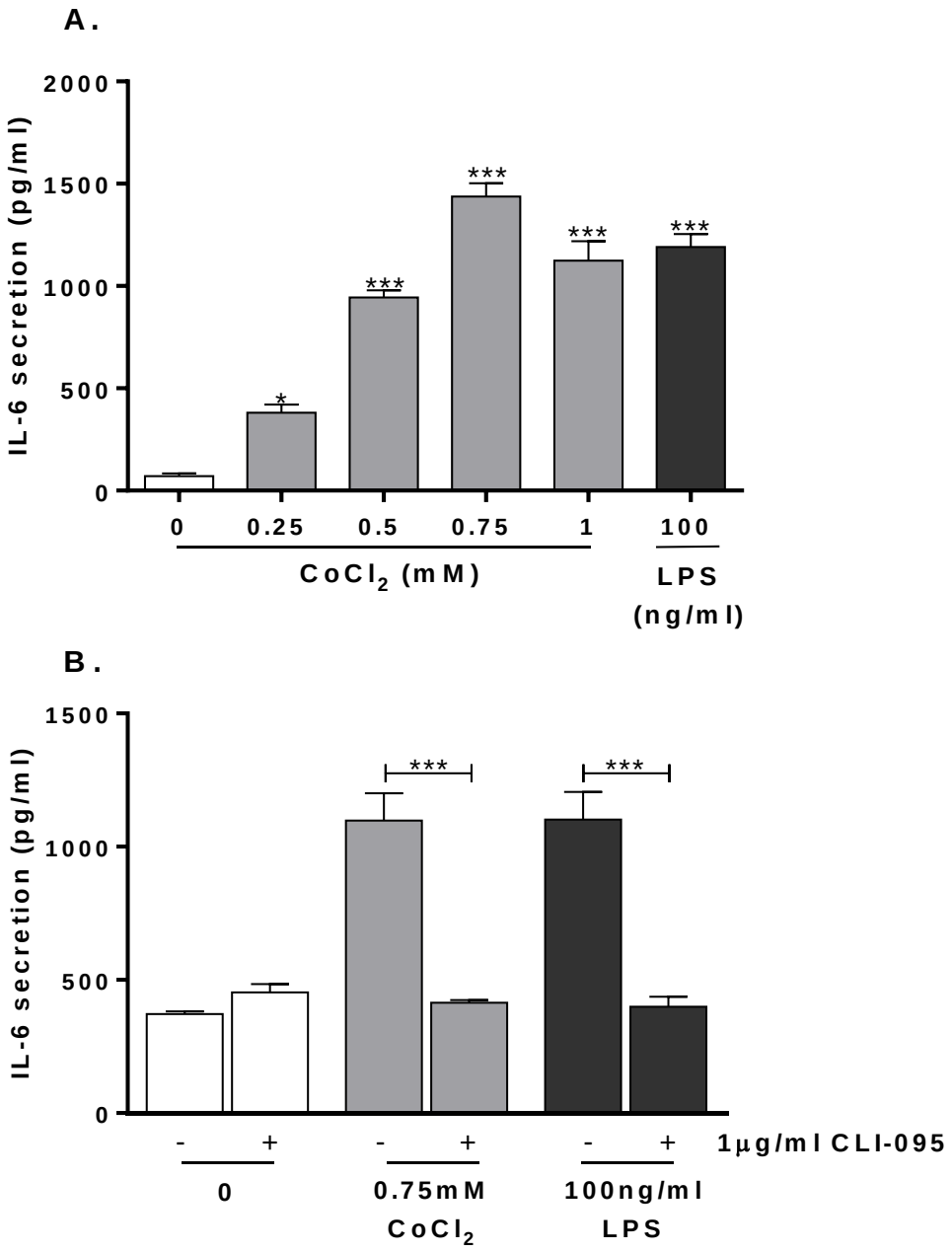

Figure 5.5: Effect of $CoCl_2$ and TLR4 inhibition on IL-6 secretion by HMEC-1
A. HMEC-1 were stimulated with 0.25-1mM $CoCl_2$ or 100ng/ml LPS for 24h before supernatant was collected and IL-6 levels measured by ELISA. Statistical significance was calculated by one-way ANOVA with Dunnett's multiple comparisons test comparing treated samples to the untreated control **B.** HMEC-1 were pre-treated with 1µg/ml CLI-095 prior to 24h stimulation with 0.75mM $CoCl_2$ or 100ng/ml LPS. IL-6 secretion was measured by ELISA. Statistical significance was calculated by one-way ANOVA with Tukey's test for multiple comparisons comparing all samples to each other. All data is representative of three independent experiments.

5.4.3. Effect of cobalt ions on adhesion molecule expression

HMEC-1 responded to $CoCl_2$ and LPS by secreting inflammatory cytokines in a TLR4-dependent manner and were therefore selected for further investigation into the effect of cobalt ions on adhesion molecule expression. Although ICAM-1 and VCAM-1 expression is most frequently studied in endothelial cells, monocytes and macrophages also express adhesion molecules to coordinate cell communication (Thornton and McDaniel, 2005). MonoMac 6 cells have previously been used for studying adhesion (Erl et al., 1995) and so they were investigated alongside HMEC-1 in this chapter.

A preliminary assay was conducted to assess the effect of cobalt ions on expression of the adhesion molecules *ICAM1* and *VCAM1* in HMEC-1 and MonoMac 6 cells. Cells were treated with 0.75mM $CoCl_2$ for 24h before RNA was isolated. cDNA was synthesised using the Maxima First Strand cDNA synthesis kit (**Section 2.6.2.2**) and *ICAM1* and *VCAM1* expression was analysed by qRT-PCR. *VCAM1* expression could not be detected in untreated and $CoCl_2$-treated HMEC-1 and MonoMac 6 cells, and Ct values were >35 in $CoCl_2$-stimulated samples (data not shown). 0.75mM $CoCl_2$ caused a 15-fold upregulation in *ICAM1* expression in MonoMac 6 cells (p=0.0010) (**Figure 5.6A**) and a 3-fold upregulation in HMEC-1 (p=0.0034) (**Figure 5.6B**). *ICAM1* expression was considerably higher (C_t<30) than *VCAM1* in unstimulated and stimulated samples from both cell lines and as a result further investigations focused on *ICAM1*.

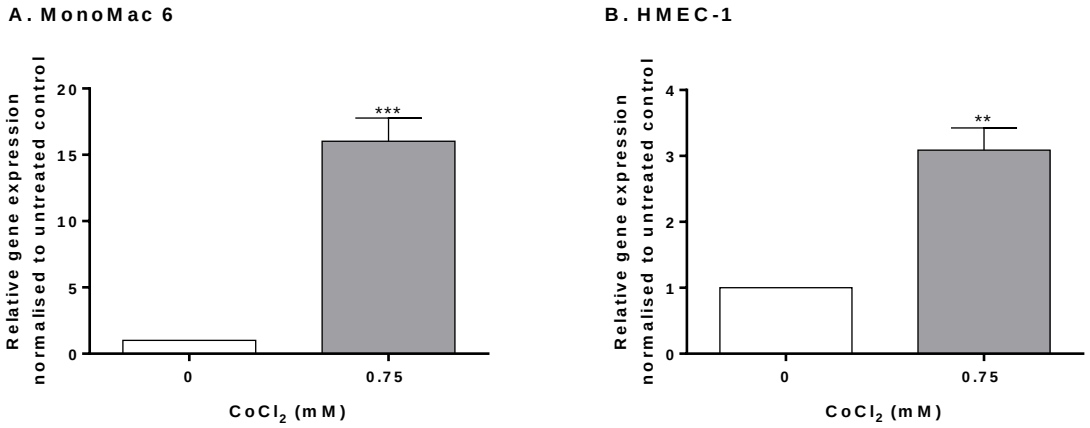

Figure 5.6: Effect of $CoCl_2$ on *ICAM1* expression
A. MonoMac 6 and **B.** HMEC-1 cells were stimulated with 0.75mM $CoCl_2$ for 24h before RNA was isolated and cDNA synthesised by reverse transcription. *ICAM1* expression was quantified by qRT-PCR. Data is representative of at least two independent experiments and statistical significance was calculated by Student's *t*-test comparing the treated sample to the untreated control.

5.4.4. Effect of TLR4 inhibition on adhesion molecule expression

The role of TLR4 in cobalt upregulation of *ICAM1* was investigated using CLI-095. MonoMac 6 cells and HMEC-1 were pre-treated for 6h with 1µg/ml CLI-095 before stimulation with 0.75mM $CoCl_2$ or 100ng/ml LPS for 24h. As previously, *ICAM1* expression was assessed by qRT-PCR. There was a significant increase in *ICAM1* expression by cobalt-stimulated MonoMac 6 cells ($p<0.001$) although the 40-fold increase was larger than that observed in preliminary experiments (**Figure 5.7A**). In the presence of CLI-095 there was a significant reduction in *ICAM1* expression ($p<0.001$), suggesting that the changes occur through cobalt activation of TLR4. As in previous assays LPS provided an effective positive control for TLR4 activation because *ICAM1* expression was also decreased in LPS-treated cells pre-incubated with CLI-095 ($p=0.0185$) (**Figure 5.7A**). Similar responses were observed in HMEC-1; *ICAM1* was upregulated 3-fold by $CoCl_2$ ($p=0.0132$) and this was inhibited by CLI-095 ($p<0.001$) (**Figure 5.7B**). LPS again provided an effective positive control for TLR4 regulation of adhesion molecule expression; there was an 8-fold upregulation in expression by LPS-stimulated HMEC-1 ($p<0.001$) and this was significantly inhibited by CLI-095 ($p<0.001$) (**Figure 5.7B**). Unlike in MonoMac 6 cells, untreated HMEC-1 cells downregulated *ICAM1* in the presence of CLI-095 although this did not reach statistical significance ($p=0.4707$) (**Figure 5.7B**).

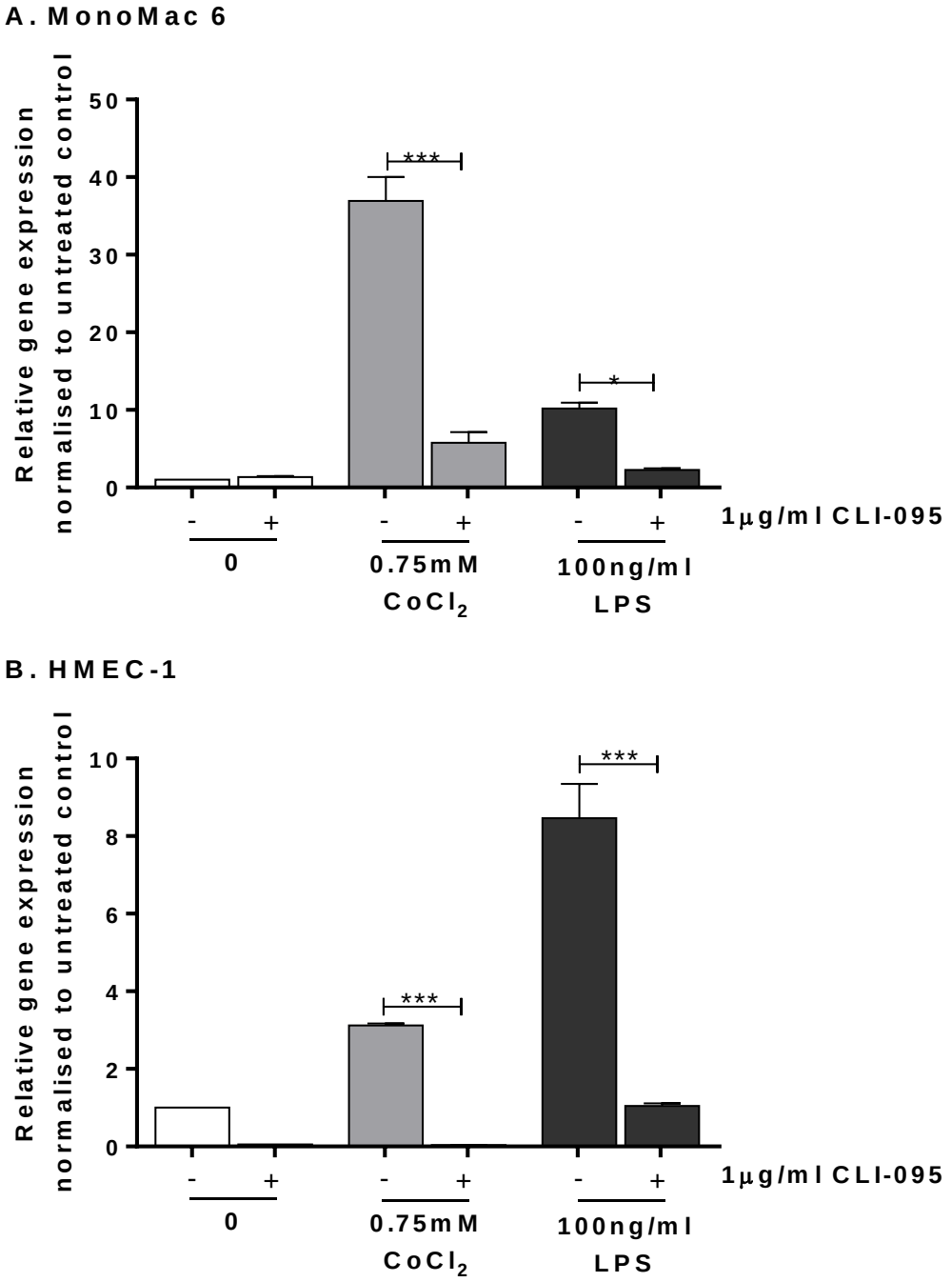

Figure 5.7: Effect of TLR4 inhibition on *ICAM1* expression
A. MonoMac 6 cells or **B.** HMEC-1 were pre-treated with 1µg/ml CLI-095 for 6h followed by 24h stimulation with 0.75mM $CoCl_2$ or 100ng/ml LPS. RNA was isolated and cDNA synthesised by reverse transcription. *ICAM1* expression was assessed by qRT-PCR. Data is representative of three independent experiments and statistical significance was calculated by one-way ANOVA with Tukey's test for multiple comparisons comparing all samples to each other.

5.4.5. Effect of $CoCl_2$ on soluble ICAM-1 secretion

The data described so far in this chapter shows that cobalt ions can regulate *ICAM1* gene expression through TLR4 activation, which has not previously been demonstrated. A number of other studies have shown that cobalt ions and wear debris can affect expression of membrane-bound adhesion molecules (Ninomiya et al., 2013) but information regarding their effect on soluble forms of adhesion molecules is limited. The Proteome Profiler arrays described in chapter 3 showed a small increase in secretion of soluble ICAM-1 (sICAM-1), a secreted form of membrane-bound ICAM-1 (mICAM-1). Given this result together with the changes in *ICAM1* expression following cobalt stimulation, sICAM-1 secretion by MonoMac 6 cells and HMEC-1 was investigated.

MonoMac 6 cells were stimulated with 0.25-1mM $CoCl_2$ or 100ng/ml LPS for 24h and sICAM-1 secretion was measured by ELISA. There was a dose-dependent increase in sICAM-1 secretion in response to $CoCl_2$ ($p<0.001$ for all concentrations except for 1mM where $p=0.0080$) **(Figure 5.8A)**. sICAM-1 release was also assessed in HMEC-1 using the same method; HMEC-1 significantly increased sICAM-1 secretion following stimulation with 0.5, 0.75 and 1mM $CoCl_2$ and 100ng/ml LPS (all $p<0.001$) but not with 0.25mM ($p=0.6142$) **(Figure 5.8B)**. The trend of sICAM-1 secretion by HMEC-1 was slightly different from that of MonoMac 6 cells, with peak $CoCl_2$-induced sICAM-1 secretion occurring at 0.5mM in MonoMac 6 cells (1000pg/ml) and 1mM in HMEC-1 (800pg/ml). In both cells lines LPS induced more sICAM-1 secretion than $CoCl_2$ stimulation.

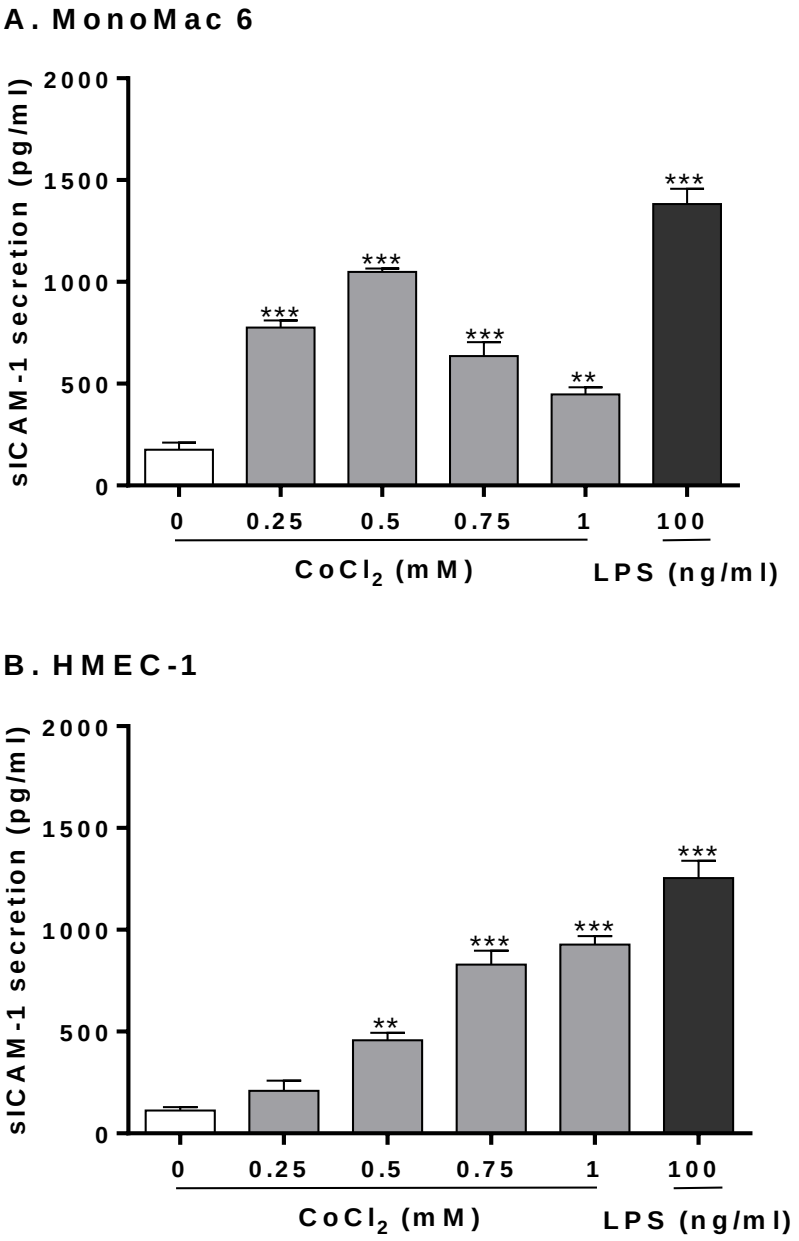

Figure 5.8: Effect of $CoCl_2$ on sICAM-1 secretion
A. MonoMac 6 cells or **B.** HMEC-1 were stimulated with 0.25-1mM $CoCl_2$ or 100ng/ml LPS for 24h before supernatant was collected. sICAM-1 secretion was measured by ELISA. Data is representative of three independent experiments and statistical significance was calculated by one-way ANOVA with Dunnett's test for multiple comparisons comparing all samples to each other. These experiments were conducted in collaboration with MRes student Sami Anjum.

The role of TLR4 in $CoCl_2$ and LPS-mediated sICAM-1 secretion was assessed using CLI-095. MonoMac 6 cells and HMEC-1 were pre-treated with 1µg/ml CLI-095 for 6h followed by stimulation with 0.75mM $CoCl_2$ or 100ng/ml LPS for 24h. In MonoMac 6 cells CLI-095 significantly decreased sICAM-1 secretion by $CoCl_2$-stimulated cells from approximately 900pg/ml to 400pg/ml ($p<0.001$) **(Figure 5.9A)**, which is comparable to sICAM-1 release by untreated cells. In HMEC-1 sICAM-1 secretion following $CoCl_2$ treatment was also significantly inhibited by CLI-095 ($p<0.001$), decreasing sICAM-1 release from 1200pg/ml to approximately 100pg/ml (**Figure 5.9B**). In this case sICAM-1 secretion by LPS-treated cells was lower than in $CoCl_2$-stimulated cells but CLI-095 was still able to inhibit its production ($p<0.001$) (**Figure 5.9B**). Unlike *ICAM1* expression, there was no difference in sICAM-1 secretion by unstimulated HMEC-1 with or without the TLR4 inhibitor ($p=0.9981$) (**Figure 5.9B**).

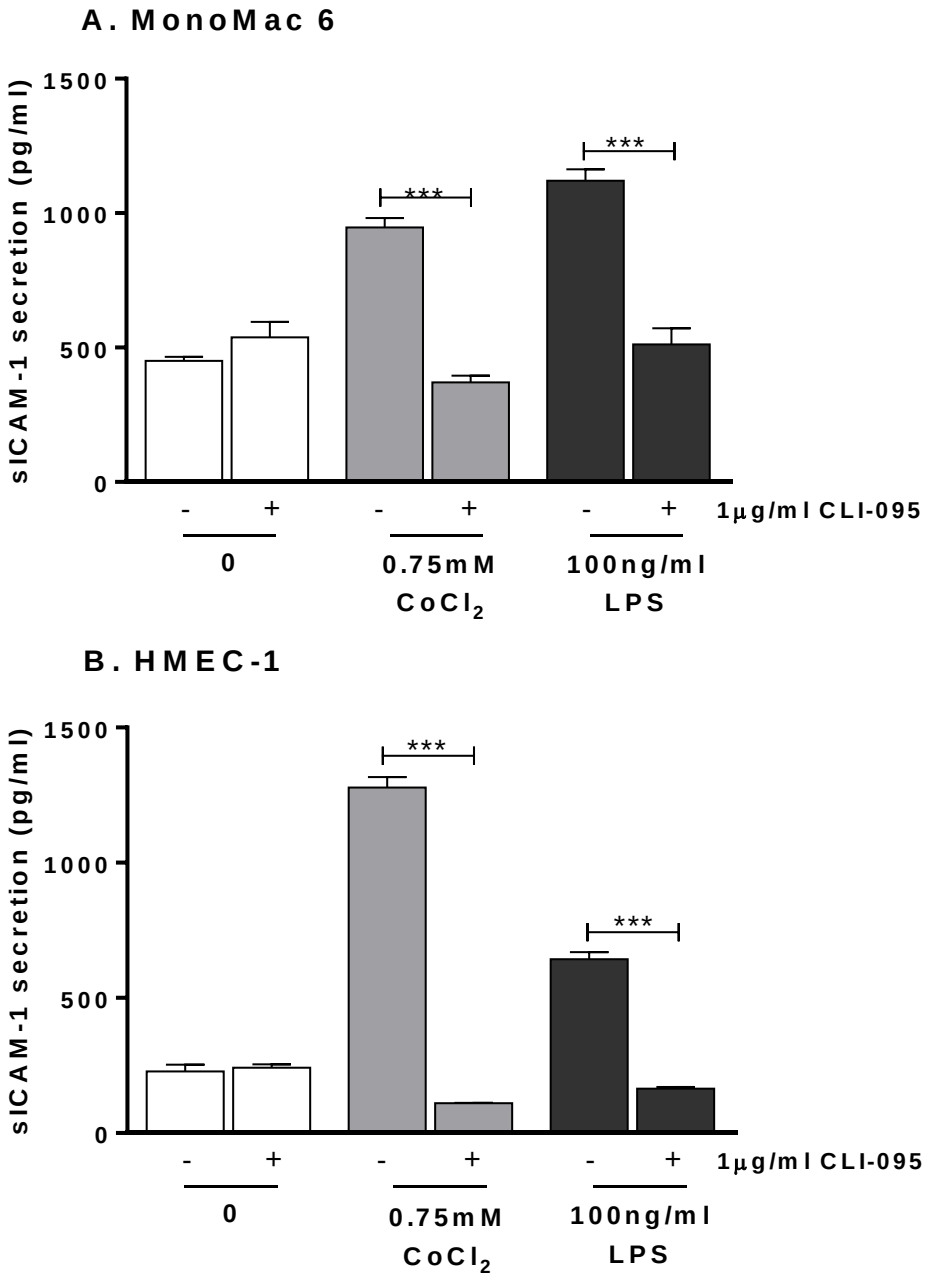

Figure 5.9: Effect of TLR4 inhibition on sICAM-1 secretion
A. MonoMac 6 cells or **B.** HMEC-1 were pre-treated with 1µg/ml CLI-095 for 6h followed by 24h stimulation with 0.75mM $CoCl_2$ or 100ng/ml LPS. Supernatant was collected and sICAM-1 secretion was measured by ELISA. Data is representative of three independent experiments. Statistical significance was calculated by one-way ANOVA with Tukey's multiple comparisons test comparing all samples to each other. These experiments were conducted in collaboration with MRes student Sami Anjum.

5.4.6. Effect of $CoCl_2$ on *CD11a* and *CD49d* expression

The effect of cobalt ions on subunits of LFA-1 and VLA-4 was investigated as they are ligands for ICAM-1 and VCAM-1 respectively. MonoMac 6 cells were chosen for this assay because as macrophages they express LFA-1 and VLA-4 (Erl et al., 1995) so that they can bind to endothelial cells expressing ICAM-1 or VCAM-1 for efficient extravasation. MonoMac 6 cells were treated with 0.75mM $CoCl_2$ or 100ng/ml LPS for 24h before *CD11a* (LFA-1 subunit) and *CD49d* (VLA-4 subunit) expression was assessed by qRT-PCR. *CD11a* expression was significantly increased following LPS stimulation ($p<0.001$) although the fold-change was very small (1.2-fold). *CD11a* was downregulated in response to cobalt ions ($p<0.001$) **(Figure 5.10A)**. *CD49a* expression was significantly decreased in response to both $CoCl_2$ and LPS (both $p<0.001$) **(Figure 5.10B)**.

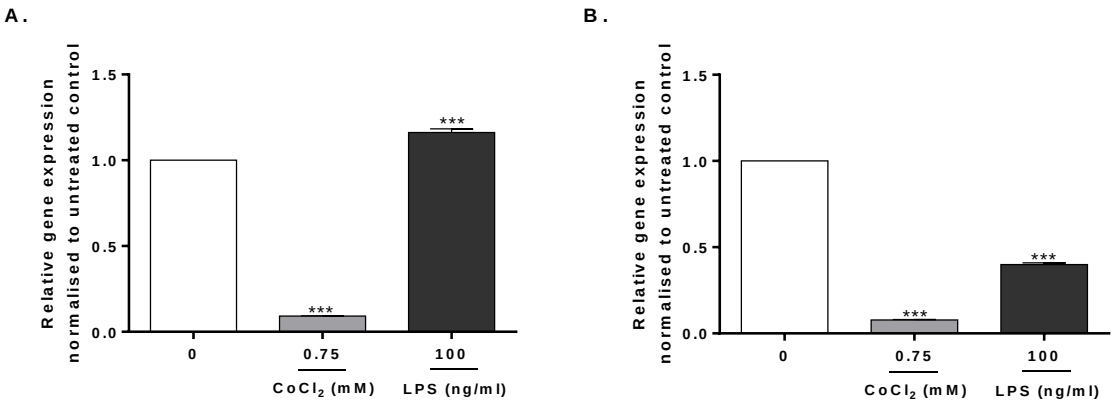

Figure 5.10: Effect of cobalt and LPS on *CD11a* and *CD49d* expression
MonoMac 6 cells were stimulated with 0.75mM $CoCl_2$ or 100ng/ml LPS for 24h. RNA was isolated and cDNA synthesised by reverse transcription. **A.** *CD11a* and **B.** *CD49a* gene expression was measured by qRT-PCR. Data is representative of two independent experiments and statistical significance was calculated by one-way ANOVA with Dunnett's test for multiple comparisons comparing treated samples to the untreated control. These experiments were conducted in collaboration with MRes student Sami Anjum.

5.4.7. Role of TLR4 in cobalt-mediated monocyte migration

A transwell chemotaxis assay was undertaken to determine the effect of cobalt on immune cell migration. Monocytes were selected for this assay because monocyte and macrophage infiltration has been observed in tissues surrounding failed MoM joints (Jämsen et al., 2014). Previous studies have also reported elevated secretion of monocyte-specific chemokines such as CCL2 in response to MoM debris (Queally et al., 2009, Devitt et al., 2010).

Data obtained in this study shows that chemokine release in response to cobalt ions can be blocked using a TLR4 antagonist. The same antagonist, CLI-095, was therefore used in the chemotaxis assays to assess its effect on monocyte migration. MonoMac 6 cells were pre-treated with 1µg/ml CLI-095 for 6h followed by 24h stimulation with 0.75mM $CoCl_2$ or 100ng/ml LPS. A transwell migration assay was then conducted using monocytes isolated from the whole blood of a healthy volunteer. Migrated monocytes were stained with haematoxylin and counted at x40 magnification (**Figure 5.11A)**. Monocyte chemotaxis was significantly increased in response to $CoCl_2$ conditioned media ($p=0.0290$) but in the presence of the inhibitor was significantly reduced ($p<0.001$) **(Figure 5.11B)**. Conditioned media from LPS-treated MonoMac 6 cells (with and without CLI-095) provided an effective positive control for both TLR4 activation and the specificity of CLI-095 in blocking TLR4 ($p<0.001$) (**Figure 5.11B**). There was no significant difference in the response to conditioned media from untreated cells with or without CLI-095 ($p>0.99$) showing that the inhibitor does not affect monocyte migration on its own. Two additional negative controls were included composed of complete media, and complete media with cobalt ions to ensure any migration was not due to a cobalt concentration gradient. There was no cell migration observed in either of these controls (data not shown).

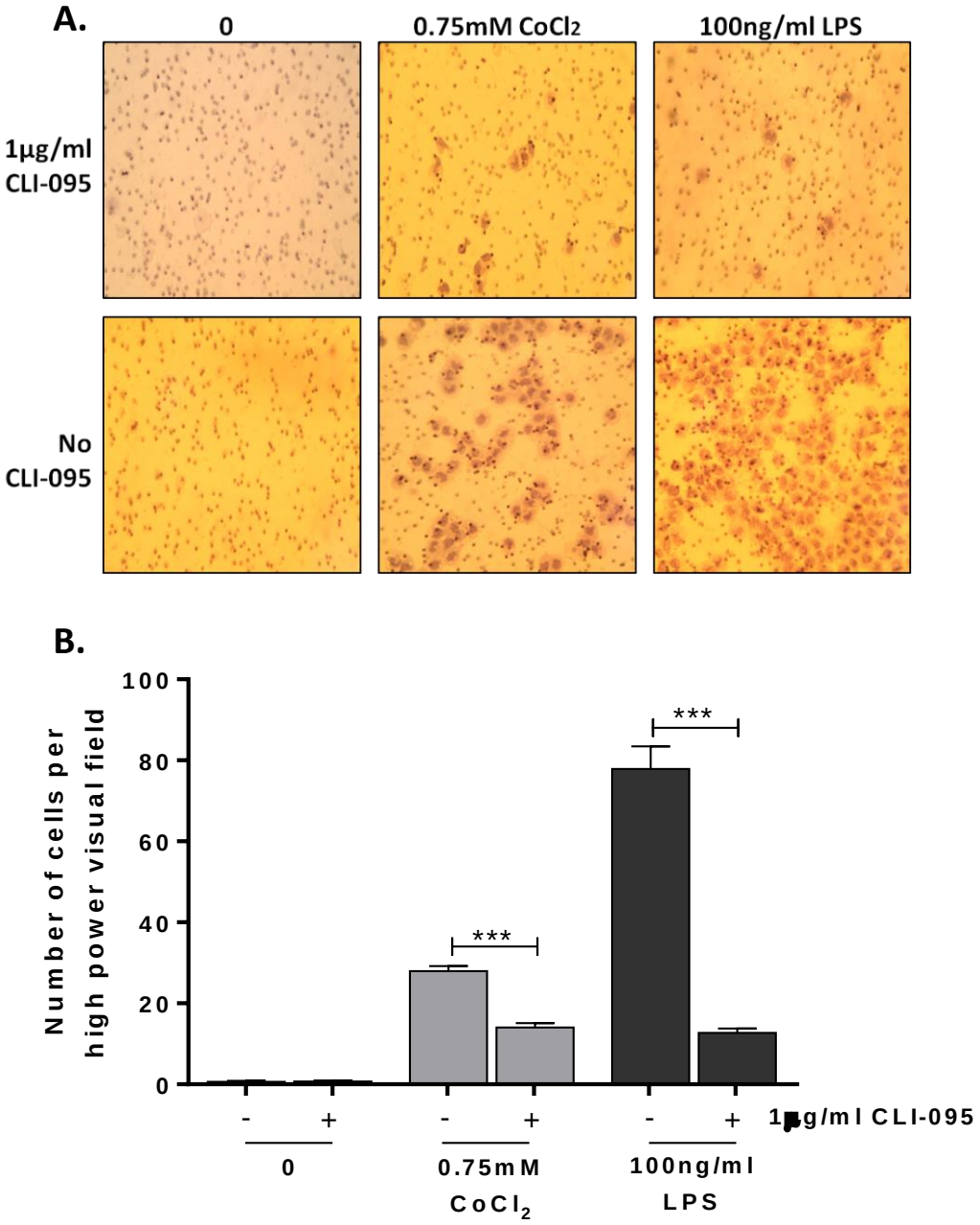

Figure 5.11: Effect of $CoCl_2$ and TLR4 inhibition on monocyte migration
MonoMac 6 cells were pre-treated with 1µg/ml CLI-095 for 6h prior to stimulation with 0.75mM $CoCl_2$ or 100ng/ml LPS. A monocyte chemotaxis assay was conducted using conditioned media. **A.** Filters showing monocyte migration in response to each of the stimuli at x40 magnification. **B.** Migrated monocytes were counted at x40 magnification. >10 visual fields were counted for each treatment condition. Data is representative of four independent experiments each using a different cell donor. Statistical significance was calculated by one-way ANOVA with Tukey's test for multiple comparisons comparing all samples to each other.

5.4.8. Role of TLR4 in cobalt-mediated neutrophil chemotaxis

MonoMac 6 cells were pre-treated with 1µg/ml CLI-095 for 6h followed by stimulation with 0.75mM $CoCl_2$ or 100ng/ml LPS for 24h. Conditioned media was collected and its neutrophil chemotactic properties assessed by transwell neutrophil chemotaxis assay. Migrated neutrophils were stained on filters with haematoxylin and counted at x40 magnification **(Figure 5.12A)**. There was a significant increase in neutrophil chemotaxis in response to cobalt conditioned media compared to untreated controls ($p<0.001$). This was significantly abrogated when MonoMac 6 cells were pre-treated with CLI-095 ($p<0.001$) **(Figure 5.12B)**, reducing neutrophil migration to levels similar to those in untreated samples. Conditioned media from LPS-stimulated MonoMac 6 cells provided a positive control. Neutrophil chemotaxis was increased in response to LPS conditioned media ($p<0.001$) and significantly decreased by CLI-095 pre-treatment ($p<0.001$). Conditioned media from untreated cells did not affect neutrophil migration and there was no significant difference in the presence of CLI-095 ($p>0.999$). As for monocyte migration, additional negative controls were included containing HBSS or complete media with and without cobalt. No migration was observed in these controls (data not shown).

A small preliminary study was also conducted using the method described above to determine the ability of anti-TLR4 neutralising antibodies MAb2-hTLR4 and PAb-hTLR4 (Error! Reference source not found.) to inhibit cobalt-mediated neutrophil migration. Both antibodies successfully inhibited migration with a greater effect observed for the monoclonal antibody than the polyclonal (**Appendix G**).

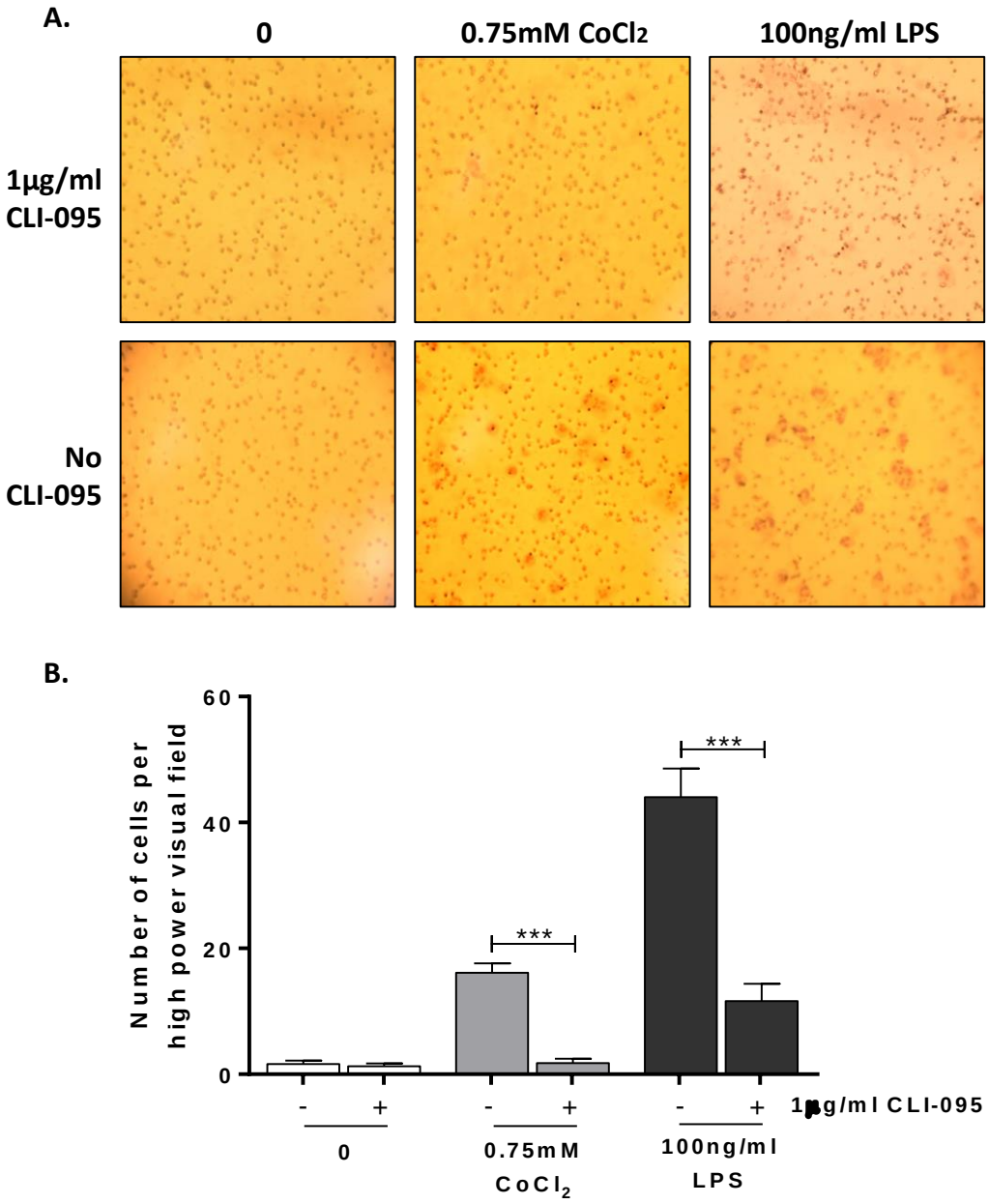

Figure 5.12: Effect of $CoCl_2$ and TLR4 inhibition on neutrophil migration
MonoMac 6 cells were pre-treated with 1µg/ml CLI-095 for 6h prior to stimulation with 0.75mM $CoCl_2$ or 100ng/ml LPS for 24h. A neutrophil chemotaxis assay was conducted using conditioned media. **A.** Haematoxylin-stained filters showing neutrophil migration in response to each of the stimuli at x40 magnification. **B.** Migrated neutrophils in >10 different visual fields were counted at x40 magnification. Data is representative of three independent experiments each using a different cell donor. Statistical significance was calculated by one-way ANOVA with Tukey's multiple comparisons test comparing all samples to each other.

5.5. Discussion

Inflammatory cytokine secretion by endothelial cells

This chapter aimed to investigate the effect of cobalt ions on inflammatory cell adhesion and migration, as well as on immune responses in endothelial cells. Cell adhesion and migration are of interest to the present study because they are both required for the immune cell infiltration of failed MoM peri-implant tissues. Furthermore, some of the cytokines and chemokines identified as being regulated by cobalt ions in earlier chapters contribute to cell adhesion and/or migration. For example, IL-8 is a potent neutrophil chemokine (Baggiolini et al., 1989) and is also associated with increased cell adhesion through its upregulation of ICAM-1 and VCAM-1 (Kuai et al., 2012). IL-6 can upregulate ICAM-1 expression resulting in ICAM-1-dependent cell migration (Lin et al., 2013). Additionally, TLR4 positive cells have been identified in the vascular endothelium surrounding aseptically-loosened MoM hips, showing the potential for an immune response to TLR4 ligands (Pajarinen et al., 2010). Taken together this information made cell adhesion and migration relevant aspects for investigation in the present study.

HUVEC are widely used as an endothelial cell model for studying the inflammatory effects of PAMPs, including LPS. Consequently they were selected as a potential model for investigating inflammatory and adhesion molecule responses to cobalt stimulation. Surprisingly, and in contrast to previous studies (Sultana et al., 1999, Ninomiya et al., 2013), there was only a small increase in IL-8 secretion with 24h of 1mM $CoCl_2$ stimulation, and no increase in secretion with longer timepoints or increased $CoCl_2$ concentrations. IL-6 secretion was also measured but no increases were noted in $CoCl_2$-stimulated cells. The data suggests that the effect of cobalt ions is cell line-specific and supports the work of Raghavan *et al* who showed that cobalt stimulated inflammatory responses in dendritic cells but not in keratinocytes (Raghavan et al., 2012).

ELISA analysis revealed a decrease in IL-8 and IL-6 secretion by $CoCl_2$-treated HUVEC compared to untreated cells. This suggests that cobalt may cause cytotoxicity in HUVEC, which contradicts the work of Ninomiya *et al* who did not observe any cytotoxic effects even at 4mM $CoCl_2$, and also found increased cytokine secretion at these high concentrations (Ninomiya et al., 2013). The variation in cytokine secretion observed between studies may arise from different levels of TLR4 expression as HUVEC are primary cells and in this study they were pooled from multiple donors. Differences in cytokine release between endothelial cell lines and primary endothelial cells has previously been reported (Lidington et al., 1999). This is particularly likely to be the case in the present study because the HUVEC showed little response to LPS as a positive control, suggesting that expression of TLR4 itself or a component of the

signalling pathway is reduced in comparison to other cells. The variation in cytotoxic cobalt concentrations is less likely to arise from TLR4 expression and may be specific to the primary cells used in each study. As the focus of this study is on the role of TLR4 in cobalt-mediated inflammatory responses rather than the cell-specific nature of inflammation, HUVEC were not used in further analysis and an alternative endothelial cell line was sought.

HMEC-1 were selected as an endothelial cell line model for this study because they express TLR4 and are responsive to LPS (J. Kirby, Institute of Cellular Medicine, Newcastle University, personal communication) (Faure et al., 2000). They are derived from the microvasculature and as cobalt is present in the blood of many MoM implant patients they are a good model for the study of ARMD. In contrast to HUVEC, HMEC-1 showed increased secretion of both IL-8 and IL-6 following $CoCl_2$ and LPS stimulation. This could be prevented with CLI-095 showing that secretion occurs through cobalt activation of TLR4. The ability of cobalt to induce inflammation in microvasculature-derived cells is interesting in the clinical context of this study because soft tissue necrosis is a common feature of ARMD. Soft tissue necrosis occurs through vasculitis (blood vessel inflammation) restricting blood flow to the joint tissues. If cobalt can cause inflammation in blood vessels through TLR4 activation then it could potentially contribute to vasculitis, soft tissue necrosis, and ARMD.

Adhesion molecule expression

VCAM1 expression was not detected in untreated MonoMac 6 cells and HMEC-1, which supports previous studies showing that it is not constitutively expressed (Panés and Granger, 1998). $CoCl_2$ stimulation did not bring *VCAM1* expression into the detection range of the qRT-PCR assay and nor did LPS treatment (data not shown). More sensitive assays would be required to determine whether or not cobalt ions affect *VCAM1* but its low expression levels raise questions of the physiological relevance for this study and therefore *ICAM1* was the focus of further work.

ICAM1 expression was increased by $CoCl_2$ and LPS; this is a direct consequence of TLR4 activation by the ligands because CLI-095 blocked the response. This supports similar work conducted using nickel ions; Tsou *et al* showed that up to 1.5mM $NiCl_2$ upregulated IL-8 and ICAM-1 expression in HUVEC. Knockdown of MyD88 with siRNA and inhibition of NFκB through overexpression of IκBα both inhibited nickel-mediated ICAM-1 expression showing that they are both essential to the response(Tsou et al., 2013). ICAM-1 and IL-8 expression was also found to be TLR4-dependent because they could be inhibited by CLI-095. The results presented in the present study also suggest that changes in adhesion molecule expression

observed in previous studies could be a result of TLR4 activation by cobalt ions. This is particularly the case for the work of Ninomiya *et al* who showed that cobalt ions have an inflammatory effect, for example increasing the secretion of IL-8 and CCL2, and upregulating adhesion molecule expression (Ninomiya et al., 2013, Tyson-Capper et al., 2013b).

As mentioned previously, adhesion molecules are regulated by cytokines such as IL-8, IL-6, IL-1β and TNFα. It is therefore conceivable that cobalt promotes adhesion molecule expression by stimulating TLR4-dependent cytokine release which then feedbacks to increase ICAM-1 expression. For example, IL-1β upregulates ICAM-1 but has little effect on VCAM-1 (Hosokawa et al., 2006). Interestingly, upregulated *IL1B* (gene encoding IL-1β) expression was detected by the qRT-PCR arrays and together with the increased IL-8 and IL-6 secretion it may contribute to the elevated *ICAM1* expression described in this chapter.

The $CoCl_2$-mediated increase in *ICAM1* shown in this chapter suggests that leukocyte adhesion can occur more readily in the presence of cobalt ions. This is similar to responses that develop during pathogen challenge and is indicative of a drive towards inflammation. Increased leukocyte adhesion favours extravasation and once inside the tissues leukocytes can perform inflammatory functions such as further cytokine secretion and activation of the adaptive immune system. The effect of cobalt on these processes could potentially contribute to ARMD development.

sICAM-1

This is the first study to show that cobalt and LPS upregulate sICAM-1 secretion in MonoMac 6 cells and HMEC-1. As with ICAM-1 protein and gene expression, sICAM-1 release can be regulated by cytokines and chemokines; sICAM-1 has previously been reported to be induced by HMGB1 activation of TLR4 and its secretion may therefore be the result of feedback mechanisms (Bauer et al., 2012). However the effect is TLR4-dependent as secretion of sICAM-1 by both cell lines was prevented by the small molecule TLR4 antagonist CLI-095. Further work is required to establish whether this is a result of direct cobalt-mediated TLR4 activation or an indirect result of feedback mechanisms.

sICAM-1 is a soluble form of membrane-bound ICAM-1 (mICAM-1) and was first identified in patients with leukocyte adhesion deficiency (Rothlein et al., 1991). Although some studies have indicated that there is a direct correlation between mICAM-1 and sICAM-1 expression (Kjærgaard et al., 2013), others suggest that sICAM-1 does not always reflect levels of mICAM-1 (Giorelli et al., 2002). Despite these differences sICAM-1 has been proposed as a biomarker in several inflammatory diseases, including systemic inflammatory response

syndrome (De Pablo et al., 2013) and systemic sclerosis (Hasegawa et al., 2014). sICAM-1 is shed from endothelial cells following cell adhesion and this is an important step in the development of an immune response. It results in sICAM-1 accumulation in serum, thus making it a potential marker of inflammation. However the results of this study indicate that sICAM-1 is not just a by-product of the adhesion process because even in the absence of cell-cell interactions in an *in vitro* cell culture assay there was still a TLR4-dependent increase in its release. Considerable further work is therefore required before sICAM-1 could be considered a marker of ARMD and MoM implant failure.

sICAM-1 may be shed from the endothelial cell surface as a protective mechanism to dampen the inflammatory response, acting as a decoy molecule for the membrane-bound adhesion molecules (Garton et al., 2006). However there is increasing evidence that sICAM-1 can also be pro-inflammatory as mice overexpressing sICAM-1 have an exaggerated immune response and show increased monocyte and neutrophil recruitment to the site of inflammation (Mendez et al., 2011). Consequently the TLR4-dependent sICAM-1 release by MonoMac 6 cells may contribute to the monocyte and neutrophil migration described elsewhere in this chapter. In addition sICAM-1 can stimulate macrophage activation, increasing NFκB activity and pro-inflammatory cytokine secretion (Schmal et al., 1998). This suggests that it could contribute to the cellular response to cobalt via feedback mechanisms that promote further cytokine release.

sICAM-1 also plays a role in cell proliferation (Takahara et al., 2013), endothelial cell migration and angiogenesis (Gho et al., 1999). As such it is regarded as a key factor in cancer metastasis and tumour formation, which is of interest to the present study because of the pseudotumour development associated with ARMD. The increased expression of pro-angiogenic factors like sICAM-1 could promote blood vessel formation to support pseudotumour growth, making sICAM-1 an interesting factor for further investigation in ARMD.

LFA-1 and VLA-4

This study investigated the effects of cobalt and LPS on integrin subunits of LFA-1 and VLA-4. Expression of *CD11a*, a subunit of LFA-1, showed a small but significant upregulation in response to LPS but was downregulated following cobalt stimulation. *CD49d* (VLA-4 subunit) expression was downregulated in response to both TLR4 agonists. This is surprising as a recent study has shown that both LFA-1 and VLA-4 can be upregulated via a TLR4-dependent mechanism (Liu et al., 2015). However the study did not investigate the effect of LPS on the individual subunits of LFA-1 and VLA-4 and it is possible that the subunits may be differentially regulated. It should also be acknowledged that cell adhesion is a rapid process

and molecules are up- or downregulated within a very short time frame to allow efficient extravasation. The decrease in subunits of LFA-1 and VLA-4 could therefore be a transient effect occurring as part of the complex adhesion process. It is interesting that LPS induced the same effect as $CoCl_2$ for *CD49a* but the opposite for *CD11a*; this may highlight key differences in cellular responses to the two ligands.

CD11 and CD18, both of which are subunits for ICAM-1 ligands, have recently been proposed as biomarkers of THR failure due to their correlation with aseptic loosening (Ovrenovits et al., 2015). Interestingly, the study found that the markers were downregulated in patients with aseptically loosened hip implants compared to the healthy control and septic loosening groups. This shows the potential of integrins for future study in the pathology of MoM implant failure.

Cell migration

This study focused on monocyte and neutrophil chemotaxis because TLR4 is an innate immune receptor and both monocytes and neutrophils are involved in innate immunity. Furthermore, data presented in chapters 3 and 4 showed increased expression and/or secretion of monocyte and neutrophil chemotactic factors, including IL-8. Monocyte and neutrophil migration was significantly upregulated following $CoCl_2$ and LPS stimulation of MonoMac 6 cells, showing that the cytokine and chemokine profile secreted by cobalt-stimulated cells favours immune cell migration. Monocyte migration was found to be TLR4-dependent as it was inhibited by CLI-095. A previous study has shown that cobalt induction of IL-8 results in neutrophil chemotaxis (Kim et al., 2006) but this is the first study to identify a role for TLR4 in the response.

In both the monocyte and neutrophil assays there was considerably more cell migration in response to LPS than $CoCl_2$. Data previously presented in this study shows that levels of cytokine and chemokine secretion are comparable between $CoCl_2$ and LPS so this is a surprising observation. The effect is likely to be due to different combinations of chemokines secreted in response to each of the ligands, perhaps generating a more potent signal for LPS than for $CoCl_2$.

Monocyte and neutrophil chemotaxis assays were conducted using supernatant from cobalt-treated cells. *In vivo*, a carefully coordinated chemokine gradient is established that directs migrating cells towards the site of inflammation. The *in vitro* assay method means that all of the chemokines are combined in each well and any potential gradient is lost. However this assay does demonstrate that the overall effect of the chemokine profile induced in response to cobalt is pro-migratory.

The results presented in this chapter may provide an explanation for the different inflammatory cell profiles observed in MoM peri-implant tissues. The reactions are often divided into two categories; ALVAL, i.e. a lymphocyte-dominated cellular infiltrate that resembles a type IV hypersensitivity response, and a monocyte/macrophage-dominated infiltrate that has been described as a 'generalised' inflammation and is sometimes attributed to cytotoxicity (Mahendra et al., 2009). It is possible that cobalt activation of TLR4 results in macrophage-specific chemokines that guide cells to the peri-implant tissues where they identify cobalt as a pathogen and mount an inflammatory response. Macrophage recruitment is a key step in the development of osteolysis because migrated macrophages can be activated by cytokines and chemokines in the peri-implant environment, which can drive their differentiation into osteoclasts. Immunohistochemical studies into MoM implant failure report that the macrophage-dominated infiltrate can also contain fibroblasts and neutrophils. Neutrophils are much more common in septic loosening than aseptic (Pajarinen et al., 2010) and therefore it was interesting in this study to observe the ability of cobalt conditioned media to recruit neutrophils as well as monocytes. The low numbers of neutrophils around MoM implants may be due to their relatively short lifespan compared to macrophages (Summers et al., 2010) but they could aid in the recruitment of other immune cells to prolong the response.

CCL2, a macrophage chemokine, has been the focus of many studies into adverse reactions to wear debris because of the high numbers of monocytes and macrophages seen around failed hip implants. Several studies have identified a central role for CCL2 in recruitment of macrophages to the site of wear particle-induced inflammation (Queally et al., 2009, Akbar et al., 2012) and as such CCL2 has recently been the target of therapies to prevent these adverse reactions (Yao et al., 2014). A mutant form of CCL2, known as 7ND, has been developed to block CCR2, the receptor for CCL2 (Yao et al., 2014). This *in vitro* study showed that 7ND blocked CCL2-induced migration of THP-1 macrophages. In the present study it is particularly interesting to observe cobalt-mediated monocyte migration despite the downregulation of *CCL2* expression described in earlier chapters, and its apparent central role in wear debris responses. However Huang *et al* found that CCL2 inhibition did not prevent macrophage migration following wear particle stimulation and concluded that other chemokines must be involved in the response (Huang et al., 2010); the data in the present study seems to support this theory. This highlights the redundancy of cytokines and chemokines and shows that it is their cumulative effect that is important to the overall inflammatory response. Functional studies such as migration assays are therefore particularly useful in the study of adverse responses to MoM implants. Cell migration is clearly a major aspect of ARMD development and work is ongoing to target macrophage

recruitment in ARMD prevention (Goodman et al., 2014). A better understanding of how recruitment is regulated could lead to improved therapeutic options.

5.5.1. Future work

ICAM1, *CD11a* and *CD49d* gene expression is altered by cobalt ions but it is not clear whether or not these changes also occur at the protein level. Analysis by flow cytometry, Western immunoblotting or immunohistochemistry would provide insight into this. It would also be interesting to investigate expression of these factors in tissue samples from MoM implant patients to see if it correlates with the *in vitro* data.

To determine the functional consequences of *ICAM1* gene expression changes, adhesion assays could be undertaken. This could include the use of a Cellix platform which can be used to investigate cell adhesion under flow conditions and represents shear stress along a blood vessel. Chips used in these assays can be coated either with recombinant adhesion molecules or with endothelial cells (stimulated or unstimulated) while immune cells (again stimulated or unstimulated) can be flowed across the chip and cell-cell adhesion is measured. Co-culture cell models could also be used to further investigate cell-cell interactions (e.g. macrophages and endothelial cells) and how this impacts upon adhesion molecule expression in the presence of cobalt ions.

To test the effects of cytokines and TLR4 inhibitors on regulation of adhesion molecule responses, HMEC-1 could be incubated with conditioned media from cobalt-treated cells such as MonoMac 6. However this assay would require careful optimisation as in this study conditioned media from MonoMac 6 cells still contained the stimulus (e.g. $CoCl_2$ or LPS). This would need to be removed before the assay could be performed.

The chemotaxis assay data presented in this chapter clearly demonstrates the potential for cobalt ions to recruit immune cells across a porous membrane in response to a chemokine stimulus. To increase the physiological relevance of the assay and represent the endothelial cell barrier present *in vivo*, an endothelial cell layer could be grown across the filters. As described earlier the conditioned media generated in this study contains cobalt and as the cell-coated chemotaxis filters come into contact with the media optimisation would be required to determine how this could affect the cells.

The use of primary monocytes and neutrophils means that there may be patient variability in the chemotaxis response to conditioned media because chemokine receptor expression differs between individuals as well as in different disease states (Henneken et al., 2005). As patient

variability increases the complexity of ARMD it would be interesting to correlate the extent of cell migration with levels of chemokine receptors such as CXCR2 (IL-8 receptor) to determine whether or not they influence the response. The use of antibodies against specific chemokine receptors would also provide further insight into the chemokines responsible for the effect (e.g. monocyte or neutrophil migration).

5.5.2. Conclusion

In summary the data presented in this chapter shows that there are differences in the endothelial cell response to cobalt ions; HMEC-1 secrete cytokines following cobalt treatment but HUVEC do not. HMEC-1 were chosen as an endothelial cell model and were found to upregulate expression of the adhesion molecule *ICAM1* in a TLR4-dependent manner. A similar effect was observed in MonoMac 6 cells. Cobalt activation of TLR4 also increased secretion of a soluble adhesion molecule, sICAM-1, in both HMEC-1 and MonoMac 6 cells. Given the changes in *ICAM1* expression it was surprising to find that expression of subunits of ICAM-1 and VCAM-1 ligands (LFA-1 and VLA-4) was downregulated by cobalt ions. Finally, conditioned media from cobalt-treated MonoMac 6 cells proved to be chemotactic for both primary monocytes and neutrophils, suggesting that the secreted chemokine profile is pro-migratory as well as TLR4-dependent.

Overall this data indicates that cobalt ions could promote inflammatory cell adhesion and migration, and therefore inflammation, through upregulation of adhesion molecule and cytokine expression. TLR4 plays a central role in these responses because they were inhibited by CLI-095. Clinically the results presented here suggest that cobalt ions released into the joint space and blood by MoM bearing articulation could drive inflammatory responses via TLR4, and contribute to the inflammatory cell infiltrate observed in failed MoM peri-implant tissues.

Chapter 6. Patient variability: investigating cellular responses to cobalt ions

6.1. Introduction

Many previous MoM implant and ARMD investigations have focused on the biomechanics and biocompatibility of MoM implants to determine the events that lead to their failure. Some have concluded that there are links between high device wear rates or increased metal ion levels, and subsequent ARMD development (Glyn-Jones et al., 2011, Hailer et al., 2014). Despite such conclusions these factors cannot fully explain ARMD because implant wear rates and metal ion levels can be similar between two patients and yet one will develop ARMD while the other will not (Campbell et al., 2014). While it is clear from the data presented so far in this study that cobalt can induce inflammation by activating TLR4, it is also evident that this activation alone cannot be responsible for every ARMD case; all healthy individuals express functional TLR4 as an essential component of the innate immune response and therefore have the potential to respond to cobalt ions. However ARMD does not develop in every patient with a MoM hip replacement (Meding et al., 2012), nor is the inflammatory response identical in those who ultimately progress to ARMD (Perino et al., 2014). Consequently other biological factors must be involved that contribute to promoting the inflammatory response in susceptible individuals, or inhibiting mechanisms that prevent the response in non-ARMD patients.

There are numerous biological factors that could influence a patient's susceptibility to ARMD. However the discovery of cobalt as a TLR4 ligand allows a more focused investigation into these factors as much is known about variables that influence the TLR4 signalling pathway.

6.1.1. Genetic variation in ARMD

Genetic variation has been investigated for its potential role in ARMD. Several studies have identified single nucleotide polymorphisms (SNPs) that may increase susceptibility to ARMD or adverse responses to other wear debris. These SNPs are mainly in genes encoding inflammatory signalling factors such as IL-6 and TNF (Gallo et al., 2009), as well as effector molecules like matrix metalloproteases (e.g. MMP-1) (Malik et al., 2007).

Mutations within the TLR4 signalling pathway are known to inhibit receptor activation by nickel and cobalt ions; Oblak *et al* showed that mutant MD2 inhibits NFκB activation following metal ion stimulation (Oblak et al., 2015). The same study showed that mutations can have different effects on TLR4 ligands; mutation of the F440 residue in the TLR4 sequence significantly abrogated LPS activation of the receptor but had little impact on cobalt and nickel-

mediated activation (Oblak et al., 2015). This indicates that genetic factors could potentially influence an individual's response to metal ions.

Two common TLR4 SNPs have been shown to affect the response to TLR4 ligands. Asp299G (referred to as D299G) and Thr399Ile (T399I) are both missense mutations. D299G is an aspartic acid to glycine change, while T399I is a threonine to isoleucine change. Both of these SNPs have a population prevalence of approximately 10% (Awomoyi et al., 2007). Guo *et al* found that murine hepatic stellate cells expressing D299G or T399I TLR4 exhibited hyporesponsiveness to LPS stimulation compared to wildtype TLR4 (Guo et al., 2009). The observed effects were not due to alterations in TLR4 expression levels in the D299G TLR4-expressing cells and therefore are more likely to be a result of molecular or structural effects that lead to diminished LPS responses. Similarly, Figueroa *et al* demonstrated reduced LPS responsiveness in D299G TLR4-expressing HEK cells compared to wildtype TLR4-expressing cells (Figueroa et al., 2012). Using co-immunoprecipitation they showed that D299G TLR4 has a reduced ability to recruit the MyD88 and TRIF adaptor proteins compared to wildtype TLR4. LPS-mediated induction of MyD88-dependent *IL8* expression was also inhibited in D299G TLR4-expressing cells (Figueroa et al., 2012). In contrast to the inhibitory effects of D299G, T399I-expressing cells did not show any reduction in MyD88 or TRIF-dependent responses to LPS. These effects occur despite the fact that LPS does not bind directly to the site of the mutation (Long et al., 2014).

6.1.2. Role of other TLR4 ligands

Receptor and ligand synergy can both affect the outcome of TLR4 signalling. For example activated TLR4 can synergise with TLR2 to promote inflammation (Sato et al., 2000). However for the purposes of this study it is the potential synergistic effects of TLR4 ligands that are of greatest interest.

6.1.2.1. Bacterial endotoxin

Aseptic loosening is the leading cause of joint implant failure but bacterial infection around a joint can also cause inflammation. If the infection is deep within the bone it may result in removal of the implant to allow treatment to take place (Haddad and Bridgens, 2008). Infection rates following THR with any implant material are generally low, at around 1% for primary THR and 2% for revision procedures (Blom et al., 2003), although infection is more common following MoM hip replacement or resurfacing (Wyles et al., 2014). The extent of the contribution of infection to joint implant failure is disputed. A recent study showed no association between bacterial infection and risk of revision in THR (Boot et al., 2015) but this

contradicts the results of many other studies that have shown that antibiotic prophylaxis can improve the longevity of a joint replacement (Espehaug et al., 1997, Engesæter et al., 2003). Infection can complicate ARMD diagnosis as the two conditions have very similar clinical presentations (Mikhael et al., 2009, Blumenfeld et al., 2010, Judd and Noiseux, 2011). It is also possible that infection and ARMD can occur at the same time and one may influence the biological and immunological response to the other.

Infection is associated with bacterial endotoxin and is of interest to the present study because endotoxin (LPS) can activate TLR4. This is an important aspect of patient variability, particularly in the case of low-grade, sub-clinical levels of infection that can go undetected and untreated. It is estimated that up to 10% aseptic loosening cases could in fact have low-grade infection (Moojen et al., 2010). Bacterial endotoxin can also adhere to wear debris and promote inflammatory cytokine secretion and osteoclast differentiation through activation of pattern recognition receptors such as the TLRs (Bi et al., 2001, Brooks et al., 2002). Bacterial biofilms can form on the surface of orthopaedic implants (Heim et al., 2015) and may become detached during the wear process, allowing them to travel systemically and activate the immune system. For MoM implants understanding the true impact of infection is made more complex by the ability of cobalt and chromium ions to inhibit bacterial growth in some cases (Hosman et al., 2009).

TLR4 ligands can synergise to alter the inflammatory outcome of receptor activation. HMGB1 and LPS combine to increase NFκB activation, cytokine secretion, and MMP activity in synovial fibroblasts (He et al., 2013b). Recent research has demonstrated that titanium particles enhance LPS-mediated nitric oxide, IL-6 and TNFα production by macrophages (Bianchi et al., 2015). However despite the clinical relevance of LPS and cobalt to ARMD, no studies have shown whether there is a synergistic relationship between LPS and cobalt in terms of TLR4 activation.

6.1.2.2. Endotoxin tolerance

Another mechanism that modulates TLR4 activation is repeated receptor exposure to a particular ligand which can result in tolerance. TLR4 tolerance to LPS is a well-documented effect and occurs through a range of mechanisms including increased IRAK-M expression (Escoll et al., 2003). This prevents formation of the IRAK complex that mediates the MyD88-dependent pathway. As a result, cells that have already been exposed to LPS/endotoxin show reduced cytokine secretion when they are subsequently re-challenged with the ligand (Sun et al., 2014), a phenomenon that was first observed in sepsis patients and can result in

immunodeficiency (Ertel et al., 1995). In contrast, TLR4 can also become sensitised to ligands. For example, in murine macrophages pre-exposure to LPS resulted in an exaggerated inflammatory response to subsequent nickel challenge (Pestka and Zhou, 2006). This aspect of TLR4 signalling is a potential source of patient variability because other TLR4 ligands, such as LPS, could be present in the joint alongside cobalt ions.

6.1.2.3. Nickel

The National Joint Registry reports that women, particularly those under 55 years old, have a higher revision rate due to MoM implant failure than men (Powers-Freeling, 2015). Although the reasons behind this are not fully understood, several theories have been suggested including a smaller joint size leading to increased implant wear and resulting metal ion release (Reito et al., 2013). Other studies have suggested that women are more prone to metal sensitisation prior to receiving their hip implant, for example through wearing jewellery containing nickel (Pandit et al., 2008). This latter point is of particular interest to the current study as nickel also activates TLR4 (Schmidt et al., 2010). A recent study using a mouse model of metal allergy showed that nickel can act as an adjuvant during cobalt sensitisation, resulting in increased numbers of B and T lymphocytes and an enhanced inflammatory response (Bonefeld et al., 2015). This is unlikely to be a TLR4-specific effect because murine Tlr4 cannot be activated by nickel or cobalt (Tyson-Capper et al., 2013a), but it does demonstrate a potential interaction between metal ions *in vivo*. As in the case of LPS there is little known about synergy between nickel and cobalt in their activation of human TLR4.

6.1.3. Inflammatory responses to other orthopaedic biomaterials

The need for improvements in the biological and mechanical properties of hip replacements means that the materials used in implants are constantly evolving. New materials include bioactive glass which is considered less susceptible to biofilm formation than other orthopaedic materials (Rahaman et al., 2014). As well as preventing infection, a major aspect of biocompatibility is the successful integration of the hip implant into the bone without causing an inflammatory response. It is therefore important to investigate the inflammatory properties of all orthopaedic materials and not just those that are released from the bearing surfaces during implant wear.

As well as nickel and cobalt, work has shown that palladium ions can activate TLR4 (Rachmawati et al., 2013). Palladium is found in dental implants but is not usually present in MoM joints. However this raises the question of whether other orthopaedic metals may contribute to the inflammatory response to MoM implants. In orthopaedic devices cobalt is

usually found as an alloy with chromium and sometimes molybdenum. Although many studies have identified inflammation induced by CoCr debris, the use of the alloy makes it difficult to determine which metal is causing the immune response, or whether they are working in combination. Chromium concentrations can vary between implants depending on their design, as well as in tissue samples from individual patients (Rodríguez De La Flor et al., 2013), and therefore may influence inflammatory responses to MoM implants. Strontium is chemically similar to calcium and consequently is used in joint replacement cements and bearing surface to promote peri-implant bone growth and integration. As a relatively new orthopaedic material strontium is not universally used and research into its effectiveness is ongoing (Lu et al., 2015). This introduces an element of variability that is of interest in the context of the present study. .

6.2. Objectives

- To generate stable cell lines expressing wildtype and D299G forms of human TLR4
- To evaluate the effect of these SNPs on the cellular response to cobalt ions using IL-8 as a marker of TLR4 activation
- To investigate the effect of LPS and nickel on cobalt-mediated cellular responses
- To investigate the potential of chromium and strontium ions to activate human TLR4

6.3. Specific materials and methods

The methods described below are based on a study by Long *et al* investigating the effect of TLR4 SNPs on the downstream TLR4 signalling pathway (Long et al., 2014), and were adapted to suit the requirements of this study.

6.3.1. 293-MD2-CD14 cells

293-hMD2-CD14 cells (Invivogen) are human embryonic kidney (HEK) 293 cells transfected with human *MD2* and *CD14* genes but lacking TLR4 expression. Cells were cultured in DMEM 4.5g/L glucose supplemented with 10% v/v FBS, 50U/ml penicillin, 50µg/ml streptomycin, 2mM L-glutamine and 100µg/ml Normocin (Invivogen). Cells were passaged by washing in PBS followed by scraping as trypsin can affect cell activity. After 2 passages 50µg/ml HygroGold selection antibiotic (Invivogen) was added to growth medium to maintain the plasmid encoding *MD2* and *CD14*.

6.3.2. Plasmid preparation

hu-TLR4 299 snp-flag pDEST40 and hu-TLR4 cDNA wt pDEST40 plasmids were gifts from Scott Friedman (Addgene plasmid #42647 and #42646 respectively). Full details of each plasmid are shown in **Table 6.1**.

TLR4 SNP	ID	Tag	Vector backbone	Bacterial resistance	Selectable marker	Laboratory source
Wildtype	42646	Flag	pcDNA DEST40	Ampicillin	Neomycin	Scott Friedman (Mount Sinai School of Medicine, USA)
Asp299Gly (D299G)	42647	Flag	pcDNA DEST40	Ampicillin	Neomycin	Scott Friedman

Table 6.1: TLR4 plasmids used in the transfection of 293-MD2-CD14 cells
Wildtype and D299G TLR4 plasmids were a gift from Scott FriedmanThe Flag tag sequence encodes a protein tag that can be used for isolation or purification of proteins. The vector backbone refers to the main part of the vector, which contains gene promoters, selectable markers and bacterial resistance genes.

Lysogeny broth (LB) agar plates were made using 35g/L LB agar dissolved in distilled H_2O. LB media was made by dissolving the following in 1L distilled H_2O; 10g tryptone/peptone, 5g yeast extract and 10g sodium chloride. *E. coli*were cultured on agar overnight at 37^{o}C and four individual colonies were selected followed by overnight incubation at 37^{o}C in an orbital shaker at 200rpm. Plasmids were isolated from bacterial cultures using a PureYield Plasmid MiniPrep System (Promega, Madison, Wisconsin, USA) according to the manufacturer's protocol.

6.3.3. Transfection procedure

293-MD2-CD14 cells were transfected using GeneJuice (Novagen, Darmstadt, Germany) according to manufacturer's protocol. Briefly, 3 days before transfection, cells were seeded at 250,000 cells per well of a 6 well plate. Four transfections were performed using DNA isolated from the four different bacterial cultures and control cells were treated with either GeneJuice only or left untreated. The volumes of reagents and/or plasmid DNA used in each well are detailed in **Table 6.2**. Six hours after transfection 3% v/v FBS was added to each well to support cell growth. Twenty four hours following transfection the transfection media was removed and replaced with complete media.

	Cells per well	Growth medium (ml)	OptiMEM (µl)	GeneJuice (µl)	Plasmid DNA (µg)
Wildtype TLR4	2.5×10^5	2	100	3	1
D299G TLR4	2.5×10^5	2	100	3	1
Vehicle	2.5×10^5	2	100	3	0
Untreated	2.5×10^5	2	100	0	0

Table 6.2: Reagent volumes used for transfection of MD2-CD14 cells

6.3.4. Selection of transfected cells

Seventy two hours after transfection the media on cells transfected with wildtype TLR4 and D299G TLR4 was changed to complete media supplemented with 1mg/ml G418 (Sigma Aldrich). G418 is a neomycin analogue that kills untransfected cells that do not carry the neomycin selectable marker. Cells were maintained in G418-supplemented media throughout subsequent experiments. After four weeks the G418 concentration was reduced to 500µg/ml and after 6 weeks this was reduced again to 100µg/ml. The control cells (vehicle only and untreated) were not treated with G418. Media on all cells was changed every 2-3 days.

6.3.5. Immunofluorescence

293-MD2-CD14 cells were seeded in complete media on glass coverslips in 12-well culture plates and cultured to approximately 70% confluency. Coverslips were removed from the plate and fixed in ice-cold 100% methanol for 10 minutes. Coverslips were air-dried overnight at room temperature.

To block non-specific binding 10% v/v normal goat serum in Tris-buffered saline (TBS) (0.1M Tris, 0.05M NaCl, at pH 7.6) was added to each coverslip and incubated at room temperature for 10 minutes. Following removal of the blocking agent, an anti-TLR4 rabbit primary antibody (Santa Cruz Biotechnology, Dallas, USA, catalogue number sc-10741) was diluted 1:100 in the

blocking agent, added to coverslips and incubated at 4ºC overnight. The primary antibody was removed and coverslips were washed 3 times (2x 5 minutes washes and 1x 15 minutes) in TBS. A rhodamine-conjugated anti-rabbit secondary antibody diluted 1:40 in TBS was added to each coverslip and incubated in the dark at room temperature for 30 minutes. Coverslips were then stained with 4',6-diamidino-2-phenylindole (DAPI) nuclear stain for 5 minutes and mounted using fluorescent mounting media (Dako, Cambridge, UK). Imaging was performed using a Zeiss Axio Imager II in the Bio-imaging Unit at Newcastle University.

6.3.6. HEK-Blue-hTLR4 reporter cell assay

HEK-Blue-hTLR4 cells (Invivogen, San Diego, USA) are human embryonic kidney (HEK) 293 cells transfected with genes encoding human TLR4 and its co-receptors MD2 and CD14. In addition these cells contain a secreted alkaline phosphatase (SEAP) reporter gene controlled by NFκB. Consequently when TLR4 is activated, NFκB is also activated and secretion of SEAP is induced. HEK-Blue-hTLR4 cells were cultured in DMEM 4.5g/L glucose supplemented with 2mM L-glutamine, 10% v/v FBS, 50U/ml penicillin and 50µg/ml streptomycin.

HEK-Blue-hTLR4 reporter cells were used to determine activation of human TLR4 by different potential ligands. The assay has previously been optimised to demonstrate TLR4 activation by cobalt ions (Tyson-Capper et al., 2013a) and therefore the same method was used in this study. Cells were seeded at 20,000 cells per well of a 96-well plate and treated with chromium chloride hexahydrate ($CrCl_3$) (Sigma-Aldrich) or strontium chloride hexahydrate ($SrCl_2$) (Sigma-Aldrich) in complete media for 24h. Supernatant was collected and SEAP secretion measured using QUANTI-Blue detection medium (Invivogen) according to the manufacturer's protocol. Absorbance was read at 620nm.

6.4. Results

6.4.1. Generation of stable TLR4 transfectants

293-MD2-CD14 cells were transfected as described in **section 6.3.3** and maintained in culture media supplemented with selection antibiotics. 1mg/ml G418 killed most cells in wells transfected with the wildtype (WT) TLR4 or D299G TLR4 plasmids, suggesting that transfection efficiency was low. However lower concentrations of G418 were ineffective at inducing cell death (data not shown). Cells were passaged as normal upon reaching confluency.

Basic culture of the transfected cells proved challenging. One of the major problems with the 293-MD2-CD14 cells line was that the cells clumped together as a sheet during passage and could not be separated by gentle pipetting. In similar cases trypsin would often be used for separation but it is not recommended by the manufacturer for these cells as it can alter their behaviour. Consequently the cells were difficult to count and seed. This made assessing TLR4 expression more complex; flow cytometry was a possible method for analysis but the clumped cells would likely block the channels of the flow cytometer. To overcome this issue TLR4 expression was assessed by immunofluorescence and qRT-PCR.

Immunofluorescence analysis demonstrated TLR4 protein expression in both WT and D299G TLR4 transfected cells (**Figure 6.1**). The staining showed mainly surface receptor expression although some intracellular staining was also noted in both cell lines. qRT-PCR analysis demonstrated increased *TLR4* expression by transfected cells compared to untransfected controls (**Appendix H**). In cases where all four replicate transfectants reached confluency and were suitable for analysis there were significant differences in *TLR4* expression levels between the replicates (**Appendix H)**.

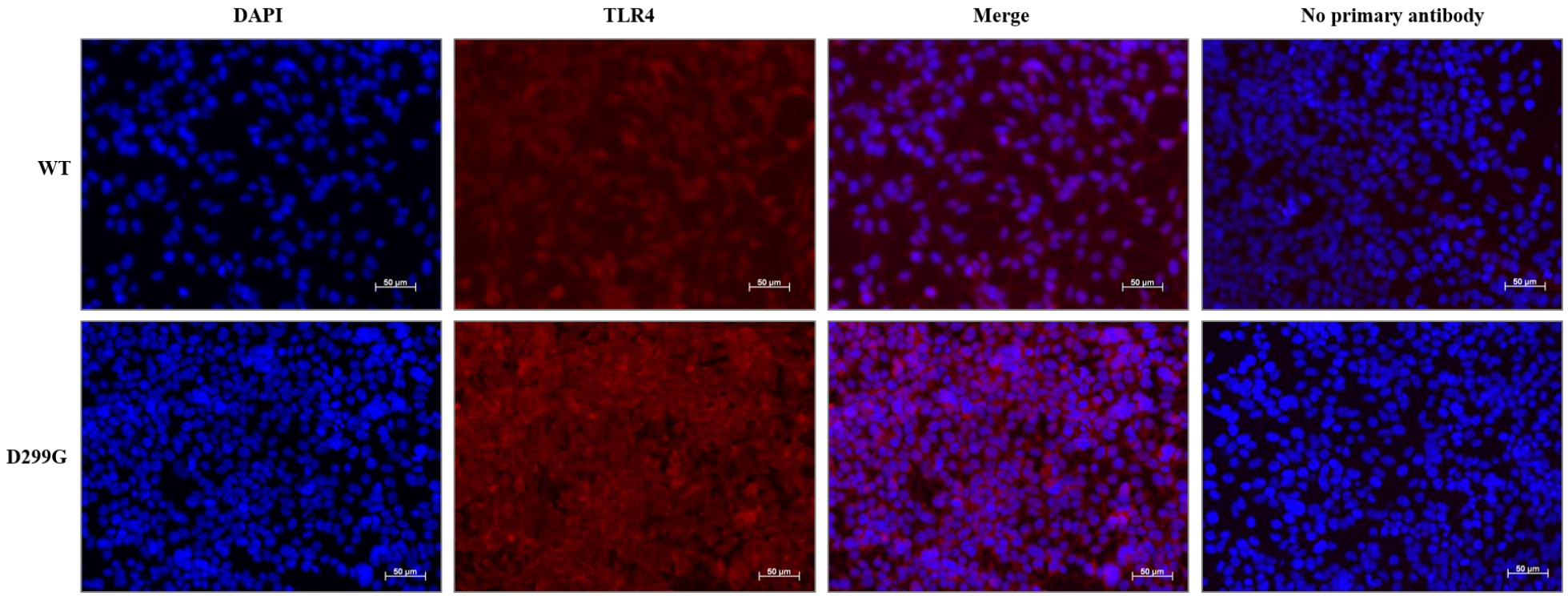

Figure 6.1: Immunofluorescent staining of transfected 293-MD2-CD14 cells
WT and D299G TLR4 transfected cells were assessed for TLR4 expression using immunofluorescent staining. A no primary antibody control was included for each assay. Images show separate DAPI and TLR4 staining and the merged images. Data is representative of two independent experiments in which four replicate transfections were performed for each SNP.

6.4.2. IL-8 expression and secretion by transfected and untransfected cells

The main aim of this aspect of the study was to determine how SNPs affect the cellular response to cobalt ions. IL-8 secretion has been used as a marker of cobalt activation of TLR4 throughout this study and was therefore deemed an appropriate marker for investigation into 293-MD2-CD14 cellular responses. As an initial control experiment untransfected 293-MD2-CD14 cells were stimulated with 0.5-1.5mM $CoCl_2$ or 100ng/ml LPS for 24h before supernatant was collected and IL-8 release measured by ELISA. A broader range of $CoCl_2$ concentrations was selected because no previous studies have investigated the effect of cobalt ions on 293-MD2-CD14 cells. Interestingly, despite the lack of TLR4 expression by these cells there was a small but significant increase in IL-8 secretion to 500pg/ml with 0.5 and 1mM $CoCl_2$ as well as with 100ng/ml LPS (all $p<0.001$) (**Figure 6.2**). At 1.5mM $CoCl_2$ there was no change in IL-8 secretion compared to the untreated control ($p=0.8351$) **(Figure 6.2)**.

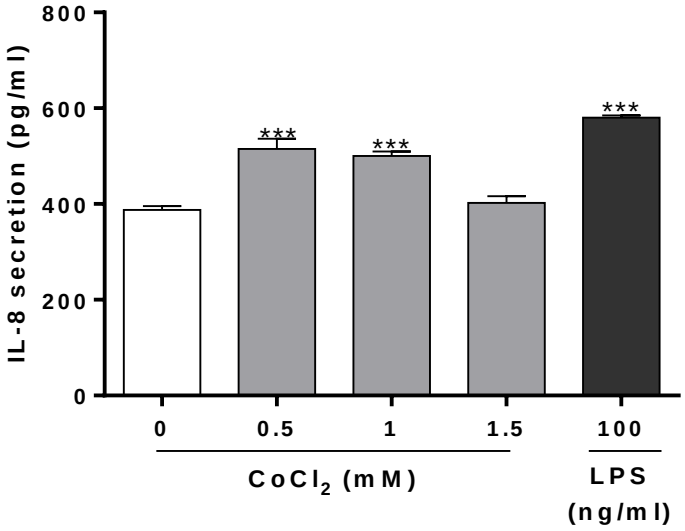

Figure 6.2: Effect of $CoCl_2$ on IL-8 secretion by untransfected 293-MD2-CD14 cells
Untransfected 293-MD2-CD14 cells were stimulated with 0.5-1.5mM $CoCl_2$ or 100ng/ml LPS for 24h and IL-8 release was measured by ELISA. n=1. Statistical significance was calculated by one-way ANOVA with Dunnett's test for multiple comparisons comparing stimulated samples to the unstimulated control.

6.4.3. IL-8 secretion and expression by TLR4 transfectants

TLR4 function in the transfected cells was assessed using IL-8 as a marker of receptor activation. Unfortunately the wildtype TLR4 transfectants died before they could be analysed, although the reasons for this were unclear. The remaining assays were conducted using only the D299G TLR4 transfectants. As well as the LPS positive control assay, a second experiment was conducted to investigate the inflammatory effect of cobalt ions on the transfected cells. D299G TLR4 transfectants were stimulated with 0.75mM $CoCl_2$ or 100ng/ml LPS for 24h before IL-8 secretion was measured by ELISA. Treatment with $CoCl_2$ and LPS increased IL-8 secretion by the D299G TLR4 transfectants to approximately 1000pg/ml ($p<0.001$) (**Figure 6.3**). The variation in *TLR4* expression by the four different cell lines did not correlate with IL-8 secretion levels (**Appendix H, Figures H.1 and H.2**).

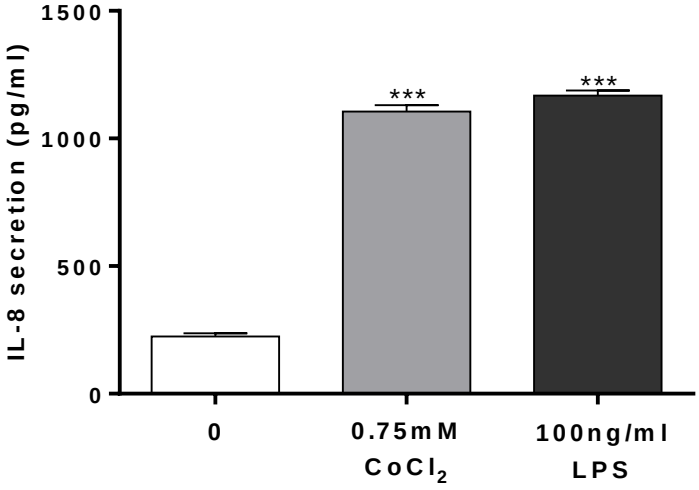

Figure 6.3: Effect of $CoCl_2$ on IL-8 secretion by D299G TLR4 transfectants
D299G TLR4 cells were stimulated with 0.75mM $CoCl_2$ or 100ng/ml LPS for 24h and IL-8 secretion was measured by ELISA. Four replicate experiments were conducted. Statistical significance was calculated by one-way ANOVA with Dunnett's test for multiple comparisons comparing treated samples to the untreated control.

6.4.4. Effect of co-exposure to LPS and cobalt ions

Another aim of this chapter was to identify how co-stimulation with cobalt ions and other TLR4 ligands (i.e. LPS and nickel ions) affects cytokine secretion. IL-6 and IL-8 secretion were selected for investigation. IL-8 was chosen because it has been used as a marker of TLR4 activation throughout this study. IL-6 was selected because its release is significantly increased following LPS activation of TLR4 but the response to $CoCl_2$ is much smaller than that of IL-8, although it is still TLR4-dependent (**Figure 4.9B**). *IL8* and *IL6* expression was also assessed, together with *CCL2* which is downregulated by $CoCl_2$ in the MonoMac 6 cell line model **(Chapter 3)**.

MonoMac 6 cells were stimulated with 0.25-1mM $CoCl_2$ with 100ng/ml LPS (referred to as $CoCl_2$-LPS) for 24h to investigate potential synergy between the two ligands. An LPS-only control was also included. RNA and supernatant were collected following stimulation. IL-6 and IL-8 secretion was measured by ELISA. IL-8 secretion was significantly upregulated to 5000pg/ml with LPS stimulation alone ($p<0.001$) but there was no further increase in IL-8 release when $CoCl_2$ was added at any concentration (all $p>0.9$ compared to LPS-only control) **(Figure 6.4A)**.

Cellular secretion of IL-6 was upregulated to approximately 1600pg/ml following stimulation with 100ng/ml LPS ($p<0.001$) **(Figure 6.4B)**. Co-stimulation with $CoCl_2$-LPS caused a significant decrease in IL-6 secretion compared to the LPS-only control (all $p<0.001$). This was a dose-dependent effect, reducing IL-6 levels from 1000pg/ml with 0.25mM $CoCl_2$-LPS to 150pg/ml with 1mM $CoCl_2$-LPS (**Figure 6.4B**).

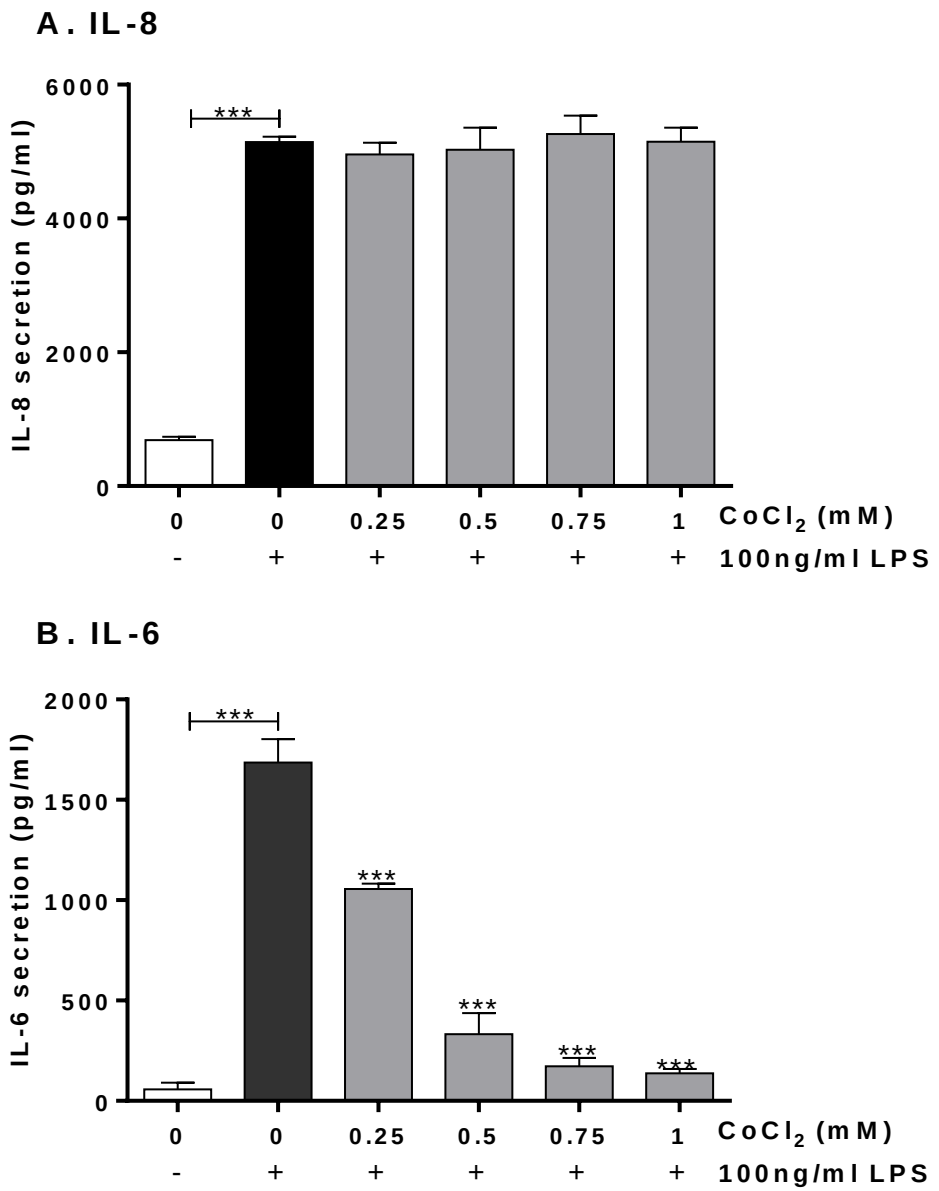

Figure 6.4: Effect of $CoCl_2$-LPS on IL-8 and IL-6 secretion
MonoMac 6 cells were stimulated with 0.25-1mM $CoCl_2$ with or without 100ng/ml LPS for 24h. Supernatant was collected and analysed by ELISA for **A.** IL-8 and **B.** IL-6 secretion. Data is representative of three independent experiments and statistical significance was calculated by one-way ANOVA with Tukey's test for multiple comparisons comparing all samples to each other. Bars show statistical difference between untreated and LPS samples. Other asterisks indicate differences between LPS and $CoCl_2$-LPS samples.

Expression of *IL6, CCL2* and *IL8* by $CoCl_2$-LPS-stimulated MonoMac 6 cells was analysed by qRT-PCR. LPS upregulated *IL6* expression by more than 3000-fold ($p<0.001$) but this was significantly abrogated by all concentrations of $CoCl_2$ (all $p<0.001$) **(Figure 6.5A)**, which reflects the pattern of IL-6 secretion shown in **Figure 6.4B**. A similar trend was observed for *CCL2* expression, with a 12-fold upregulation in expression following LPS stimulation ($p<0.001$) (**Figure 6.5B**). This was significantly decreased by $CoCl_2$ across all concentrations to expression levels lower than the untreated control (all $p<0.001$) **(Figure 6.5B)**.

IL8 expression showed a different pattern to that of *IL6* and *CCL2*. While LPS treatment alone still caused a significant 140-fold upregulation in *IL8* expression ($p<0.001$) **(Figure 6.5C)**, the addition of $CoCl_2$ had no synergistic effect at 0.25mM ($p=0.2593$) and 0.5mM ($p=0.4028$). At 0.75mM and 1mM $CoCl_2$-LPS there was a significant decrease in *IL8* expression compared to the LPS only control (both $p<0.001$) (**Figure 6.5C**) which is a slightly different trend from the IL-8 secretion profile shown in **Figure 6.4A**. However the decrease was fairly small compared to that which was observed for *IL6*. *IL8* expression decreased from 140-fold (LPS only) to 107-fold for 0.75mM $CoCl_2$, and 70-fold for 1mM $CoCl_2$. In contrast, *IL6* expression decreased from 3000-fold (LPS only) to 80-fold with the addition of 0.25mM $CoCl_2$.

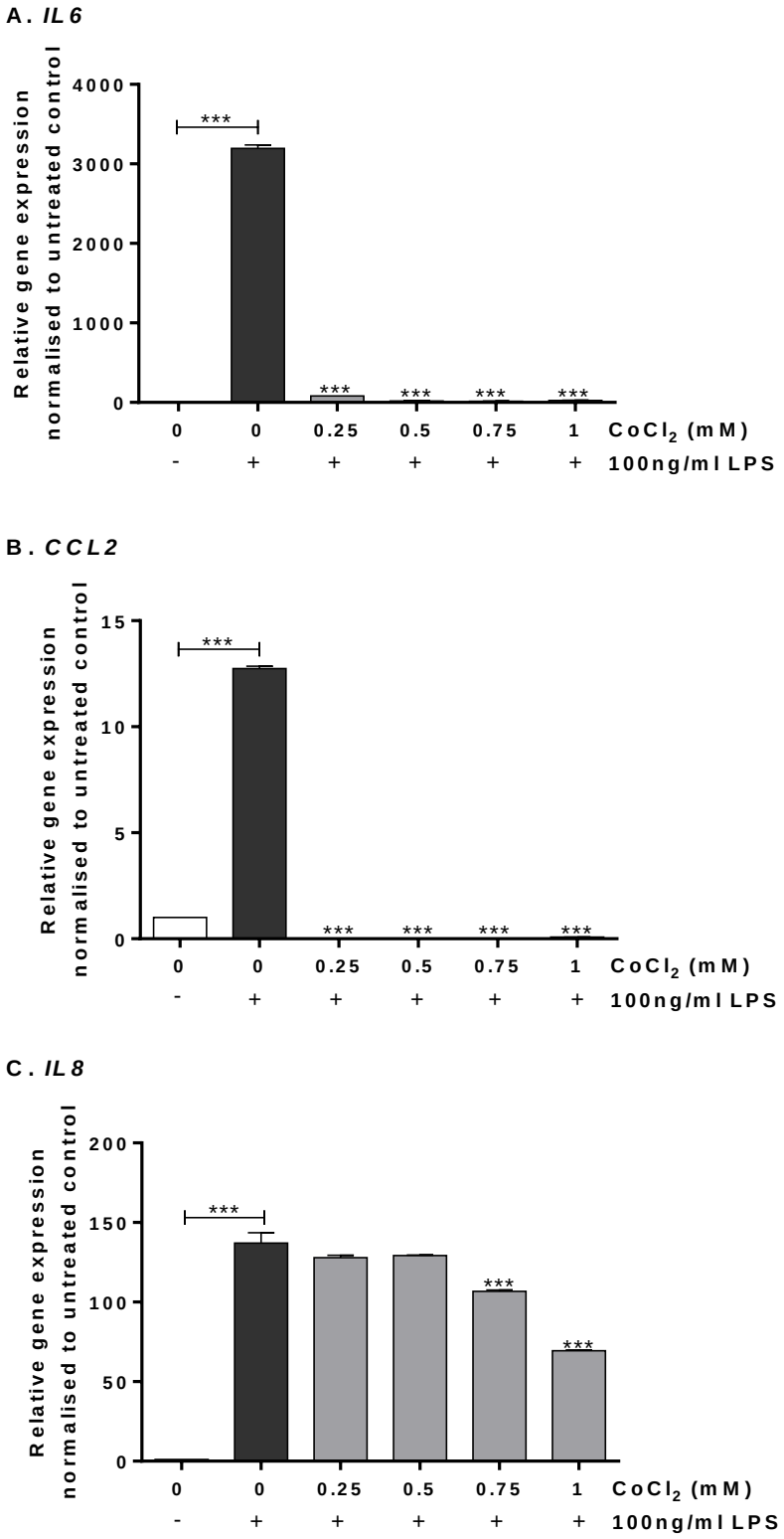

Figure 6.5: Effect of $CoCl_2$-LPS on inflammatory gene expression
MonoMac 6 cells were stimulated with 100ng/ml LPS and 0.25-1mM $CoCl_2$ in combination for 24h. RNA was isolated and cDNA synthesised by reverse transcription. qRT-PCR was performed for **A.** *IL6* **B.** *CCL2* and **C.** *IL8*. Data is representative of three independent experiments and statistical significance was calculated by one-way ANOVA with Tukey's test for multiple comparisons comparing all samples to each other. Bars show significance between LPS only and untreated control. Other asterisks show $CoCl_2$-LPS samples compared to the LPS only sample.

In addition to the target gene expression changes observed following $CoCl_2$-LPS stimulation, it was also noted that the combination of treatments caused a downregulation in expression of the housekeeping gene *GAPDH* **(Figure 6.6A)**. *GAPDH* Ct values were approximately 19 for untreated samples and increased significantly and dose-dependently with the addition of LPS and $CoCl_2$ (all $p<0.001$), peaking at 21 with 1mM $CoCl_2$-LPS. This was of concern because housekeeping gene expression should be consistent across all samples to provide an appropriate control for normalisation. Beta actin (*ACTB*) expression was also assessed to see if this effect occurred with other housekeeping genes and showed a very similar pattern to *GAPDH* **(Figure 6.6B)**. Changes in housekeeping gene expression, particularly downregulation, can often be attributed to cell stress and RNA degradation (Hilmi et al., 2003, Snider et al., 2011). To check RNA integrity in the $CoCl_2$-LPS samples RNA was separated by agarose gel electrophoresis. There did not appear to be any loss of RNA integrity that may account for the decrease in *GAPDH* expression (**Figure 6.7)**. RNA was not quantified prior to loading onto the gel which accounts for the differences in band intensity between the samples.

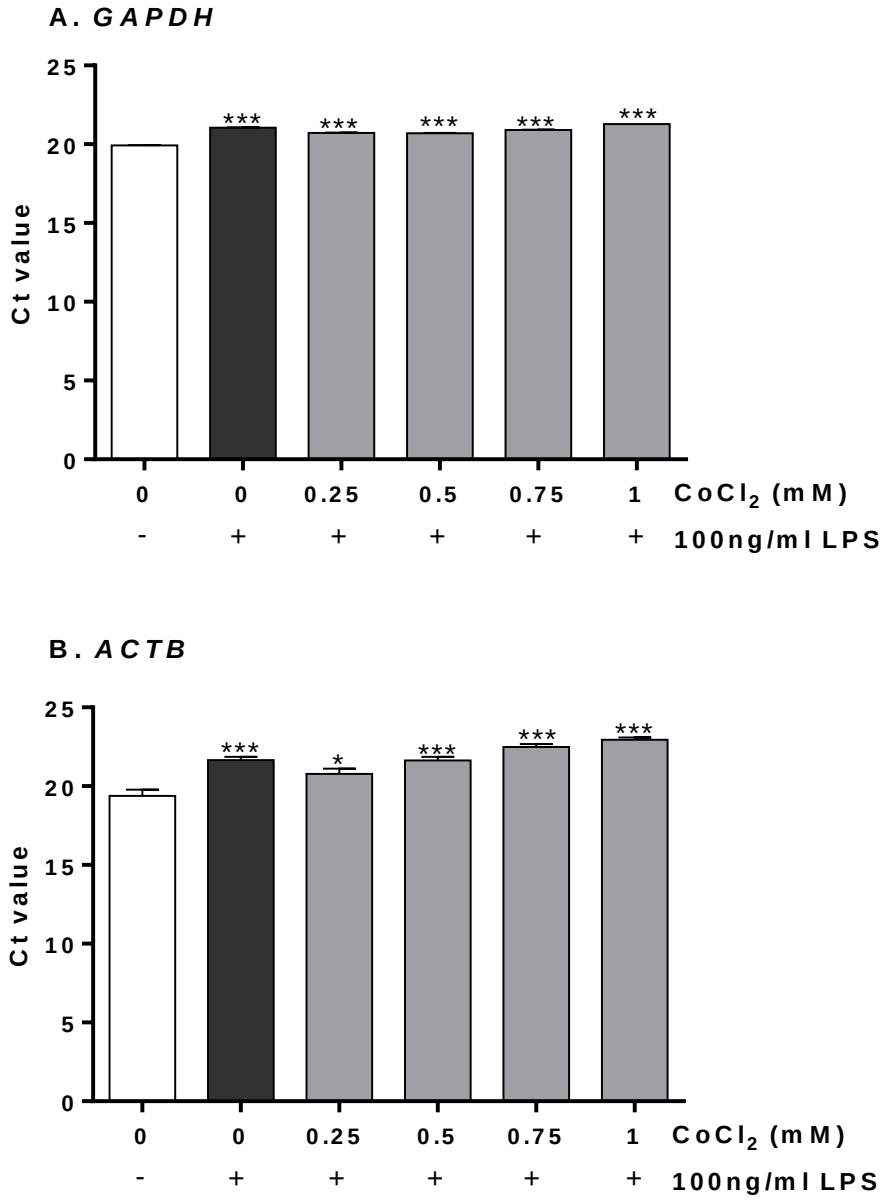

Figure 6.6: Effect of $CoCl_2$-LPS on *GAPDH* and *ACTB* expression
MonoMac 6 cells were stimulated with 100ng/ml LPS together with 0.25-1mM $CoCl_2$ for 24h. RNA was isolated and cDNA synthesised by reverse transcription. **A.** *GAPDH* and **B.** *ACTB* expression was analysed by qRT-PCR. Data is representative of three independent experiments and statistical significance was calculated by one-way ANOVA with Dunnett's test for multiple comparisons comparing treated samples to the untreated control.

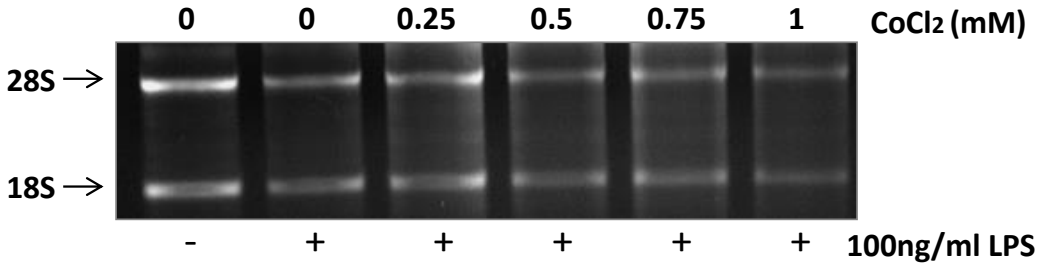

Figure 6.7: Effect of $CoCl_2$-LPS on RNA integrity
RNA integrity from $CoCl_2$ and LPS-treated MonoMac 6 cells was assessed by electrophoresis separation on a 1.2% w/v agarose gel.

6.4.5. Effect of co-exposure to cobalt and nickel ions

MonoMac 6 cells were stimulated with 0.25-1mM $CoCl_2$ together with 0.75mM $NiCl_2$ for 24h (referred to as $CoCl_2$-$NiCl_2$). Control cells were treated with 0.75mM $NiCl_2$ only or left untreated. Following stimulation RNA was isolated and supernatant was collected. IL-6 and IL-8 secretion was quantified by ELISA.

In response to 0.75mM $NiCl_2$ there was a significant increase in IL-8 secretion by MonoMac 6 cells, reaching approximately 5000pg/ml ($p<0.001$) (**Figure 6.8A**). With the addition of 0.25, 0.5 and 0.75mM $CoCl_2$ there was no significant change in IL-8 secretion ($p>0.2$ in all cases) by co-stimulated cells. When cells were stimulated with 1mM $CoCl_2$-$NiCl_2$ there was a significant decrease in IL-8 release to approximately 3500pg/ml ($p=0.0300$) (**Figure 6.8A**). IL-6 secretion was significantly increased to just over 2000pg/ml by treatment with 0.75mM $NiCl_2$ ($p<0.001$) (**Figure 6.8B**). $CoCl_2$-$NiCl_2$ co-stimulation resulted in abrogation of IL-6 release across all $CoCl_2$ concentrations (all $p<0.001$), reducing IL-6 levels to approximately 100pg/ml (**Figure 6.8B**).

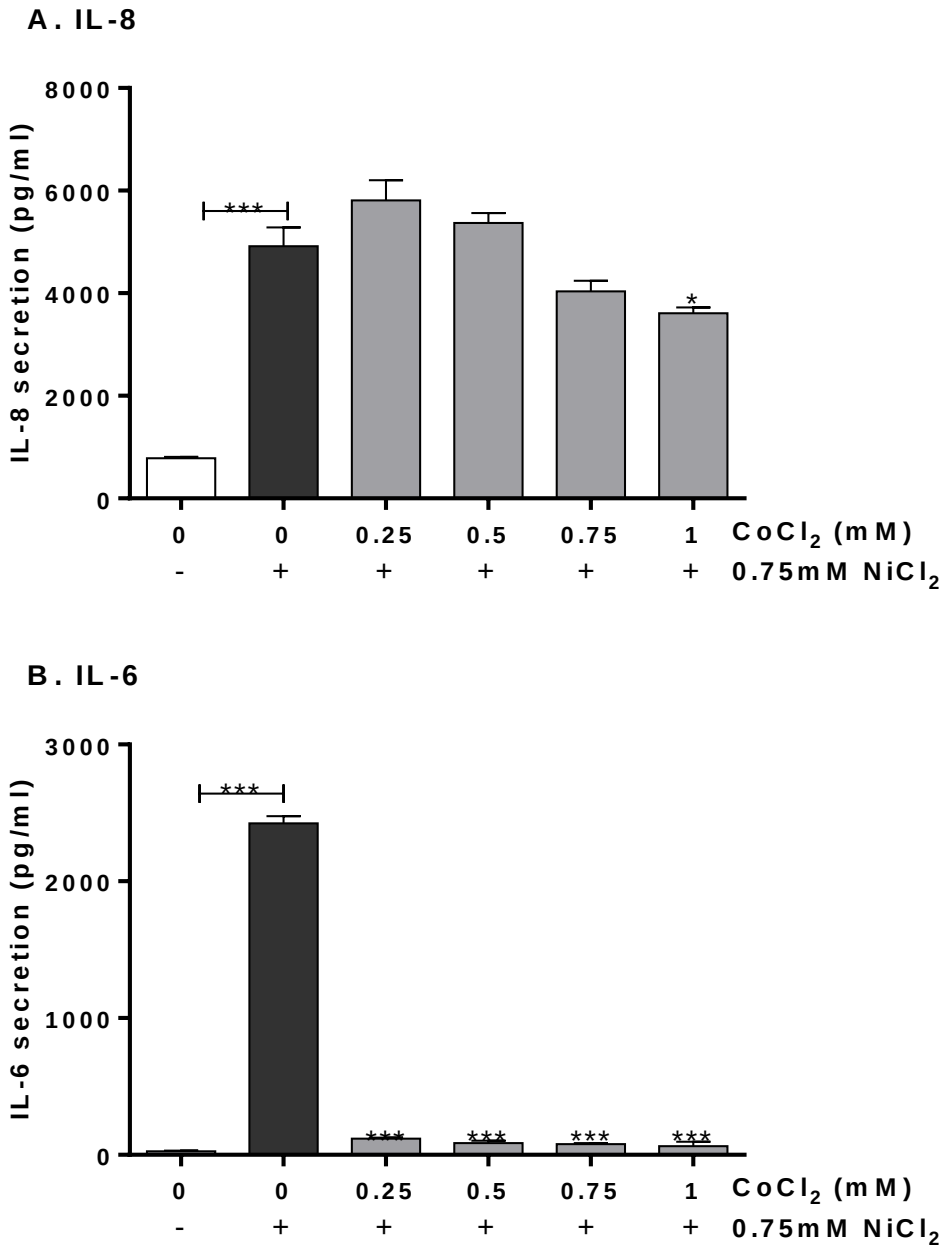

Figure 6.8: Effect of $CoCl_2$-$NiCl_2$ on IL-8 and IL-6 secretion
MonoMac 6 cells were stimulated with 0.75mM $NiCl_2$ and 0.25-1mM $CoCl_2$ in combination for 24h. Supernatant was collected and analysed by ELISA for **A.** IL-8 and **B.** IL-6 secretion. Data is representative of three independent experiments and statistical significance was calculated by one-way ANOVA with Tukey's test for multiple comparisons comparing all samples to each other. Bars show statistical difference between untreated and $NiCl_2$ only samples. Other asterisks indicate differences between $NiCl_2$ and $CoCl_2$-$NiCl_2$ samples.

Following $CoCl_2$-$NiCl_2$ stimulation *IL6, IL8* and *CCL2* expression was assessed by qRT-PCR. *IL6* secretion was significantly upregulated (more than 3000-fold) by $NiCl_2$ compared to the untreated control ($p<0.001$) (**Figure 6.9A**). The addition of $CoCl_2$ significantly decreased *IL6* expression in response to $NiCl_2$ (all $p<0.001$ compared to $NiCl_2$-only control) (**Figure 6.9A**). In contrast to the upregulation in *IL6* expression there was a significant decrease in *CCL2* expression following $NiCl_2$ stimulation ($p<0.001$ compared to untreated control) (**Figure 6.9B**). With the addition of 0.25mM $CoCl_2$ there was a further reduction in *CCL2* expression ($p=0.0424$ compared to $NiCl_2$-only control) but it increased again with 0.75mM and 1mM $CoCl_2$. Although this was a significant increase from the $NiCl_2$ sample (both $p<0.001$) it did not reach the *CCL2* expression levels detected in untreated cells. *IL8* expression was upregulated approximately 150-fold by $NiCl_2$ ($p<0.001$ compared to untreated control) (**Figure 6.9C**). When $CoCl_2$ was added, *IL8* expression decreased across all concentrations (all $p<0.001$ compared to $NiCl_2$-only control) although it was still upregulated 50-fold in these samples compared to the untreated control (all $p<0.05$). However there was no evidence of a dose-dependent response (**Figure 6.9C**).

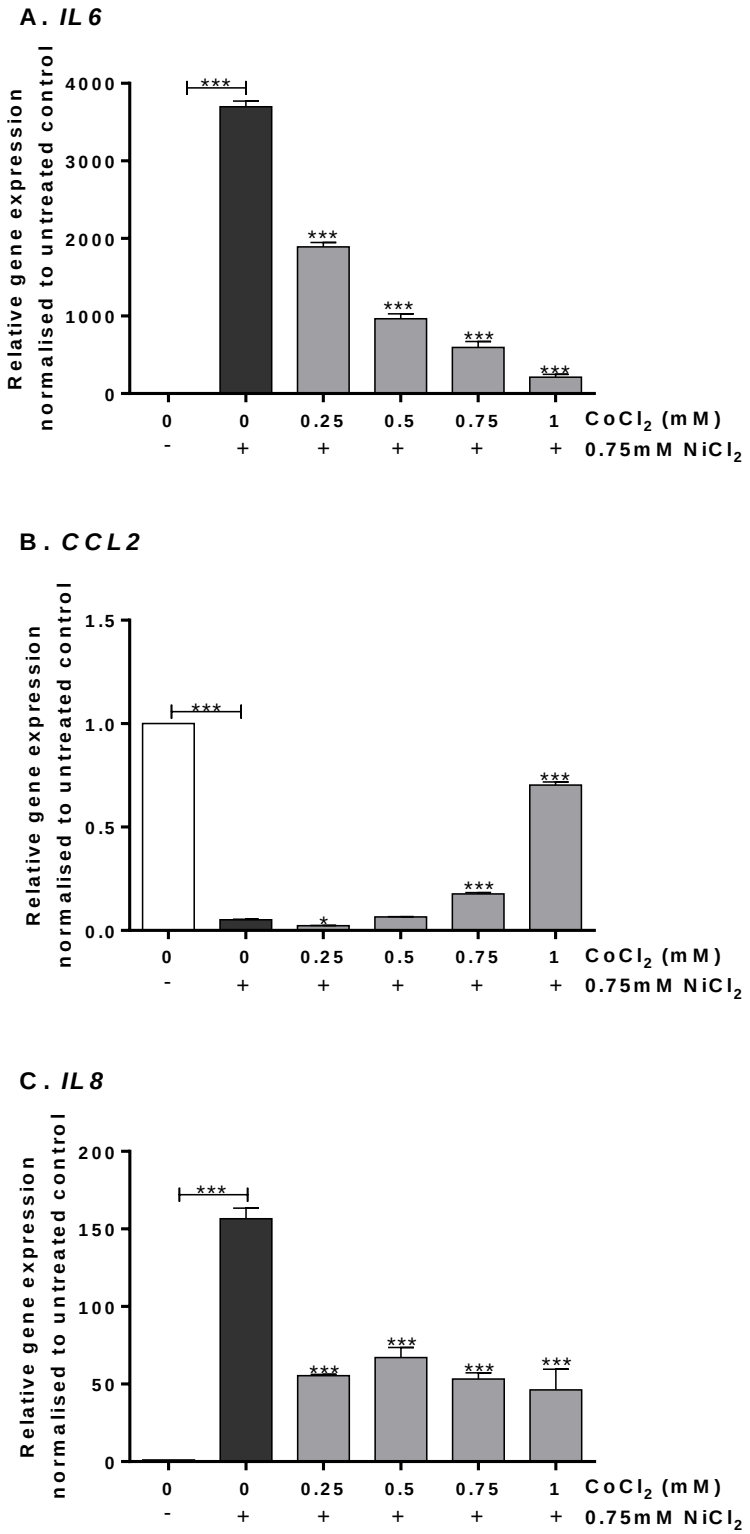

Figure 6.9: Effect of $CoCl_2$-$NiCl_2$ on inflammatory gene expression
MonoMac 6 cells were stimulated with 0.75mM $NiCl_2$ and 0.25-1mM $CoCl_2$ in combination for 24h before RNA was isolated and cDNA synthesised by reverse transcription. **A.** *IL6* **B.** *CCL2* and **C.** *IL8* gene expression was analysed by qRT-PCR. Data is representative of two independent experiments and statistical significance was calculated by one-way ANOVA with Tukey's test for multiple comparisons comparing all samples to each other. Bars show statistical difference between $NiCl_2$ only samples and the untreated control. Other asterisks show significance of $CoCl_2$-$NiCl_2$ samples compared to the $NiCl_2$ only sample.

As with the $CoCl_2$-LPS assays, closer analysis of the qRT-PCR data highlighted considerable changes in *GAPDH* expression between $CoCl_2$-$NiCl_2$-treated cells and untreated controls. The Ct value for *GAPDH* in untreated cells was approximately 19 and this increased dose-dependently to 24 in 1mM $CoCl_2$-$NiCl_2$ samples (all $p<0.001$ compared to the untreated control) (**Figure 6.10**). In this case *ACTB* expression was not assessed because in the $CoCl_2$-LPS assays it showed a similar pattern to that of *GAPDH* (**Figure 6.6)**.

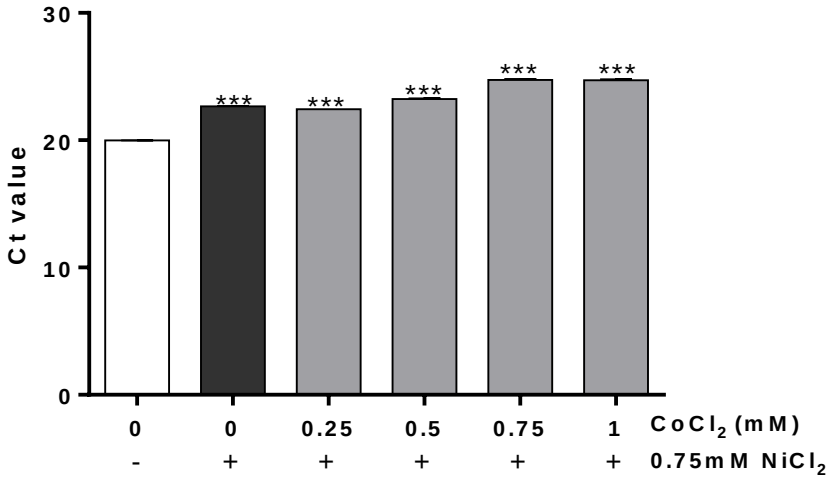

Figure 6.10: Effect of $CoCl_2$-$NiCl_2$ on *GAPDH* expression
MonoMac 6 cells were stimulated with 0.25mM-1mM $CoCl_2$ in combination with 0.75mM $CoCl_2$. RNA was isolated and cDNA synthesised by reverse transcription. *GAPDH* expression was quantified by qRT-PCR. Data is representative of two independent experiments and statistical significance was calculated by one-way ANOVA with Dunnett's test for multiple comparisons comparing all samples to the untreated control.

To determine whether or not the changes in *GAPDH* expression were due to RNA degradation induced by the $CoCl_2$-$NiCl_2$ cell stimulation, RNA was separated by electrophoresis on a 1.2% w/v agarose gel to assess integrity. There was no evidence of RNA degradation in any of the samples indicating that this is not the cause of downregulated *GAPDH* expression (**Figure 6.11**).

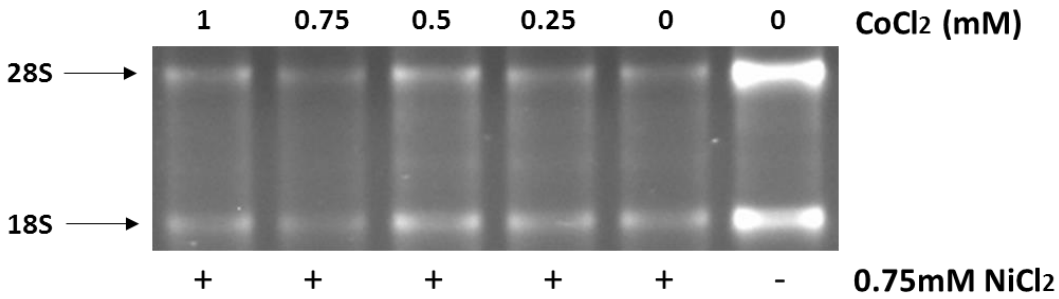

Figure 6.11: Effect of $CoCl_2$-$NiCl_2$ on RNA integrity
RNA from MonoMac 6 cells stimulated with $CoCl_2$-$NiCl_2$ was separated by electrophoresis on a 1.2% w/v agarose gel to assess integrity.

6.4.6. Chromium and strontium ions do not activate human TLR4

Chromium and strontium are present in CoCr alloys and bone cement respectively, but their effect on TLR4 is unknown. HEK-Blue-hTLR4 cells were stimulated with 0.5-1.5mM $CrCl_3$ or $SrCl_2$ for 24h before TLR4 activation was assessed by SEAP reporter assay, as described previously (Tyson-Capper et al., 2013a). There was no increase in SEAP secretion by the cells following metal ion challenge (all $p>0.3$), indicating that neither chromium nor strontium activates TLR4 (**Figure 6.12A&B**). There was increased SEAP secretion in response to the positive control LPS which is indicative of TLR4 activation ($p<0.001$). To further support this data MonoMac 6 cells were incubated with 0.5-1.5mM $CrCl_3$ or $SrCl_2$ for 24h, and IL-6 and IL-8 secretion was measured by ELISA. No increase in IL-8 secretion was detected with either $CrCl_3$ or $SrCl_2$ stimulation (all $p>0.6$) (**Figure 6.12C&D**), nor was there any change in IL-6 release (all $p>0.9$) (**Figure 6.12E&F**). LPS again provided an effective positive control by increasing both IL-8 and IL-6 secretion (all $p<0.001$).

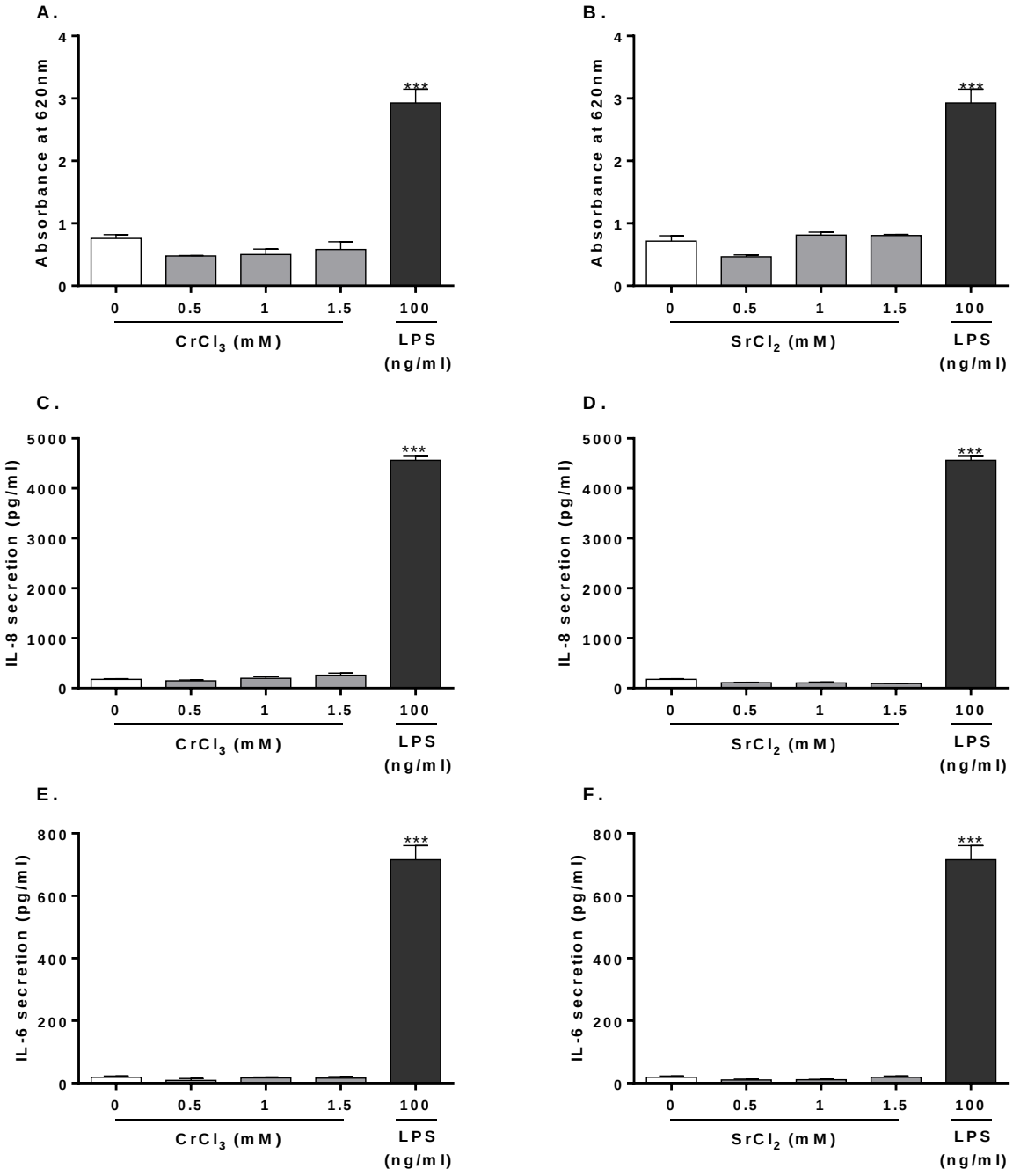

Figure 6.12: Effect of chromium and strontium ions on TLR4 activation
HEK-Blue-hTLR4 cells were stimulated with **A.** 0.5-1.5mM $CrCl_3$ or **B.** 0.5-1.5mM $SrCl_2$ and SEAP release was measured by reading absorbance at 620nm. MonoMac 6 cells were then stimulated with the same concentrations of metal ions and LPS, and IL-8 or IL-6 secretion was quantified by ELISA. **C.** IL-8 secretion following $CrCl_3$ stimulation. **D.** IL-8 secretion following $SrCl_2$ stimulation. **E.** IL-6 secretion following $CrCl_3$ stimulation. **F.** IL-6 secretion following $SrCl_2$ stimulation. n=2 for reporter assays and n=1 for ELISAs. Statistical significance was calculated by one-way ANOVA with Dunnett's test for multiple comparisons comparing stimulated samples to the unstimulated control.

6.5. Discussion

Effect of TLR4 SNPs on cobalt response

There are many polymorphisms that influence the outcome of TLR4 signalling pathway activation and contribute to its regulation of inflammation, including mutations in signalling factors and inflammatory cytokines. TLR4 SNPs were investigated in this study firstly because cobalt activates TLR4 and secondly because the SNPs can affect the response to other TLR4 ligands including LPS (Figueroa et al., 2012, Long et al., 2014).

293-MD2-CD14 cells were selected for this aspect of the study as they express the key TLR4 co-receptors MD2 and CD14 but do not express TLR4 itself. A preliminary investigation was conducted to assess IL-8 secretion by untransfected 293-MD2-CD14 cells in response to $CoCl_2$ and LPS. There was a small (100pg/ml) but significant increase in IL-8 secretion following treatment with 0.5mM and 1mM $CoCl_2$ as well as with 100ng/ml LPS. Given that the $CoCl_2$-mediated IL-8 release observed so far in this study has been attributed to TLR4 activation it is surprising that the cells released IL-8 when they do not express TLR4. However it is possible that this is the result of a generalised inflammation that occurs when cells are challenged with a pathogen. It would also provide an explanation for the cobalt-induced inflammatory responses detected in animal models (Masui et al., 2005, Akbar et al., 2012) that lack the TLR4 histidine pocket for cobalt binding and receptor activation.

As described in **section 6.4.2** there were some difficulties in assessing TLR4 expression levels following transfection of the 293-MD2-CD14 cells and these were mainly caused by cell clumping. As a result, flow cytometric analysis of TLR4 expression was not possible; to try and prevent cell clumping in future assays the cells could be incubated with ethylenediaminetetraacetic acid (EDTA), or sub-cultured at a reduced confluency. Caution would be required with the EDTA method because it can chelate metal ions which may affect the outcome of assays using cobalt ions.

For this study immunofluorescence was selected as an alternative method to flow cytometry. This revealed TLR4 expression by cells transfected with the WT TLR4 and D299G TLR4 plasmids. Further analysis of *TLR4* expression by qRT-PCR confirmed receptor expression by the transfectants. To confirm that cells expressed functional TLR4, IL-8 secretion was assessed. Although the D299G TLR4 polymorphism can abrogate inflammatory responses (e.g. cytokine secretion and NFκB activity (Arbour et al., 2000)) to LPS, Figueroa *et al* showed that it only partially inhibited IL-8 secretion (Figueroa et al., 2012), meaning that LPS-mediated IL-8 release is still an indicator of TLR4 activation. D299G TLR4 cells secreted IL-8 following

stimulation with LPS, showing that the cells were expressing functional TLR4. Following $CoCl_2$ treatment the cells also increased IL-8 release; this demonstrates that the D299G TLR4 variant does not completely prevent $CoCl_2$ activation of TLR4. Unfortunately the WT TLR4 transfectants died before further assays could be conducted and therefore the D299G transfectant response cannot be compared to a wildtype control. The reasons for the differences in cell survival between the two groups are not clear but could be due to increased susceptibility of the WT TLR4 cells to low levels of contaminants (e.g. endotoxin) in the culture media. However this means that there is no indication of whether or not the response to cobalt ions is abrogated in D299G TLR4 cells and consequently further optimisation of these assays is required.

The genetic aspect of this study is a preliminary investigation into the effect of TLR4 mutations on the inflammatory response to cobalt ions. A protocol was developed that can be used for studying other mutations in TLR4 and other aspects of the TLR4 signalling pathway. For a more comprehensive analysis of the effect of D299G TLR4, quantitation of TLR4 expression would be required to determine the amount of IL-8 released in direct relation to the amount of TLR4 expression. This will be discussed in more detail in **section 6.5.1**.

Effect of LPS and $NiCl_2$ on $CoCl_2$-mediated responses

The immunological effect of cobalt co-stimulation with other TLR4 ligands was also investigated. Co-stimulation of MonoMac 6 cells with $CoCl_2$-LPS did not significantly increase cellular IL-8 secretion compared to treatment with $CoCl_2$ alone. *IL8* expression also decreased with increasing $CoCl_2$-LPS concentrations but remained elevated compared to the untreated control. In contrast, although IL-6 secretion was upregulated by LPS, the addition of $CoCl_2$ resulted in a dose-dependent decrease in cytokine secretion. Expression of *IL6* and *CCL2* showed a similar trend to IL-6 release, although the $CoCl_2$-dependent decrease was greater at gene level. The large increase in LPS and $NiCl_2$-mediated *IL6* expression occurred because *IL6* in untreated cells had a Ct value of >37, which increased to approximately 27 following ligand stimulation.

The $CoCl_2$-$NiCl_2$ assays revealed a similar pattern of IL-6 secretion to that observed for $CoCl_2$-LPS. IL-8 secretion showed a slight increase with 0.25mM $CoCl_2$-$NiCl_2$ but this dose-dependently decreased with increased $CoCl_2$ concentrations. *IL6* and *IL8* expression was upregulated by $NiCl_2$ alone and abrogated with the addition of $CoCl_2$. Unlike LPS there was no increased *CCL2* expression following $NiCl_2$ stimulation, although the addition of $CoCl_2$ caused a small dose-dependent increase in expression towards untreated levels. The results of these

assays suggest that the effect of $CoCl_2$ dominates over those of LPS and $NiCl_2$. For example LPS alone upregulates *CCL2* expression while cobalt ions alone cause downregulation (described elsewhere in this study and in **Appendix I**). Co-stimulation with both ligands results in decreased *CCL2* expression. Similar effects were also observed for IL-6 secretion and expression.

Of the three cytokines assessed in this aspect of the study, IL-8 was perhaps most likely to show synergistic upregulation because LPS, $NiCl_2$ and $CoCl_2$ can all individually activate TLR4 and increase its secretion. However no such effects were observed. This could be due to receptor saturation preventing any further cytokine secretion, particularly in the case of $NiCl_2$ as $CoCl_2$ and $NiCl_2$ activate TLR4 via the same mechanism (Raghavan et al., 2012). It is also conceivable that the two ligands in combination increase cytotoxicity which could mask any synergy. To overcome this problem cytotoxicity assays similar to those performed elsewhere in this study could be undertaken. Alternatively total protein assays would allow correlation of cell numbers with cytokine secretion levels.

Cytotoxicity may also have affected the expression of housekeeping genes *GAPDH* and *ACTB* which were both downregulated with increasing concentrations of $CoCl_2$ in combination with LPS or $NiCl_2$ (*GAPDH* only). These changes were much smaller than those observed for the target cytokine genes and are therefore unlikely to have notably affected the assay results. The downregulation could not be attributed to loss of RNA integrity but is still significant and consequently further optimisation is required to provide a solution. This could involve using alternative housekeeping genes such as hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) to identify a gene that is not affected by the co-stimulation treatments.

As well as receptor saturation and cytotoxicity there may be other underlying molecular mechanisms contributing to the response observed in the co-stimulation assays. Recent work investigating the combined effects of $NiCl_2$ and LPS showed that nickel ions cause downregulation of LPS-mediated IL-6 expression by decreasing expression of an IL-6 mRNA stabiliser normally induced in response to LPS (Asakawa et al., 2015). In the present study there was no evidence of total RNA degradation with different treatment combinations but this does not mean that the mRNA of an individual gene is not affected. Other potential mechanisms could include TLR4 tolerance developing in response to challenge with multiple ligands, or cell-mediated downregulation of cytokines and chemokines for protection against uncontrolled inflammation.

A potential role for hypoxia

The results presented in this chapter raise questions about the role of hypoxia in cellular responses to cobalt. Cobalt is widely used in laboratories as a hypoxia mimetic (Salnikow et al., 2000) and data presented here shows similar patterns to hypoxic responses detected by other studies. Hypoxia results in activation of the transcription factor hypoxia inducible factor 1 (HIF1). HIF1 upregulates the expression of genes that can counteract hypoxic conditions by promoting angiogenesis, erythropoiesis, and increased iron metabolism; these include erythropoietin and vascular endothelial growth factor (VEGF) (Forsythe et al., 1996). Cobalt stabilises hypoxia inducible factor 1α (HIF1α), a subunit of HIF1, resulting in increased gene transcription. A preliminary assay in this study showed that there is a small but significant upregulation in *HIF1A* (gene encoding HIF1α) expression by MonoMac 6 cells stimulated with 0.75mM $CoCl_2$ **(Appendix J)**, suggesting that hypoxia and particularly HIF1α may be involved in some of the cellular responses described in this study. However this is purely speculative and further analysis would be required to support the hypothesis.

Binding of NFκB to the *IL8* promoter induces *IL8* gene transcription. The hypoxia response element (HRE) is adjacent to the NFκB binding site and therefore when HIF1α binds to the HRE, *IL8* transcription is also induced (Kim et al., 2006). This may account for the difference between *IL8* expression patterns and those of *IL6* and *CCL2* when cells were treated with $CoCl_2$-LPS. It may also explain why there were such large differences in the detected levels of cytokines in earlier chapters; IL-8 secretion following cobalt stimulation often reached >5000pg/ml whereas with the same concentrations of $CoCl_2$ IL-6 and CCL20 secretion was usually <1000pg/ml. Although this effect has been reported in LPS-stimulated cells in other studies (Sawa et al., 2008) it is conceivable that it is potentiated by HIF1α.

HIF1α appears central to cobalt-mediated inflammatory responses because HIF1α-deficient mice display a Th2 (immunomodulatory) phenotype compared to the Th1-dominated response in their wildtype counterparts (Saini et al., 2010a). However there are mixed reports regarding the effect of HIF1α on the secretion of specific inflammatory cytokines. CCL2 release can be upregulated in hypoxia as its promoter region contains several binding sites for HIF1α (Mojsilovic-Petrovic et al., 2007). Other work has indicated that $CoCl_2$-induced hypoxia downregulates CCL2 secretion (Negus et al., 1998, Eleftheriadis et al., 2011) which is consistent with the results obtained in this study for both $CoCl_2$ and $CoCl_2$-$NiCl_2$ stimulation. The variability between studies could arise due to different cell lines or cobalt concentrations used which could be an interesting avenue for further investigation.

Studies have also shown conflicting results when investigating the effect of hypoxia on LPS-mediated inflammation. Jantsch *et al* reported that LPS can stabilise HIF1α via a TLR4, MyD88 and NFκB-dependent signalling pathway (Jantsch et al., 2011). However other studies have observed anti-inflammatory responses to LPS and hypoxia; under hypoxic conditions LPS-induced TLR4 activation and secretion of IL-8 and IL-6 is attenuated (Hu et al., 2014) and NFκB signalling is inhibited (Müller-Edenborn et al., 2015). In the case of IL-6 expression and secretion this appears to occur through downregulation of TLR4 expression (Shirasuna et al., 2015).

The potential importance of cobalt and hypoxia in MoM implant failure has recently been highlighted. In a study comparing patients with MoM implants to those with MoP devices, Samelko *et al* found increased HIF1α expression in the peri-implant tissues and synovial fluid of MoM patients (Samelko et al., 2013). Nyga *et al* reported dose-dependent increases in HIF1α stabilisation and expression in macrophages stimulated with cobalt nanoparticles from MoM implants (Nyga et al., 2015). Increased HIF1α production was also noted following cobalt ion treatment, although to a lesser extent than with nanoparticles (Nyga et al., 2015). Taken together this research suggests that there may be a link between cobalt, hypoxia, and TLR4 activation, which would warrant further investigation.

Chromium and strontium

Chromium and strontium ions were investigated for their ability to activate TLR4 and induce inflammatory cytokine secretion. Chromium was chosen because it is found as an alloy with cobalt and sometimes molybdenum in many MoM hip implants. Strontium is used in the cement that fixes hip replacements into the bone and was selected because hip implants can be cemented or uncemented, which introduces another aspect of patient variability. There was no evidence of TLR4 activation by either chromium or strontium ions using a HEK-Blue-hTLR4 reporter cell assay. This assay does not exclude their ability to activate cells by other mechanisms, such as activation of different PRRs. Consequently MonoMac 6 cells were stimulated with the metal ions to investigate their inflammatory properties but no IL-6 or IL-8 secretion was detected. The assay still does not entirely eliminate the possibility of immune activation by different pathways that are beyond the scope of this *in vitro* model. However IL-6 and IL-8 are secreted via multiple signalling pathways and therefore their absence suggests that a significant inflammatory response is not taking place. It also supports the TLR4-dependent nature of cobalt-mediated inflammatory responses, as without TLR4 activation there was no evidence of inflammatory cytokine secretion.

It is perhaps not surprising that strontium does not cause an inflammatory response because one of its beneficial properties is its chemical similarity to calcium; it is absorbed via the same mechanisms and therefore used in bone cement to promote bone growth, osseointegration, and help prevent osteolysis. The outcome of the assays conducted in the present study also support the work of Ninomiya *et al* who found that in comparison to the inflammatory effects of cobalt, chromium ions do not induce IL-8 or CCL2 secretion in endothelial cells, nor do they affect lymphocyte chemotaxis and adhesion molecule expression (Ninomiya et al., 2013). From the results described in the literature and in the present study it appears that cobalt is more involved in inflammatory responses than chromium or strontium.

6.5.1. Future work

293-MD2-CD14 cells, whilst good models for transfection studies, are not immune cells nor would they be found around a failed joint replacement. They also lack many of the typical characteristics of inflammatory cells including expression of a wide range of inflammatory cytokines and chemokines, and phagocytic capabilities. It would therefore be interesting to induce the D299G TLR4 mutation or other TLR4 SNPs in cells such as MonoMac 6 macrophages to determine how this affects immune cells. The effects of SNPs on secretion of different inflammatory cytokines could also be investigated.

The treatment combinations ($CoCl_2$-LPS and $CoCl_2$-$NiCl_2$) represent a good starting point for modelling the effect of infection and nickel exposure on the TLR4 response to cobalt ions. However more work is required to refine the assays to better replicate the *in vivo* scenario; for example, pre-treating the cells with either nickel ions or LPS may alter the cellular response, as may changing the agonist concentrations used. These assays provide an appropriate *in vitro* model for further investigation into TLR4 tolerance and sensitisation; TLR4 can become sensitised or desensitised to ligands such as LPS, but it is not known whether or not metal ions can produce similar effects. Preliminary investigations were conducted in this regard using MonoMac 6 cells and HMEC-1, and LPS tolerance was established in both cell lines (data not shown). However the matter of cobalt cytotoxicity must be resolved before a complete study can be performed.

As described earlier, simultaneous activation of TLR4 and other TLRs can enhance secretion of inflammatory cytokines. For example co-stimulation of dendritic cells with LPS and a TLR7 agonist results in increased IFN-β secretion compared to treatment with individual ligands (Mäkelä et al., 2011). Synergistic effects can be the result of activation of multiple TLR-related signalling pathways, e.g. the MyD88-dependent and TRIF-dependent pathways (Ting Tan et

al., 2013). It would be interesting to investigate the effect of other TLR ligands on the inflammatory response to cobalt ions, and this could also provide a greater understanding of endogenous and exogenous factors that may be implicated in ARMD.

The role of hypoxia in inflammation is complex and the effect of PAMPs such as LPS is yet to be fully elucidated. However there are similarities between the responses to cobalt reported in the present study and hypoxic effects described in others, including downregulation of *CCL2*. It is therefore conceivable that some of the inflammatory effects described in this study could be the result of hypoxia. Further work is required to determine whether HIF1α and other hypoxia mediators are regulated by cobalt activation of the TLR4 signalling pathway, and if they in turn can modulate cobalt-mediated TLR4 activation. This could be done using the TLR4 inhibitors optimised in chapter 4 (CLI-095, MAb2-hTLR4 and PAb-hTLR4).

This chapter has begun to investigate potential factors involved in ARMD patient variability but there is a considerable number remaining that have not yet been studied. It would be particularly interesting to investigate the role of hormones on inflammatory responses to metal ions as ARMD is more common in women than men, and the TLR4 signalling pathway can be modulated by hormones; progesterone inhibits LPS-mediated immunity by upregulating inhibitory suppressor of cytokine signalling-1 (SOCS-1) expression and preventing NFκB activation (Su et al., 2009). The effect of hormonal regulation on cobalt-mediated TLR4 activation is unknown.

6.5.2. Conclusion

The results obtained in this chapter show the development of an assay designed to test the effects of a TLR4 SNP on inflammatory responses to cobalt ions. A common TLR4 SNP, D299G, did not inhibit IL-8 secretion following cobalt stimulation although comparison to the wildtype TLR4 response was not possible. The protocol used for these assays can be refined and used to investigate other polymorphisms within the TLR4 signalling pathway.

The data presented here also shows that co-stimulation of cells with cobalt and LPS causes a significant decrease in *IL6* and *CCL2* expression, as well as in IL-6 secretion. A similar effect is observed when nickel and cobalt treatment is combined, although nickel does not upregulate *CCL2* expression as LPS does. The results are an interesting effect given that individually the TLR4 ligands mainly exert pro-inflammatory responses. Further optimisation of these assays is required given the decrease in housekeeping gene expression caused by the combined treatments. Investigation into potential links between hypoxia and cobalt-mediated TLR4 activation would also be of interest.

The final aspect of patient variability investigated in this chapter was the inflammatory effect of other orthopaedic biomaterials, specifically chromium and strontium. Neither of these metal ions activated TLR4, nor did they induce inflammatory cytokine secretion in MonoMac 6 cells. Consequently it can be concluded that chromium and strontium do not have the same TLR4-stimulating properties as cobalt.

The results of this chapter show that there are numerous factors that could affect inflammatory responses and contribute to the patient variability in ARMD development. The assays described in this chapter can be optimised further to allow for detailed investigation into endogenous and exogenous factors affecting the outcome of cobalt-mediated TLR4 signalling.

Chapter 7. Conclusions

7.1. Aims and outcomes

The hypothesis of this study was that cobalt activation of human TLR4 leads to cellular inflammatory responses that may play a role in adverse reactions to metal debris. The main aims and outcomes of this study were:

A. To investigate the overall effect of cobalt ions on cellular immune responses

The effect of cobalt ions on MonoMac 6 macrophages was investigated using SYBR Green qRT-PCR arrays and Proteome Profiler cytokine arrays. Cobalt ions upregulated the expression of numerous inflammatory genes, including *IL8*, *IL1A*, *CCL20* and *CCL25*. These results were validated by TaqMan-based qRT-PCR. Secretion of inflammatory cytokines such as IL-8 and CXCL1 was also increased. There were notable differences in cytokine secretion profiles between $CoCl_2$ and LPS-stimulated MonoMac 6 cells, particularly for the CCL chemokines including CCL2 and CCL3.

B. To determine the role of TLR4 in observed responses

IL-8 was selected as a marker of cobalt-mediated cellular responses based on the array data described in chapter 3. Further analysis by ELISA showed that cobalt ions increased IL-8 secretion by MonoMac 6 and THP-1 macrophages, and U2OS osteoblasts. Using a small molecule TLR4 antagonist this was shown to be TLR4-dependent. TLR4-dependent *CXCL10* gene expression and IL-6 and CCL20 secretion were also observed in cobalt-stimulated MonoMac 6 cells. The potential of monoclonal and polyclonal anti-TLR4 neutralising antibodies to inhibit the response to cobalt was assessed using IL-8 as an inflammatory marker; the monoclonal antibody successfully inhibited cobalt-mediated IL-8 expression and secretion but the polyclonal antibody failed to do so. However both antibodies inhibited expression of other inflammatory genes; MAb2-hTLR4 inhibited expression of both *CCL20* and *IL1A* following cobalt treatment while PAb-hTLR4 prevented *CCL20* expression but not *IL1A*.

C. To investigate the effect of cobalt ions on cell adhesion and immune cell migration

The effect of $CoCl_2$ on endothelial cells was assessed using IL-8 as a marker of TLR4 activation. HUVEC were unresponsive to $CoCl_2$ stimulation with no IL-8 secretion detected across a broad range of concentrations and timepoints. Similar effects were observed for IL-6 secretion. However a second endothelial cell line, HMEC-1, secreted IL-8 and IL-6 in a cobalt/TLR4-dependent manner. qRT-PCR revealed upregulated expression of the adhesion molecule *ICAM1* in cobalt-treated MonoMac 6 cells and HMEC-1, and this was attributed to TLR4 activation by cobalt ions. A TLR4-dependent increase in sICAM-1 release by both cell lines

following cobalt stimulation was also observed. Conditioned media from cobalt-stimulated MonoMac 6 cells was found to be chemotactic for primary human monocytes and neutrophils. TLR4 was again implicated as the response was prevented by CLI-095.

D. To investigate factors that may impact upon patient variability in adverse reactions to metal debris

293-MD2-CD14 cells were successfully transfected with D299G and WT TLR4 as ascertained by immunofluorescence and qRT-PCR. However issues with cell maintenance and passage prevented thorough analysis of the WT TLR4 cells. D299G TLR4 cells increased IL-8 secretion when stimulated with $CoCl_2$ or LPS. This preliminary result suggests that the mutation does not abrogate inflammatory responses to cobalt ions, although comparison to WT TLR4 cells would be beneficial. Co-stimulation of MonoMac 6 cells with $CoCl_2$-LPS and $CoCl_2$-$NiCl_2$ elicited some surprising results, including decreased *IL6* expression compared to LPS or $NiCl_2$ alone. The inflammatory properties of orthopaedic metals chromium and strontium were also investigated. These metals did not activate human TLR4, nor did they induce IL-6 and IL-8 secretion by MonoMac 6 cells.

7.1.1. Overall conclusions

The data presented in this study demonstrates that cobalt activation of human TLR4 regulates expression of inflammatory cytokines and chemokines in macrophages and endothelial cells. Responses are dependent on the cell line used as HUVEC did not respond to $CoCl_2$ while all other cell lines showed inflammatory effects. IL-8 is an effective marker of TLR4 activation and was used in this study to assess the potential of TLR4 inhibitors in preventing receptor activation by $CoCl_2$. A small molecule TLR4 antagonist and a monoclonal anti-TLR4 neutralising antibody proved effective at abrogating cellular responses to cobalt ions while a polyclonal antibody provided less inhibition. Although the array data showed that cobalt affected both pro- and anti-inflammatory cytokine expression, further analysis of adhesion molecule expression and immune cell migration suggested a pro-inflammatory and TLR4-dependent response. Surprisingly investigation into factors influencing ARMD patient variability indicated that nickel and LPS downregulate cobalt-mediated expression of inflammatory cytokines including *IL6*, although further investigation is required. In summary, activation of TLR4 by cobalt ions results in an inflammatory response that is similar but not identical to that which is induced by LPS. The TLR4 signalling pathway is therefore an interesting avenue for further investigation into the cell and molecular biology of ARMD, as well as an exciting potential therapeutic target.

7.2. Final discussion

Since the emergence of adverse reactions to metal debris following MoM hip replacement there have been many studies investigating cellular responses to metal debris and ions. The majority of these studies reveal an inflammatory response to the metals, including cobalt, characterised by elevated secretion of pro-inflammatory cytokines and chemokines. *In vivo*, persistent peri-implant inflammation is a key factor in osteoclastogenesis, osteolysis and aseptic implant loosening, which are major features of ARMD. The initial stimulus for this inflammation has previously been attributed to PRR activation by endogenous DAMPs released by stressed or damaged cells, or to endotoxin coating of wear debris leading to immune system activation (Greenfield et al., 2010). However the present study provides a new perspective on the mechanisms that lead to cobalt-mediated inflammation and ARMD; cobalt activation of TLR4 may be an additional (or initial) stimulus that drives inflammatory responses or perpetuates existing inflammation.

Macrophages and endothelial cell lines were used throughout this study and were selected to model the peri-implant environment. Macrophages are found in synovial fluid and peri-implant tissues, and much of the inflammation associated with ARMD is attributed to macrophage infiltration and activation (Nich et al., 2013). Endothelial cells line blood vessels surrounding the joint and are also present in the pseudotumour vasculature. Both cell types displayed increased TLR4-dependent inflammatory cytokine secretion and adhesion molecule expression when challenged with cobalt ions *in vitro*, suggesting that the tissues surrounding the implant may be responsive to cobalt. This could account for the increased cytokine and chemokine levels detected around failed MoM implants (Singh et al., 2015), as well as being a potential stimulus for osteoclastogenesis, osteolysis, and implant loosening. Some of the cytokines secreted (e.g. CCL20) by cobalt-stimulated activated macrophages are also characteristics of M1 pro-inflammatory macrophages (Martinez et al., 2006), which suggests that the overall cellular response may be pro-inflammatory.

Cytokine and chemokine secretion is a direct consequence of TLR4 activation by cobalt ions. Adhesion molecule expression was also TLR4-dependent but it is not clear whether this is a direct result of cobalt binding to TLR4 or if it occurs through TLR4-dependent cytokine secretion; ICAM-1 and VCAM-1 expression can be increased by cytokines including IFNγ and TNFα (Ren et al., 2010). The observed monocyte and neutrophil migration is modulated by chemokines because the chemotaxis assays were performed using conditioned media from cobalt-stimulated cells. However the exact chemokines responsible for each effect have not been identified. Further work to resolve this would be beneficial as it would provide more

insight into the individual factors that regulate inflammatory responses following TLR4 activation.

This study has also identified differences in the cellular inflammatory responses to LPS and $CoCl_2$, including higher fold changes in gene expression (e.g. *ICAM1*) and increased monocyte and neutrophil migration with LPS compared to cobalt. Although these changes could be attributed to different ligand chemistry and concentrations, opposing effects such as downregulation of *CCL2* in the presence of $CoCl_2$ but upregulation in response to LPS indicate that immune regulation by the two ligands is not identical. This also shows that the $CoCl_2$ solutions used throughout this study are endotoxin-free, otherwise similar responses would be anticipated between the LPS and $CoCl_2$ groups.

Other studies have identified inflammatory responses to cobalt ions, but have not investigated the role of TLR4 in the observed effects. For example Ninomiya *et al* showed increased adhesion molecule expression and leukocyte binding capacity in cobalt-stimulated endothelial cells (Ninomiya et al., 2013), which correlates with results presented in this study and consequently they may be TLR4-mediated (Tyson-Capper et al., 2013b). This could be assessed using the chemotaxis and CLI-095 assays detailed in chapter 5. Further studies have shown increased TLR4 expression in cells from aseptically-loosened joint replacements (Lähdeoja et al., 2009) and this has been attributed to either sub-clinical levels of infection or endotoxin coating of wear debris, both of which activate PRRs including TLR4 (Greenfield et al., 2010, Bonsignore et al., 2013). However although endotoxin can coat most biomaterials (Greenfield et al., 2005), revision rates and incidence of adverse reactions are considerably higher for MoM implants than they are for ceramic-on-polyethylene and metal-on-polyethylene devices (Powers-Freeling, 2015). Additional factors must therefore be involved and it is conceivable that cobalt activation of TLR4 exacerbates other adverse reactions.

A working model based on the results of this study and the current literature is shown in **Figure 7.1**.

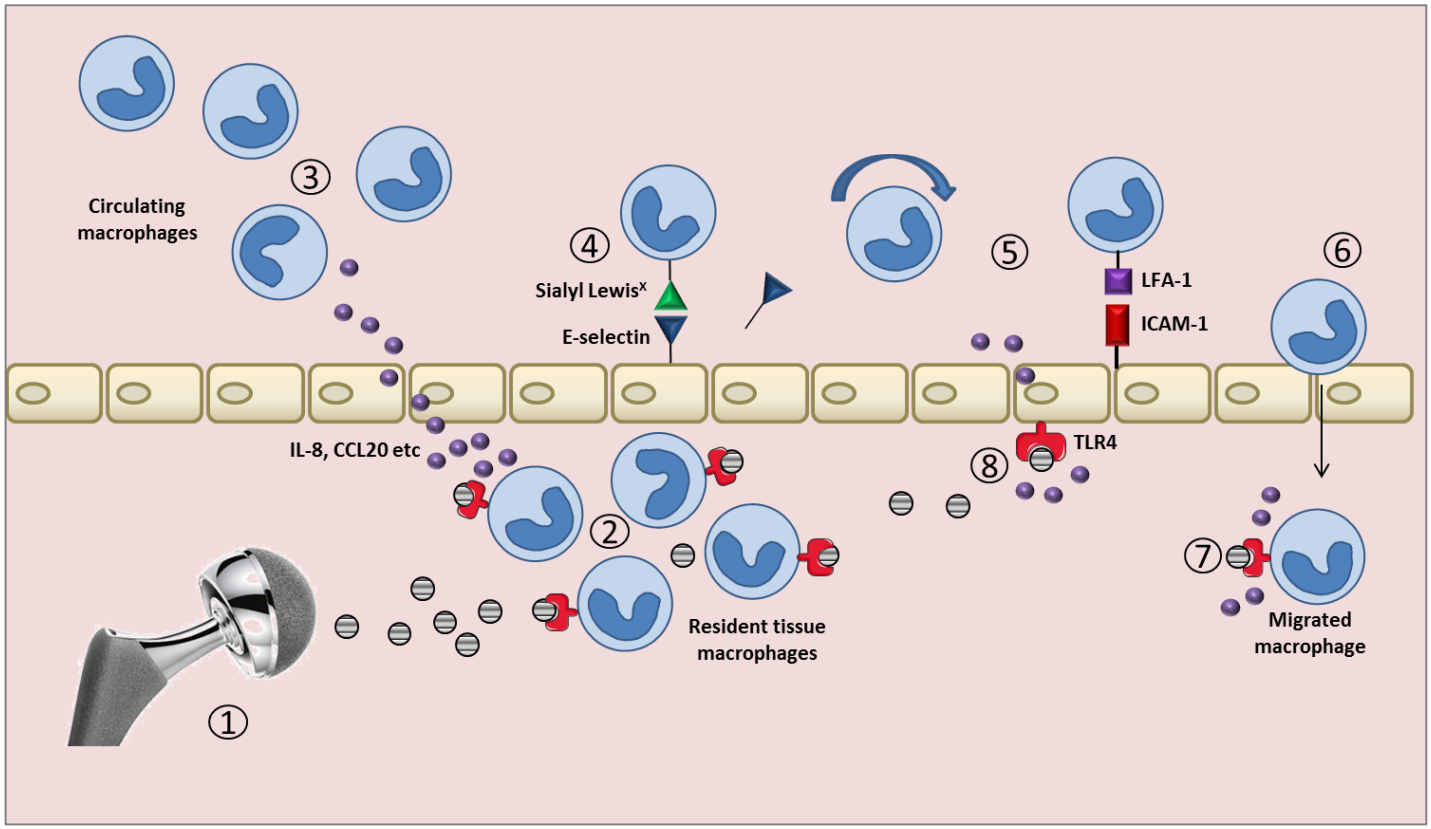

Figure 7.1: Working model
1. A metal-on-metal hip implant releases cobalt ions and debris into the joint environment. **2.** Cobalt activates TLR4 on resident tissue macrophages resulting in increased cytokine and chemokine secretion, including IL-8 and CCL20. **3.** Chemokines induce circulating leukocyte migration towards the site of inflammation. **4.** Engagement of Sialyl Lewis^X on leukocytes with E-selectin on endothelial cells slows leukocyte passage through the blood vessel; this is rolling adhesion. **5.** E-selectin is shed by the endothelial cell allowing the leukocyte to transition to firm adhesion via engagement of LFA-1 (leukocyte) with ICAM-1 (endothelial cell). **6.** Firm adhesion increases the ability of leukocytes to pass through the endothelial cell barrier and into the tissues. **7.** TLR4 on migrated macrophages is also activated by cobalt ions, resulting in further cytokine and chemokine release. **8.** TLR4-expressing endothelial cells are activated by cobalt from the MoM implant, and increase cytokine secretion and adhesion molecule expression. This also contributes to the adhesion described in **step 5**.

7.2.1. Study limitations

Specific limitations for the individual assays conducted are discussed in their respective chapters. The main overall limitation of this study is that cell lines were used throughout, with the exception of the primary HUVEC used for the adhesion studies in chapter 5, and the primary monocytes and neutrophils used in the migration assays in the same chapter. The cell lines were appropriate for use in this study because the primary aim was to investigate the effect of cobalt on TLR4 signalling, downstream signalling pathway factors and the inflammatory outcome (i.e. cytokine and chemokine secretion), without the complexity of patient variability when considering the use of inhibitors and neutralising antibodies. However, a limitation of cell lines is that they do not provide the opportunity to study patient variability, for example investigating patient-specific differences in cytokine secretion levels in response to the same stimulus. To study aspects of patient variability in-depth, PBMC from patients and healthy volunteers could be used, but this was beyond the scope of the present study given the numbers required for comprehensive analysis.

An additional limitation of cell lines is that they may not express all of the factors for investigation, particularly when studying a broad range of molecules such as in the arrays. It was particularly notable that the MonoMac 6 cells did not express high levels of TNFα in response to either LPS or cobalt, which is surprising because it is one of the key cytokines released when TLR4 is activated. Further work would be required to establish whether or not this response is unique to MonoMac 6 cells, and this is where the use of primary cells would again be advantageous.

The study is also limited by the cobalt concentrations and stimulation times used. The cobalt concentrations were carefully optimised based on previous studies using *in vitro* assays but cytotoxicity was observed which may contribute to the cellular response. The use of a small range of timepoints and concentrations was necessary due to the scope of the study, particularly for the arrays in chapter 3, but could also prevent detection of temporal or dose-dependent inflammatory responses.

The antagonists used in the study (CLI-095 and anti-TLR4 neutralising antibodies) do not exclude the possibility of TLR4 activation by endogenous DAMPs or exogenous ligands such as LPS. However previous studies have shown that mutation of the cobalt binding site in human TLR4 abrogates cobalt-mediated inflammatory responses (Raghavan et al., 2012) and murine TLR4 is not activated by cobalt, suggesting that cytokine release only occurs when cobalt binds directly to the receptor. Furthermore, endotoxin assays were negative (data not shown),

eliminating the possibility of cell culture contamination by endotoxins which could produce comparable inflammatory responses to cobalt. There were also differences in the LPS and cobalt-mediated inflammatory responses, such as increased *CCL2* expression in LPS-stimulated MonoMac 6 cells, but a decrease in expression of the same gene in response to cobalt ions. If the metal ion solutions were contaminated by endotoxin, the response of the cells to LPS and cobalt would be very similar.

The final major limitation of this study is that it is based on *in vitro* assays. This was important to the study because of the specificity of cobalt in binding to human TLR4, thus eliminating the possibility of using animal cells or models. The investigation is also in the early stages in terms of the use of inhibitors (for example) and therefore it would not be feasible to introduce animal models. However the use of *in vitro* assays does mean that the cell-cell interactions (e.g. antigen presentation by macrophages to lymphocytes) that would take place *in vivo* cannot be studied. It is also not possible to identify any systemic or physiological effects of cobalt ions, including how the initial innate immune response mediated by TLR4 could be promoted or dampened by other aspects of the immune system. An appropriate animal model for further study could be mice expressing humanised TLR4, although given the species differences in receptor activation by cobalt, caution must be exercised when considering the validity of such models for furthering the work described in the present study.

7.3. Clinical implications

MoM hip replacement and resurfacing use has declined dramatically since the emergence of the side effects now termed adverse reactions to metal debris. However there are still more than one million MoM hips implanted worldwide and the unpredictability of ARMD means that reactions can present at any time. An ARMD therapy could reduce the requirement for complex and costly revision surgery, particularly among an elderly and more vulnerable population. Moreover, CoCr alloys are used in trunnions in MoP and ceramic implants and therefore therapeutic prevention of the inflammatory activity of cobalt ions could also prevent adverse reactions in these implants.

There are many factors to be considered in the development of a therapy targeting TLR4 for ARMD prevention and several are discussed elsewhere in this thesis (Chapter 4). An additional factor to be considered in the development of a therapy targeting TLR4 for ARMD prevention is the extent of the role TLR4 plays in adverse reactions. It is clear from the data presented in this study that cobalt activation of TLR4 can induce a cellular inflammatory response. TLR4 antagonism by neutralising antibodies or a small molecule inhibitor abrogated many of the effects. In a lot of cases this was a near-complete blockade, for example CLI-095 reduced IL-8 secretion in MonoMac 6 cells nearly to the levels of untreated controls, which suggests that the response is entirely TLR4-dependent. In other cases there was only partial inhibition of responses in the presence of an inhibitor or antibody which may be the result of feedback loops whereby cytokines promote further inflammation, compensatory cytokine secretion, or responses entirely independent of TLR4 activation. There must be additional factors involved in ARMD as TLR4 is universally expressed but not every MoM implant patient develops ARMD. As previously mentioned it could be that cobalt-mediated TLR4 activation acts as a 'trigger' that combined with other stimuli (e.g. antigen presentation to adaptive immune cells) results in inflammatory responses to metal ions. The response could also be influenced by cobalt ion concentrations, carrier proteins, endogenous factors such as hormones, or the presence of other metal ions, wear debris or PAMPs like LPS.

A greater understanding of the inflammatory mechanisms underlying ARMD could also allow the identification of factors influencing patient variability, which is currently a major confounding factor in ARMD development. Not all patients experience adverse reactions to their MoM hip implant and this suggests that MoM implants could work very well in the right patient group. A long-term clinical goal of research such as this is to identify factors that influence patient variability, allowing improved stratification of patients receiving hip replacements and as a result reducing the incidence of adverse reactions and revision surgery.

This could include investigating the relationship between ARMD, pre-existing medical conditions (e.g. rheumatoid arthritis) and their associated treatments, as well as immune regulation by hormones and other endogenous factors, and genetic variation. Given the number of potential causes of patient variability the TLR4 signalling pathway provides an excellent starting point for such investigations.

The benefits of understanding the inflammatory effects of cobalt ions are not limited to MoM hip replacements as cobalt is also used in other medical devices such as dental implants, spinal hardware and coronary stents. Although these devices do not articulate and therefore release less debris than hip implants, they are still associated with increased serum metal ion levels (Cundy et al., 2014) and can cause adverse reactions which often resemble those linked to MoM implants (e.g. lymphocytic infiltrate or osteolysis) (Guyer et al., 2011, Zairi et al., 2013, Shang et al., 2014). Furthermore, other metal ions that are used in medical implants such as nickel and palladium are known TLR4 ligands **(Figure 7.2)**. Nickel's use is more widespread than that of cobalt as it is present in everyday items like coins, jewellery and cooking utensils, and is associated with allergic contact dermatitis. Palladium is used in dental implants, which have also been linked to hypersensitivity and other adverse reactions (Khamaysi et al., 2006, Muris et al., 2015). A greater understanding of the immunological effects of cobalt could therefore contribute to wider biomaterial research and benefit a broad range of patients receiving medical implants. In addition it could also help individuals who are exposed to metal ions via other means, for example by industrial exposure. There are many other metals used in orthopaedic devices that should be investigated for their inflammatory properties, including titanium, zirconium and rhodium. To date none of these metals are known to activate TLR4 although titanium enhances the inflammatory effects of LPS suggesting that it may influence the TLR4 signalling pathway (Bianchi et al., 2015).

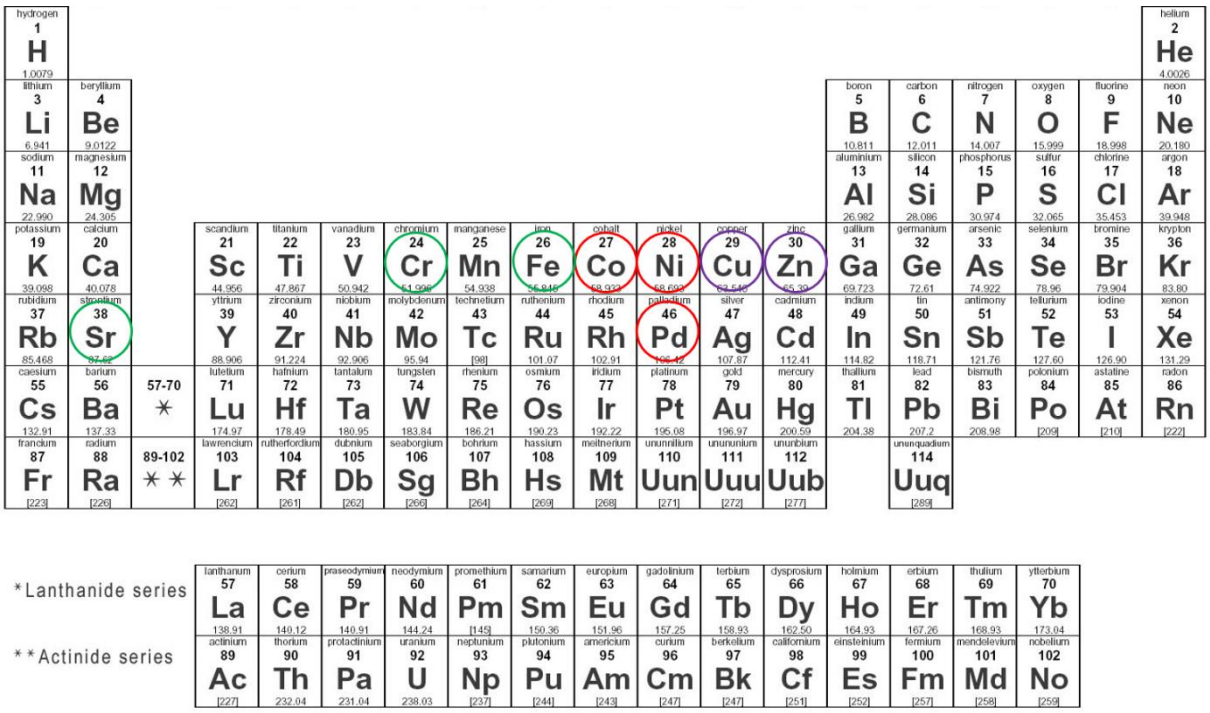

Figure 7.2: Metal ions known to activate TLR4 and other potential ligands
Cobalt, nickel and palladium (red) are known TLR4 ligands. Copper and zinc (purple) can induce low level inflammatory cytokine secretion but it is not clear if this is TLR4-dependent (Rachmawati et al., 2013, Tsou et al., 2013). Chromium, iron and strontium have been investigated but were not found to activate TLR4 (present study and (Rachmawati et al., 2013)).

7.4. Future directions

This study investigated the inflammatory effects of cobalt ions, with a specific focus on the immunological outcomes of cobalt-mediated TLR4 activation. Many inflammatory factors were found to be regulated by cobalt including cytokines, chemokines and adhesion molecules. All of these factors have different functions which highlights numerous avenues for potential functional studies including cell adhesion experiments using a Cellix platform, and transendothelial migration assays.

The assays developed during this study can be used to investigate the inflammatory effects of other biomaterials including metals like titanium and molybdenum, as well as polyethylene and ceramic. Given the specific nature of cobalt and nickel binding to TLR4, it is unlikely that a material as chemically different as UHMWPE will activate the same receptor but the subsequent assays, such as the chemotaxis and adhesion molecule assays could be used to identify the potential immunological properties of existing and newly developed orthopaedic materials.

The work contained in this thesis could be furthered considerably by analysis of tissue samples from patients with MoM hip implants. For example quantification of cytokines in synovial fluid could be assessed alongside cobalt concentrations and TLR4 expression levels to determine the role of the TLR4 signalling pathway in ARMD. Genetic variation (e.g. in TLR4 or related signalling factors) could also be investigated through analysis of DNA extracted from blood samples.

In summary this study demonstrates that cobalt ions from MoM hip implants can activate the innate immune receptor TLR4, resulting in cellular inflammatory responses such as cytokine secretion and adhesion molecule expression. These may create a pro-inflammatory environment that favours the adverse reactions associated with MoM hip failure including osteolysis and aseptic loosening. A greater understanding of the effect of cobalt on the TLR4 signalling pathway will allow further studies into ARMD patient variability, as well as the development of novel therapeutics that could prevent ARMD development and allow cobalt to be used as a safe and effective biomaterial in a wide range of orthopaedic devices.

Appendix A Toll-like Receptors and Signalling qRT-PCR array results

Abbreviation	Gene name	Fold change	p value
BTK	Bruton agammaglobulinemia tyrosine kinase	-1.49	0.2810
CASP8	Caspase 8	2.06	0.9255
CCL2	Chemokine (C-C motif) ligand 2	-410.75	0.3654
CD14	**Cluster of differentiation 14**	**-8.76**	0.0069
CD180	Cluster of differentiation 180	-9.00	0.1021
CD80	Cluster of differentiation 80	-1.15	0.4077
CD86	Cluster of differentiation 86	-2.22	0.1955
CHUK	Inhibitor of NFκB kinase subunit alpha	2.35	0.5675
CLEC4E	C-type lectin domain family 4 member E	2.01	0.4151
CSF2	Colony stimulating factor 2/Granulocyte-macrophage colony-stimulating factor (GMCSF)	2.42	0.7848
CSF3	Colony stimulating factor 3	-1.52	0.3793
CXCL10	Chemokine (C-X-C motif) ligand 10	3.28	0.7637
ECSIT	Evolutionarily conserved signaling intermediate in Toll pathway	1.12	0.8138
EIF2AK2	Interferon-induced, double-stranded RNA-activated protein kinase	-1.24	0.4321
ELK1	ETS domain-containing protein	-2.67	0.2641
FADD	**Fas-associated death domain**	**-3.70**	**0.0222**
FOS	Proto-oncogene c-FOS	-1.41	0.3164
HMGB1	High mobility group box protein 1	1.70	0.4836
HRAS	GTPase HRas	-3.05	0.2659
HSPA1A	**Heat shock protein A1A (70kDa)**	**202.40**	**0.0022**
HSPD1	Heat shock protein (60kDa), Hsp60	28.04	0.1072
IFNA1	Interferon alpha 1	1.56	0.6302
IFNB1	Interferon beta 1	-3.21	0.3791
IFNG	Interferon gamma	-10.96	0.3738
IKBKB	Inhibitor of NFκB kinase, subunit beta	1.86	0.8123
IL10	Interleukin 10	-1.61	0.4795
IL12A	Interleukin 12, subunit alpha	-5.46	0.3645
IL1A	**Interleukin 1 alpha**	**17.11**	**0.0255**
IL1B	Interleukin 1, beta	4.32	0.3126
IL2	Interleukin 2	-32.56	0.3739
IL6	Interleukin 6	1.48	0.481027
IL8	**Interleukin 8**	**40.91**	**0.0006**
IRAK1	Interleukin 1 receptor-associated kinase 1	-1.08	0.5917
IRAK2	**Interleukin 1 receptor-associated kinase 2**	**3.17**	**0.0133**
IRAK4	**Interleukin 1 receptor-associated kinase 4**	**-2.18**	**0.0200**
IRF1	Interferon regulatory factor 1	1.94	0.8094
IRF3	Interferon regulatory factor 3	1.14	0.5888

JUN	**Transcription factor AP-1**	**4.47**	**0.0159**
LTA	Leukotriene A	-1.53	0.4304
LY86	Lymphocyte antigen 86	1.41	0.2815
LY96	Lymphocyte antigen 96, MD2	1.57	0.5099
MAP2K3	Mitogen-activated protein kinase kinase 3	1.34	0.1374
MAP2K4	**Mitogen-activated protein kinase kinase 4**	**-2.02**	**0.0204**
MAP3K1	Mitogen-activated protein kinase kinase kinase 1	-1.37	0.3713
MAP3K7	Mitogen-activated protein kinase kinase kinase 7	1.05	0.8917
MAP4K4	Mitogen-activated protein kinase kinase kinase kinase 4	1.70	0.6470
MAPK8	Mitogen-activated protein kinase 8	1.41	0.7777
MAPK8IP3	Mitogen-activated protein kinase 8 interacting protein 3	1.66	0.8183
MYD88	Myeloid differentiation protein 88	-3.36	0.0601
NFKB1	**Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1**	**7.62**	**0.0060**
NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2	1.03	0.5086
NFKBIA	**Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha**	**7.81**	**0.0435**
NFKBIL1	Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor-like 1	-3.18	0.4328
NFRKB	Nuclear factor related to kappaB binding protein	-1.97	0.0605
NR2C2	Nuclear receptor subfamily 2 group C member 2	1.05	0.7292
PELI1	**Pellino homologue 1**	**4.42**	**0.0359**
PPARA	Peroxisome proliferator-activated receptor alpha	-1.54	0.0755
PRKRA	Protein kinase interferon-inducible double stranded RNA dependent activator	-1.56	0.1023
PTGS2	Prostaglandin-endoperoxide synthase 2	1.55	0.1948
REL	**V-rel reticuloendotheliosis viral oncogene homologue (avian)**	**2.32**	**0.0285**
RELA	V-rel reticuloendotheliosis viral oncogene homologue A (avian)	1.19	0.9052
RIPK2	Receptor-interacting serine-threonine kinase 2	2.25	0.629152
SARM1	Sterile alpha and TIR motif containing 1	-1.04	0.6075

SIGIRR	Single immunoglobulin and toll-interleukin 1 receptor domain	-2.39	0.3113
TAB1	**TGF-beta activated kinase 1**	**-3.01**	**0.0042**
TBK1	TANK-binding kinase 1	2.28	0.1925
TICAM1	**Toll-like receptor adaptor molecule 1**	**3.68**	**0.0394**
TICAM2	Toll-like receptor adaptor molecule 2	4.92	0.1963
TIRAP	Toll-interleukin 1 receptor (TIR) domain containing adaptor protein	3.47	0.9698
TLR1	Toll-like receptor 1	-33.91	0.1837
TLR10	Toll-like receptor 10	3.20	0.1737
TLR2	**Toll-like receptor 2**	**1.90**	**0.0235**
TLR3	Toll-like receptor3	-3.48	0.2466
TLR4	Toll-like receptor 4	1.01	0.4157
TLR5	Toll-like receptor 5	-3.23	0.1672
TLR6	**Toll-like receptor 6**	**-2.75**	**0.0251**
TLR7	Toll-like receptor 7	1.48	0.5228
TLR8	Toll-like receptor 8	-12.88	0.3591
TLR9	Toll-like receptor 9	-2.41	0.3893
TNF	Tumour necrosis factor	1.75	0.2205
TNFRSF1A	Tumor necrosis factor receptor superfamily member 1A	1.66	0.3946
TOLLIP	Toll interacting protein	-1.03	0.6979
TRAF6	TNF receptor-associated factor 6	-1.02	0.9210
UBE2N	Ubiquitin-conjugating enzyme E2N	5.04	0.8119
ACTB	Beta actin	-	-
B2M	Beta-2-microglobulin	-	-
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	-	-
HPRT1	Hypoxanthine phosphoribosyltransferase 1	-	-
RPLP0	Ribosomal protein, large, P0	-	-
HGDC	Human genomic DNA control	-	-
RTC	Reverse transcriptase control	-	-
PPC	Positive PCR control	-	-

Table A.1: Full list of genes, fold changes and p values from Toll-like receptor signalling RT^2 Profiler qRT-PCR arrays (PAHS-018Z).

Appendix B Inflammatory Cytokines and Receptors qRT-PCR array results

Gene	Gene name	Fold change	p value
ABCF1	**ATP-binding cassette sub-family 4, member 1**	**-2.57**	**0.0360**
BCL6	B-cell CLL/lymphoma 6	1.88	0.3650
C3	Complement component 3	7.20	0.8531
C4a	Complement component 4a	-1.79	0.5547
C5	Complement component 5	-2.12	0.1571
CCL1	Chemokine (C-C motif) ligand 1	-1.36	0.5889
CCL11	Chemokine (C-C motif) ligand 11	-3.82	0.2048
CCL13	Chemokine (C-C motif) ligand 13	-3.96	0.1527
CCL15	Chemokine (C-C motif) ligand 15	-4.10	0.2200
CCL16	Chemokine (C-C motif) ligand 16	-1.82	0.2631
CCL17	Chemokine (C-C motif) ligand 17)	-1.82	0.5610
CCL18	Chemokine (C-C motif) ligand 18	-1.02	0.6818
CCL19	Chemokine (C-C motif) ligand 19	1.71	0.6805
CCL2	Chemokine (C-C motif) ligand 2	-6.81	0.3378
CCL20	**Chemokine (C-C motif) ligand 20**	**34.96**	**0.0020**
CCL21	Chemokine (C-C motif) ligand 21	1.19	0.6931
CCL23	Chemokine (C-C motif) ligand 23	-2.05	0.2375
CCL24	Chemokine (C-C motif) ligand 24	-1.51	0.2616
CCL25	Chemokine (C-C motif) ligand 25	7.71	0.8192
CCL26	Chemokine (C-C motif) ligand 26	1.02	0.7988
CCL3	Chemokine (C-C motif) ligand 3	1.71	0.7312
CCL4	Chemokine (C-C motif) ligand 4	10.27	0.5958
CCL5	Chemokine (C-C motif) ligand 5	-1.06	0.6667
CCL7	Chemokine (C-C motif) ligand 7	-9.11	0.4229
CCL8	Chemokine (C-C motif) ligand 8	-4.10	0.4464
CCR1	Chemokine (C-C motif) receptor 1	-9.43	0.2044
CCR2	Chemokine (C-C motif) receptor 2	-77.10	0.1127
CCR3	Chemokine (C-C motif) receptor 3	-1.02	0.7038
CCR4	Chemokine (C-C motif) receptor 4	-1.09	0.7666
CCR5	Chemokine (C-C motif) receptor 5	-2.33	0.2005
CCR6	Chemokine (C-C motif) receptor 6)	-4.35	0.3660
CCR7	Chemokine (C-C motif) receptor 7	-1.04	0.3856
CCR8	Chemokine (C-C motif) receptor 8	-1.73	0.2276
CCR9	Chemokine (C-C motif) receptor 9	-1.99	0.3338
CEBPB	CCAAT/enhancer binding protein (C/EBP) beta	-1.61	0.7641
CRP	C-reactive protein, pentraxin-related	-4.10	0.2200
CX3CR1	Chemokine (C-X3-C motif) receptor 1	-3.25	0.3205
CXCL1	Chemokine (C-X-C motif) ligand 1	1.32	0.5776
CXCL10	Chemokine (C-X-C motif) ligand 10	1.04	0.4815
CXCL11	Chemokine (C-X-C motif) ligand 11	-1.25	0.5168
CXCL12	Chemokine (C-X-C motif) ligand 12	-3.03	0.1976
CXCL13	Chemokine (C-X-C motif) ligand 13	-2.66	0.2746
CXCL14	Chemokine (C-X-C motif) ligand 14	2.96	0.0837
CXCL2	Chemokine (C-X-C motif) ligand 2	11.21	0.0629
CXCL3	Chemokine (C-X-C motif) ligand 3	15.14	0.0879

CXCL5	Chemokine (C-X-C motif) ligand 5	-1.76	0.4191
CXCL6	Chemokine (C-X-C motif) ligand 6	-2.53	0.4169
CXCL9	Chemokine (C-X-C motif) ligand 9	-1.12	0.8718
CARD18	Caspase recruitment domain family member 18	-4.42	0.2117
IFNA2	Interferon alpha 2	1.50	0.4962
IL10	Interleukin 10	-5.50	0.4154
IL10RA	Interleukin 10 receptor alpha	1.09	0.8428
IL10RB	**Interleukin 10 receptor beta**	**-2.10**	**0.0006**
IL13	Interleukin 13	2.98	0.3070
IL13RA1	Interleukin 13 receptor alpha 1	-2.02	0.0993
IL17C	Interleukin 17C	1.63	0.4937
IL1A	Interleukin 1 alpha	1.83	0.2512
IL1B	Interleukin 1 beta	2.11	0.4250
IL1F10	Interleukin 1 family member 10 (theta)	-5.47	0.2784
IL36RN	Interleukin 36 receptor antagonist	-1.06	0.8036
IL36A	Interleukin 36 alpha	-6.25	0.3278
IL37	Interleukin 37	-1.14	0.4714
IL36B	Interleukin 36 beta	-3.60	0.2305
IL36G	Interleukin 36 gamma	2.73	0.9206
IL1R1	Interleukin 1 receptor type I	2.76	0.2213
IL1RN	Interleukin 1 receptor antagonist	-2.86	0.1482
IL22	Interleukin 22	-1.88	0.6038
IL5	Interleukin 5 (colony-stimulating factor, eosinophil)	-2.82	0.5378
IL5RA	Interleukin 5 receptor alpha	2.74	0.3518
IL8	**Interleukin 8**	**14.53**	**0.0236**
CXCR1	Chemokine (C-X-C motif) receptor 1	-2.10	0.3066
CXCR2	Chemokine (C-X-C motif) receptor 2	-2.50	0.1204
IL9	Interleukin 9	-1.52	0.5004
IL9R	Interleukin 9 receptor	-1.28	0.4003
LTA	Lymphotoxin alpha (TNF superfamily member 1)	2.53	0.0918
LTB	Lymphotoxin beta (TNF superfamily member 3)	-5.91	0.0817
LTB4R	Leukotriene B4 receptor	-11.04	0.1395
MIF	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	-1.75	0.3582
AIMP1	**Aminoacyl tRNA synthetase complex-interacting multifunctional protein 1**	**-2.58**	**0.0452**
SPP1	Secreted phosphoprotein 1	1.07	0.6038
TNF	Tumor necrosis factor	-3.25	0.0773
CD40LG	CD40 ligand	-11.78	0.2447
TOLLIP	Toll interacting protein	-2.54	0.1069
XCR1	Chemokine (C motif) receptor 1	-1.52	0.5463
B2M	Beta-2-microglobulin	-	-
HPRT1	Hypoxanthine phosphoribosyltransferase 1	-	-
RPL13A	Ribosomal protein L13a	-	-
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	-	-
ACTB	Beta actin	-	-
HGDC	Human Genomic DNA Contamination	-	-

RTC	Reverse Transcription Control	-	-
PPC	Positive PCR Control	-	-

Table B.1: Full list of genes, fold changes and p values from Inflammatory Cytokines and Receptors RT^2 qRT-PCR arrays (PAHS-011C)

Appendix C *GAPDH* Ct values following cell stimulation

Cell stimulation (24h)	Mean *GAPDH* Ct value
0.75mM $CoCl_2$	18.3
100ng/ml LPS	18.6
0	18.9

Table C.1: Effect of $CoCl_2$ on *GAPDH* Ct values
MonoMac 6 cells were stimulated with 0.75mM $CoCl_2$ or 100ng/ml LPS for 24h. RNA was isolated and cDNA synthesised by reverse transcription. *GAPDH* expression was quantified by qRT-PCR. Table shows mean Ct values from an assay performed in triplicate. Data is representative of five independent experiments.

Appendix D CLI-095 cytotoxicity assay

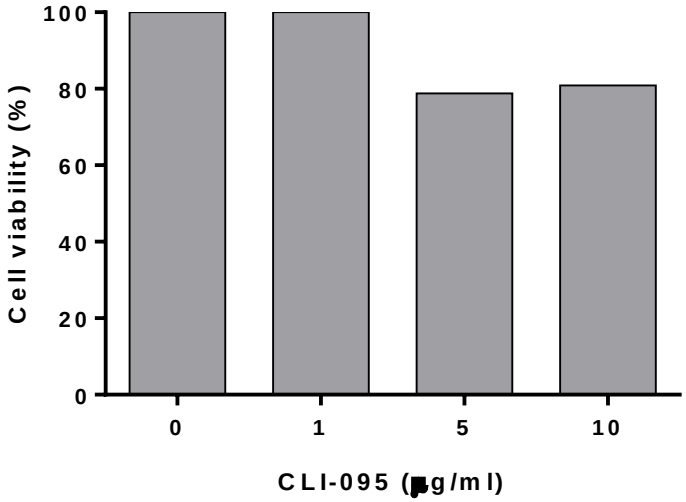

Figure D.1: Effect of CLI-095 on MonoMac 6 cell viability
MonoMac 6 cells were stimulated with 1, 5 or 10µg/ml CLI-095 for 30h (time represents a 6h pre-incubation followed by 24h cell treatment) and cytotoxicity was assessed by trypan blue staining. A Luna II automated cell counter was used to take images of the stained cells and they were then counted manually due to the issues described in **section 2.5**. n=1**.**

Appendix E *ICAM1* dose response curve

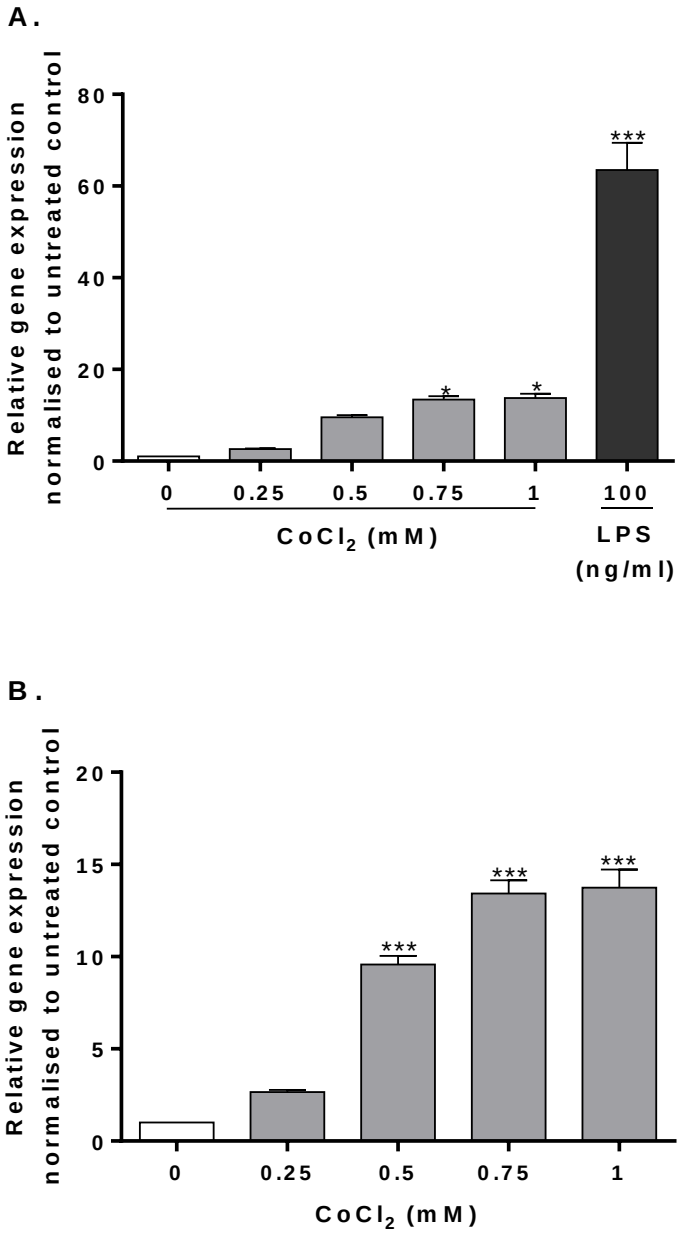

Figure E.1: Effect of $CoCl_2$ on *ICAM1* expression
A. MonoMac 6 cells were stimulated with 0.25-1mM $CoCl_2$ or 100ng/ml LPS for 24h. RNA was isolated and cDNA synthesised by reverse transcription. *ICAM1* expression was quantified by qRT-PCR. **B.** *ICAM1* expression shown without LPS, as LPS can mask the effects of $CoCl_2$. n=1. Statistical significance was calculated by one-way ANOVA with Dunnett's test for multiple comparisons comparing treated samples to the untreated control. This experiment was conducted in collaboration with MRes student Sami Anjum.

Appendix F CLI-095 optimisation in HMEC-1

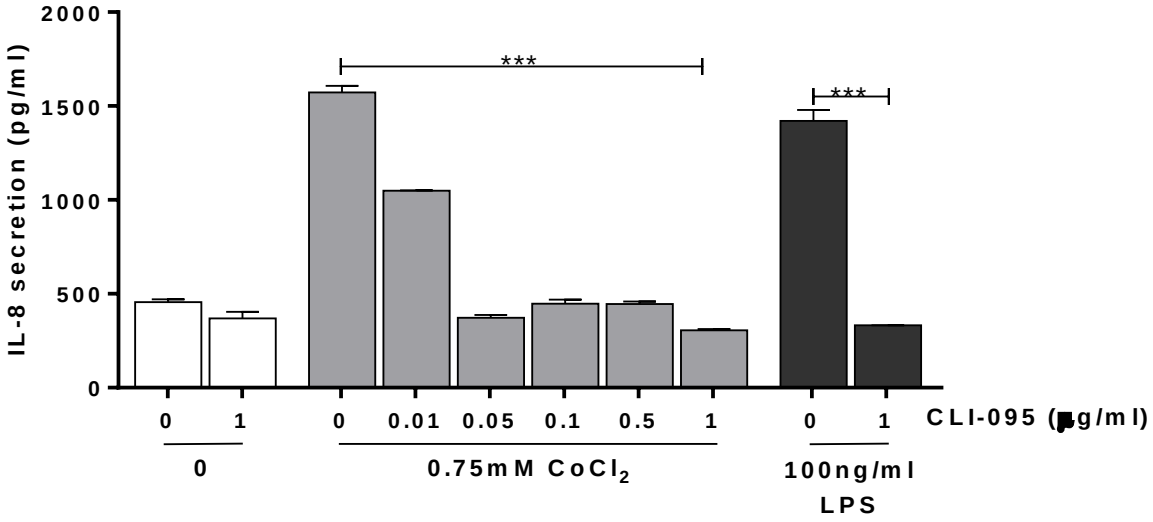

Figure F.1: CLI-095 optimisation in HMEC-1
HMEC-1 were pre-treated with 0.01-1µg/ml CLI-095 for 6h before 24h stimulation with 0.75mM $CoCl_2$. Controls were a 6h pre-treatment with 1µg/ml CLI-095 followed by stimulation with 100ng/ml LPS (positive control) or untreated (negative control) for 24h. Supernatant was collected and IL-8 secretion measured by ELISA. n=1. Statistical significance was calculated by one-way ANOVA with Tukey's test for multiple comparisons comparing all samples to each other. This experiment was conducted in collaboration with MRes student Sami Anjum.

Appendix G Neutrophil chemotaxis and anti-TLR4 neutralising antibodies

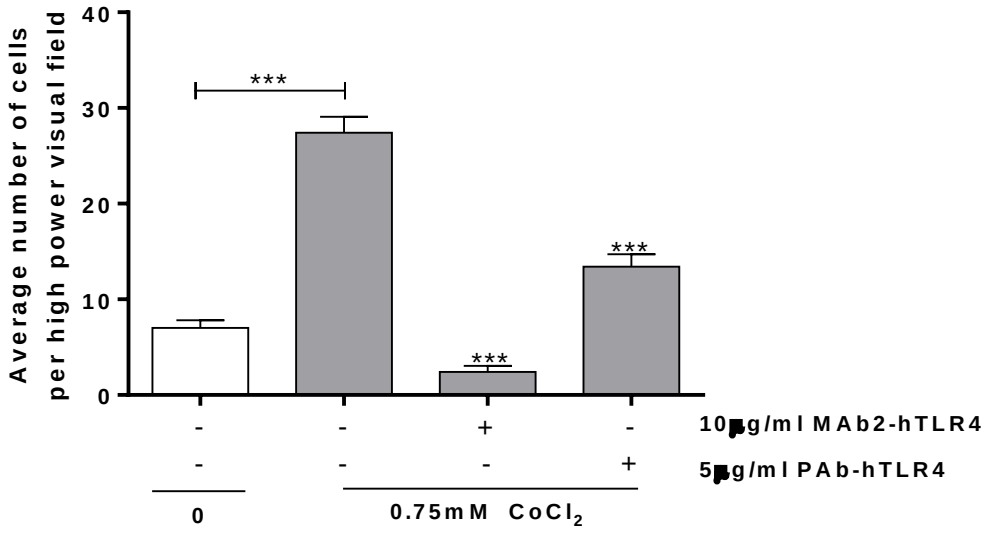

Figure G.1: Effect of anti-TLR4 neutralising antibodies on neutrophil migration
MonoMac 6 cells were stimulated with 10µg/ml MAb2-hTLR4 for 1h or 5µg/ml PAb-hTLR4 for 10 minutes followed by stimulation with 0.75mM $CoCl_2$ for 16h. Supernatant was collected and its chemotactic properties assessed by transwell neutrophil migration assay. Data is representative of three independent experiments each using a different neutrophil donor. Statistical significance was calculated by one-way ANOVA with Tukey's test for multiple comparisons comparing all samples to each other. *** indicates significance compared to cobalt-only control except where bar is shown. *This data appears in Lawrence* et al (Lawrence et al., 2016).

Appendix H D299G TLR4 transfectants

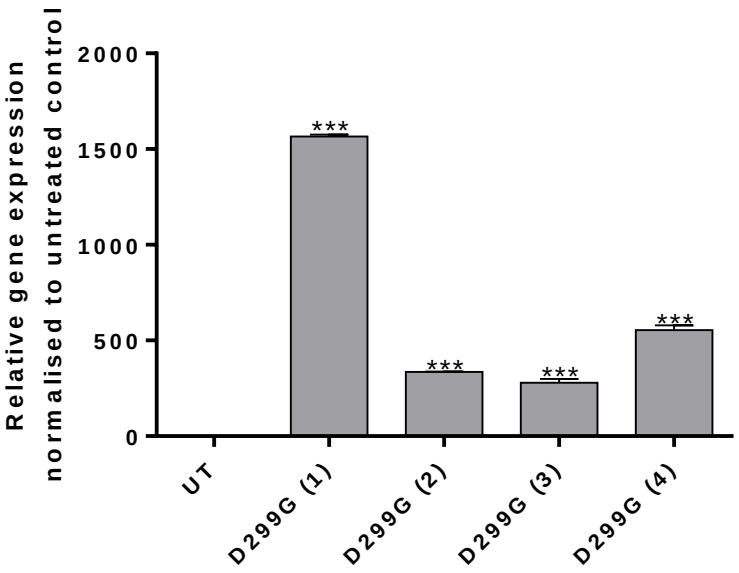

Figure H.1: *TLR4* expression by D299G TLR4 transfectants
TLR4 expression by 293-MD2-CD14 transfected with plasmid DNA encoding the D299G TLR4 SNP. The DNA used was from four different bacterial colonies (1-4) and *TLR4* expression was compared to untransfected 293-MD2-CD14 cells. UT=untreated cells.

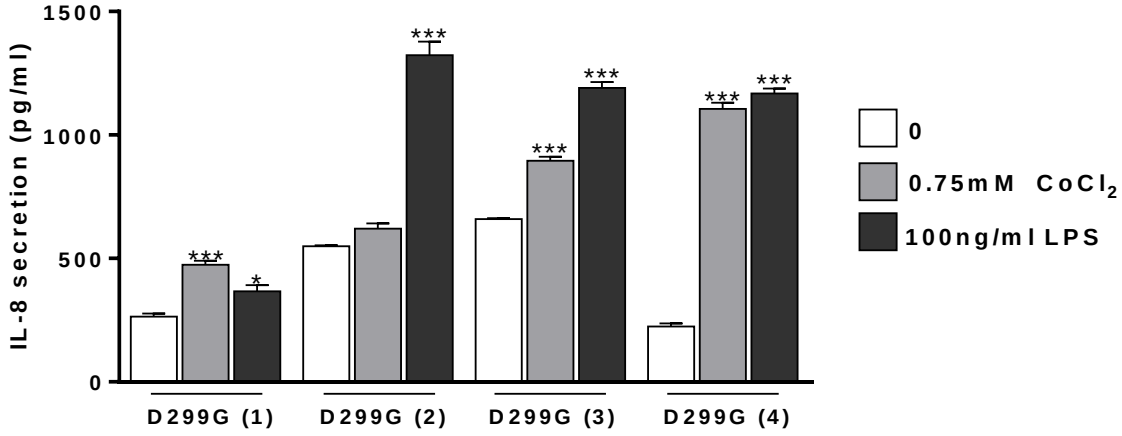

Figure H.2: IL-8 secretion by D299G TLR4 transfectants
D299G TLR4 transfectants were stimulated with 0.75mM $CoCl_2$ or 100ng/ml LPS for 24h before supernatant was collected and IL-8 release quantified by ELISA. Statistical significance was calculated by one-way ANOVA comparing stimulated samples to the untreated control within each transfectant group (e.g. D299G (1)).

Appendix I Effect of cobalt on *IL6* and *CCL2* expression

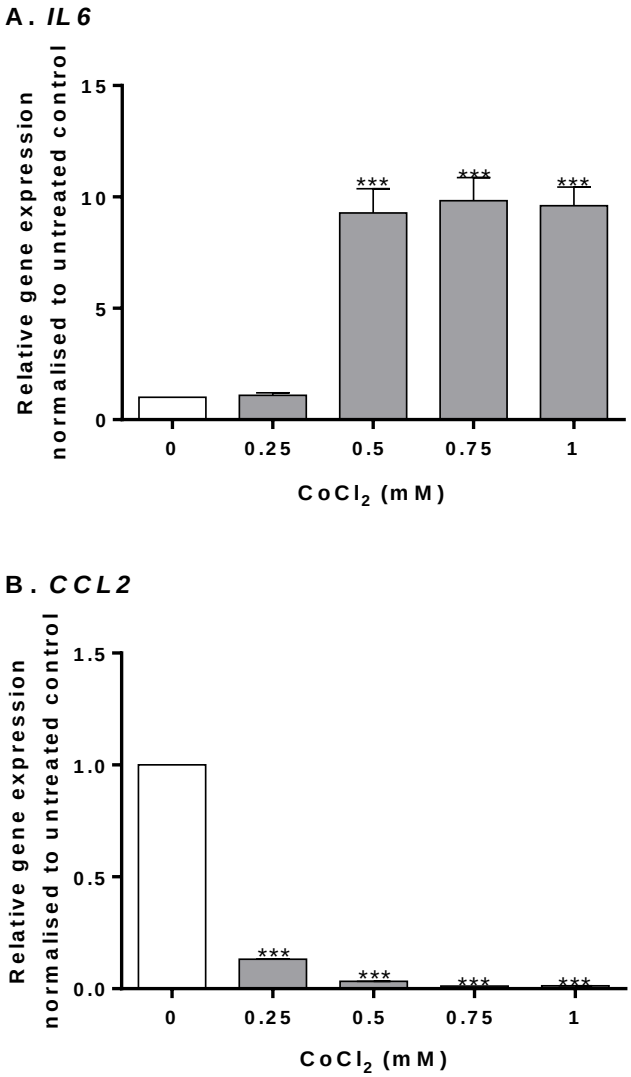

Figure I.1: Effect of $CoCl_2$ on *IL6* and *CCL2* expression
MonoMac 6 cells were stimulated with 0.25-1mM $CoCl_2$ for 24h before RNA was isolated and cDNA synthesised by reverse transcription. **A.** *IL6* and **B.** *CCL2* expression was quantified by qRT-PCR. n=1. Statistical significance was calculated by one-way ANOVA comparing stimulated samples to the unstimulated control.

Appendix J Effect of cobalt on *HIF1A* expression

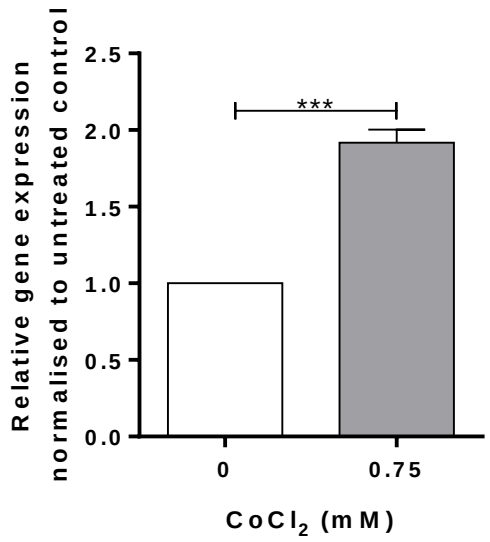

Figure J.1: Effect of $CoCl_2$ on *HIF1A* expression
MonoMac 6 cells were treated with 0.75mM $CoCl_2$ for 24h before RNA was isolated and cDNA synthesised by reverse transcription. qRT-PCR was performed to quantify *HIF1A* expression. Statistical significance was calculated by Student's *t* test. n=1.

Appendix K Publications, presentations and prizes

Publications

Lawrence H*, Mawdesley AE*, Deehan DJ, Holland JP, Kirby JA, Tyson-Capper AJ (2016) 'Preventing the inflammatory response to cobalt ions'. *Oncotarget* 7(7):7578-7585 (*joint first authors)

Lawrence H, Deehan DJ, Holland JP, Kirby JA, Tyson-Capper AJ. (2014) 'Immunobiology of cobalt: demonstration of a potential aetiology for inflammatory pseudotumours after metal-on-metal replacement of the hip'. *Bone and Joint Journal* 96-B:1172-1177

Lawrence H, Deehan DJ, Holland JP, Kirby JA, Tyson-Capper AJ. (2014) Can cobalt from metal-on-metal joints activate human TLR4 and cause an inflammatory response? *Bone and Joint Journal* 96-B (Supp 11):292

Tyson-Capper AJ, **Lawrence H**, Holland JP, Deehan DJ, Kirby JA. (2013). 'Molecular mechanism of cell activation by cobalt ions. Comment on Ninomiya *et al*.: Metal ions activate vascular endothelial cells and increase lymphocyte chemotaxis and binding'. *Journal of Orthopaedic Research* 31(11):1859

Manuscripts submitted

Lawrence H, Deehan DJ, Holland JP, Kirby JA, Tyson-Capper AJ 'Cobalt ions recruit inflammatory cells *in vitro* through human Toll-like receptor 4'. *Submitted to Biochemical and Biophysical Research Reports*, February 2016

Presentations

February 2016	*Cell biology and tissue analysis in retrieval studies* Oral presentation, Northern Retrieval Centre annual meeting, Newcastle, UK
September 2015	*Role of TLR4 in the immunological response to cobalt* Oral presentation, European Orthopaedic Research Societies meeting, Bristol, UK
June 2015	*Role of TLR4 in adverse reactions to cobalt ions* Oral presentation, Immunology North East Annual Symposium, Durham University, UK
June 2015	*Role of TLR4 in adverse reactions to biomaterials* Oral presentation, NIHR Postgraduate Research Day, Newcastle, UK
May 2015	*Role of TLR4 in adverse reactions to cobalt ions* Oral presentation, American-British-Canadian Travelling Orthopaedic Fellows meeting, Hexham, UK
May 2015	*Fooled: How metal hips can confuse our immune system* Oral presentation, INSIGHTS Public Lecture Series, Newcastle University, UK (*Faculty of Medical Sciences public speaking prize)* Available online at http://www.ncl.ac.uk/events/public-lectures/item.php?three-tales-from-the-biomedical-frontier3
April 2015	*Fooled: How metal hips can confuse our immune system* Oral presentation, INSIGHTS Public Lecture Prize heats and ICM poster evening, Newcastle University, UK
October 2014	*Metal-on-metal joints: cobalt can cause an inflammatory response through human TLR4* Oral presentation, North East Postgraduate Conference, Newcastle, UK
June 2014	*Metal-on-metal joints: cobalt can cause an inflammatory response through human TLR4* Poster presentation, Immunology North East Annual Symposium, Northumbria University, UK
June 2014	*Metal-on-metal joints: cobalt can cause an inflammatory response through human TLR4* Poster presentation, ICM Research Day, Newcastle University, UK
May 2014	*Metal-on-metal joints: cobalt can activate TLR4 and cause an inflammatory response* Oral presentation, ICM Seminar Program, Newcastle University, UK
October 2013	*Can cobalt from metal-on-metal joints activate human TLR4 and cause an inflammatory response?*

	Oral presentation, Combined meeting of Orthopaedic Research Societies, Venice, Italy
June 2013	*Can failing metal-on-metal joints cause inflammation via an endotoxin-like response?* Poster presentation, NIHR Training Camp, Berkhamsted, UK
June 2013	*Do failing metal-on-metal joints cause inflammation via an endotoxin-like response?* Oral presentation, ICM Research Day, Newcastle University, UK

Prizes and Awards

August 2015	Faculty of Medical Sciences Graduate School Travel Award to attend the European Orthopaedic Research Societies meeting
May 2015	Faculty of Medical Sciences postgraduate public speaking prize (INSIGHTS public lecture series)
June 2014	2nd prize, Immunology North East Annual Symposium poster competition, Northumbria University
June 2014	2nd prize, ICM Research Day poster competition, Newcastle University

Other co-authored abstracts

Mawdesley AE, Anjum SA, **Lawrence H**, Deehan DJ, Kirby JA, Tyson-Capper AJ
Preventing the inflammatory response to cobalt ions
Presented at the North East Postgraduate conference, Newcastle, UK, October 2015 (Oral presentation, British Society for Immunology prize)

Anjum SA, Mawdesley AE, **Lawrence H**, Deehan DJ, Kirby JA, Tyson-Capper AJ
The effect of cobalt on inflammatory cytokine and adhesion molecule expression
Presented at the British Orthopaedic Research Society meeting, Liverpool, UK, September 2015 (Oral presentation)

Anjum SA, Mawdesley AE, **Lawrence H**, Deehan DJ, Kirby JA, Tyson-Capper AJ
The effect of cobalt on inflammatory cytokine and adhesion molecule expression
Presented at the European Orthopaedic Research Societies meeting, Bristol, UK, September 2015 (Oral presentation)

Mawdesley AE, Anjum SA, **Lawrence H**, Deehan DJ, Kirby JA, Tyson-Capper AJ
Preventing the inflammatory response to cobalt ions
Presented at the European Orthopaedic Research Societies meeting, Bristol, UK, September 2015 (Elevator pitch presentation, runner-up prize)

Anjum SA, Mawdesley AE, **Lawrence H**, Deehan DJ, Kirby JA, Tyson-Capper AJ
The effect of cobalt on inflammatory cytokine and adhesion molecule expression
Presented at the Immunology North East Annual Symposium, Durham, UK, June 2015 (Poster presentation)

Mawdesley AE, Anjum SA, **Lawrence H**, Deehan DJ, Kirby JA, Tyson-Capper AJ
Preventing the inflammatory response to cobalt ions
Presented at the Immunology North East Annual Symposium, Durham, UK, June 2015 (Poster presentation)

Lawrence H, Deehan DJ, Holland JP, Deehan DJ, Kirby JA, Tyson-Capper AJ
Metal-on-metal joints: cobalt can activate TLR4 and cause an inflammatory response
Presented at the 6th Advanced Hip Resurfacing course, Ghent, Belgium, May 2014 (Oral presentation)

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