STUDY OF TOLL-LIKE RECEPTOR EXPRESSION AND ACTIVITY IN HIP AND KNEE OSTEOARTHRITIS

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Abstract

The Toll-like receptors (TLRs) are a family of highly conserved pattern recognition receptors involved in the detection of pathogenic invasion and tissue damage. Activation of TLRs can occur through binding to pathogen associated molecular patterns (PAMPs) but also host derived damage associated molecular patterns (DAMPs), which are highly abundant in the OA joint (Sokolove and Lepus, 2013). Originally thought of as a disease of wear and tear, osteoarthritis (OA) is better described as a disease of chronic inflammation, with the production of pro-inflammatory and degenerative products perpetuating disease activity.

Resident joint cells can react through TLRs to these damage products and thereby induce the production and secretion of pro-inflammatory cytokines and matrix degradative enzymes. This study sought to identify TLR expression and activity within synovial fibroblasts categorised by joint location and cartilage sub-categorised by OA progression. TLR induced pro-inflammatory cytokine induction from these cells along with matrix degenerative enzyme production was analysed to determine the pathogenic contribution of the cell types.

Significant differences in expression of TLR2, TLR3 and TLR4 were noted between hip and knee derived synovial fibroblasts. Increased expression correlated to a higher pro-inflammatory cytokine production from knee opposed to hip derived synovial fibroblasts. This difference in inflammatory cytokines production comparing knee to hip became even more apparent when observing spontaneous production from synovial membrane cultures that demonstrated TNF, IL-6 and IL-8 to be significantly higher in cultures from

the knee opposed to hip. Knee synovial fluid was also noted to have a significantly lower level of MMP1 compared with hip derived samples supporting a hypothesis that knee and hip osteoarthritis pathogeneses may differ.

Analysis of articular chondrocytes at different stages of disease progression demonstrated altered phenotypes. Chondrocytes isolated from regions of degeneration had an altered TLR expression pattern and subsequently produced significantly elevated IL-6 and MMP13 following TLR stimulation relative to those isolated from intact regions of cartilage.

These data suggest that TLR-induced cytokine secretion by synovial fibroblasts and chondrocytes potentially contribute to disease pathology and hypothetically have two separate phenotypes of disease progression. It also highlights the possible need for tailored treatment of OA dependent on joint location providing some explanation of differences in efficacy of trialled biologic therapies.

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List of abbreviations

AP-1	Activator protein-1
APC	Allophycocyanin
APS	Ammonium Persulphate
CpG	Cytosine-phosphate-guanine
DAMP	Damage associated molecular pattern
DAPI	4',6-diamindino-2-phenlyindole
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FITC	Fluorescein isothiocyanate
FSL-1	Pam2CGDPKHPKSF
FwB	FACS wash buffer
G0	Grade 0 (healthy) cartilage
G1	Grade 1 (early stage OA) cartilage
G2	Grade 2 (late stage OA) cartilage
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HC	Healthy control
HEK	Human embryonic kidney cells
HPRT1	Hypoxanthine phosphoribosyltransferase
HRP	Horse radish peroxidase
IFN	Interferon

IL-6	Interleukin 6
IL-8	Interleukin 8
kDa	Kilodaltons
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
Mal	MyD88 adaptor like
MFI	Mean fluorescent intensity
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary response 88
NF-kB	Nuclear factor kappa beta
NP-40	Nonylphenoxypolyethoxyethanol
NSAID	Non-steroidal anti-inflammatory drug
OA	Osteoarthritis
OACh	OA chondrocyte
OASF	OA synovial fibroblast
ODN	Oligodeoxynucleotide
PAM3	Pam3CysSerLys4
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PBS-T	PBS + Tween 20
PCR	Polymerase chain reaction
PE	Phycoerythrin
Poly(I:C)	Polyinosinic-polycytidylic acid
PRR	Pattern recognition receptor
qPCR	quantitative PCR
R-848	Resiquimod
RA	Rheumatoid arthritis

RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute (medium)
RT	Reverse transcription
SDS	Sodium Dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
ssDNA	Single stranded deoxyribonucleic acid
ssRNA	Single stranded ribonucleic acid
TAK1	Tumour growth factor-B-activated kinase 1
TBE	Tris acetate-EDTA buffer
TBK1	TANK binding kinase 1
TBS	Tris-buffered saline
TBS-T	TBS + Tween 20
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor alpha
TRAF	TNF receptor associated factor
TRAM	TIR domain containing adapter molecule 2
TRIF	TIR domain containing adapter inducing interferon B
v/v	Volume per volume
w/v	Weight per volume

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This is, and always will be for, Mum and Dad and me.

"L'apprendimento non esaurisce mai la mente"

(Learning never exhausts the mind)

-Leonardo da Vinci

<u>Declaration</u>

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

Signed

Matthew Jon Craig Stephens

Dated

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Chapter one - Introduction

1.1. Overview

The pathogenesis of Osteoarthritis (OA) was believed to be a general process of wear and tear associated with age (Wright, 1944). It has been more recently viewed as an inflammatory disease of the joint, characterised by chronic low level inflammation and the production of matrix degradative enzymes, which contribute to disease progression (Sokolove and Lepus, 2013). Activation of a family of innate immune receptors, the Toll-like receptors (TLRs), has been suggested by many to contribute to the progression of OA through induction of cytokine secretion following stimulation of synovial fibroblasts, chondrocytes and infiltrating cells of the joint (Iqbal et al., 2016, Qin et al., 2014, Sillat et al., 2013).

Activation of TLRs can be triggered through the recognition of pathogen associated molecular patterns (PAMPs) but also by damage associated molecular patterns (DAMPs). DAMPs are found in abundance within the diseased joint space and are produced by, but not limited to, the degradation of the cartilage matrix (Okamura et al., 2001, Termeer et al., 2002, Sohn et al., 2012).

Whilst there are numerous TLRs as well as potential DAMPs reported in the joint as a whole, the characterisation of their ability to perpetuate disease activity is poorly defined. This thesis demonstrates new evidence suggesting the contribution of two types of cells within the OA joint (synovial fibroblasts and articular chondrocytes) to disease activity through TLR stimulation.

1.2. __Osteoarthritis__

OA is the most common musculoskeletal disease in the western world affecting a third of people over 45 in the UK and almost half of the population aged 65 and over (F et al., 2011). In the Global Burden of disease 2010 study, hip and knee OA was ranked as the 11th highest contributor to global disability and as the 38th highest in disability-adjusted life years (Murray et al., 2012). Predominant in the aged and obese population, OA has been correlated with years of severe mechanical stress, sporting injuries or accidents, suggesting OA as a disease of age and injury (Saxon et al., 1999).

The disease manifests first as a molecular derangement (abnormal joint tissue metabolism) followed by anatomic and/or physiologic derangements (characterised by cartilage degradation, bone remodelling, osteophyte formation, joint inflammation and loss of normal joint function), that can culminate in illness (Lohmander, 2000). The observed heterogeneity of the disease has meant that research and treatment is difficult. Prioritisation of research focused on pain mechanisms in OA, improving the understanding of inter-tissue communication and early intervention strategies have since led to a series of biologic therapies (Ohtori et al., 2015, Detert et al., 2014, Grunke and Schulze-Koops, 2006, Guler-Yuksel et al., 2010, Kingsbury et al., 2013, Li et al., 2011).

In the late 19th century physicians and researchers identified primary familial OA associated Heberden's nodes, swollen nodules on the interphalangeal joints, as a hereditary disease that appears at an increased rate in members

of the same family (Duckworth, 1899, J, 1881). Duckworth's observations of a strong female predominance of the disease through four generations of a family hinted to a single autosomal gene that was dominant in women. Stecher's formal study into Heberden's nodes showed a much higher incidence of occurrence in mothers and siblings of affected individuals. Whilst primary familial OA was noted through the Heberden node distribution in family groups, multi-joint OA characteristics were determined through radiographic analysis (Stecher and Hersh, 1944).

Greater evidence towards of a role of genetics in the pathogenesis of OA has been highlighted through epidemiological studies of family history, clustering, twin studies and exploration of rare genetic disorders related with OA (Tak and Firestein, 2001). Single nucleotide polymorphisms (SNPs) in genes encoding cartilage oligomeric matrix protein, collagen type II alpha 1, transforming growth factor-β signalling, interleukin-1 and interleukin-4 receptor have been implicated as genetic variation markers for predisposition to OA (Mabuchi et al., 2001, Ikeda et al., 2002, Valdes et al., 2007, Stern et al., 2003, Vargiolu et al., 2010) (Table 1.1).

Gene	Phenotype	Reference
COL1A1	Reduction in incidence of hip OA females	(Loughlin et al., 1994)
COL2A1	Early onset OA	(Loughlin et al., 1994, Zhai et al., 2004, Valdes et al., 2007)
IL1	Knee and hip OA	(Meulenbelt et al., 2004)
IL4	Hip OA females	(Forster et al., 2004)
IL6	Hip OA	(Pola et al., 2005)
AGC1	Hand OA	(Aigner and Dudhia, 2003)
ADAM12	Knee OA	(Valdes et al., 2006)
CALM1	Hip OA Japanese population	(Valdes et al., 2007)
BMP2	Reduced incidence of knee OA female	(Valdes et al., 2006)
COMP	Early onset hip OA	(Valdes et al., 2007)

Table 1.1: A selection of mutations involved in the progression of OA. Genes encoding products of inflammatory or degenerative pathways and those involved in cartilage protection have been included. COL1A1 (collagen type I), COL2A1 (collagen type II), AGC1 (Aggrecan), ADAM12 (Disintegrin and metalloproteinase domain-containing protein 12), BMP2 (Bone morphogenic protein 2) and COMP (Cartilage oligomeric matrix protein).

Biochemical, cellular and structural changes in the synovium, bone, ligaments, supporting musculature and meniscal tissue are observed in the progression of OA (Ayral et al., 2005, Ajuied et al., 2014, Edd et al., 2015, Verdonk et al., 2016). Chronic inflammation within the synovial membrane (synovitis) has also been shown to contribute to OA pathogenesis, through the production of pro-inflammatory cytokines and matrix degradative enzymes which increase the overall rate of cartilage erosion (Scanzello and Goldring, 2012). First line treatments for OA include non-steroidal anti-inflammatory drugs (NSAIDS) which are available over the counter (e.g. ibuprofen or aspirin) or prescribed (e.g. naproxen). However, NSAIDs only alleviate the inflammatory pain and do not prevent ongoing OA associated cartilage degradation (da Costa et al., 2016, Richette et al., 2015). Corticosteroid injections offer fast relief from joint associated pain and inflammation through their inhibitory effect upon the enzyme phospholipase A_2 which is required in the supply of arachidonic acid, a component essential in the formation of inflammatory mediators (Wang and He, 2015, Soriano-Maldonado et al., 2016). Hyaluronic acid injection is another popular therapy in treatment of OA aiding in reducing inflammation in the joint by providing additional lubrication for the affected cartilage (Altman et al., 2015). Whilst abating many symptoms of OA progression, none of these procedures have any long term preventative effect on cartilage destruction with a high proportion of patients requiring joint surgery.

OA is not just a physical manifestation but also an emotionally debilitating condition leaving those who have it with a reduced sense of self-worth (Vriezekolk et al., 2010). The pain caused by the inflammation and the

associated joint damage often limits movement to a point where physiotherapy, a method of reducing the progression of OA, becomes unbearable and the disease progresses rapidly (Page et al., 2011, Bennell, 2013). In an ageing Western society, the burden that OA puts on health care systems is large with such economic expenditures including additional health care, medication, surgery and loss of economic productivity by patients being out of the work place (Chen et al., 2012).

1.3.   <u>Toll-like receptors</u>

The inflammation related to OA may, in part, be regulated by a family of innate immune receptors known as the Toll-like receptors (TLRs). TLRs are evolutionary conserved type I membrane pattern recognition receptors (PRRs) that are located at the cell membrane or within endosomes (Botos et al., 2011). To date 10 TLRs have been identified in humans which recognise bacterial, viral and fungal PAMPs and host derived DAMPs (Table 1.2) (Yu et al., 2010).Toll-like receptors, named for their similarity to the *D melanogaster* protein Toll, was first described for its importance in dorso-ventral development of *D melanogaster* embryos (Anderson et al., 1985). Toll was later noted to aid in fungal infection resistance in adult flies. Induction of NF-kB cytokines through activation of Toll resulted in the expression of antifungal peptide gene drosomycin (Lemaitre et al., 2012). Discovery of TLRs in mice, along with advances in the understanding of activation of innate immunity, led Bruce Beutler, Jules Hoffmann and Ralph Steinman to win the collaborative Nobel prize in Physiology in 2011.

TLR	Location	PAMPs	DAMPs	Synthetic agonists
TLR1/2	Cell membrane	Peptidoglycan, lipoproteins, LTA, Zymosan	Heat shock proteins, HMGB1	Pam$_3$Cys
TLR2/6	Cell membrane	Lipoproteins	Heat shock proteins, HMGB1	MALP2
TLR3	Endosome	dsRNA	Self dsRNA	Poly(I:C)
TLR4	Cell membrane	LPS, mannan, RSV fusion protein	Heat shock proteins, Fibrinogen, heparin sulphate, fibronectin, hyaluronic acid	Lipid A derivatives
TLR5	Cell membrane	Flagellin	-	-
TLR7/TLR8	Endosome	ssRNA	Self ssRNA	R848 (TLR7/8), Imiquimod (TLR8)
TLR9	Endosome	CpG DNA, hemozoin	Self DNA	CpG-ODNs

Table 1.2: TLRs and examples of known natural and synthetic agonists. dsRNA – double stranded ribonucleic acid, ssRNA – single stranded ribonucleic acid, DNA – deoxyribonucleic acid, ODNs – oligodinucleotides, LPS – lipopolysaccharides.

TLRs, primarily form homodimers (TLR3, TLR4, TLR5, TLR7, TLR8 and TLR9) or heterodimers (TLR1/2 and TLR2/6). Regulation of activation is controlled through a ligand-dependent union of the toll-interleukin receptor (TIR) domains, an important step required for the recruitment of adaptor molecules, that downstream signalling is activated. Some TLRs depend on other co-receptors for full ligand sensitivity, such as the recognition of lipopolysaccharide (LPS) by TLR4, which requires at least the presence of Lymphocyte antigen-96 (MD-2) (Kim et al., 2007). Additionally, CD14 and LPS-Binding Protein (LBP) also facilitate the presentation of LPS to MD-2 by acting as accessory molecules. TLR4 can also undergo endocytosis and signal though acidification of the endosome (Gangloff, 2012). TLR signalling utilises either the myeloid differentiation primary response gene 88 (MyD88)-dependent or MyD88 independent pathway following activation (Premkumar et al., 2010).

Of the four main adaptor proteins MyD88, toll-interleukin 1 receptor adaptor protein (MAL), TIR-domain-containing adapter-inducing interferon-β (TRIF), TRIF-related adapter molecule (TRAM), MyD88 is utilised by all of the receptors except TLR3, which at this time is only known to use TRIF (Yamamoto et al., 2002). TLR signalling pathways lead to induction of transcription factors including; NF- kB, interferon regulatory factors (IRFs) and activator protein-1 (AP-1) (Kenny and O'Neill, 2008) (Figure 1.1 and 1.2).

Mammalian TLR3, TLR7, TLR8 and TLR9 are endosomal receptors that sense nucleic acids from endocytosed viruses, bacteria or parasites (Beignon et al., 2005, Triantafilou et al., 2005, Ahmad-Nejad et al., 2002, Parroche et al.,

2007). Self-nucleic acids are not usually recognised by these TLRs due to regulation events including receptor compartmentalisation, proteolytic processing and trafficking (Ewald et al., 2008). TLR3, 7, 8 and 9 commonly reside within the endoplasmic reticulum (ER) however, their activation through ligand binding only occurs within acidified endolysosomal compartments (Hacker et al., 1998, Funami et al., 2004, Gibbard et al., 2006). There are several chaperon proteins that traffic these TLRs to the endosomes including UNC93B1 (Kim et al., 2008). The ectodomains of TLR7 and TLR9 are proteolytically cleaved by cysteine endoproteases to activate downstream signalling (Ewald et al., 2008, Ewald et al., 2011, Kanno et al., 2013). A similar cleavage of TLR3 has been reported, though conflicting studies demonstrated the N-terminal region of TLR3 was implicated in ligand binding suggesting TLR3 may function as a full-length receptor (Liu et al., 2008, Tokisue et al., 2008).

The first example of a non-microbial particulate activating a TLR response was heat shock protein 60 (HSP60) which was demonstrated to induce a cytokine response via TLR4 activation in mouse macrophages and more recently through TLR2 and TLR4 in human cardiomyocytes. The activation of TLR4 by HSP60 was ascribed to LPS contamination due to its bacterial origin. However, several follow on studies have confirmed their observations and the activation of TLR4 through this DAMP (Ohashi et al., 2000, Tian et al., 2013, Ueki et al., 2002). Other such examples of DAMPs that could be important in OA include; High mobility group box - 1 (HMGB1), RNA and DNA immune complexes, calcium-containing crystals and Tenascin-C which elicit immune responses,

usually in response to necrotic cells debris resultant from tissue damage (Lotze and Tracey, 2005, Midwood et al., 2009).

Endogenous ligands generated through matrix degradation and cell death have been linked to a several TLR-dependent inflammatory responses in OA. Necrosis of chondrocytes, synoviocytes infiltrating cells, releases host nucleic acids into the synovial cavity. This process has been observed to activate rheumatoid arthritis synovial fibroblasts via TLR3, an affect that was attenuated through treatment with the potent nuclease, benzonase (Brentano et al., 2005). These findings, even though documented in a different disease suggest the possibility that mRNA can act as an endogenous ligand for TLR3, supports the hypothesis that several TLRs play a potential role in the inflammation associated with OA (Kariko et al., 2004).
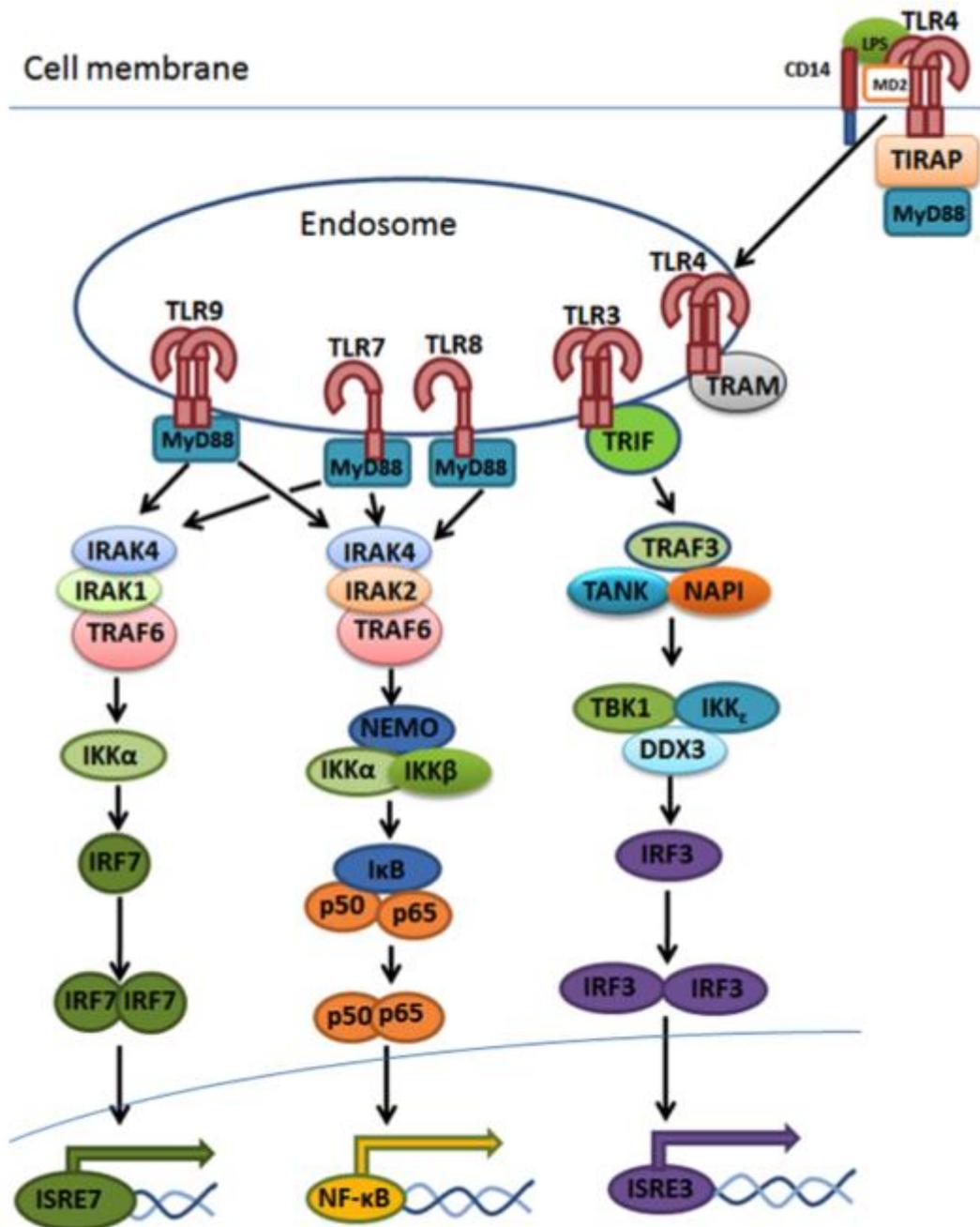
Figure 1.1: Signalling pathways of endosomal TLRs. TLR3, TLR7, TLR8 and TLR9 are known to be primarily expressed and activated within the endosomal compartment of the cell. MyD88 is utilised by all TLRs bar TLR3 which uses TRIF for signalling activating both ISRE3 and NF-kB transcription factors. TLR4 homodimer formation and activation can occur on the cell surface membrane or can be internalised after TIR dimerisation and signal through TRAM on the endosome rather than through MyD88. MKK, MAP kinase kinase; RIP1, receptor-interacting protein 1; TAB, TAK1-binding protein; TAK, TGFβ-activated kinase; TBK1, TANK-binding kinase 1. TRADD, TNFRSF1A-Associated Via Death Domain. ERK, Extracellular signal-regulated kinases. DDX3, Dead box RNA helicase. IRF7, interferon regulatory factor 7. TRIF, TIR-domain-containing adaptor-inducing interferon-β. TRAM, Toll-like receptor 4 adaptor protein. NEMO, NF-kappa-B essential modulator. Adapted from (Kawai and Akira, 2006).
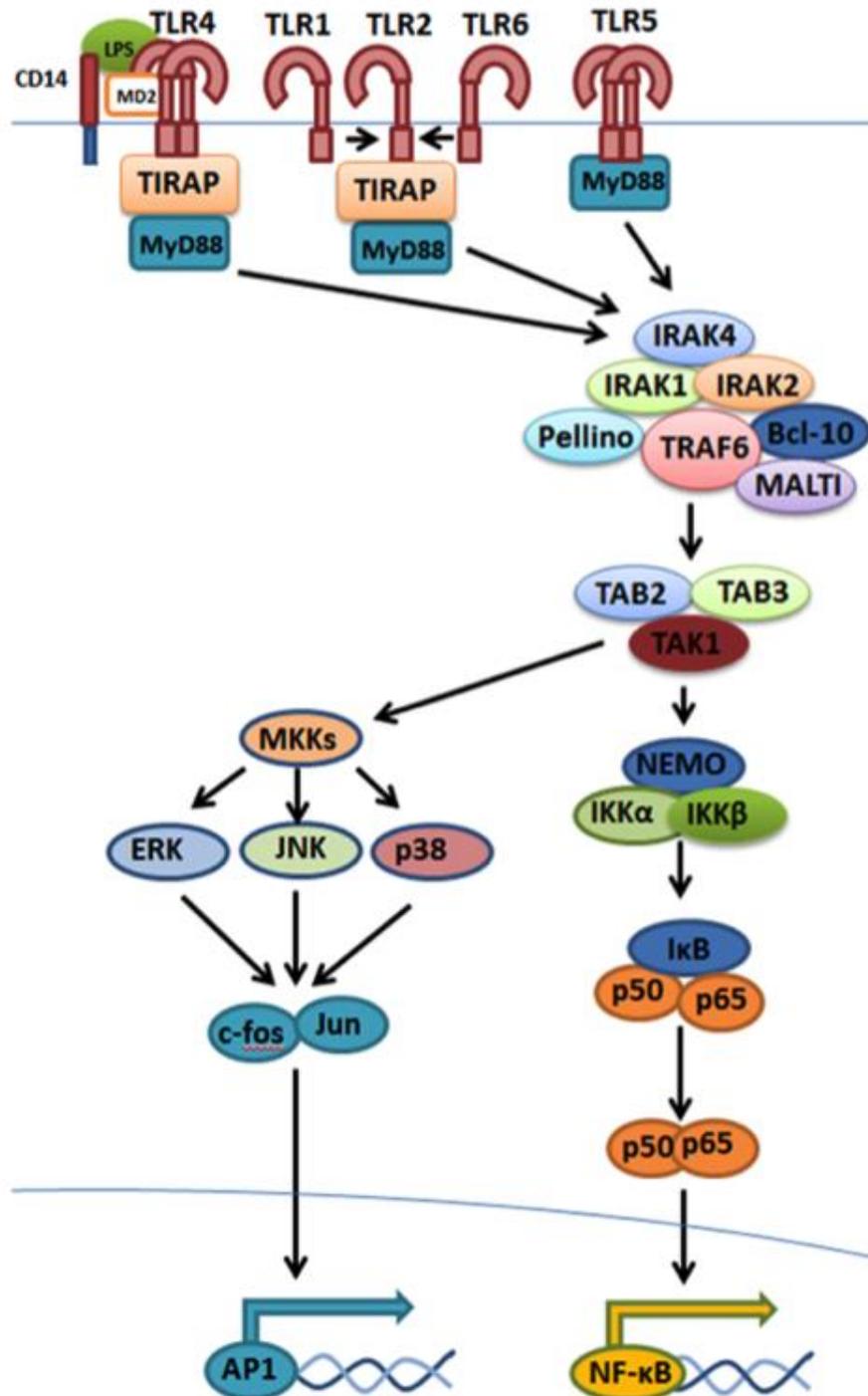
Figure 1.2: Signalling pathways of cell surface membrane TLRs. Following ligands binding, the TIR domains of the TLRs dimerise allowing for the recruitment of MyD88 and/or TIRAP. Both combinations signal through the TRAF6 complex. This activates either: the Map kinases (MKKs) resulting in the phosphorylation of ERK, JNK or p38 in turn releasing AP-1 which is translocated to the nucleus; or the IKK complex resulting in dissociated of IkB from NF-kB (p50/p65) which can then translocate to the nucleus and initiate gene transcription. Adapted from (Kawai and Akira, 2006).

1.4.   Toll-like receptors in osteoarthritis

There have been several suggestions regarding the role of TLRs in the pathogenesis of OA, with much of the research focusing on the production of pro-inflammatory cytokines and matrix degenerative enzymes. TLRs recognise a wide array of PAMPs and DAMPs present within the joint, which can induce the production of pro-inflammatory cytokines, chemokines and type I interferons leading to the recruitment and activation of immune cells (Kawai and Akira, 2006). With the range of endogenous TLR ligands currently expanding, OA is viewed as an auto-inflammatory disease where host cells overstimulated by DAMPs create a sterile inflammatory response, cartilage erosion and bone remodelling (Berenbaum, 2013).

Many TLRs are constitutively expressed by immune cells including monocytes and macrophages but they are also present by, and can be induced in, non-immune cell types such as synovial fibroblasts and articular chondrocytes in response to TLR ligands (Hu et al., 2014, Bobacz et al., 2007). Synovial membranes from OA biopsies contain high levels of TLR4 expression (Nair et al., 2012). The expression and associated cytokine production is much lower than that of RA cultures, due to a lower infiltration of peripheral monocytes into the synovial lining and associated synovitis in OA. However, several cytokine levels are significantly greater in the synovial fluid of OA patients compared to healthy controls (Sauerschnig et al., 2014).

However, the limited sample size of some studies, and outdated techniques, suggest the results require validation in a larger, more reliable, patient cohort (Kuroki et al., 2010). Lesioned areas of cartilage with advance OA have been

demonstrated to have increased levels of TLR2 and TLR4 present on their chondrocytes (Sillat et al., 2013). Whilst, isolated OA synovial fibroblasts respond to a variety of synthetic and naturally occurring TLR ligands with cytokine production from these cells higher than healthy controls (Hu et al., 2014).

TLR signalling has been reported to show cross-talk with complement signalling and interestingly, elevated complement components C3, C5 and their derivatives, have been reported in OA patient synovial fluid (Wang et al., 2011). This increased complement presence and subsequent activity in conjunction with OA produced DAMPs may lead to an elevated inflammatory response in patients with the disease.

Many inflammatory/degenerative arthrides including OA and RA involve NF-kB dependent cytokines and matrix degenerative enzymes, consequently TLRs became of interest for study due to their ability to activate and perpetuate inflammatory responses. Research into synovial fibroblast NF-kB signalling revealed that the production of IL-6, IL-8 and MMP13 was dependent on NF-kB induction (Tak and Firestein, 2001).

Several population studies into the genetics of TLR expression have revealed insight into variation between populations. For example, a study into TLR expression indicated a predisposition of a subset of the Taiwanese population to knee OA who had a SNP variation in an upstream TLR3 promoter element (Yang et al., 2013). Another group discovered promoter polymorphisms in TLR9 (-1486T/C) in a large Chinese cohort study suggesting the potential link between mutant TLR expression and disease activity (Su et al., 2012). Whilst

confined to a specific joint (knee) and population (Taiwanese/Chinese), these studies and others have illustrated the possibility of a genetic TLR link to disease predisposition and activity for which further research is underway.

Increasing evidence points toward TLR2 and TLR4 as being two of the most ubiquitous TLRs involved in sensing tissue damage, responding to several endogenous ligands produced through tissue destruction (Ohashi et al., 2000, Smiley et al., 2001, Taylor et al., 2007). TLRs have been previously implicated with the progression of arthritis models and sterile inflammatory diseases. For example, TLR4$^{-/-}$ total knockout mice demonstrated impaired cytokine production under surgically induced meniscal damage. This observation has been theorised to be caused through production of biologically active extracellular membrane (ECM) components reducing normal inflammatory response through receptor antagonistic competition (Taylor et al., 2007). Endogenous DAMPs, capable of activating TLRs, can be produced by the ongoing destruction of the OA joint and have been shown to lead to sterile inflammation. They include hyaluronan, fibrinogen, high-mobility group box1 protein (HMGB1) and heat shock protein 60 (HSP60) (Beg, 2002).

Fibronectin, an ECM component of cartilage, has an alternative form in damaged and aged tissue. This form, as well as several other splice variants, has been shown to stimulate a TLR4 dependent pathway in TLR4 transformed HEK cells preferentially to normal fibronectin (Lasarte et al., 2007, Okamura et al., 2001), Gondokaryono et al., 2007). Additionally, hyaluronan is identified as a DAMP for TLR4. A large multimeric protein, hyaluronan can fragment through enzymatic digestion or mechanical forces. A study into hyaluronan-TLR4 interaction has demonstrated that enzymatically

digested fragments of specific length (9-12kDa) inhibit cytokine production by recruitment of an alternate accessory molecule. Whilst these fragments of hyaluronan bind to TLR4 they do not associate using CD14 or LBP, but rather by CD44 (Kawana et al., 2008). This binding supresses further signalling (Figure 1.3).

Hyaluronic acid injection into an OA affected joint is a common and popular therapy for alleviating OA associated inflammation and pain and is thought that this CD44-TLR4 mediates this activity. CD44-TLR4 interaction also reduces MMP13 production from human OA and RA chondrocytes through a similar mechanism which, in turn, limits disease progression by reducing cartilage degradation (Julovi et al., 2011).
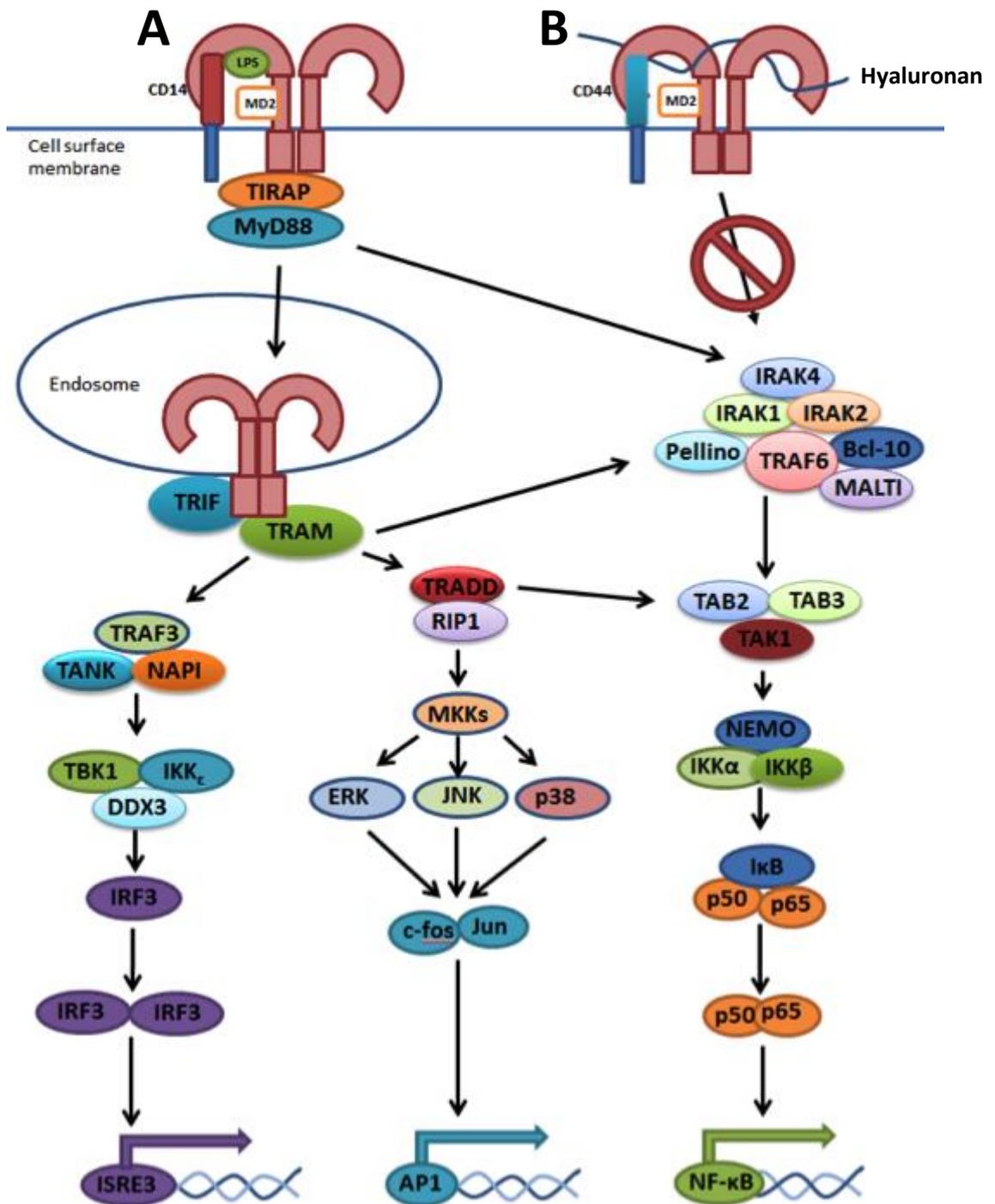
Figure 1.3: Signalling pathways of TLR4. A) Signalling cascades for TLR4 activation mediated through CD14 and MD2. Binding of LPS to the leucine rich region of TLR4 initiates the bringing together of the toll-interleukin receptor (TIR) region and MAL/TIRAP. Signalling can also occur after endocytosis of TLR4 which switches signalling from MyD88 dependent to utilise TRIF or TRAM. Activation results in the formation of the TRAF6 complex culminating in NF-kB activation. Additionally, TRAM signalling can activate ISRE3 transcription factor activation or AP-1 through TRADD/RIP1. B) Recruitment of CD44 through hyaluronan fragment binding results in no further signalling.

TLRs 2 and 4 are expressed and functional in human osteoarthritic synovial membranes (Ospelt et al., 2008). A conditional TLR4 knock-out mouse model demonstrated reduced cartilage and bone erosion associated with the progression of RA, whilst TLR2 knock out increased disease severity suggesting a role for TLRs in mediating cartilage turnover and erosion in experimental arthritis (van Lent et al., 2008). Expression of TLR2 and TLR4 has been demonstrated to be significantly increased in chondrocytes of patients with OA, particularly at sites of lesions within the cartilage (Kim et al., 2006). This increase in TLR2 and TLR4 receptor levels confirmed through immuno-histochemical analysis indicated the potential for TLRs to act as a marker for disease progression with TLR overexpression resulting in over production of functional inflammatory products including IL-1. Within the affected tissue, increased levels of pro-inflammatory cytokines, (e.g. IL-6) chemo-attractive factors (e.g. IL-8) and MMPs result in a more catabolic environment, contributing to disease progression (Zhang et al., 2008, Kim et al., 2006).

1.4.3 Damage associated molecular patterns (DAMPs)

Synovial fluid and joint tissue of OA patients contain significantly increased levels of DAMPs such as HMGB1, many of these molecules are shown to activate the innate immune system including several TLRs (Table 1.2). These molecules, released both through trauma and/or ageing cells, are both strong risk factors for developing OA such as is the case with tenascin-C release from human and canine joint damage (Chockalingam et al., 2013). Additionally, necrosis of cells through cellular trauma or caspase mediated cell death,

releases intracellular components into the extracellular environment with the potential to initiate a sterile response (Wang et al., 2016). These findings continually demonstrate the potential for the synovial fluid to perpetuate disease activity through the presence of, and ability to induce, catabolic factors.

1.5   <u>Synovial membrane</u>

The synovial membrane is a balloon-like fibrous tissue that encapsulates the joint. Comprised of several cell types, its major constituent are synovial fibroblasts which aid in the regulation of the elastic matrix of collagens. Synovitis is common in patients with inflammatory joint disease and is characterised by the inflammation of the synovial membrane primarily caused by the infiltration of peripheral blood cells into the membrane which, if activates, release pro-inflammatory factors. Synovitis is common in OA, with 1 in 2 patients undergoing total knee replacement (TKR) showing signs (Ayral et al., 2005, Goldring and Otero, 2011, Li et al., 2016, Wenham and Conaghan, 2010). Whilst the most definitive method of diagnosis is histological analysis, X-ray and MRI analysis of the affected joint, is effectively used to diagnose OA-related synovitis (Li et al., 2016).

In classical inflammatory arthrides, synovitis of the joint is easily detectable and has a wide heterogeneity. The synovium of patients with OA demonstrates a wide array of physical changes, although inflammation much less than found in RA (Lo et al., 2006). Changes include hyperplasia of the lining, due to infiltrating lymphocytes and monocytes, but also a thickened fibrotic tissue membrane caused by increased fibrin deposition within the synovium (Loeuille

et al., 2005). This synovial inflammation, in knee OA, tends to be diffuse and not isolated to an area of chondral defect (Loeuille et al., 2005). However, co-localisation of specific chondral defects and associated synovitis in the medial tibiofemoral compartment of the knee has been reported by others (Ayral et al., 2005). Several groups have also shown that inflammation is a prerequisite for cartilage damage, where an MMP3 knockout mouse model of OA demonstrated that macrophage activation in the synovium is essential for cartilage damage via the production of other matrix metalloproteases (Blom et al., 2007).

1.6   Chondrocytes and cartilage degradation

Cartilage degradation is a characteristic trait of OA progression. Following cartilage erosion, bone, the synovial membrane and ligature are damaged, resulting in increased pain and joint inflammation (Abraham et al., 2014, Edd et al., 2015, Fernandes et al., 2002). Articular cartilage has only one resident cell type, the chondrocyte, which regulates cartilage turnover (McElligott and Collins, 1960). Encased in a collagen, aggrecan and hyaluronan based matrix, chondrocytes exist in a challenging environment (Figure 1.4). The macro-structure of articular cartilage ECM is like that of a sponge; absorbing nutrient rich tissue fluid into the ECM during rest and flushing out waste products into the synovial cavity under load (Reeves, 1876, Mac, 1951). Chondrocytes readily absorb water during resting stages expanding in size due to osmolarity changes with their structural integrity maintained by the elastic yet restrictive forces of the ECM (Urban et al., 1993). Chondrocytes exist in a nutrient deficient, hypoxic environment with

oxygen tension varying from 1-7% through the tissue but have adapted for purpose (Rajpurohit et al., 1996). In arthritic joints, oxygen exchange is poor due to the a-vascular nature of the cartilage and the deep placement of capillaries within the synovial membrane (Silver, 1975). It is therefore presumable that the joint of a person affected by OA is more hypoxic than normal due to the increased metabolism occurring within the joint (Stevens et al., 1991). Alterations in this environment has been demonstrated to alter growth factor production (Tumour Growth Factor-TGF, Bone Morphogenic Protein-BMP, Cartilage Oligomeric Matrix Protein-COMP and Vascular Endothelial Growth Factor-VEGF) in OA tissue resulting in mass morphological and biochemical changes of the cell (Frenkel et al., 2000, Wang and Chen, 2016, Yi et al., 2014, Beckmann et al., 2014).

Articular cartilage itself can be described separated into the superficial, middle/transitional and deep zones which border subchondral bone through a calcified layer. Cell density is highest in the superficial layer forming a clustered layer of cells, whilst cells in the middle zone are semi-elliptical, those of the deep zone line up in columnar fashion. Progenitor cells are debated to be found both within the superficial and deep zone and are derived from bone mesenchymal stem cells (Solchaga et al., 2011) (Figure 1.5).
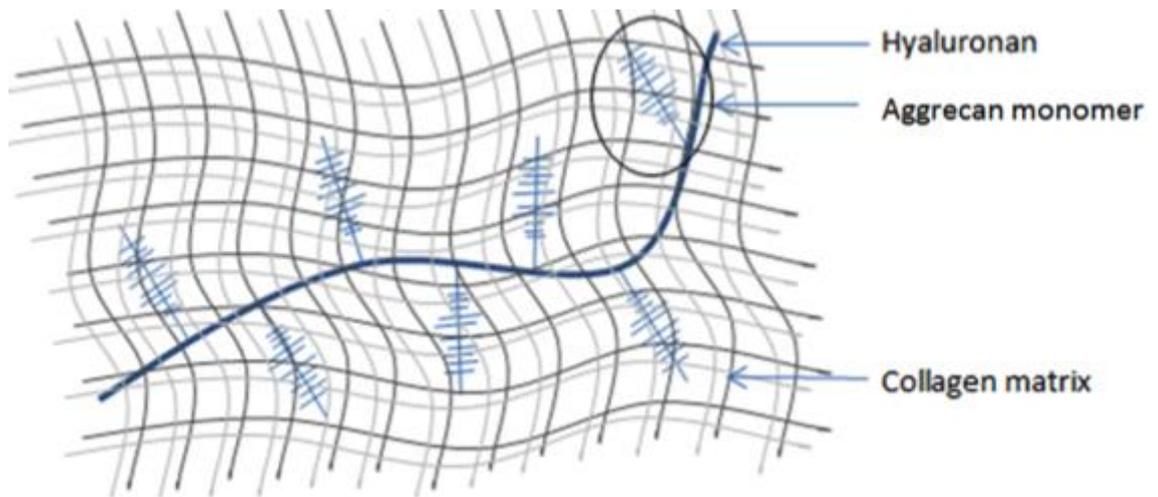
Hyaluronan

Aggrecan monomer

Collagen matrix

Figure 1.4: A schematic representation of the macro-matrix structure of articular cartilage. Large hyaluronan polymer chains which readily exceed 1MDa in size act as a base scaffold for aggrecan monomers which branch off of the main structure. These structures are contained within the collagen based matrix and comprise up to 90-95% of the dry weight of cartilage the remaining percentage being the chondrocytes themselves.

A — Superficial / Middle / Deep

B — Superficial / Middle / Deep
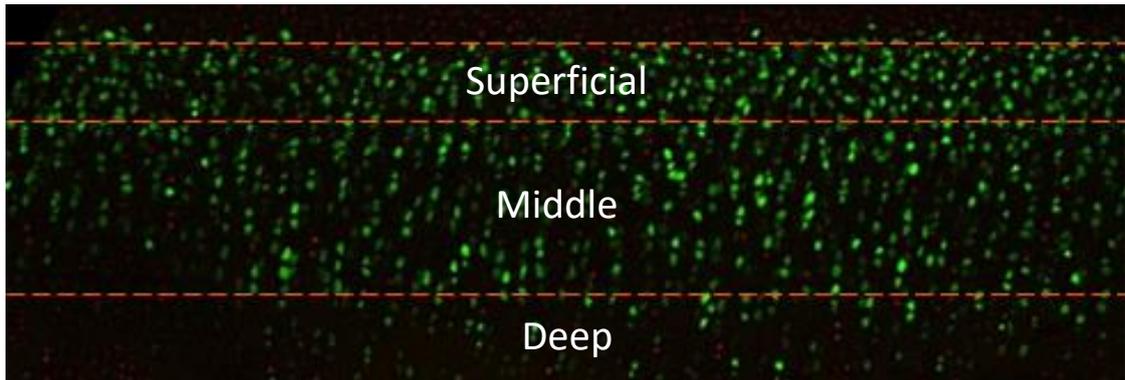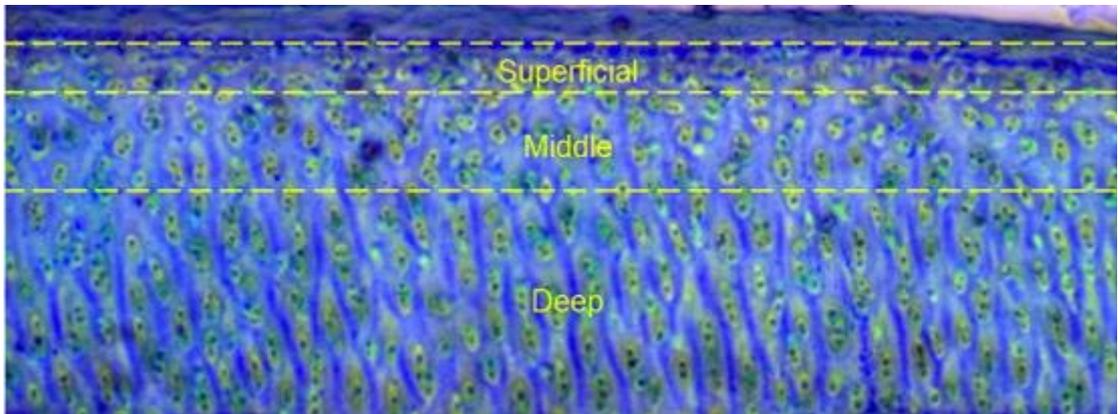
Figure 1.5: The three major zones of articular cartilage. **A)** An acetomethoxy derivate of calcein (calcein AM) and propidium iodide cell viability stain on a section of bovine articular cartilage. Sample was imaged at x200 magnification using Leica SP5 confocal microscope (methods 2.10.5). Live chondrocytes (green) dead cells created through isolation of sample (red). **B)** Toluidine blue/Fast green staining of bovine cartilage. Toluidine blue is a metachromatic dye staining dependent on pH. Nuclei (dark blue) cartilage proteoglycans (blue to purple) background cellular composites (green). Imaged on Leica white light and microscope x200 magnification.

Articular cartilage in adults is completely devoid of blood vessels or lymphatics, relying primarily on anaerobic respiration and mechanical diffusion of nutrients (Sophia Fox et al., 2009). Articular ECM is composed of a fine matrix that has an approximate pore size of ~6nm, which allows for the rudimentary screening of biological molecules by size and charge as well as limiting pathogen invasion (Buckwalter and Rosenberg, 1988, Linn and Sokoloff, 1965, Mow et al., 1992). This screening provides a physical barrier between chondrocytes and the synovial fluid DAMP/PAMP rich environment whilst also retaining many chondrogenic regulatory factors that are produced, by chondrocytes, to maintain cellular phenotype (Loeser, 1997).

After enzymatic single cell isolation and 2D culture, chondrocytes undergo a de-differentiation process by which they lose their chondrocytic phenotype and become fibroblastic like in appearance (Maroudas and Bullough, 1968). This process is characterized by the altered expression of many ECM proteins such as type I and type II collagen and consequently results in a change of cellular phenotype to a fibroblastic nature (Hong and Reddi, 2013). Primary chondrocyte phenotypic restoration can be achieved by suspension of the cells within a 3D matrix culture system, such as alginate beads or agarose gels (Ma et al., 2013). However, whilst these methods of culturing have been shown to restore the phenotype markers of the original cell, whether they truly resemble the original phenotype is still debated (Caron et al., 2012). Effects upon the expression of TLRs and other innate receptors of differing culture conditions are yet to be studied in detail. Rather than using a 3D culture system, chondrogenic differentiation media can been used as an alternative in order to maintain

primary isolated cells (Solchaga et al., 2011).

1.7   Osteoarthritic synovial fluid

Synovial fluid maintains a lubricating environment for articular joints whilst also acting as a nutrient medium, which maintains the cellular population (Smith, 2011). However, in OA, synovial fluid is infiltrated by leukocytes that perpetuate an inflammatory environment through activation of pattern recognition receptors such as the TLRs (Huss et al., 2010). Infiltrating cells, fragile to the constant stresses of the diseased joint, undergo osteo- or avascular necrosis attributing disease promoting factors to the synovial fluid. RNA released from these necrotic cells has shown to activate synovial fibroblasts of RA patients via TLR3 and it is theorised that TLR3 could be activated in a similar manner in OA (Brentano et al., 2005). Several complement components including C3a and soluble MAC complex have also been demonstrated to be significantly increased in quantity in early and late stage OA compared to healthy controls suggesting a role for complement in disease progression (Wang et al.,2011). With the documented cross-talk between TLR and complement signalling, observations like these make investigation of synovial fluid and joint cell interactions prudent.

1.8   Inflammatory cytokines in the pathogenesis of osteoarthritis

The pathogenesis of OA is poorly understood; however, the inflammation associated with the disease may have a key role and has the potential to be therapeutically targeted. Thus, to improve the prognosis and treatment of OA, a better understanding of the mechanisms driving the disease is needed. OA is now no longer viewed as a disease of wear and tear but as a disease of

chronic inflammation (Berenbaum, 2013). Production and modulation of pro-inflammatory cytokines and MMPs from the synovium have become the area of most intensive research with TNF and IL-1 seen as the most feasible targets for therapeutic treatment (Scanzello et al., 2008, Grunke and Schulze-Koops, 2006, Jotanovic et al., 2012). However, variable efficacy of anti-TNF therapies for OA treatment have been observed, with some success in treatment of inflammatory knee OA but a failure to reduce the progression of OA in the hand (Alice, 2012), Grunke and Schulze-Koops, 2006, Miwa and Song, 2001). Tissue derived DAMPs and plasma proteins activate resident cells through TLR2 and TLR4 which induces both chemo- attractive and pro-inflammatory cytokines such as IL-6, IL-8 and IL-1β increasing inflammation within the joint which can be observed in the synovial fluid (Sohn et al., 2012). The lack of consistency in treatment efficacy demonstrates that little is known about the role inflammation plays in the diseases progression. Furthering the understanding of these mechanisms may be a key factor in understanding and developing preventative measures for the future.

1.8.1 Interleukin 6

Dichotomous roles have been described for IL-6, which can promote fever in autoimmune, infectious or non-infectious disease through its pyrogenic activity (Dinarello et al., 1991). IL-6 is primarily produced by monocytes and macrophages in response to other inflammatory cytokines such as IL-11 and TNF-beta but is also produced by non-immune cells, including fibroblasts and chondrocytes, in response to NF-kB/AP-1 driven transcription factor activation (Guerne et al., 1990, Matsuoka et al., 1996, Pelletier et al., 1997b). IL-6

participates in the short-term defence against infection or injury and alerts the active immune system towards the source of inflammation (Gauldie et al., 1985). However, defective regulation in the production of IL-6 can result in chronic inflammation, like that seen in OA (Wojdasiewicz et al., 2014). The monoclonal antibody biologic Tocilizumab, which targets IL-6-receptor, has been studied as to its effect in chronic inflammatory disorders including RA, and is licensed for treatment, but with limited success in the treatment of OA it is not currently used.

1.8.2   Interleukin 8

Interleukin-8 (IL-8) is a chemotactic factor which attracts neutrophils, basophils and T-cells but not monocytes and has the additional function of neutrophil activation (Baggiolini and Clark-Lewis, 1992). It is secreted by several cell types in response to inflammatory stimuli including non-immune cells such as synovial fibroblasts (Alaaeddine et al., 1999). The presence of neutrophils within the synovial joint fluid of patients with RA and to a lesser extent OA is thoughts to be due to the activity of chemotactic factors released by activated cells (Balakrishnan et al., 2014a). IL-8 is thought to be one of these factors and can be promoted by several innate receptors present in cells of the joint. Its secretion leads to: peripheral cell infiltration, increased metabolic activity, reduced oxygen content and a higher rate of mechanical lysis and necrotic cell debris formation, all of which have been noted to exacerbate inflammatory cytokine production through the innate immune response (Goldring and Otero, 2011). The additional function of neutrophils to secrete proteolytic enzymes, in order to remove intercellular connections aiding in its ability to travel through

tissue has potential activation properties on pro-form cytokines and matrix proteases which theoretically can potentiate disease activity (Gupta et al., 2007). The importance of these chemotactic factors is thought to be an early stage of disease pathology with increased infiltration of mononuclear cells and inflammatory markers in arthritic joints at early OA, rather than late stage cases (Ene et al., 2015).

1.8.3 Tumour Necrosis Factor

Although OA is not commonly seen as an inflammatory disease, early characterisation of the synovial cells contribution to low level chronic inflammation demonstrated that TNF levels produced by synovial isolates were comparable with those extracted from RA joints (Brennan et al., 1989). The spontaneity of cytokine induction from the cell isolates was attributed originally to LPS contamination of collagenase enzymes produced before the discovery of TLR4. However, more recent published work has since demonstrated the same phenomenon in a cleaner system, although endotoxins may have been present (Sacre et al., 2008). Increasing amounts of evidence point to TNF having an important role, not only in inflammatory arthritis but also in degenerative joint disease. However, effects of anti-TNF therapy have been continually variable with efficacy entirely dependable on joint location (Brennan et al., 1989, Guler-Yuksel et al., 2010, Chevalier et al., 2015).

1.9   **Matrix metalloproteases**

MMPs are a group of zinc dependent endopeptidases that play an important role in matrix regulation and wound healing. In OA the degradation of cartilage

due to MMPs through aggrecan and collagen digestion leads to the breakdown of articular cartilage and the exposure of the subchondral bone resulting in joint destruction and bone remodelling (Poole et al., 1995). MMPs rely on calcium or zinc cofactors for activity. Whilst levels of zinc remain stable between joint aspirations of OA and healthy controls (HC) (Yazar et al., 2005) levels of calcium crystals present in synovial fluid are up to 40% higher than normal (Yavorskyy et al., 2010). These changes noted in OA synovial fluid suggest an environment exists for increased MMP activity within the OA joint and are theorised to promote disease state.

A prominent feature for the development of OA is a plethora of physical articular anomalies that arise due to tissue destruction and alteration in joint structure. During the early stages of cartilage degradation aggrecan within the ECM is the first to be removed, loosening the normally dense matrix (Ali and Evans, 1973, Fosang et al., 1996). This altered permeability severely modifies the concentrations of growth factors and cytokines that diffuse into the cartilage from the synovial fluid (Hooiveld et al., 2003). Not only are the cells now exposed to foreign influences usually devoid in the shielded environment of the chondrocyte, but now chondrocyte derived factors are free to diffuse into the joint space.

Several genes that act as potent chondrogenic modulators including bone morphogenic protein-2, hypoxia inducible factor 1-alpha and transient receptor potential cation channel, subfamily V are demonstrated to be significantly higher in concentration in diseased OA tissue opposed to HC (Muramatsu et al., 2007, Zhou et al., 2015). Other modulators and growth factors include:

vascular endothelial growth factor, collagen type I, type II and sex determining region Y-box 9. They have been shown to be significantly altered in expression compared to healthy controls (Pelletier et al., 1997a, Kobayashi et al., 2005).

It has been theorised that the increased permeability of the matrix and exposure to synovial fluid may be a contributing factor to cellular differentiation and chondrocyte apoptosis and senescence, a common stage in progressive cartilage destruction (Price et al., 2002). The altered cellular environment, created through the breakdown of the ECM results in a variety of abnormal cell responses. One such alteration in cellular response is the increased production of MMPs which effect the successful processing of the cartilage, shifting towards a more degenerative environment (Loeser, 2009). It has long been thought that these cells entering senescence are one of the leading risk factors in developing OA, a hypothesis supported by the correlation between old age and likelihood of developing OA (Price et al., 2002). Subdivided into several categories, MMPs process a wide array of matrix components (Table 1.3). The most widely studied groups in OA pathogenesis are the collagenases (MMP1, 8 and 13), the gelatinases (MMP2 and 9) and the stromelysins (MMP3, 10 and 11), as they can process all components of articular cartilage and are heavily implicated in OA pathogenesis (Pelletier et al., 1983, Ali and Evans, 1973, Rose and Kooyman, 2016, Zeng et al., 2015).

Group name	Constituents	Example Substrates
Collagenases	MMP1 MMP8 MMP13	Collagen I, II, III, VII, VIII, X, gelatin, aggrecan (Yang et al., 2013, Sires et al., 1995, Fosang et al., 1993)
Matrilysin	MMP7 MMP26	Fibronectin, laminin, Col IV and gelatin. (Fosang et al., 1993)
Metalloelastase	MMP12	Elastin, fibronectin and Col IV. (Ohuchi et al., 1997, Chandler et al., 1996, Murphy et al., 1991)
Gelatinases	MMP2 MMP9	Gelatin, Col I, II, III, IV, VII, X (Sires et al., 1995)
Enamelysin	MMP20	Amelogenin (Llano et al., 1997)
Stromelysins	MMP3 MMP10 MMP11	Gelatin, fibronectin, laminin aggrecan, elastin, Col II, IV, IX, X and XI. (Fosang et al., 1993, Nicholson et al., 1989, Ohuchi et al., 1997, Miyazaki et al., 1990, Fosang et al., 1994)
Membrane-type MMPs	MMP14 MMP15 MMP16 MMP17	Gelatin, fibronectin and laminin (Pei and Weiss, 1996, d'Ortho et al., 1997)
Other	MMP19 MMP21 MMP23A MMP23B MMP27 MMP28	A mix of secreted and membrane associated MMPs (Llano et al., 1997, Kolb et al., 1997, Cossins et al., 1996)
Inhibitors	TIMP1 TIMP2 TIMP3 TIMP4	MMP inhibitors

Table 1.3: Matrix metalloprotease and their inhibitors. MMP4, MMP5 or MMP6 are omitted from the table as their activities could not be ascribed to a specific gene product. MMP-18 is only known to be present in Xenopus so is also excluded. MMP22, 24 and 25 are excluded from this list as their substrate and function are unknown at this time.

The categorisation of MMPs is based on domain organisation and substrate preference. The human collagenases (MMP1, MMP8, and MMP13) cleave interstitial collagen types I, II and III but also have the ability to digest several other ECM components. MMP2 and MMP14 have been noted to have collagenolytic activity but are characterised into the other groups due to their domain compositions (Nagase et al., 2006). MMP2 in particular can digest collagen type I, II and III in a similar manner to the collagenases but has a greatly reduced activity and as such deemed to be not as potent as the collagenases MMP1 and MMP13 which are seen by many as key drivers of cartilage destruction in OA (Wang et al., 2013, Wu et al., 2008).

The stromelysins (MMP3, MMP10 and MMP11) have a domain arrangement similar to that of the collagenases but do not cleave interstitial collagens, however their expression is documented to be related to disease activity (Chen et al., 2014). Whilst the MMPs are highly destructive to the joint, several act in an anti-inflammatory manner. MMP1, 2 and 9 aid in the degradation of IL-1β whilst MMP1, 2, 3, 13 and 14 aid in the degradation of monocyte chemoattractant protein-3 aiding in an anti-inflammatory state (Westermann et al., 2011, Ito et al., 1996).

Interstitial collagenase Matrix Metalloprotease 1 (MMP1) also known as fibroblast collagenase cleaves several types of collagen (Table 1.3). The structure of MMP1 is distinct in the presence of two domains, the catalytic N-terminal, and the C-terminal which is involved in substrate specificity and binding to tissue inhibitor of metalloproteinases (TIMPs). A conserved cysteine present in the cysteine-switch motif binds catalytic zinc ions, thus inhibiting the enzyme. Dissociation of the cysteine from the zinc ion upon cleavage of pro-MMP1 allows activation (Williams and Olsen, 2009).

Due to the abundance of these matrix proteins within the joint, ECM collagenases play a key role in the pathogenesis of OA through the imbalance in production of degenerative enzymes such as MMP1 and reduced collagen production from resident cells (Tchetverikov et al., 2005). MMP1 has been noted in proteomic studies to be upregulated in the synovial fluid of patients with OA compared to healthy controls pointing to a relationship between MMP1 and disease activity (Tchetverikov et al., 2005). Several cell types produce MMP1 although the highest contributors are seen to be the articular chondrocytes which regulate the cartilage matrix through production of several of MMPs and counterbalanced matrix protein production (Loeser, 2009).

MMP3 has shown some limited activity to degrade collagen types II, III, IV, IX and X but, more importantly for this study, can activate other collagenases through cleavage of the pro-domain (Pei and Weiss, 1995). The multifactorial effects of MMP3 has meant its involvement in the pathogenesis of OA has been widely studied. However, due to its reduced effect on interstitial collagen its activity in the progression of OA was theorised to be limited to the activation

of other more potent MMPs rather than direct ECM targeting itself (Troeberg and Nagase, 2012).

The most ubiquitous of the collagenases, MMP13 demonstrates the ability to degrade many components of the ECM especially those found in the cartilage (Fosang et al., 1996). Its activity towards collagens type I and II along with aggrecan make it a potent marker for early stage disease progression and predictably a potent MMP in OA (Wang et al., 2013, Li et al., 2011). Alterations in cell proliferation (chondrocyte clonal clusters), and cell death (necrosis induced by mechanical and chemical/oxidative stress), combined with expression of matrix components negatively affect the anabolic-catabolic homeostasis maintained by the chondrocytes, ultimately shifting it towards a degenerating state (Poole et al., 2002, Poole et al., 1991, Kouri et al., 1998, Notoya et al., 2000).

1.10 Complement, OA and TLRs

The complement system consists of a large variety of proteins including serine proteases, that trigger an enzymatic cascade. This yields active complement components which promote opsonisation, chemotaxis and cell lysis. The three distinct pathways of complement activation depend upon the molecules causing initiation of the cascade. These occur either through: the classical pathway, which is activated through direct binding of antibody or complement component C1q to the pathogen's surface; the mannose binding-lectin pathway whereby MBL binds encapsulated bacteria; or by the alternative pathway, which is triggered through spontaneous hydrolysis of C3 or binding directly to pathogen surfaces (Janeway, 2012) (Figure 1.6).These processes

are regulated by naturally occurring inhibitors such as factor H which aid in keeping the complement system in equilibrium (Visse and Nagase, 2003). When this system is out of balance however, a process designed to protect may become the means of tissue destruction.
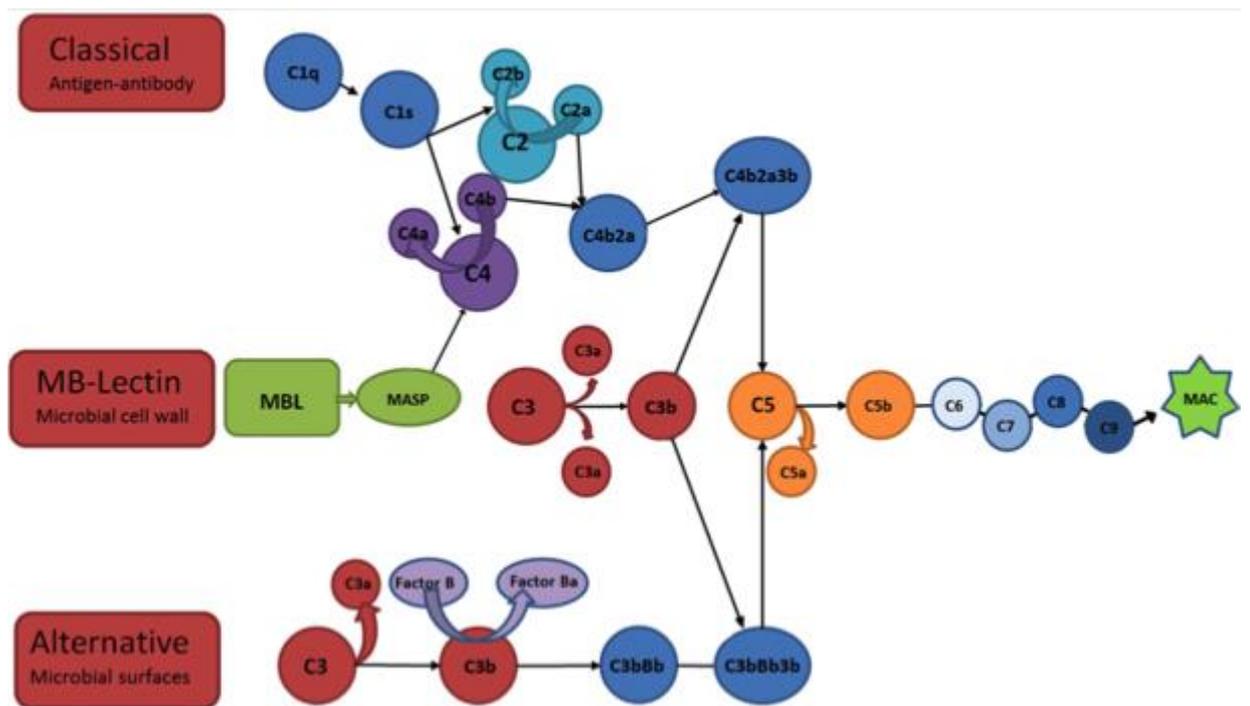
Figure 1.6: The complement system. Activating through either the classical, mannose binding lectin or alternative pathways activation converges on the C3 protein. C3 cleavage products, C3a and C3b contribute to the activation of C5 which is cleaved into C5a and C5b. C5a contributes to a local inflammatory response whilst C5b forms the basis for the membrane attack complex (C5b-9). Several membrane and soluble proteins regulate complement activity either upstream of membrane attack complex formation through C3 interference or through inhibition of the membrane attack complex itself.

In addition to the contribution of PRRs, activation of the complement system represents yet another innate immune mechanism by which OA inflammation and cartilage damage may be propagated. Several proteomic analyses revealed increased levels of complement anaphylatoxins, and their peptide components, in OA compared with healthy synovial fluid (Table 1.4) (Smith, 2011, Ross et al., 2012). Complement plays a crucial role in host defence. Support for the role of complement in OA, originally was documented where mice deficient in complement components C5 and C6 which were protected in an experimental collagen-induced OA model (Okamura et al., 2001).

Complement component	Observation	Reference
C3a	Increased in synovial fluid of OA patients	(Wang et al., 2011)
C3	Significantly increased in OA synovial fluid compared to healthy controls.	(Gobezie et al., 2007)
C4b	Significantly increased in OA synovial fluid compared to healthy controls.	(Gobezie et al., 2007)
C5	Knockout mice showed protection against OA.	(Wang et al., 2011)
C5b-9 (MAC)	Increased in synovial fluid of OA patients compared to healthy controls.	(Wang et al., 2011)
CD59	Knockout of this complement inhibitor lead to more severe OA in mice.	(Wang et al., 2011)

Table 1.4: Components of the complement system that have a putative role in osteoarthritis. OA: Osteoarthritis, MAC: membrane attack complex.

1.10.1 C3a/C5a

The term anaphylaxis comes from the Greek, Ana (against) and phylaxis (protection) and is denoted by the sudden and rapidly progressing, life-threatening allergic reaction that can result from the immune system responding to otherwise harmless substances from the environment (Mace, 1908). The factors responsible of this potentially fatal immune response are a group of complement peptide fragments known as anaphylatoxins. The three different anaphylatoxins, C3a, C4a and C5a induce inflammation through several different pathways. C3a induces inflammation through binding cell membrane C3aR receptors, which are expressed on almost all cell types in the body and stimulate chemotaxis, granule release and superoxide production (Zwirner et al., 1999). C5a has the highest specificity of the anaphylatoxin family which acts directly upon neutrophils and monocytes to speed up the phagocytosis of pathogens (Jacob, 1983, Regal et al., 1980). C3a and C5a work in tandem to recruit antibodies, activate mast cells, increase inflammatory and chemotactic cytokines and increase fluid within the tissue, all of these factors aid in the activation of the adaptive immune response.

Little is known about the specific action of C4a although its elevated levels have been correlated with autoimmune diseases including SLE (Moulds et al., 1993). The increasingly notable role of complement with TLR signalling cascades emerged in the past decade with the cross-talk altering MAPK phosphorylation in stimulated cells (Holst et al., 2012, Song, 2012, Raby et al., 2011). This effect, caused by the shared pathways between the two receptor families has, to our knowledge, still to be investigated in synovial fibroblasts,

which share an environment increasingly saturated with complement protein fragments.

1.10.2 CD46/CD55/CD59

Membrane cofactor protein (MCP/CD46) is a ubiquitously expressed C3b-binding protein that acts primarily as a co-factor in the Factor-I mediated proteolytic cleavage of C3b and C4b (Oglesby et al., 1992, Cardone et al., 2011). The vital role it plays in preventing host deposition of MAC complex has been noted by deficiency studies indicating predisposition of several disease conditions arising from its absence (Geis et al., 2010). In relation to disease, the role of CD46 in regulation of complement activity has been evidenced in several pathologies including the findings of a heterozygotic mutation causing atypical haemolytic uremic syndrome (Brocklebank et al., 2014). Contrastingly, several tumours are known to overexpress CD46 to increase resistance to complement driven attack (Geis et al., 2010). Decay accelerating factor (DAF/CD55) inhibits complement activity through interference with C3 and C5 convertase activity (Miwa and Song, 2001). MAC-inhibitory protein (CD59) regulates complement mediated cell lysis through binding either complement component C8 and C9 during MAC assembly thereby limiting incorporation of C9. CD59 also plays a role in signal transduction within T cells in conjunction with CD2 (Deckert et al., 1995).

The formation of the membrane attack complex (MAC) has been observed to be upregulated in human and mouse OA tissue and disease was aggravated in mice deficient in CD59 (van der Kraan and van den Berg, 2012). Activation of complement has been shown to be induced through ECM components

aggrecan and fibromodulin which induced the assembly of the MAC, further exacerbating cartilage destruction through chondrolysis and further cyclical complement activation (van der Kraan and van den Berg, 2012). The MAC arm of the complement system has been shown to co-localise with MMP-13 and activated extracellular signal-regulated kinase (ERK) in chondrocytes of human OA cartilage suggesting a cross-talk or convergence of signalling paths between complement receptors and drivers of inflammation such as TLRs (van der Kraan and van den Berg, 2012).

Binding of the ECM component fibromodulin to C1q has been demonstrated to upregulate MAC formation in synovial tissue of OA patients (van der Kraan and van den Berg, 2012). Additionally, other ECM components interact with complement peptides including: oligomeric matrix protein, osteoadherin and chondroadherin, products and molecules produced by chondrocytes (Acharya et al., 2014, Happonen et al., 2009, Happonen et al., 2010, Sjoberg et al., 2009)

These findings indicate a potential role of complement or several of its components in the activation of pathogenic mediators associated with OA. It is hypothesised that the formation of MAC complexes may be detrimental to chondrocytes which, if lysed, would release endogenous material into the synovial cavity (Yoshioka and Shichikawa, 1987). This debris has been shown to activate several TLRs including TLR3 and TLR4 which in-turn can exacerbate the degradation process through production of pro-inflammatory modulators and matrix destructive enzymes (Fernandes et al., 2002, Hu et al., 2014).

1.11 <u>Pharmacological therapeutic intervention in OA</u>

Corticosteroids have long since been used as a method of delaying joint replacement procedures of those with OA. However, the short term benefits of the treatment, along with the loss in efficacy over time, means that it is only a stop gap in disease pathology, merely alleviating some inflammation and pain associated with the disease (Ayhan et al., 2014). Hyaluronic acid injections aid in the function of the joint by improving lubrication, reducing inflammation and promoting cartilage matrix regeneration increasing the time until necessary joint replacement (Ong et al., 2016). Joint hydra-effusion therapy and visco-supplementation has been shown to help, and systematic analysis comparing effects showed a marked improvement with long term benefits (Wang and He, 2015). There has however, been several attempts to emulate the success of biologic treatment of RA through targeting of poignant cytokines.

1.11.1   anti-TNF

Tumor necrosis factor is noted as one of the master cytokines involved in a myriad of processes within the body, not least being its role in inflammation. Part of the TNF family of cytokines that can cause cell death through apoptotic pathways, TNF is a monocyte-derived cytotoxin that has been implicated in several inflammatory diseases (Bradley, 2008, Esposito and Cuzzocrea, 2009). The successful treatment of RA with anti-TNF agents such as etanercept (Enbrel) aids in reducing TNF presence functioning as a soluble decoy (Grunke and Schulze-Koops, 2006).

Etanercept is a fusion protein of the TNF receptor and the Fc portion of IgG1, creating a highly stable and active molecule able to blockade TNF (Peppel et al., 1991). Since approval for medical use in late 1998 the use of anti-TNF therapies now encompasses the treatment of RA, Polyarticular Juvenile RA, Psoriatic Arthritis and Ankylosing Spondylitis to name but a few (Haraoui and Bykerk, 2007, Hung and Huang, 2005, Kivelevitch et al., 2014, Frech, 2007).

Several previous trials of anti-TNF treatment in OA have shown variable results with the treatment of certain forms of OA proving to be mildly successful whilst others had no significant beneficial effects compared with placebo. A pilot study of infliximab treatment of hand OA investigated the association between systemic and local inflammation and incident progression of secondary OA. The treatment greatly reduced the chance of secondary OA over a three-year period independent of a decrease in inflammation. This finding suggested that there may be other non-inflammatory mechanistic effects of TNF in the disease type (Guler-Yuksel et al., 2010). However, due to the study identifying secondary OA only, further studies into populations of primary OA would need to be undertaken.

A recently published comparative study demonstrated the efficacy of direct injections of etanercept. A small cohort of 39 patients was subdivided into treatment (etanercept) and hyaluronic acid joint supplementation groups. The data illustrated the potential beneficial effect of the treatment in alleviating joint pain in moderate and severe OA, however, the short half-life of etanercept, culminated in variable results (Ohtori et al., 2015).

As well as being a promising treatment for RA, these findings highlight the possibility for the involvement of TNF in what is currently viewed by many, as a non-TNF driven disease. Blockade with antagonists such as infliximab, etanercept and adalimumab were noted to reactivate latent tuberculosis and hepatitis B amongst other serious infections and sepsis induced fatalities showing the dangers of cytokine therapy and the need to develop alternatives (Li et al., 2009). However, due to the immense cost of cytokine blockade therapy, it would likely cripple the health service if given as a front-line therapy.

1.11.2   anti-IL-1 therapy

Dose responsive studies in dogs demonstrated that with local administration of IL-1Ra, occurrence of macroscopic and microscopic lesions in knee OA was reduced (Caron et al., 2012). Additional benefits of this treatment include, reduced inflammation within the synovium (identified through biopsied sample comparison) and reduced tenderness was identified in treated horses (Ross et al., 2012). Several human studies have confirmed the potential for IL-1 blockade for the treatment of OA *in vitro* resulting in reduced cartilage destruction (Kobayashi et al., 2005).

However, whilst studies in the laboratory and animal counterparts proved successful, intra-articular injections of Anakinra, a recombinant form of IL-1Ra, proved inconclusive in the treatment of patients with knee OA (Chevalier et al., 2009). A later, long-term blinded study of 160 patients verified no improvement in knee pain, function, stiffness or cartilage health compared with control (Chevalier et al., 2015). However, hope remained as statistically significant improvements were observed within the first 4 days of treatment suggesting

that, the lack of improvement over an increased time period was perhaps in part due to the short half-life of the drug mentioned earlier (Chevalier-Ruggeri and Zufferey, 2016).

Diacerein is a slow-acting drug widely used in collaboration with NSAIDs for treatment of early stage OA due to its high gastro-intestinal tolerance and cost-effectiveness. It acts by inhibiting the synthesis of IL-1B and has been demonstrated to reduce symptoms associated with disease activity in a significant manner in both animal models and humans proving the importance of IL-1 in disease, associated inflammation, and pain (Li et al., 2015, de Isla and Stoltz, 2008).

1.11.3 Hydroxychloroquine

The anti-malarial and anti-parasitic drug, chloroquine (CQ) inhibits the pathogen by impeding the digestive vacuole of a cell. Entering through passive diffusion, CQ becomes pronated (CQ^{2+}) through the acidic nature of the vacuole and becomes unable to leave through diffusion. This accumulative effect results in the starvation of the cell triggering apoptosis (Sams, 1967). In rheumatic diseases, such as RA and SLE, chloroquine and its derivatives have been used for their ability to mildly supress the immune system, reducing the disease pathology. However, adverse side effects are widespread and immunosuppression is known to allow opportunistic infections to take hold. The use of CQ in osteoarthritis treatment has had little effect, with studies focusing primarily on hand OA showing no effect in easing pain or function in affected joints (Kingsbury et al., 2013, Detert et al., 2014).

1.12 <u>Project aims and hypotheses:</u>

Advances have been made in the therapeutic treatment of inflammatory arthrides such as RA. However, little progress has been made in the treatment of OA, with treatments remaining stagnant for several decades. In a heterogeneous disease, such as OA, the heterogeneity presents a large array of targets and pathways for potential therapeutic assault.

In the previous sections the current understanding of the role of Toll-like receptors in OA are discussed and demonstrate the potential for TLR mediated pathogenesis. As potent drivers of cytokine production, the involvement of these receptors is increasingly thought to be attributed to disease progression from multiple cells of the joint. However, in an age of tailored treatment, discrimination between joints is lacking and could potentially shine some light on variances between findings documented by others.

The project first aim was to investigate the involvement of the TLRs in driving inflammation and degradation from two resident cells of the joint, specifically the synovial fibroblasts and articular chondrocytes. Broad aims of the project are outlined below:

- To measure TLR expression in synovial fibroblasts and chondrocytes of the OA joint.

- To measure TLR induced pro-inflammatory cytokine and matrix degenerative enzyme secretion from these cell types.

- To determine the effect of synovial fluid upon TLR induced inflammation in synovial fibroblasts.

- To determine whether there is a crosstalk between anaphylatoxins and TLR signalling in synovial fibroblasts.

- To observe the effect of TLR and anaphylatoxin stimulation upon membrane bound complement regulators in chondrocytes.

It was theorised that increased TLR expression would correlate with increased cytokine and MMP production and that TLR expression may vary dependent upon disease severity (chondrocytes). Additionally, it was postulated that a difference in TLR driven inflammation may be observed between knee and hip derived synovial fibroblasts and that OA synovial fluid would trigger TLR activation in an inflammatory manner. Finally, with published data indicating increased MAC deposition in OA tissue, it was hypothesised that membrane bound complement regulators, involved in prevention of host-deposition of MAC complex, would be reduced in regions of cartilage severely affected by the progression of OA in comparison to unaffected regions.

Chapter 2 - Materials and Methods

2.1 Ethical approval and patients

Joint tissue was collected from OA patients undergoing elective joint replacement procedures. Ethical approval for this study was granted by Brighton and Sussex (REC ref 10/H1107/8) and Wales REC 7 (14/WA/1016). Informed written consent was obtained from all participants. All elective joint replacement procedures were either hip replacements (femoral head) or knee arthroplasty (both hemispheres of the joint including: lateral and medial condyle and the tibial plateau). Patients undergoing a partial replacement or with a history of a separate inflammatory joint disease were excluded, as were those with clinical or serological evidence of chronic or recent acute infections.

2.1.1 Patient inclusion /exclusion criteria

Patients referred to the clinic for assessment and treatment of osteoarthritis, other joint procedures. Participants must have been able to give informed consent and be over the age of 30. Adults that are unable to provide informed consent, are pregnant, have an acute or chronic infection such as HIV, anti-HCV antibodies, hepatitis B or syphilis were excluded from recruitment.

2.1.2 Patient characteristics

A total of 44 patients were recruited onto the study. 43 patients had clinical OA with no previous nor other current arthrides (Table 2.1). A single patient who showed clinical characteristics of OA though was removed from the data cohort post-operative due to a secondary underlying arthrides.

Disease group	Number of samples	Male	Female	Age
Osteoarthritis	43	16	27	70 (45-95)

	Number of samples	Male	Female	Age
Knee OA	19	2	17	62-86
Hip OA	24	14	10	45-95

Table 2.1: Patients recruited on "Inflammation regulation" and "Role of inflammation in osteoarthritis". OA; Osteoarthritis

2.3 <u>Cell isolation and culture</u>

Primary human tissue/cells along with immortalised cell lines were used throughout the project (Table 2.2). All cells/tissues were incubated in a humidified environment at $37^{\circ}C$ in 5% CO_2 unless otherwise stated. Cells were stored in liquid nitrogen in a suspension of FCS + 10% DMSO (Sigma-Aldrich, Gillingham, UK). Cells were defrosted in a water bath ($37^{\circ}C$) before being re-suspended in DMEM and washed through centrifugation for 10 minutes at 300g and reconstituted in culture medium.

LAL assay was performed on collagenases used for isolation procedures to determine whether they were truly endotoxin low as stated in the manufacturers product guidelines. All collagenases were diluted as used in isolation protocols (2.3.1 and 2.3.3) and tested using a gelatification LAL assay (Lonza Cat No N183-06) with a limit of detection of 0.125 endotoxin units.

All tissue culture plastic wares were obtained through VWR (Lutterworth, UK), Nunc (Paisley, UK) or Corning (Corning, NY, USA).

Cell line/name	Origin/lineage	Culture media	Specific supplements	Comments
HEK293T	Human embryonic kidney	DMEM	10% FCS, Pen/Strep	Control, adherent
HEK293 TLR2, TLR3, TLR7, TLR8 and TLR9	Human embryonic kidney	DMEM	10% FCS, Pen/Strep Blastocidin Zeocin	SEAP NF-kB/AP-1 reporter, adherent
HEK293 TLR4	Human embryonic kidney	DMEM	10% FCS, Pen/Strep Hygragold	CD14 and MD2 co-transfected, adherent
Synovial Fibroblasts	Primary human synovium	DMEM	10% FCS, Pen/Strep (Gentamicin 1st passage)	Adherent
Articular chondrocytes	Primary human articular cartilage	DMEM	10% FCS, Pen/Strep (Gentamicin 1st passage)	De-differentiate under culture into fibroblastic phenotype

Table 2.2: Cell lines and/or names of primary cells including culture conditions and medium. FCS – Foetal calf serum, DMEM – Dubeccols modified eagle medium, Pen/Strep – Penicillin and Streptomycin, SEAP – Secreted embryonic alkaline phosphatase.

2.3.1 Isolation of primary synovial fibroblasts

Fatty tissue and extraneous cartilage were removed from the synovial membrane using a sterile scalpel and scissors. The synovial tissue was then cut into small 1-3mm^3 pieces and filter-strained (70µm filter) before being added to a 20ml volume of DMEM containing 100mg of collagenase type I and 3mg of DNase I. The tissue was then enzymatically digested at 37°C in a water bath for no more than 90 minutes to prevent dissociation of deep tissue cell types. The cell suspension was then filtered through sterile muslin to remove any extraneous material before addition of complete media to prevent further activity of the collagenase activity (Figure 2.1). Cells were washed three times in complete media before centrifugation at 300xg for 10 minutes after which, the mixed cellular population were counted using a haemocytometer and plated at 1x10^6 cells per well in a 6 well plate in 2ml of complete DMEM per well.

The use of a gentleMACs Octo dissociator (Miltenyl Biotec) automated the digestion process for multiple samples. Tissue was cut into 1-5mm pieces using sterile scissors. These pieces were then added to serum free DMEM containing 100mg of collagenase type I and 3mg of DNase I and kept at 37°C for no more than 90 minutes under agitation, in C-type tubes (Miltenyl Biotec). Cellular isolates were then filtered through sterile muslin, washed and plated as described above.

The cellular content of synovial membrane cultures was rudimentarily screened using anti-CD4 (monocyte, macrophage and dendritic cell marker) columns to determine relative amounts of CD4$^+$ (immune) and CD4$^-$ (non-

immune) cells. This was performed using antibody conjugated beads on a random selection of hip and knee samples on 1×10^6 cells (Table 2.3).

Sample type	Total amount of synovocytes obtained	Number of CD4$^+$ cells	Number of CD4$^-$ cells
Knee	12.5×10^6	0.18×10^6	0.82×10^6
Knee	24.3×10^6	0.34×10^6	0.66×10^6
Knee	8.5×10^6	0.27×10^6	0.73×10^6
Hip	7.4×10^6	0.16×10^6	0.84×10^6
Hip	11.3×10^6	0.21×10^6	0.79×10^6
Hip	17.2×10^6	0.09×10^6	0.91×10^6
Average knee	15.1×10^6	0.26×10^6	0.73×10^6
Average hip	11.9×10^6	0.15×10^6	0.84×10^3

Table 2.3: Rudimentary screening of synovial membrane isolates. Several samples of synovial membrane isolates were counted for total cell count and using anti-CD4 bead extraction two fractions of cells were obtained. CD4$^+$ cells including monocytes, macrophage, T-Helper and dendritic cells and CD4$^-$ cells including but not limited to synovial fibroblasts.
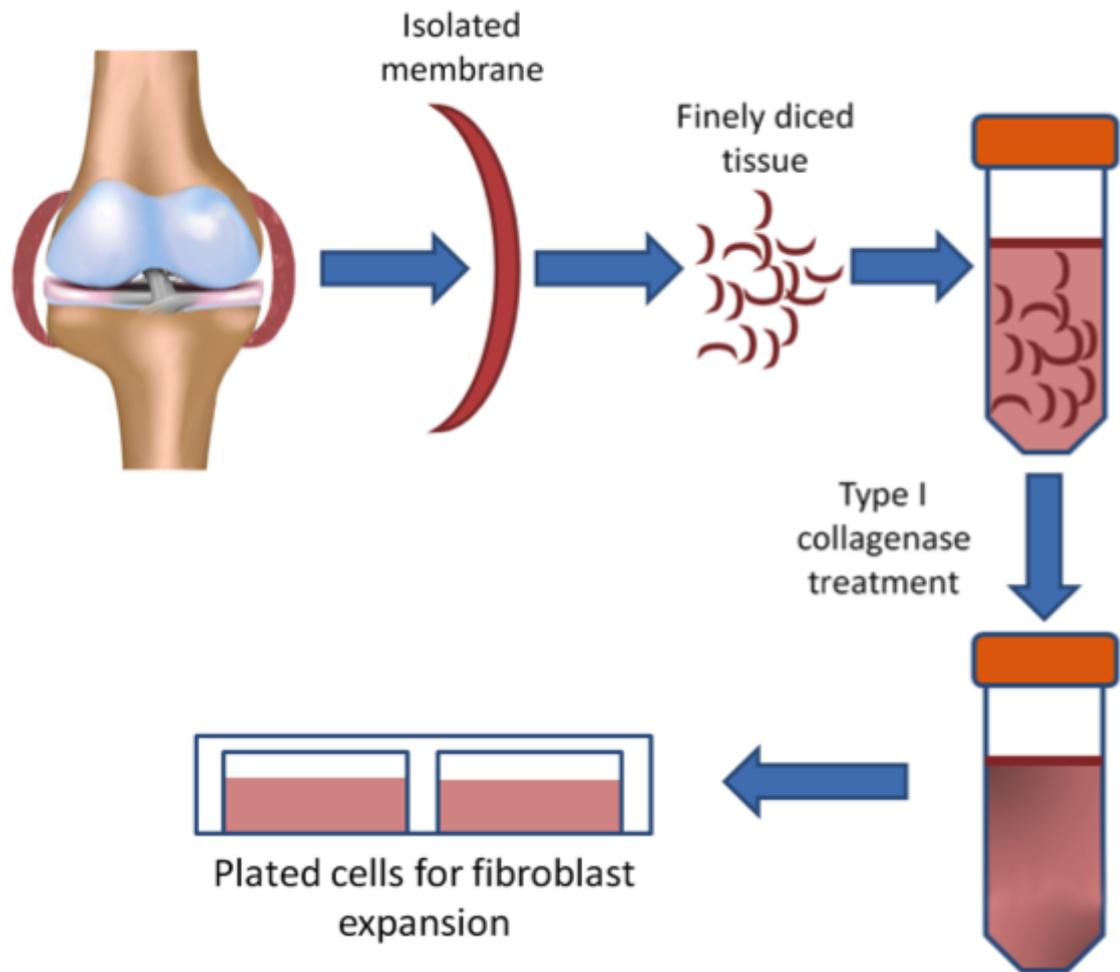
Figure 2.1: Isolation protocol of synovial fibroblasts from synovial membrane primary tissue samples. The synovial membrane from total knee and hip replacement procedures were isolated by the surgeon. Once returned to the lab the synovial tissue was finely sliced before the extra-cellular matrix is degraded through enzymatic digestion via type I collagenase. Isolated cells were then washed before being plated for fibroblast expansion.

2.3.2 Stimulation of synovial fibroblasts

After growing the synovial membrane cultures for at least three passages a population of synovial fibroblasts remained from which all stimulations denoting OASFs were performed. Cells were plated at either 10×10^4 cell per well in a 96 well plate or 2.3×10^4 cells per well in 384 cell culture plates after which they were cultured for 16 hours. Stimulations were performed in complete DMEM for 24 hours unless otherwise stated. Supernatants from the stimulations were harvested and immediately analysed or kept at -20°C until further analysis.
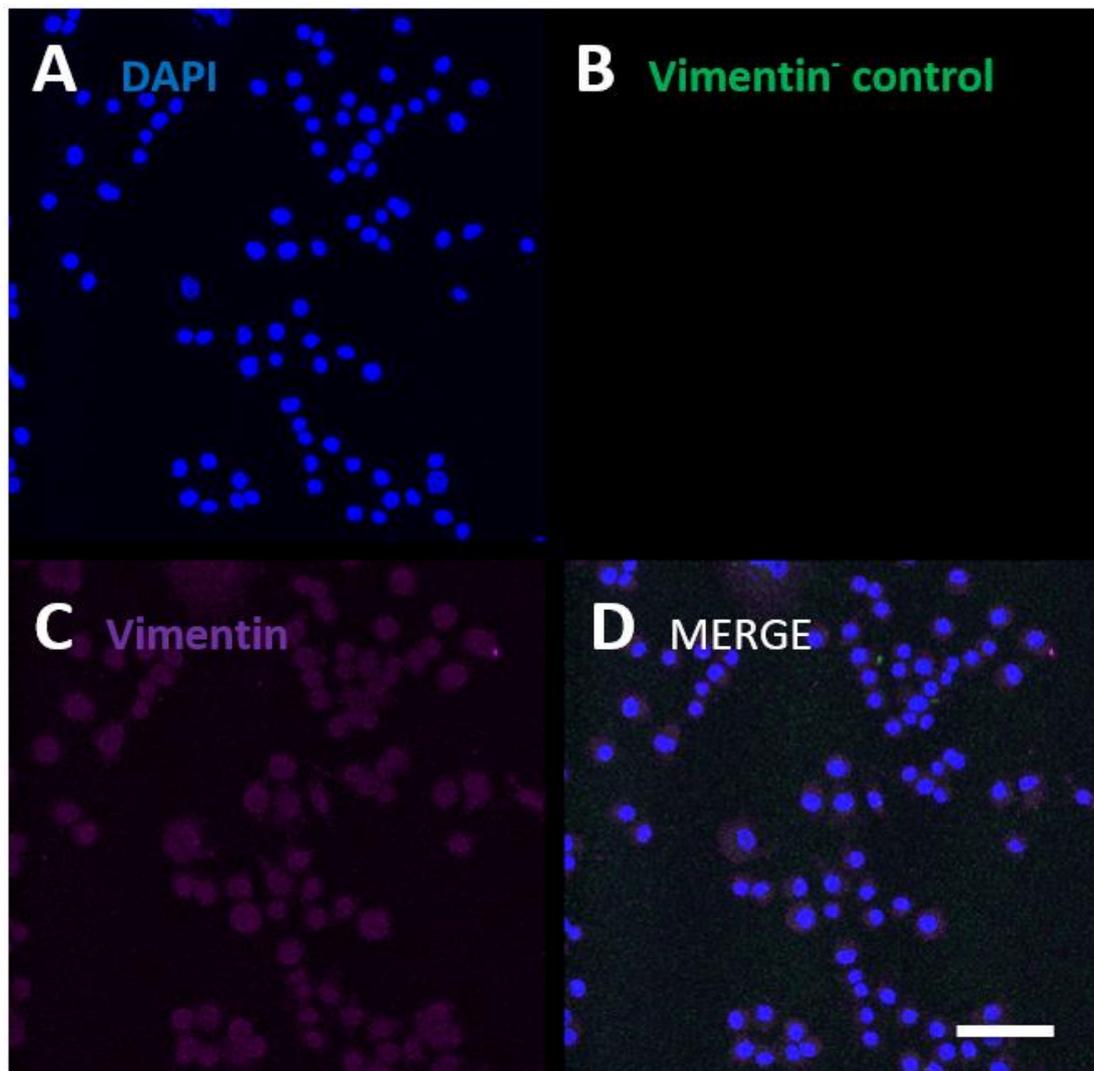
Figure 2.2: Vimentin staining of synovial fibroblasts. OASFs isolated from several patients were passaged until P3 before a random selection were isolated, plated and stained for the fibroblast marker vimentin. Isotype matched controls run alongside showed minimal cross-reactivity and allowed us to determine the use of a pure population of cells. **A)** DAPI – nuclear stain (Blue) **B)** Vimentin isotype control (Green), **C)** Vimentin (Magenta). **D)** Co-localisation (MERGE) Sample shown is representative example (knee OASF). Original magnification is x 200. Scale bar = 100μm.

2.3.3   Isolation of primary chondrocytes

Cartilage was isolated from the joint surface using a No.11 scalpel and diced into small 1-3mm^3 pieces before being digested with type II collagenase for 16 hours in serum free complete DMEM. After incubation, digestion was halted through the addition of complete media (DMEM+10%FCS) and isolated chondrocytes were pelleted. The cells were washed three times by centrifugation at 300xg for 10 minutes in complete media and counted using a haemocytometer.

2.3.4   Stimulation of chondrocytes

Enzymatically isolated cells were plated at 2.3×10^4 cells per well in 384 well cell culture plates and left to settle for 16 hours in complete DMEM before stimulation unless otherwise stated. Overnight media was removed and replaced with fresh DMEM containing TLR ligands and allowed to stimulate the cells for 24 hours unless otherwise stated.

2.3.5   Isolation and treatment of synovial fluid

Synovial fluid was removed from the joint before the synovial membrane was disrupted by a surgeon using a 12-gauge hypodermic needle. Typically, 0.5-10ml of synovial fluid was aspirated then transferred to a heparin sulphate vacutainer. All synovial fluid samples were then diluted 1:8 with complete DMEM for later use.

2.3.6 HEK293 Stimulation

HEK293 cells were stimulated in 96-well tissue culture plates (Corning Inc, Corning, NY, USA) with individual TLR ligands dependent on transfected TLR expression (Table 2.2). Cells were cultured at 40×10^4 cells per well in a 96-well plate in the presence of TLR ligands in DMEM containing 10% (v/v) FCS and 1% (v/v) Pen/Strep (PAA, Pasching, Austria). Experiments were conducted at a minimum of biological triplicates. Supernatants were harvested after 24 hours of stimulation and cell viability assays performed immediately after supernatant extraction.

2.3.7 Synovial fluid stimulation of TLR expressing HEK293 cells

Stably transfected HEK293 cells (Invivogen, USA) expressing either: TLR2, TLR3, TLR4, TLR8 or TLR9 were plated at 40×10^4 cells per well in a 96-well tissue culture plate in complete DMEM containing 10% FCS and 1 until/ml Penicillin and Streptomycin. Cells were incubated with their corresponding ligands with or without synovial fluid. Stimulations were performed in triplicate. NF-kB/AP-1 activation was measured via Quanti-blue assay (TLR2, TLR3, TLR8 and TLR9) or through IL-8 production (TLR4 activation).

	Ligand	Supplier	Working concentration
TLR1/2	Pam3CysSerLys4 (Pam3)	Axxora, Nottingham, UK	100ng/ml
TLR2/6	Pam2CGDPKHPKSF (FSL-1)	Invivogen, San Diego, CA, USA	1ng/ml
TLR3	Polyinosinic:polycytidylic acid (Poly(I:C)) HMW	Invivogen, San Diego, CA, USA	20µg/ml
TLR4	Lipopolysaccharide (LPS)	Axxora, Nottingham, UK	10ng/ml
TLR8	CL075	Source Bioscience, Nottingham, UK	1µg/ml
TLR7/8	Resiquimod (R-848)	Enzo Life Sciences, Lausen, Switzerland	2µg/ml
TLR9	ODN2216	Invivogen, San Diego, CA, USA	5mM

Table 2.4: Agonists used for stimulation of specific TLRs. Agonists for TLRs were used at stated concentrations.

2.4   Flow cytometry

2.4.1   Procedure

Primary isolated or cultured cells were blocked on ice for 30 minutes in Facs wash buffer (FwB) before incubation with primary cell surface marker antibodies or equivalent concentrations of isotype controls (Table 2.5) on ice in the dark for 30 minutes. Samples were then washed three times in 1ml FwB and centrifugation at 500xg for 3 minutes at 4°C. Cells were then fixed in 2% formaldehyde for 5 min at room temperature. Cells were again washed three times in 1ml FwB and centrifuged at 500xg for 3 min at 4°C before permeabilisation in permeabilising FACS wash buffer (pFwB) for 30 min, on ice, in the dark. Primary intracellular antibodies or isotype controls (Table 2.5) were added on ice, in the dark for 1 hour for intracellular staining. Samples were washed in pFwB and centrifuged at 500g for 3 min at 4°C before re-suspension in FwB.

2.4.2   LSR-II

Becton Dickinson LSRII flow cytometer and BD FACS Diva software (BD Biosciences, San Jose, CA, USA) were used for multi-colour analysis and data collection. Fluorophore compensation was achieved using BD Sphero™ rainbow calibration particles and BD Comp Beads (anti-mouse Ig_k or anti-rat Ig_k) (BD Biosciences, San Jose, CA, USA). Data analysis was performed using FlowJo software (Tree Star Inc, Ashland, OR, USA). Cells were gated on size and granularity using forward and side scatter analysis of a control group. The subtraction of isotype controls from positively stained controls allowed for

analysis of protein expression, both in terms of percentage of cells TLR positive and the mean fluorescence intensity (MFI).

2.4.4 Prevention of non-specific binding

In order to compensate for any non-specific binding of the Fc portion of the antibody, isotype controls were run in parallel and the shift in fluorescence due to binding was measured as ΔMFI, (the difference between antibody binding minus the isotype control binding value).

Antigen	Fluorophore	Isotype	Supplier	Quantity used per 10^6 cells
TLR1	FITC	Mouse IgG1$_\kappa$	Abcam, Cambridge, UK, Clone - GD2.F4	0.5µg/ml
TLR3	FITC	Mouse IgG1$_\kappa$	Imgenex, San Diego, CA, USA Clone - 40C1285.6	0.5µg/ml
TLR2	PE	Mouse IgG1$_\kappa$	Imgenex, San Diego, CA, USA, 121810 Clone - 1030A5.138	0.2µg/ml
TLR4	APC	Rat IgG2a$_\kappa$	BioLegend, San Diego, CA, USA 92121 Clone - HTA125	0.1µg/ml
TLR6	PE	Mouse IgG1$_\kappa$	BioLegend, San Diego, CA, USA 92121 Clone - TLR6.127	0.5µg/ml
CD46	APC	Mouse IgG1	BioLegend, San Diego, CA, USA 92121 Clone - TRA-2-10	0.5µg/ml
CD55	FITC	Mouse IgG1$_\kappa$	BioLegend, San Diego, CA, USA 92121 Clone - JS11	0.5µg/ml
CD59	PE	Mouse IgG2a$_\kappa$	BioLegend, San Diego, CA, USA 92121 Clone – p282 (H19)	0.5µg/ml
Isotype control	FITC	Mouse IgG1$_\kappa$	Imgenex, San Diego, CA, USA, Clone – P3.6.2.8.1	0.5µg/ml
Isotype control	PE	Mouse IgG1$_\kappa$	Imgenex, San Diego, CA, USA, Clone – MOPC-173	0.2µg/ml
Isotype control	APC	Rat IgG2a$_\kappa$	BD Biosciences, San Jose, CA, Clone - MOPC-21	0.1µg/ml
Isotype control	APC	Mouse IgG2a$_\kappa$	BioLegend, San Diego, CA, USA 92121 Clone – MOPC-173	0.2µg/ml

Table 2.5: Antibodies used in flow cytometric analysis of protein expression on fixed cells.

2.5   Enzyme linked immunosorbent assay (ELISA)

Supernatants from TLR stimulation assays were harvested and cytokine production was measured using ELISA on high binding ELISA plates (Corning inc, Corning, NY, USA). Plates were initially coated with the capture antibody (in PBS) (Table 2.6) overnight at 4°C washed three times in ELISA wash buffer using a Biotek plate washer (Bioteck, Winooski, VT, USA). To prevent non-specific binding to the antibody plates were incubated in 2% BSA (in PBS) for 1 hour at room temperature. Both samples and standards were diluted as indicated and added to the plate at RT for 2 hours or overnight at 4°C. After 3 further washes the plates were incubated with a biotin labelled detection antibody in 0.5% BSA containing PBS for 1 hour at RT. After another three washes the plates were incubated with streptavidin conjugated HRP (R&D systems, Minneapolis, MN, USA) in 0.5% BSA for 1 hour.

Three final washes were then performed and using a TMB microwell peroxidase substrate (KPL, Gaithersbury, MD, USA) samples were developed before having the colour change reaction stopped using an ELISA stop solution (5% H_2SO_4 diluted in H_2O). Plates were then read immediately using a plate reading spectrophotometer measuring absorbance at 450nm.

Cytokine	Capture antibody (working concentration)	Detection antibody (working concentration)	Protein standard (top concentration)
IL-6	1µg/ml *(Becton Dickinson, Oxford, UK)* **Cat # 554543** **Clone - MQ2-13A5**	0.5µg/ml *(Becton Dickinson, Oxford, UK)* **Cat # 554546** **Clone – MQ2-39C3**	10,000pg PeproTech, London,UK **Cat # 200-06**
IL-8	2µg/ml *(Becton Dickinson, Oxford, UK)* **Cat # 554716** **Clone - G265-8**	0.4µg/ml *(Becton Dickinson, Oxford, UK)* **Cat # 554718** **Clone – G265-8**	10,000pg R&D Systems, Abingdon, UK **Cat # 208-IL**
MMP1	1µg/ml *(R&D Systems, Abingdon, UK)* **Cat # DY901** **Clone - N/A**	1µg/ml *(R&D Systems, Abingdon, UK)* **Cat # DY901** **Clone - N/A**	4,000pg *(R&D Systems, Abingdon, UK)* **Cat # DY901**
MMP3	1µg/ml *(R&D Systems, Abingdon, UK)* **Cat # DY513** **Clone - N/A**	1µg/ml *(R&D Systems, Abingdon, UK)* **Cat # DY513** **Clone - N/A**	4,000pg *(R&D Systems, Abingdon, UK)* **Cat # DY513**
MMP13	1µg/ml *(R&D Systems, Abingdon, UK)* **Cat # DY511** **Clone - N/A**	1µg/ml *(R&D Systems, Abingdon, UK)* **Cat # DY511** **Clone - N/A**	4,000pg *(R&D Systems, Abingdon, UK)* **Cat # DY511**
TNF	Becton Dickinson, Oxford, UK (4µg/ml) **Cat # 551220** **Clone – MAb1**	Becton Dickinson, Oxford, UK (0.5µg/ml) **Cat # 554511** **Clone – MAb11**	PeproTech, London, UK (10ng/ml) **Cat # 554618**

Table 2.6: Antibodies used for ELISA of conditioned supernatants and synovial fluid. IL – Interleukin, MMP – Matrix Metalloprotease, TNF – Tumour necrosis factor.

2.6   Quantiblue assay

Transfected HEK293 cells stably expressing individual TLRs (Invivogen, San Diego, CA, USA) and the NF-kB/AP-1 SEAP reporters were seeded at 1×10^4 cells per well in a 96-well plate before stimulation of TLRs by corresponding ligands. After 24 hours' incubation at $37^{\circ}C$ in a humidified chamber (21% O_2, 5% CO_2), 20µl of conditioned supernatant was removed from the cells and added to 200µl pre-warmed Quantiblue solution (Invivogen, San Diego, CA, USA). Alkaline phosphatase activity was denoted by a change in colour from pink to blue over 15 minutes to 1 hour which was then quantified by being read on a plate-reader at 605nm.

2.7   MTT Assay

For the measurement of cell viability, Methylthiazolyldiphenyl-tetrazolium bromide (MTT) reagent (Sigma) was dissolved to 0.5mg/ml in the cell specific culture media. Cells were then cultured with 20µl, for 384-well plates, or 100µl, for 96 well plates, of this solution for 2 hours during which time an insoluble purple formazan precipitate formed. The precipitates were then dissolved for 1 hour with 10% (w/v) SDS in PBS at 37°C. Absorbance at 590nm was then determined by spectrophotometry using a BioTek Synergy HT Microplate reader (BioTek, Winooski, VT, USA).

2.8   Nucleic acid purifications and processing

2.8.1   RNA purification Phenol/Chloroform extraction

To isolate total RNA from synovial fluid an acid phenol chloroform extraction method was utilised. Samples were added 1:1 with QIAzol phenol reagent

before 10% (v/v) of chloroform was added and mixed by inversion and left to incubate at room temperature for 10 minutes. The homogenate was then separated at 13,000g on a benchtop centrifuge at 4°C for 10 minutes. The RNA remains in the aqueous phase due to its positive polarity, which is lost in DNA due to neutralisation with H^+ forming an interphase whilst proteins precipitate out and are pelleted in the chloroform fraction. The aqueous phase has the RNA precipitated from solution using 1:1 v/v isopropanol which is incubated on ice for 1 hour. The RNA is pelleted at 13,000g for 10 minutes at 4°C and washed three times in 90% ethanol. The pellet is then left to air dry before the RNA pellet is re-suspended in nuclease free water.

2.8.2 RNA isolated from cultured cells

Total RNA was extracted from isolated primary cells using a Promega Wizard RNA extraction kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. RNA was eluted into 30-100µl RNase free H_2O by centrifugation at 13,000g for 1 minute and quantified using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) to check for RNA purity. RNA purity was determined using 260:280 ratio calculations to check for protein or phenol contamination. Any sample with a ratio less than 1.8 or high protein contamination was re-purified or discarded.

2.8.3 RNA extraction from frozen cartilage

To Isolate RNA from cartilage, samples were macroscopically visually assessed to determine grading of the areas of the cartilage under the modified Mankin cartilage scaling system. After which samples were snap frozen in liquid nitrogen and pulverised into a powder using a liquid nitrogen cooled

pestle and mortar (Omega, Manchester, UK) before having total RNA extracted through the Plant RNAeasy isolation kit (Qiagen, Manchester, UK) according to manufacturer's protocol.

2.8.4 Production of cDNA by RT-PCR

RNA (100ng) was reverse transcribed to cDNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Paisley, UK) to the manufacturer's instructions using a Verti 96-well Thermocycler (Applied Biosystems, Paisley, UK).

2.9 <u>Quantitative polymerase chain reaction - qPCR</u>

Using a low-ROX brilliant III master mix (Agilent technologies, Wokingham, UK) and Taqman™ qPCR primer pairs (Table 2.7) (Life Technologies, Paisley, UK) reactions were performed on 96-well qPCR plates (Alpha laboratories, Eastleigh, UK). Reaction volumes were performed at 10µl with 0.1ng/µl of cDNA used as a template. Arrays were carried out for multiple patient samples on the same plate but with only one qPCR Taqman™ probe. Plates were sealed with plate caps (Alpha laboratories, Eastleigh, UK) and centrifuged for 10 seconds to accumulate all of the reaction volume at the bottom of the well. All qPCR reactions were performed using a Stratagene Mx3000 thermocycler (Agilent Technologies, Wokingham, UK). A fluorescence threshold value of 0.5 was used when analysing the raw data.

Reactions were performed under the following conditions: 95°C (10 minutes) 40 cycles of: 95°C (15 seconds) 60°C (1 minute) (Figure 2.3). Data was collected and analysed using MXPro qPCR software (Agilent Technologies,

Wokingham, UK). Fluorescence threshold value of 0.5 was used when analysing the raw data. Data was plotted using GraphPad Prism software (La Jolla, CA, USA) which was also used for statistical analysis using a paired two-tailed student t-test for data interpretation. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase (HPRT1) were used as housekeeping control genes due to their stability between patient samples determined by others and confirmed at the beginning of the project.

Gene		Invitrogen catalogue number for Taqman© probe
TLR1	Toll-like receptor 1	Hs00413978_m1
TLR2	Toll-like receptor 2	Hs01872448_s1
TLR3	Toll-like receptor 3	Hs01551078_m1
TLR4	Toll-like receptor 4	Hs00152939_m1
TLR6	Toll-like receptor 6	Hs01039989_s1
COL1A2	Collagen type I	Hs00164099_m1
COL2A1	Collagen type II	Hs00264051_m1
CD46	Membrane cofactor protein	Hs00611257_m1
CD55	Decay accelerating factor	Hs00892618_m1
CD59	MAC inhibitory protein	Hs00174141_m1
C3aR	Complement anaphylatoxin C3a receptor	Hs00269693_s1
C5aR	Complement anaphylatoxin C5a receptor	Hs00704891_s1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	Hs02758991_g1
HPRT1	Hypoxanthine Phosphoribosyltransferase	Hs02800695_m1

Table 2.7: qPCR primers used for mRNA analysis.

Cycles	Duration of cycle	Temperature
1	3 mins	95°C
40	15 s	95°C
	20 s	60°C
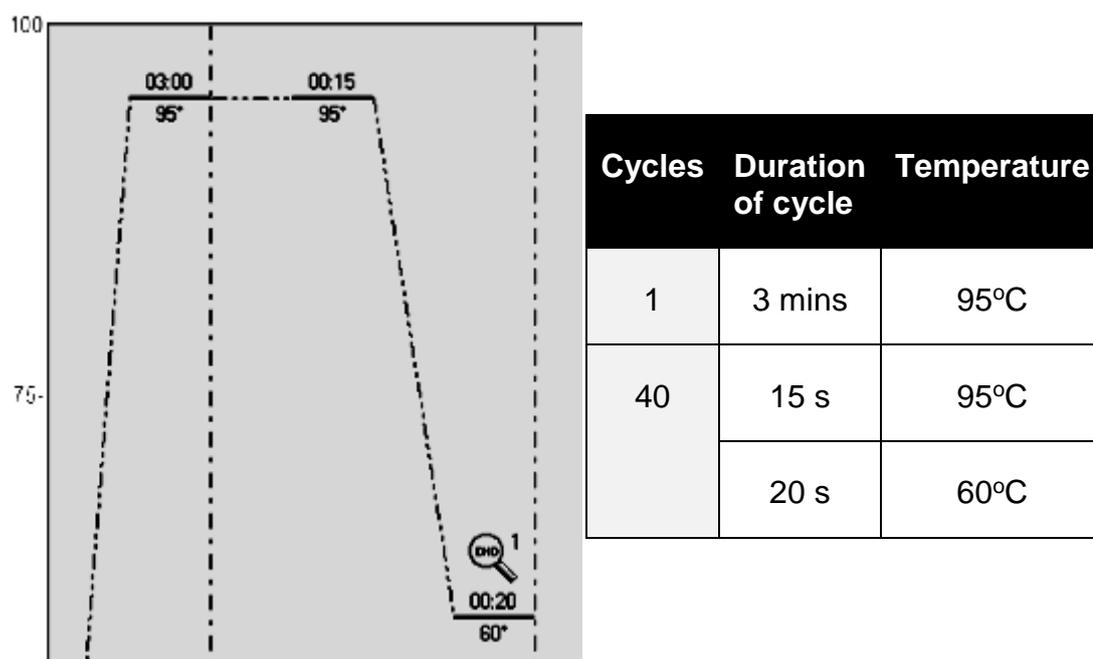
Figure 2.3: Amplification conditions for qPCR. Samples of 20µl were loaded into 96well clear bottom and capped thermocycler plates. The lid was pre-heated to 115°C followed by an initial denaturation step of 95°C for 3 minutes followed by 40 cycles of ligation and elongation. Fluorescent thresholds and raw amplification curves were analysed after each experiment.

2.9.3 Quantification (2$^{-\Delta Ct}$ Livak method)

The Livak method, more commonly known as the delta delta CT method, assumes that the amplification efficiencies of the reference control gene and the target gene of interest must be approximately equal. In essence this method assumes that each PCR cycle will exactly double the amount of material in your sample (amplification efficiency = 100%). The ratio of the target gene in sample relative to a designated control gene was then calculated by taking the 2-$^{\Delta Ct}$ (Livak and Schmittgen, 2001).

2.10 <u>Cartilage isolation</u>

A simplified version of the Mankin cartilage scaling system was used to macroscopically grade cartilage before it was isolated from the femoral head or tibial plateau of the sample. Grading's were confirmed microscopically during confocal analysis.

2.10.1 Cartilage preparation for immunohistochemistry.

Isolated cartilage samples were flash frozen in liquid nitrogen immediately after acquisition. Samples were then processed using a Leica Cryostat CM1800 where samples were cut into 10µm slices at -20°C and mounted on positively charged histology slides (Fisher scientific) before staining. Explants were sectioned transversely in order to view cells within the zones of cartilage.

2.10.2 Cartilage staining for immunofluorescence

Samples were blocked for 30 minutes in 1% BSA in PBST at 37°C in a humidified chamber. Slides were then washed three times for 1 minute in PBST before being incubated with the primary antibody added for 1 hour at 37°C in a humidified chamber (Table 2.8). After the primary incubation, samples were washed 3 times in PBST before being incubated for 1 hour at RT with the secondary, fluorophore conjugated, antibody for immunofluorescent imaging. Before mounting, slides were counterstained with 100µM DAPI, a fluorescent nuclear dye, for 1 minute before being washed once with distilled water, left to completely dry, then mounted using Vectorsheild (VectorLabs, Peterborough, UK) and a 0.13mm cover slip.

2.10.3 Toluidine Blue/Fast Green Stain for cartilage

Samples were first hydrated in distilled water before a solution of 0.04% Toluidine Blue solution (Sigma Aldrich) was added gently for 10 minutes. Samples were gently washed three times with deionised water followed immediately by counterstain with 0.02% Fast Green (Sigma Aldrich) for 3 minutes. Samples were washed twice for 30 seconds before being dehydrated with 95% ethanol for 30 seconds then with 100% ethanol for a further 30 seconds. Samples were mounted for imaging using a 0.13mm cover slip (Fisher Scientific).

	Primary Antibody	Conc μg/ml	Secondary antibody	Fluorophore excitation and emission
TLR1	(Abcam) Mouse IgG1 Cat # Ab59702 Clone – GD2.F4	0.5	(Abcam) Mouse IgG1, FITC Cat # Ab59702	488nm / 517nm
TLR2	(SantaCruz) Mouse IgG2a Cat # Sc-21759 Clone – TL2.1	0.5	(Santa Cruz) Goat anti-mouse IgG2a CFL 555 Cat # SC-395770	555nm / 568nm
TLR3	(SantaCruz) Goat IgG Cat # SC-12509 Clone – Q-18	1.5	(SantaCruz) Donkey anti-goat 488nm Cat # SC-362255	488nm / 517nm
TLR4	(SantaCruz) Rabbit IgG Cat # SC-10741 Clone – H-80	0.5	(SantaCruz) Goat anti-rabbit IgG-PerCP-Cy5.5 Cat # SC-45101	565nm / 695nm
TLR6	(BioLegend) Mouse IgG1 Cat # 334707 Clone -TLR6.127	1	(BioLegend) Mouse IgG1, PE Cat # 334707	570nm / 578nm
EEA1	(SantaCruz) Mouse IgG Cat # SC-365652 Clone -E-8	1.2	(SantaCruz) Goat anti-mouse Texas Red Cat # SC-2979	565nm / 615nm
Vimentin	(Abcam) Rabbit monoclonal Clone - EPR3776	0.5	(SantaCruz) Goat anti-rabbit IgG-PerCP-Cy5.5 Cat # SC-45101	565nm / 695nm
Other				
DAPI	Sigma Aldrich	(100μM)	-	355nm / 460nm
Propidium iodide	Life Technologies P1304MP	1	-	493nm / 636nm
Calcein AM	Life Technologies C1430	3	-	495nm / 516nm

Table 2.8: Antibodies used for fluorescent confocal microscopy. DAPI – 4',6-Diamindino-2-Phenylindole, Dihydrochloride.

2.10.4 Synovial fibroblast confocal microscopy

Synovial fibroblasts were plated at 4×10^4 cells per well in 24-well tissue culture plates with autoclaved 16mm coverslips at the bottom of the well. After being allowed to adhere for 24 hours, cells were washed in PBS and fixed using 4% paraformaldehyde for 10 minutes at RT. Cells were washed a further 3 times for 1 minute each in PBS and then blocked in 2% BSA (v/v) (PBS +/- 0.05% Triton X-100 for permeabilisation) for 1 hour at RT whilst rocking. Samples were then washed again for 1minute, 3 times in PBS before being incubated with the primary antibody for 1 hour at RT. Samples were then washed again before having the secondary antibody added for 1 hour at RT. Cover-slips were then washed with PBS before a final wash in distilled water to prevent phosphate crystal formation before being mounted on a slide using DAPI containing fluoro-preserve agent (VectorLabs).

2.10.5 Confocal microscopy image acquisition

Confocal images were obtained using a Leica SP5 confocal laser scanning microscope system (Leica, Milton Keynes, UK) and *z*-stack composite image maximising fluorescent intensity was achieved by taking 15 optical sections (2µm at x200, 1µm at x630 and x1000 magnification) spanning the entire cartilage section. All images had a maximum gain of 750V to reduce noise levels in the images. Primary antibody omitted negative controls underwent an identical imaging protocol. Images of multiple small groups or single cells were used to obtain TLR expression distribution in synovial fibroblasts.

2.11 Complement anaphlatoxin expression in HEK293T cells

2.11.1 Amplification of C3a/C5a gene fragments

Complement genes C3 and C5 were purified from plasmid expressing *E.coli* cells (Dharmacon). *E.coli* was cultured overnight in 200ml Lysogeny (LB) broth (Sigma-Aldrich (Gillingham, UK), under agitation at 37°C. Plasmids were isolated from bacterial pellets using a MaxiPrep kit (Qiagen, Manchester, UK). Once isolated, the identified C3a and C5a regions of the gene were amplified using custom primers (Table 2.9) (Sigma-Aldrich (Gillingham, UK) which added a cleavable His –tag to the N-terminus of the translated protein.

2.11.2 Gel extraction of purified samples

These regions once amplified were purified through agarose gel analysis and a gel extraction kit (Qiagen, Manchester, UK) before being cloned into the pcDNA6 plasmid (Figure 2.4) (Life Technologies).

2.11.3 Cloning into dh5a *E.coli*

The plasmid was subsequently transfected into competent *E.coli* dh5a cells (New England Biolabs) plated on 200μg/ml Carbenicillin agar plates overnight at 37°C. Five colonies were picked at random and sent for sequencing to confirm the correct insert sequence (MWG Eurofins). Clones of correct sequence were incubated for 18 hours in 200ml of LB broth at 37°C under constant agitation before the plasmid was isolated using a MaxiPrep (Qiagen, Manchester, UK).

2.11.4 Transfection procedure of HEK293

The eluted pure plasmid was then transfected at 25µg/10cm^3 cell culture dish into 2x10^6 HEK293 cells using the transfection mix (Table 2.10). The media on the cells was removed and replaced with 9ml of complete media after which the 1ml of transfection mix (Table 2.10) was added drop by drop dispersed across the plate. After 24hrs the transfection mix containing media was removed and replaced with fresh complete media containing 50ng/ml TNF to induce production of the complement protein from the plasmid (Figure 2.4). After a further 24 hours the media was removed before the cells were collected, pelleted and lysed in 500µl lysis buffer (50mM sodium phosphate pH8.0, 300mM NaCl, 0.01% Tween 20 and 1% Triton X100) and stored at -80ºC until further use.

	Forward Primer	Reverse primer
C3a	5' ctag aagctt ATG CAT CAT CAC CAT CAC CAT CTG GAA GTT CTG TTC CAG GGC CCA TCC GTG CAG CTC ACG GAG AAG CGA 3'	5' ctag ctcgag AGT CCT GGC CAG GCC CAG GTG GCT GGC 3'
C5a	5' CTAG GGATCC ATG CAT CAT CAC CAT CAC CAT CTG GAA GTT CTG TTC CAG GGC CCA ACG CTG CAA AAG AAG ATA GAA GAA 3'	5' ctag ctcgag AGT CCT TCC CAA TTG CAT GTC TTT ATG 3'

Table 2.9: Custom primers for C3a/C5a amplification. Lower case bases indicate His-tag addition to the N-terminal region.

Reagent	Volume/amount
PEI	75µl
Plasmid DNA	25µg
OPTIMEM	Up to 1ml

Table 2.10: Transfection mix for HEK293 pcDNA6 transformation. Volumes used for transfection of $2x10^6$ cells
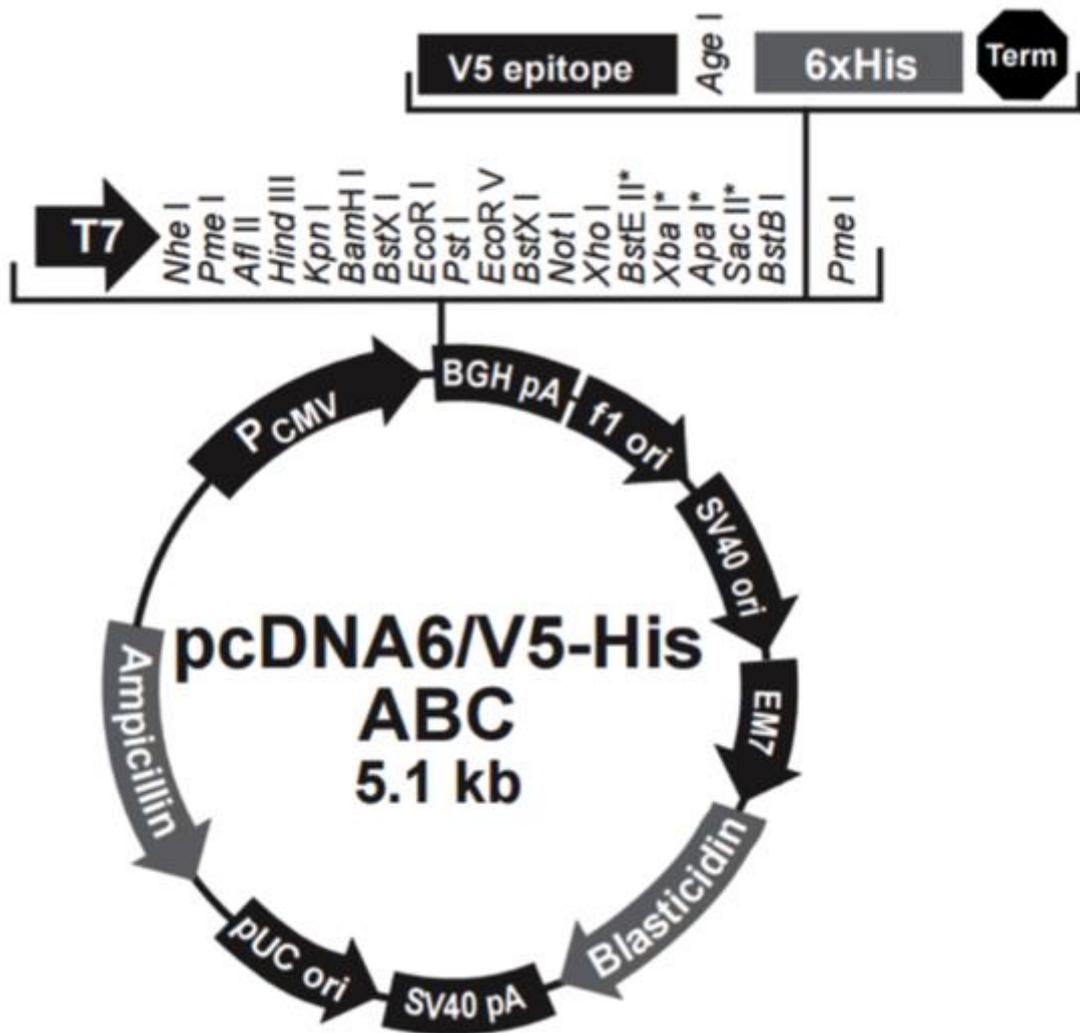
Figure 2.4: Vector map of pcDNA6 plasmid used for cloning and transfection of C3a and C5a components. During the cloning of C3a and C5a anaphylatoxins this plasmid construct was used with restriction sites; HindIII/XhoI and BamHI/XhoI used respectively. Plasmid map from ThermoFisher.

2.11.5 Nickel-His purification of protein

Cell lysates of C3a and C5a transfected HEK293 cells were centrifuged for 10 minutes at 13,000g at 4°C to remove any cell debris. Supernatants were then harvested before being added to 100µl pre-washed Nickel beads. Using a magnet, the unbound fraction was removed and kept for later analysis. The nickel beads were then washed with wash buffer (50mM sodium phosphate pH8.0, 300mM NaCl, 0.01% Tween 20 and 50mM imidazole) and then in 50mM imidazole. Beads with bound protein were then washed in 100µl elution buffer (50mM sodium phosphate pH8.0, 300mM NaCl, 0.01% Tween 20 and 300mM imidazole) for 5 minutes before being magnetically separated. All wash and eluted fractions were retained for later analysis and were run with anti-His, anti-C3a and anti-C5a western blot to confirm presence and size.

2.12 <u>Western blot / SDS-PAGE</u>

2.12.1 Gel electrophoresis

All samples were lysed in NP-40 (150mM NaCl, 1% NP-40, 50mM Tris pH8.0 and protease inhibitor cocktail (Sigma Aldrich)) or RIPA buffers (150mM NaCl, 1% Triton X-100, 0.5 sodium deoxycholate, 0.1% SDS, 50mM Tris pH8.0 and protease inhibitors) for 20 min on ice, then clarified by centrifugation at 13,000g for 10 min at 4°C. Samples were boiled at 95°C for 5 min in Laemmli buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125M Tris HCL pH6.8) prior to loading on to polyacrylamide gels (Table 2.11). Full range rainbow molecular weight marker (GE Healthcare, Little Chalfont, UK) was used for determination of band size. Gels underwent electrophoresis for 75-90 min at 120V in SDS running buffer.

SDS-PAGE gels were transferred to nitrocellulose membranes using a BioRad MiniTrans Blot cell (BioRad, Hercules, CA, USA) wet blotting system at 120V for 2 hours at 4°C in Western transfer buffer (25mM Tris, 192mM glycine, 20% methanol). Membranes were blocked in 5% BSA (PBS) in TBST for 1 hour at room temperature.

2.12.4 Antibody dilutions and incubations

Primary antibodies were incubated overnight at 4°C in BSA (Table 2.12). After incubation membranes were then washed 3 times for 5 min each in TBS + 0.05% Tween-20 on a rocking mixer before incubation with secondary HRP conjugated goat anti-mouse IgG (1: 25,000) (FAb specific) or goat anti-rabbit IgG (1:5000) (whole molecule), both from (Sigma-Aldrich Gillingham, UK), diluted in BSA or a milk solution, for 1 hour at room temperature.

2.12.5 Luminescent detection

Membranes were washed again as previously described and incubated with Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, UK). Membranes were then exposed to photographic film (GE Healthcare, Little Chalfont, UK) and developed using a Konica Minolta SRX-101A film developer and Champion Photochemistry film developer and fixer solutions (Jet X-ray, London, UK).

Running Gel:	7.5%	10%	12%	15%	Stacking Gel:
Acrylamide/ bis solution	2.5ml	3.3ml	4ml	5ml	835µl
Running buffer 4x	2.5ml	2.5ml	2.5ml	2.5ml	1.25ml
SDS (10%)	100µl	100µl	100µl	100µl	50µl
APS (10%)	100µl	100µl	100µl	100µl	75µl
Temed	10µl	10µl	10µl	10µl	5µl
H_2O	4.9ml	4.067 ml	3.4ml	2.4ml	2.86ml

Table 2.11: Composition list for mini- poly-acrylamide sodium dodecyl sulphate gels.

Immunogen Molecular (weight kDa)	Manufacturer Cat No	Working dilution	Species	Blocking solution (w/v)
MMP1 (pro-52) (mature-41)	sc-21731 Clone – 3B6 Santa Cruz Biotechnology	1:400	Mouse	5% BSA
MMP3 (Pro-60) (Mature-51)	sc-21732 Clone – 1B4 Santa Cruz Biotechnology	1:400	Mouse	5% BSA

Table 2.12: Antibodies used for western blot analysis of synovial fluids. Conditions were optimised for each antibody from conditions described by the suppliers. Both blocking and antibody solutions were in TBS/Tween.

2.13 <u>Statistical analysis</u>

Relative RNA levels were normalised to two housekeeping genes using the Livak method of analysis for comparisons of TLR expression in synovial fibroblasts and cartilage isolates (Livak and Schmittgen, 2001). Values were individually plotted with the error bars representing the mean ± standard error of the mean (SEM) (GraphPad Prism 5).

The student's t-test was performed between synovial fibroblast sample groups to determine any significant differences between knee and hip expression profiles. 2-way repeated measured Anova (Bonferroni correction test), Paired or un-paired student t-tests (2-tailed) were performed where appropriate and indicated. Levels of statistical significance are shown as $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ and $****P \leq 0.0001$. Linear regression of correlated samples was performed alongside Pearson D'Agnosto correlation analysis.

Chapter 3 - Expression and activation of Toll-like receptors in synovial fibroblasts

Hypothesis: Toll-like receptors contribute to OA progression through their ability to induce pro-inflammatory cytokine production from synovial fibroblasts.

3.1 Introduction

Conflict within the literature has meant that the expression and activation of TLRs in synovial fibroblasts is still debated due to differences in detectable mRNA and protein expression between labs (Ospelt et al., 2008, Hu et al., 2014). Analysis of the activity of cytokine induction from TLR stimulation (Figure 3.10) demonstrated only TLR1/2/3/4/6 were active and were consequently studied in depth. Quantitative expression and activation of TLR was determined by the use of qPCR, flow cytometry and ELISA. The synovium has been noted to be involved in the progression of inflammatory arthrides such as RA and OA (Blom et al., 2007, Goldring and Otero, 2011, Okada et al., 1992, Smith, 2011). This study aimed to elucidate the involvement of TLRs in the production of pro-inflammatory cytokines from OA synovial fibroblasts, which may have the potential to contribute to disease activity.

3.2 Expression of TLRs in OA synovial fibroblasts (qPCR)

Synovial fibroblasts isolated from knee and hip were cultured for 3 passages before being plated at 1×10^6 cells per well in a 6-well culture dish before having total RNA extracted without any external stimulation. Transcript levels of

TLRs1-6 were determined using qPCR. Basal expression of active TLRs were determined using the Livak ($2^{-\Delta Ct}$) method of quantification (see methods for further details).

TLR1, TLR2, TLR3, TLR4 and TLR6 expression was evident in all OASF samples tested and a significant difference in populations was noted for TLR2 expression in which hip OASFs expressed significantly less TLR2 than knee OASFs (Figure 3.1).
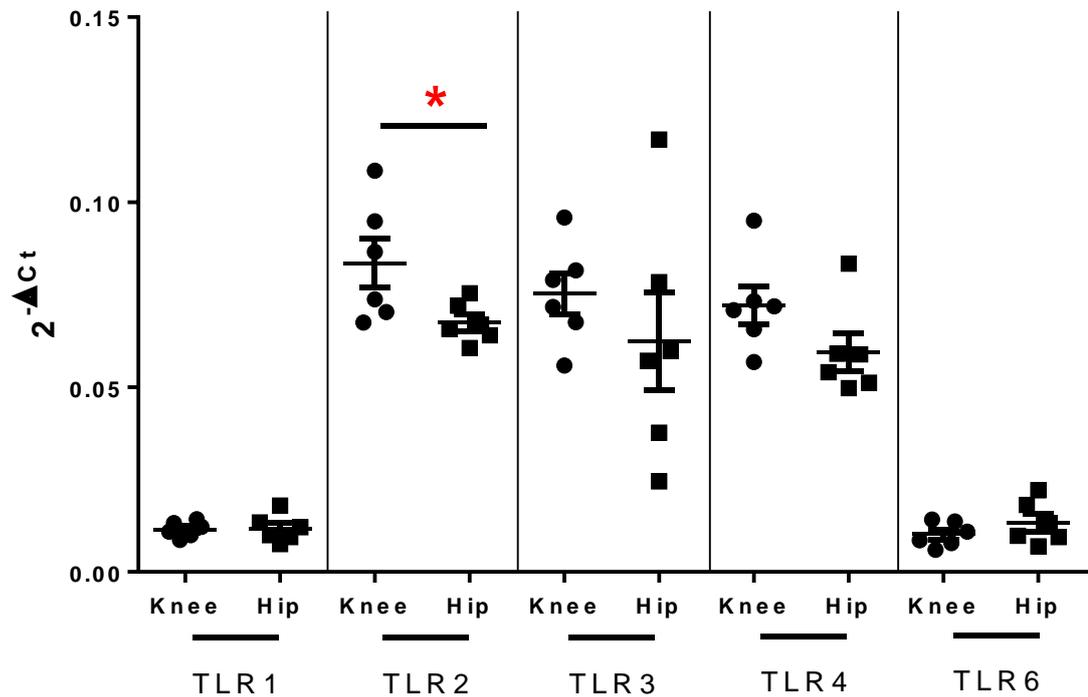
Figure 3.1: Quantitative mRNA analysis of Toll-like receptors show levels are altered in synovial fibroblasts from Knee and Hip. Unstimulated OA synovial fibroblasts were harvested at P3 and analysed for TLR mRNA expression by PCR. Values are represented as the $2^{-\Delta\Delta Ct}$ where the target gene is compared with the geometric mean of two housekeeping genes. Data from 12 patient samples are shown (6 knee OASFs, 6 hip OASFs). Each sample is the mean of triplicate assays. * P <0.05 un-paired student *t*-test, 2-tailed.

3.2.1 TLR2 mRNA is increased in knee compared to hip OASFs

Synovial fibroblasts were passaged for three population expansions before total RNA was isolated. Analysis of mRNA expression demonstrated that TLR2 expression was significantly increased at the message level in knee OASFs compared with hip OASFs (P=0.0411). Expression of TLR1 (P=0.9037), TLR3 (P=0.3095), TLR4 (P=0.0931) and TLR6 (P=0.04848) were not significantly different between patient groups.

3.3 Expression of TLRs in synovial fibroblasts

3.3.1 TLR2 co-localises with TLR1 and TLR6 whilst TLR3 co-localises with the early endosome marker (EEA1) in OASFs

Expression at the mRNA level demonstrated a significant difference in TLR2 expression between synovial fibroblasts isolated from knee and Hip with the latter expressing significantly less (P=0.0411). However, it was also important to determine the protein expression of the receptors as posttranslational regulation of their expression could be different. Therefore, flow cytometric analysis of patient fibroblasts and confocal microscopy was used to determine the distribution of the receptors and their localisation (Figure 3.2-3.5). TLR2 heterodimerises with TLR1 and TLR6 on the cell surface membrane whilst TLR3 is expressed within the endosome, which is important for activation through cathepsin cleavage. To our current knowledge of the literature, confocal analysis of TLR expression and localisation has not been performed in primary synovial fibroblasts. Whilst research into dimerisation of TLRs using primarily FRET has been performed, that method was beyond the scope of this project and in addition, it utilised TLR transfected cells rather than primary

cultures (Horvath, 2009). Therefore, confocal analysis of TLR localisation and was conducted.

The following experiments were performed on both hip and knee synovial fibroblasts which demonstrated no structural characteristics differences between groups. Therefore, a mix of both knee and hip synovial fibroblasts were used for the confocal imaging (Figure 3.2-3.6).
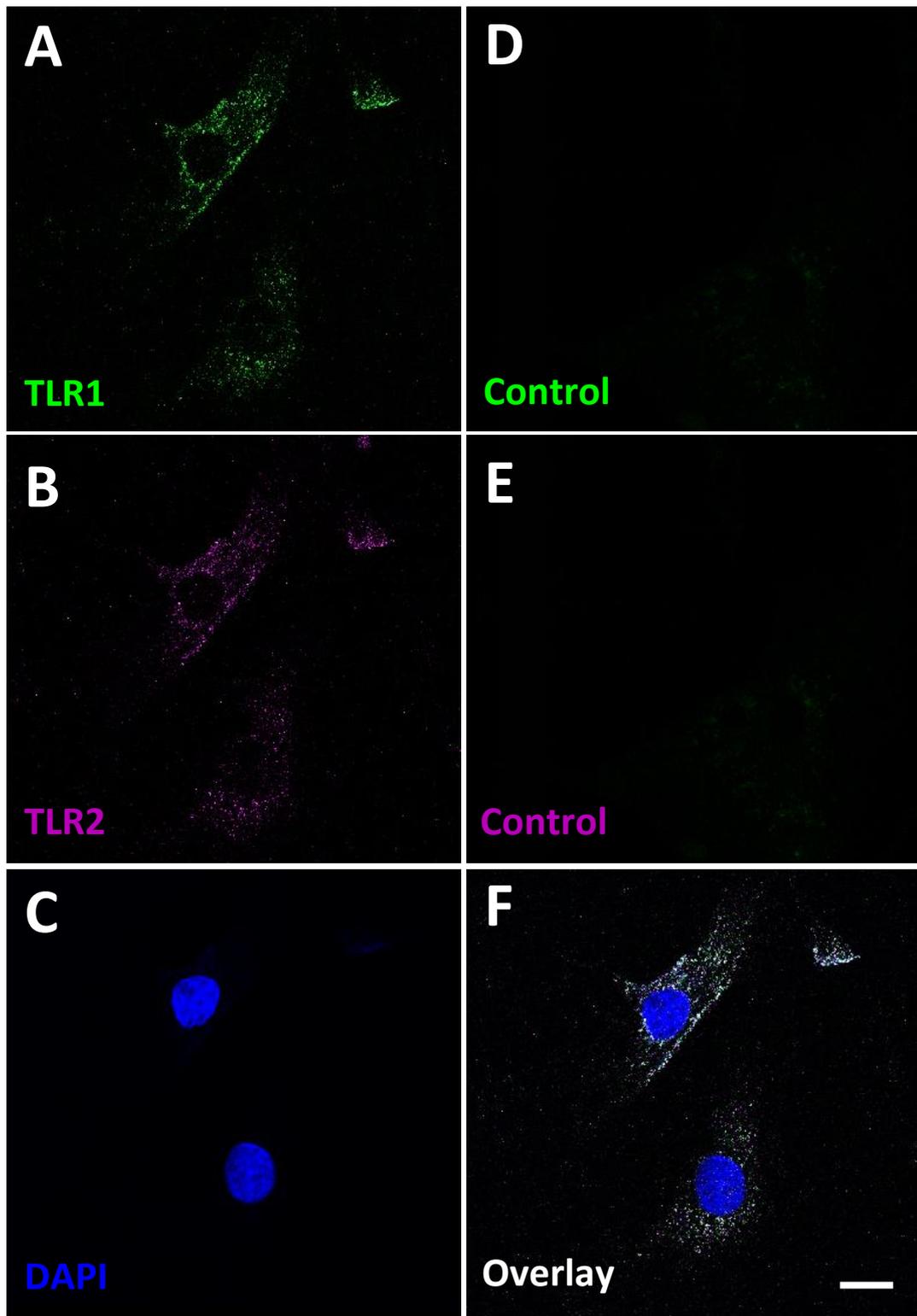
Figure 3.2: TLR1 and TLR2 are expressed in a cell membrane and cytoplasmic distribution in OASFs. Double immunofluorescent staining for TLR1 and TLR2 was performed and analysed with confocal microscopy. **A)** TLR1 - FITC (Green) and **B)** TLR2 - Alexa 555 (Magenta). **C)** DAPI – Blue **F)** (Overlay) results in a white colour. Primary antibody omitted controls (Panel **D and E**) showed minimal immunoreactivity of the secondary antibody alone. Original magnification is x 1000. Sample (knee OASFs) Scale bar = 20µm.
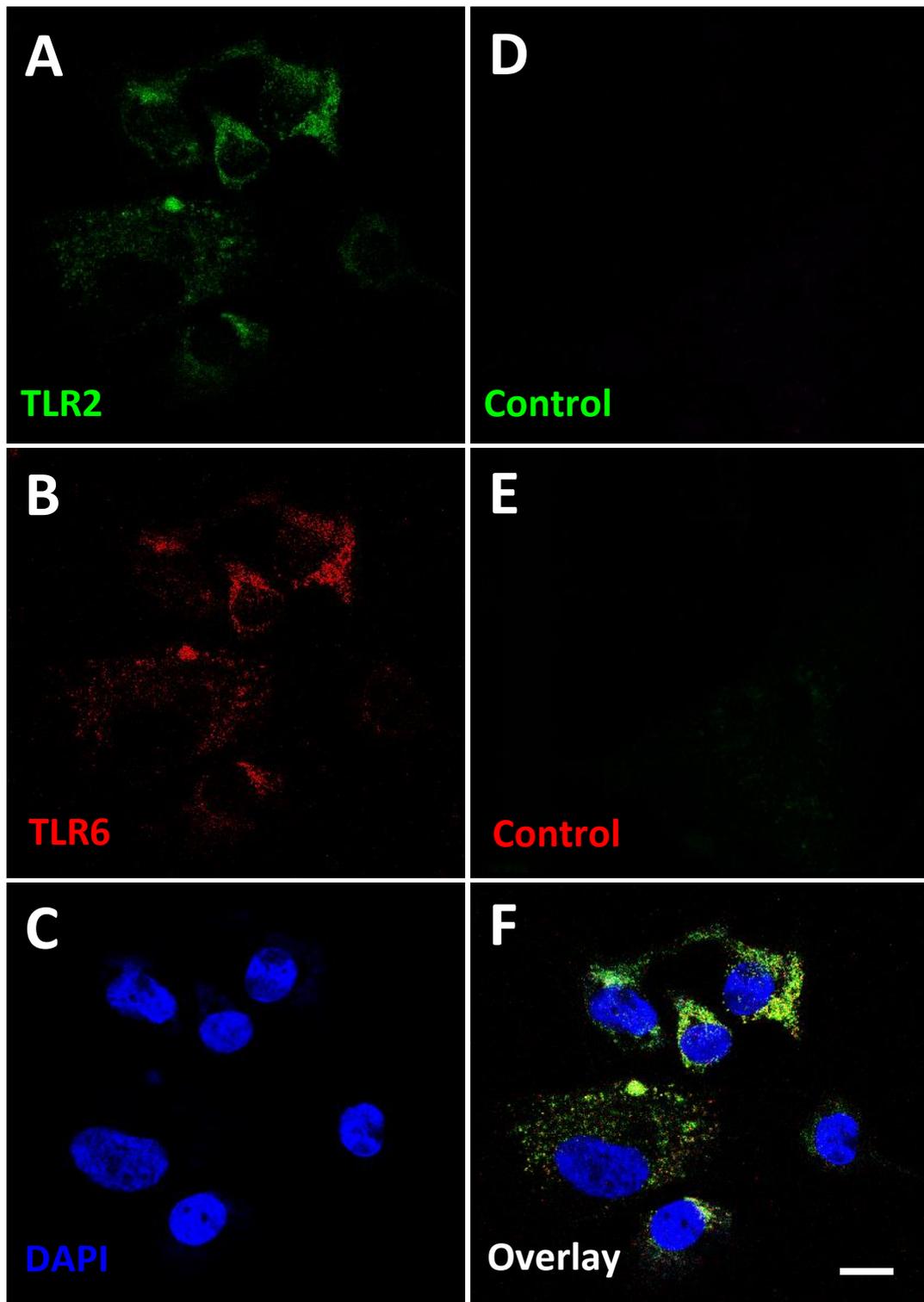
Figure 3.3: TLR2 and TLR6 are co-expressed in a cell membrane and cytoplasmic distribution in OA synovial fibroblasts. Double immunofluorescent staining for TLR2 and TLR6 was performed and analysed with confocal microscopy. **A)** TLR2 - ALEXA647 (Green) **B)** TLR6 - PE (Red). **C)** DAPI – Blue. **F)** The clear co-localisation of the two receptors (Overlay) results in a yellow colour. Primary antibody omitted controls (Panel **D and E**) showed minimal immunoreactivity of the secondary antibody alone. Original magnification is x 1000. Sample (knee OASFs). Scale bar = 20µm.
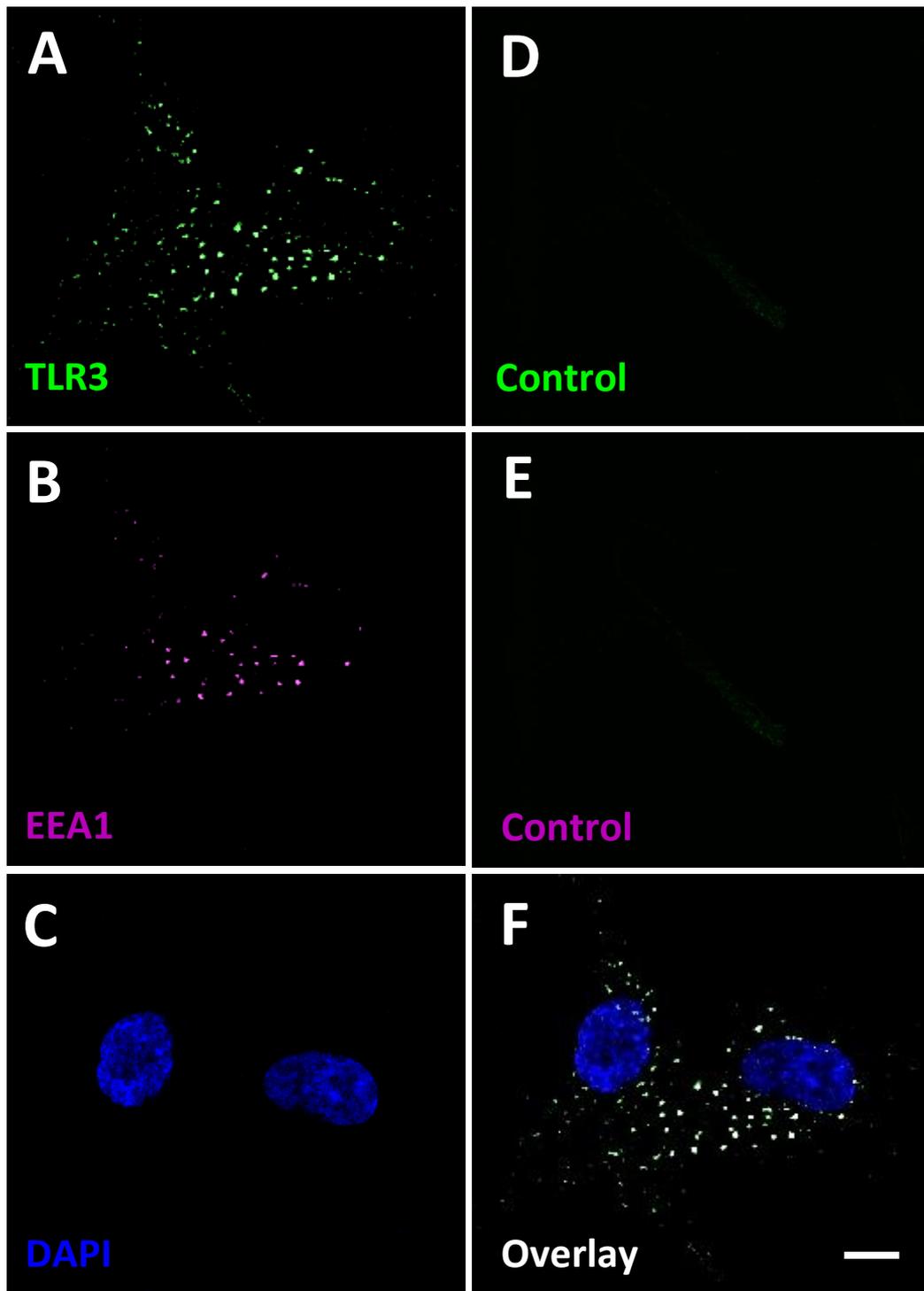
Figure 3.4: TLR3 is expressed in the endosome as indicated by the early endosomal marker EEA1 in OA synovial fibroblasts. Double immunofluorescent staining for TLR3 and early endosomal marker EEA1 was performed and analysed with confocal microscopy. **A)** TLR3 - Alexa 488 (Green) **B)** EEA1 - Texas Red (Red), **C)** DAPI- Blue. **F)** Co-localisation of the two receptors (Overlay) punctate regions indicative of endosomes. Primary antibody omitted controls (Panel **D and E**) showed minimal immunoreactivity of the secondary antibody alone. Sample (knee OASFs). Original magnification is x 1000. Scale bar = 20µm.
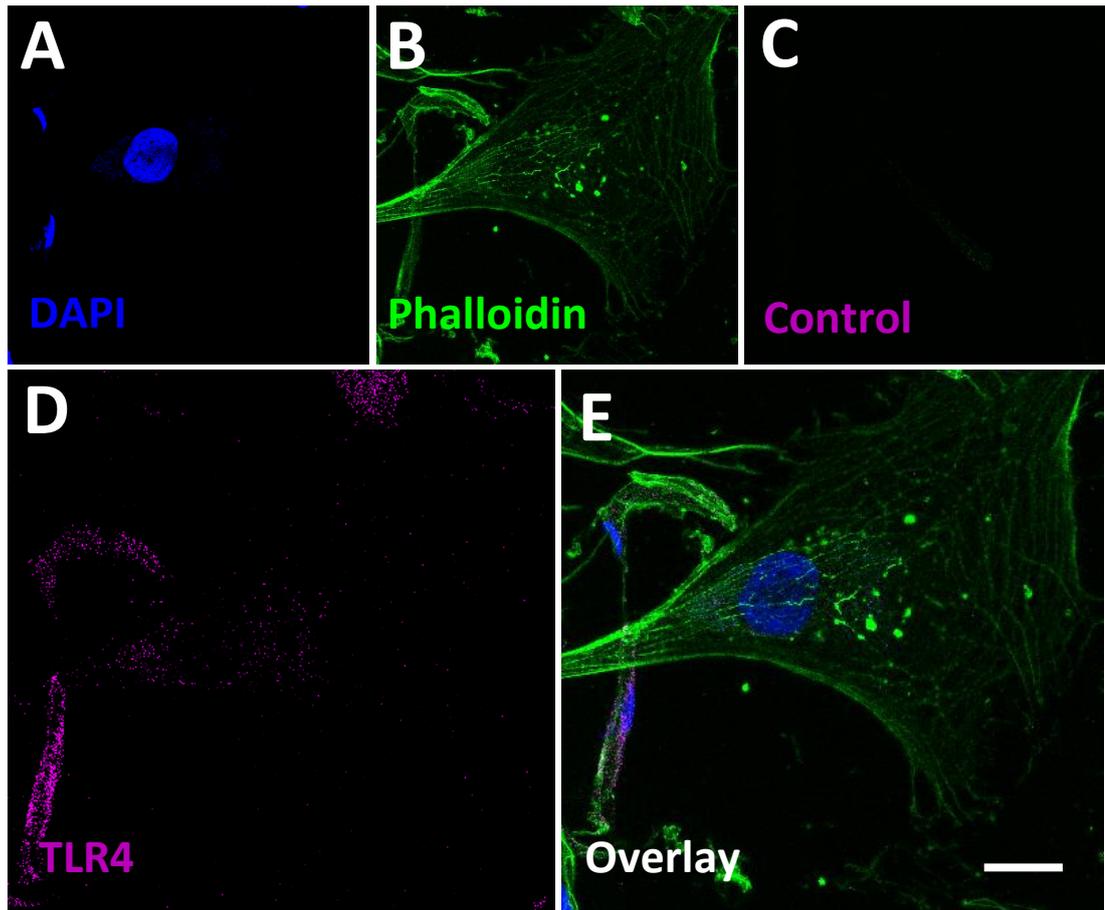
Figure 3.5: TLR4 is expressed in a cell membrane and cytoplasmic distribution in OA synovial fibroblasts. Immunofluorescent staining for TLR4 was performed and analysed with confocal microscopy. **A)** DAPI (blue) **B)** Phalloidin, anti-actin marker of cytoskeleton Alexa488 (green) **D)** TLR4 - Alexa 647 (Magenta) Primary antibody omitted controls (Panel **C**) showed minimal immunoreactivity of the secondary antibody alone. **E)** Overlay of fluorescence. Original magnification is x 1000. Sample (hip OASFs) Scale bar = 20μm.

These data confirmed expression of cell surface TLR2 that demonstrated to be co-expressed with TLR1 or TLR6. Additionally, the co-localisation of TLR3 with the early endosomal marker EEA1 demonstrated previously reported localisation of the receptor, for successful cathepsin cleavage requires the acidic environment of the endosome.

3.3.2 Flow cytometric analysis of TLR expression in OASFs

Confocal analysis of synovial fibroblasts detailed localisation of the TLRs of interest within the cells, however could not be readily quantified. To rectify this, we performed flow cytometric analysis for quantification of protein expression. With the fibroblast marker, vimentin, populations of cells (Figure 2.2), TLR expression was determined from 10 OA donors. Donors were categorised into knee and hip derived OASFs. Cell surface expression of TLR1, TLR2, TLR4 and TLR6 was evident on all synovial fibroblasts tested, as was intracellular expression of TLR3 (Figure 3.6).
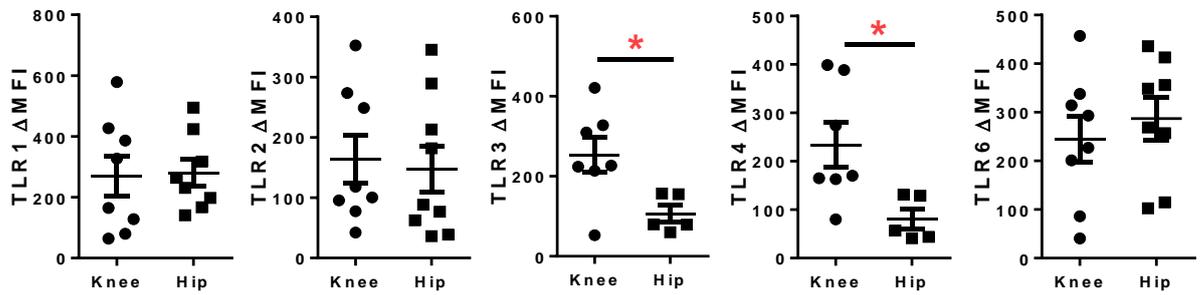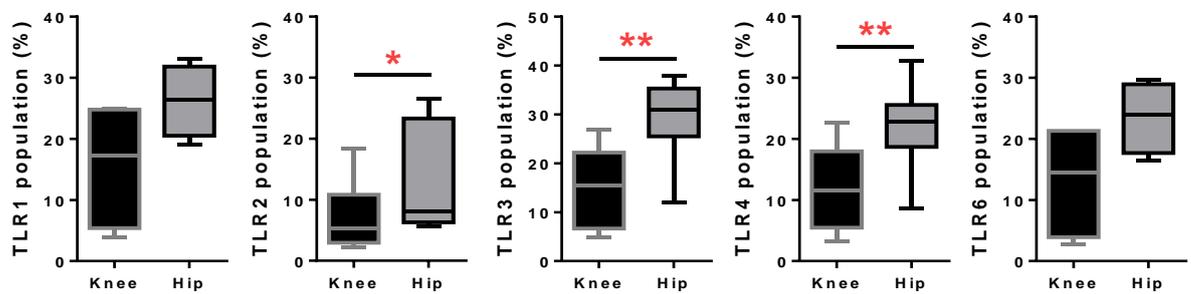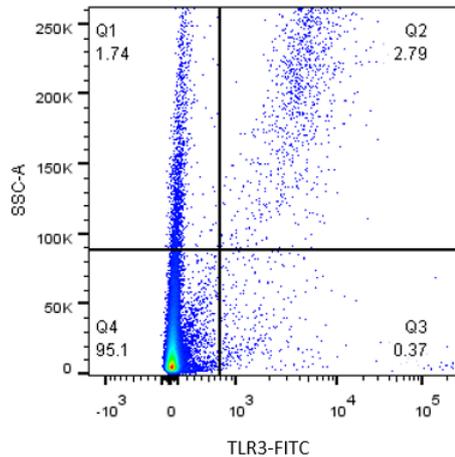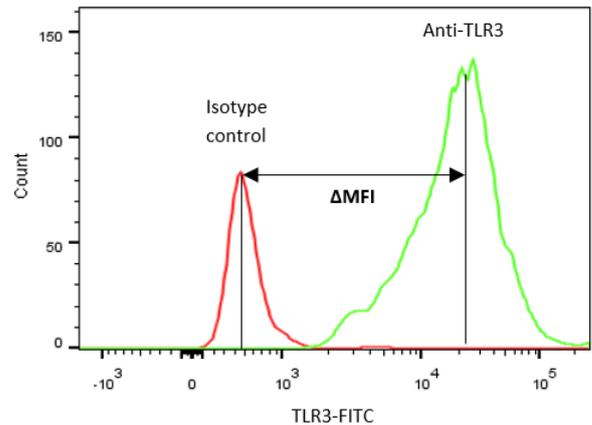
Figure 3.6: Flow cytometry analysis of TLRs in OA synovial fibroblasts. Unstimulated OA synovial fibroblasts were harvested at passage 3-4 and stained for TLR1, 2, 3, 4 or 6. **A)** TLR protein expression (ΔMFI) and **B)** TLR positive populations of cells (Q2). **C)** Samples were gated by size, granularity and single protein markers. **D)** Example Delta-MFI calculated using histogram analysis of anti-TLR antibodies and subtracting isotype controls on the same samples. Data are shown as the mean ± SEM 16 patient samples (8 x knee, 8 x hip OASFs). * P <0.05, ** P <0.01 unpaired student *t*-test, 2-tailed.

Protein expression of TLR1 was not significantly different between knee and hip OASFs (P= 0.9002) and the percentage positive cell populations were also not different (P=0.1143). TLR2 expression was additionally unchanged at the protein level (P=0.7824) although hip OASF populations were significantly increased (P=0.0487). The protein expression of TLR3 was also different between knee and hip OASFs, being significantly higher in knee (P=0.0234). However, the populations of TLR3 positive cells was markedly increased in hip compared with knee (P=0.0012). TLR4 was significantly higher in knee OASFs (P=0.0245) than in hip OASFs. Additionally, whilst protein expression of the receptor was lower, the percentage of positive cells was significantly higher (P=0.0041) in hip OASFs compared to knee OASFs. The expression of TLR6 was not different between knee or hip OASFs (P=0.5281) and there was no significant difference in the percentage positive population (P=0.1148).

3.3.3 Synovial fibroblast TLR mRNA and protein expression do not correlate

The previous observations of TLR mRNA and protein expression whilst statistically significant, demonstrated high variation between samples, showing high heterogeneity between donors. In order to determine whether qPCR mirrors TLR receptor quantity, correlations between mRNA (qPCR) and protein expression (flow cytometry) were determined. To that end, patient matched P3 synovial fibroblasts undergoing flow cytometric analysis were compared with TLR mRNA expression from the same sample (Figure 3.7).
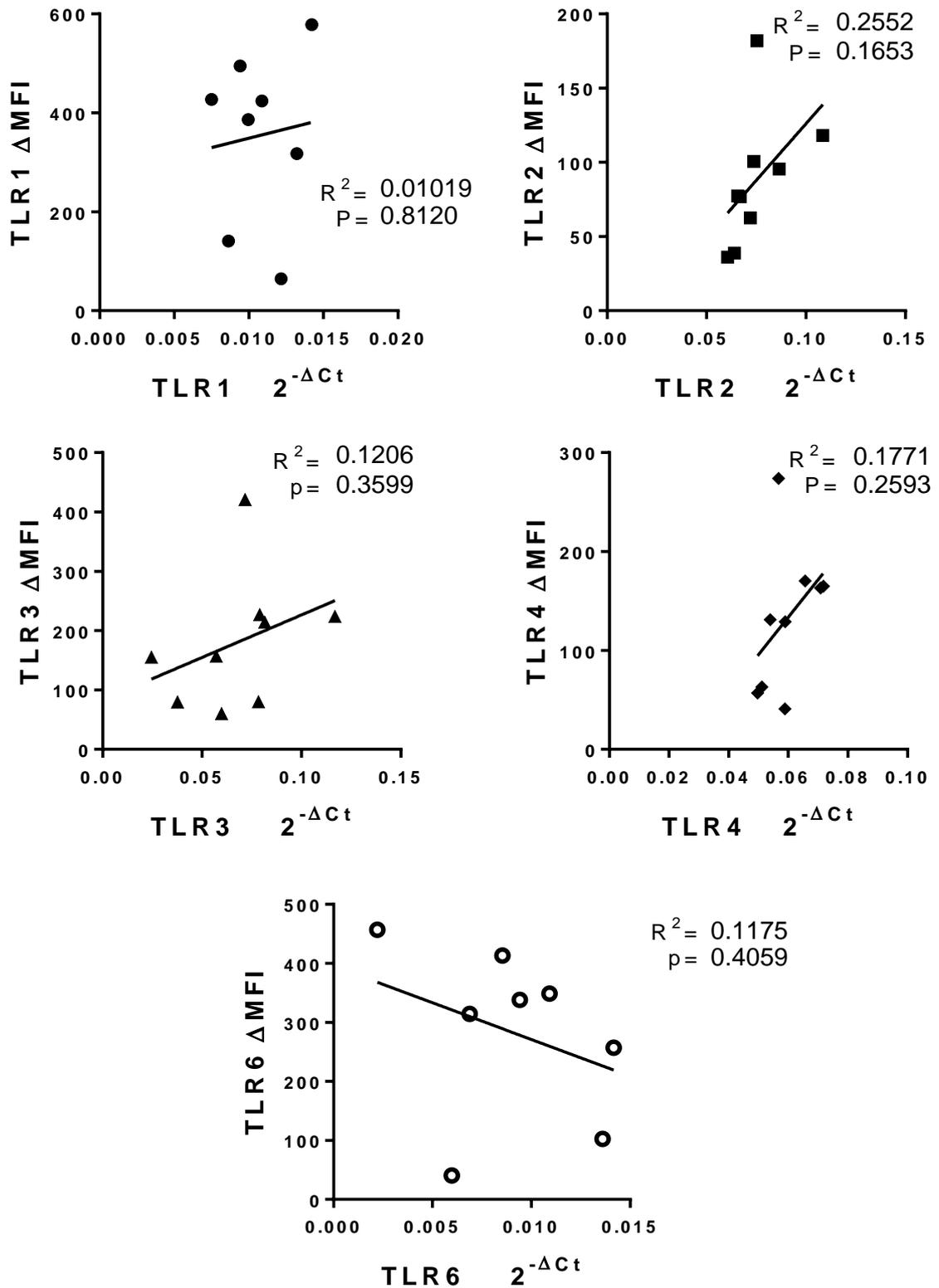
Figure 3.7: There was no correlation between mRNA and protein expression of TLRs in OASFs. Patient-matched samples undergoing flow cytometric analysis for TLR protein expression (MFI) were also tested for mRNA expression by qPCR ($2^{-\Delta CT}$). Data are from multiple experiments performed on n=9 unrelated patient samples (5 x Knee and 4 x Hip). Pearsons linear regression analysis at 95% confidence interval, 2-tailed.

Expression of the functional TLRs determined through qPCR analysis did not correlate with protein expression of the TLRs measured via flow cytometry. Samples were grouped independent of the joint location to determine whether mRNA expression reflected protein expression within the cell type. TLR mRNA expression showed no sign of correlation with protein for TLR1 (P=0.812), TLR2 (P=0.1653), TLR3 (P=0.3599), TLR4 (P=0.2593) or TLR6 (P=0.4059) (Figure 3.7).

3.3.4 TLR1, TLR3, TLR4 and TLR6 protein expression correlates with TLR positive cell populations in synovial fibroblasts

In order to determine whether knee or hip synovial fibroblasts were different in their expression of total TLRs we performed flow cytometric analysis comparing both TLR protein expression (ΔMFI) and the percentage positive populations. The expression levels were determined and then correlated (Figure 3.8).
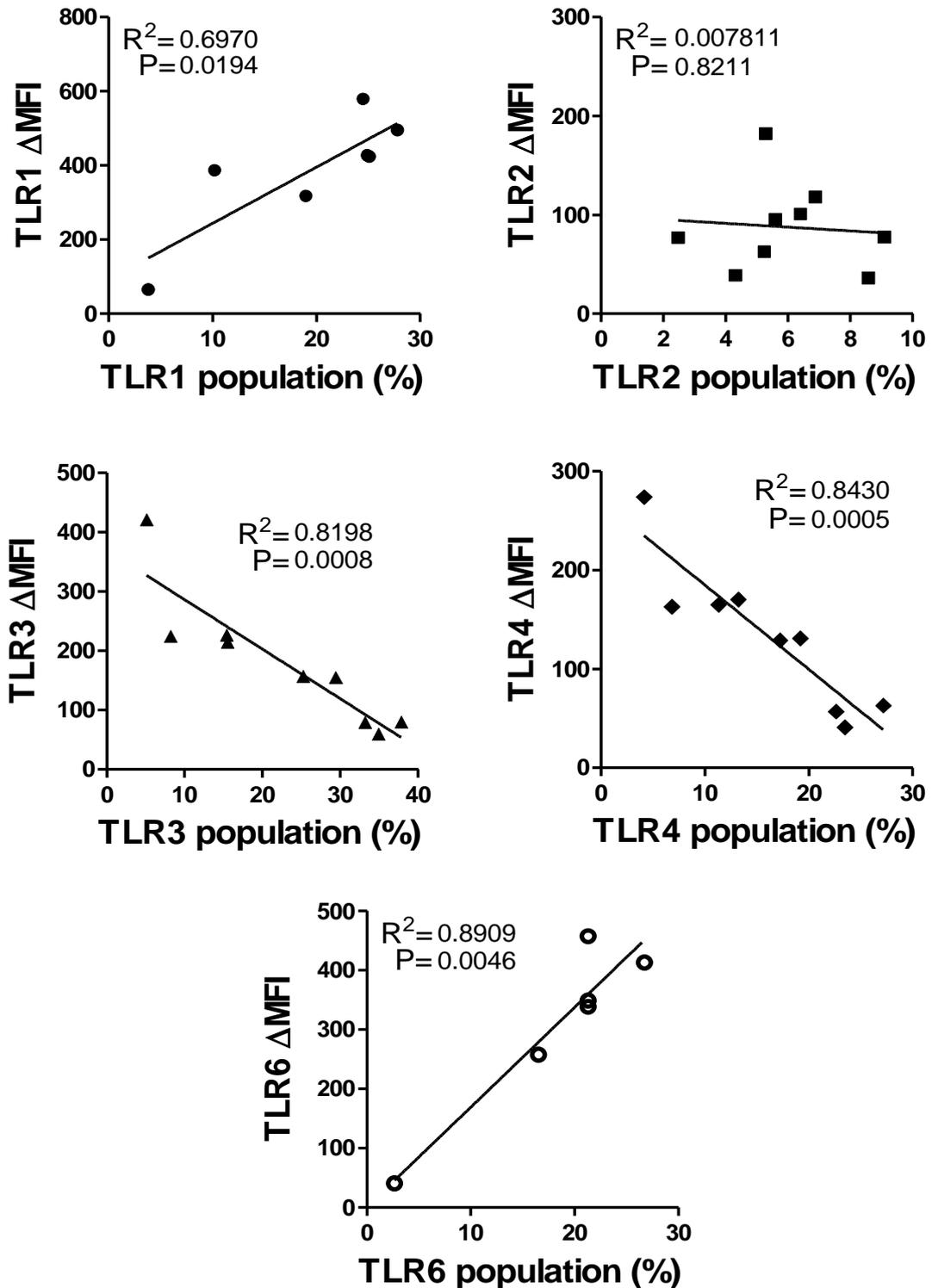
Figure 3.8: OASF TLR1, TLR3, TLR4 and TLR6 protein expression correlates with percentage of positive cells. Correlation of percentage positive population of TLR expressing cells correlated with protein expression determined through flow cytometry analysis of P3 synovial fibroblasts. Cells were gated by size and granularity before analysis of protein expression determined as the change in MFI from isotype control. Data is of 2-3 separate experiments with n=6-9 unrelated patient samples. (3-5 x Knee and 3-4 x Hip) Pearsons linear regression analysis at 95% confidence interval, 2-tailed.

TLR protein expression determined through flow-cytometric analysis of P3 OASFs demonstrated a correlation of several TLRs with positive populations. The data indicated a significant positive correlation between patient-matched samples for TLR1 (P=0.0194) and TLR6 (P=0.0046). TLR3 (P=0.0008) and TLR4 (P=0.0005) correlations were significantly negative in trend, with increased protein expression resulting in a reduced positive cell population. Interestingly, TLR2 protein expression was very stable across patients and showed no correlation to positive cell populations (P=0.8211).

Both protein expression (as MFI) and percentage of TLR+ cells were highly variable between patients demonstrating the heterogeneity of the population and also indicated that patients can have incredibly variable TLR presence. Samples were also grouped regardless of joint location to determine expression of TLRs in OASFs independent on joint location.

3.4 Knee synovial membrane cultures secrete high levels of pro-inflammatory cytokines

Research into rheumatoid arthritis commonly uses OA as a model of non-inflammatory joint arthritis (Brentano et al., 2009, Ospelt et al., 2008, Takakubo et al., 2014). Many of these studies group their joint samples together to increase sample size, irrespectively of joint location. Given the findings reported above, the spontaneous production of pro-inflammatory cytokines from OA synovial membrane in culture was investigated. In these experiments, synovial membranes isolated from knee and hip were compared, in order to determine if there was a distinct difference (Figure 3.9).

As several MMPs have been implicated in disease pathology (Sun et al., 2014, Tchetverikov et al., 2005, Goldring, 2012), I investigated the expression of MMPs: 1, 3 and 13 in the synovial membrane cultures, to establish the levels of matrix degenerative enzymes produced by synovial membrane-resident cells.
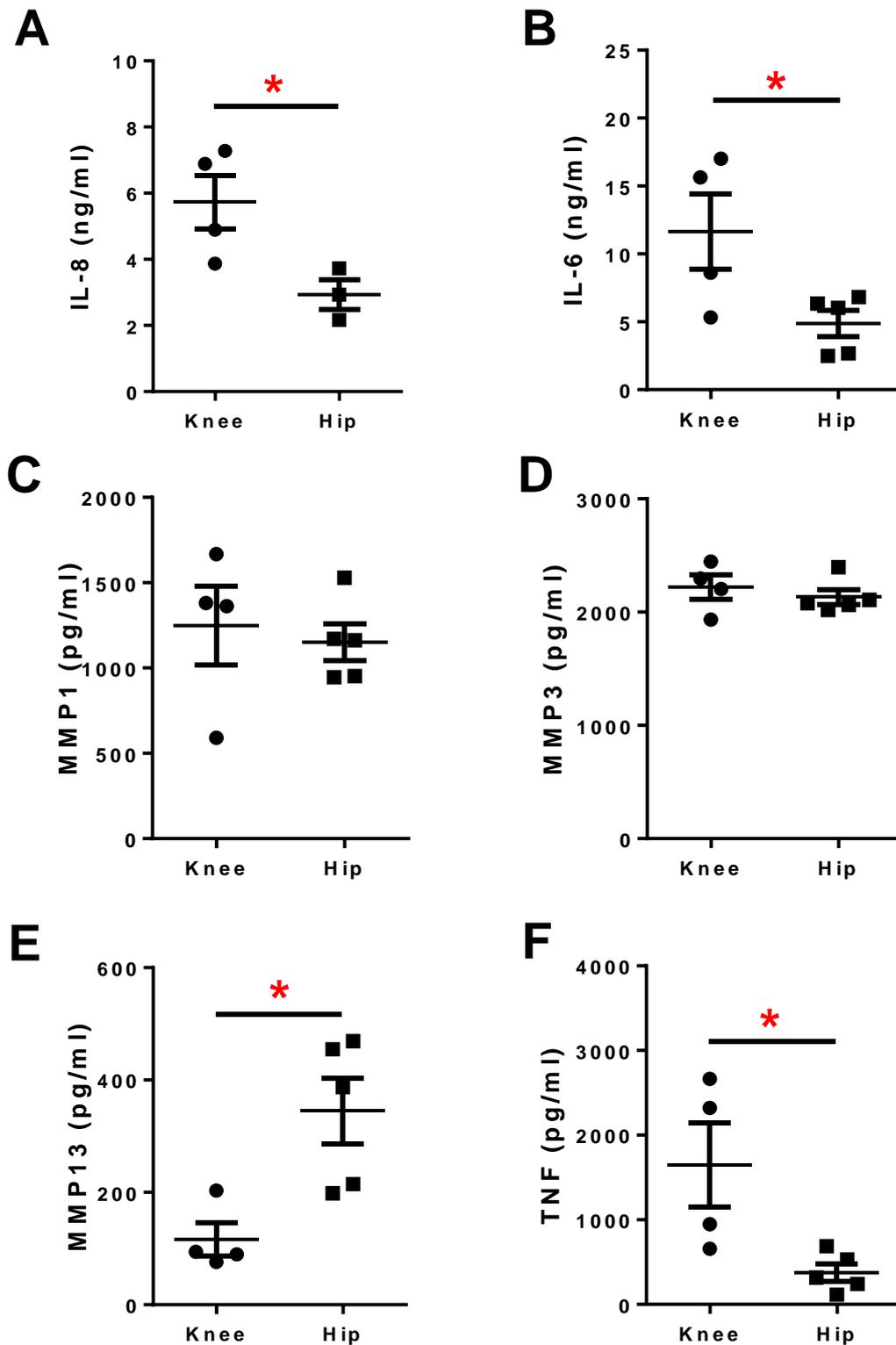
Figure 3.9: TKR-derived synovial membrane cultures produce more pro-inflammatory cytokines than those derived from THR. OA synovial membrane cells were cultured for 24 hours in the absence of any external stimuli. Supernatants were analysed for **A)** TNF, **B)** IL-6, **C)** IL-8, **D)** MMP1, **E)** MMP3 and **F)** MMP13 by ELISA. Data is shown as mean ±SEM of 4 knee and 5 hip donors assayed in triplicate. *P <0.05 unpaired student t-test.

The resident mixed cell population of the synovium were enzymatically isolated and cultured unstimulated for 24hrs before supernatants were analysed for cytokine production. These data showed an interesting bias between joint location and pro-inflammatory and MMP production. Cells derived from the knee demonstrated a much greater production of TNF, IL-6 and IL-8 pro-inflammatory and chemotactic agents whilst the hip produced significantly higher levels of MMP13 (Figure 3.9).

Spontaneous TNF was found at significantly increased levels (P=0.026) in knee synovial membrane cultures (1649±496pg/ml) compared to those from the hip (379±103pg/ml). Likewise, IL-6 production in knee (11.5±2.7ng/ml) was significantly higher (P=0.0384) than those from hip cultures (5.7±0.8ng/ml) as was the average spontaneous IL-8 production from knee (4.9±0.9ng/ml) as opposed to hip (2.9±0.5ng/ml) (P=0.0428).

Spontaneous MMP production also showed significant differences between membrane cultures from knee and hip. There was no significant difference in the spontaneous production of MMP1 in hip (1469±98.75pg/ml) and knee cultures (1152± 106.2pg/ml) (P=0.09228). Similarly, no significant difference in spontaneous production of MMP3 between knee (2220±107.8pg/ml) and hip (2194+101) (P=0.8715). There was however, a significant difference in MMP13 production, with hip cultures producing significantly more MMP13 (374±88pg/ml) then knee (86.8±5.4pg/ml) (P=0.0313).

The increased presence of pro-inflammatory cytokines, especially TNF, is thought to be produced by infiltrating peripheral monocytes, which have entered the synovium through the progression of the disease.   As

demonstrated (Table 2.3) cellular composition of CD4$^+$ cells are marginally increased in knee OASFs primary isolated cultures giving some preliminary validation to this hypothesis which could be confirmed in future studies through the flow cytometric analysis of cultures separating by several cell markers.

There is however the other possibility that the spontaneity of the cytokine release from these cells may be due to possible endotoxin contamination. Whilst, unstimulated, the cells were incubated with complete media containing 10% FCS. The FCS used in the media preparation contains, according to manufacturer's guidelines ≤10EU/ml which dilutes down to ~1EU/ml in DMEM preparations (see methods). As both knee and hip cultures were exposed to the same FCS in these primary experiments and later TLR stimulations to class this production as truly spontaneous is difficult and is one of the limitations of the experimental design.

Synovitis is a common feature of OA and commonly observed in knee but not in the hip, demonstrating a potential dichotomy in disease groups (Scanzello and Goldring, 2012, Wenham and Conaghan, 2010). These observations suggest that OA in the knee is potentially more inflammatory, while MMPs such as MMP13 may be more involved in the pathogenesis of hip OA. The results further strengthen the heterogeneity of the disease in different joint locations however, more work should be performed before definitive conclusions can be drawn.

3.4.2 Investigations on the potential endotoxin contamination of collagenase used for isolation of synovial membranes

One limitation in studies of cytokine production by synovial membranes arises from the potential endotoxin contamination of the collagenase used for tissue dissociation. Therefore, several batches of collagenase used for isolation procedures were analysed by use of gelatification LAL-test. These batches were negative by the LAL assay (Table 3.1). However, we were unable to test all the batches used through this study. Therefore, the samples whose cytokine production is reported (Figure 3.9) were those prepared using the three batches of collagenase we could analyse.

Of course, there is always the possibility that, at least in some experiments, potential contamination with endotoxin, even though below the sensitivity limit of the LAL test used, might account for part of the spontaneous cytokine production. However, treating THP-1 monocytes with a collagenase suspension for 1hr did not induce TNF or IL-6 production above background levels (P=0.3956) (Figure 3.10).
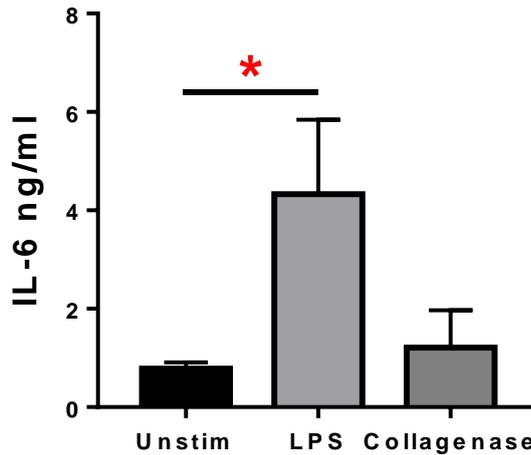
Figure 3.10: IL-6 production from THP-1 monocytes with collagenase preparations. THP-1 monocytes were plated, and then stimulated for 24hours with LPS (10ng/ml) or collagenase preparation (20mg/ml). IL-6 production was determined through ELISA. Data is mean ±SEM of 3 individual experiments. Un-paired student t-test, 2 –tailed *P <0.05.

The sensitivity of THP-1 monocytes are highly variable between laboratories concentrations of LPS needed for activation of TLR4 are higher than required to stimulate primary cells. In order to exclude the potential of endotoxin contamination, in a more sensitive cellular environment, the use of primary isolated monocytes could be done in the future.

EU (IU)/100μl			
	Test 1	Test 2	Test 3
Collagenase type I (20mg/ml)			
Batch 1	<0.06	<0.06	<0.06
Batch 2	<0.06	<0.06	<0.125
Collagenase type II (20mg/ml)			
Batch 1	<0.06	<0.06	<0.06
Batch 2	<0.06	<0.06	<0.06

Table 3.1: Endotoxin levels of 2 batches of collagenase type I and 2 batches of collagenase type II were analysed using gelatification LAL test kit (limit of detection 0.125EU). EU = Endotoxin Units, IU = International unit of endotoxin level.

3.5 Identification of active TLRs in synovial fibroblasts

Secretion of IL-6 following activation of different TLRs from synovial fibroblasts was performed using a panel of TLR ligands and concentrations. Pilot experiments (Figure 3.11) indicated that agonists for some TLRs (TLR5, TLR7, TLR8 and TLR9) did not induce cytokine secretion in the synovial fibroblasts. Flagellin stimulation of TLR5 stimulated IL-6 secretion in only one out of three patients tested and was therefore disregarded from further study to focus attention on the other more consistently active receptors. These observations are in agreement with the literature reporting that TLR7, TLR8 and TLR9 are not actively expressed in synovial fibroblasts (Ospelt et al., 2008, Hu et al., 2014), so were not measured in our project. Initially, induction of IL-6 was used to determine the concentration of TLR ligands for TLR1/2, TLR3, TLR4 and TLR2/6 to use in the study.
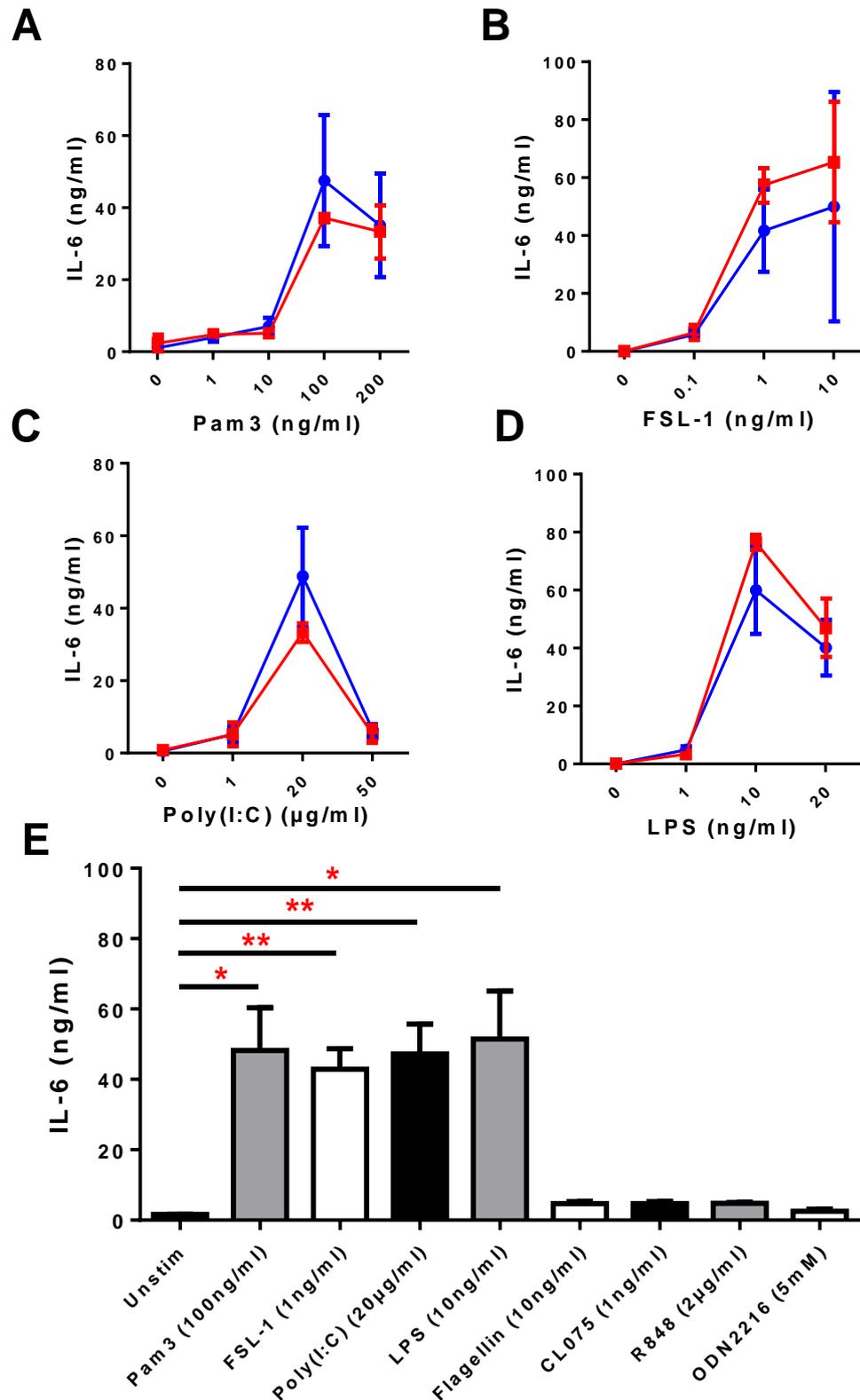
Figure 3.11: Titration of active TLR ligands in OA synovial fibroblasts.
Synovial fibroblasts at P3 were analysed for TLR activity through induction of IL-6. Several inactive TLRs were ruled out and the optimal concentration of ligand was determined fully through dilution screens. Data is mean ±SEM from 3 knee (Blue) and 2 hip (blue) donors performed in triplicate. Un-paired student t-test, 2 –tailed *P <0.05, ** P<0.01.

After established ligand concentrations were determined, TLR stimulation of P3 synovial fibroblasts were performed blindly after which IL-6 and IL-8 induction was determined through ELISA (Figure 3.12).

As TLR2 can heterodimerise with both TLR1 and TLR6, two individual ligands were used: Pam3, a triacylated lipopeptide (agonist of TLR1/2) and FSL-1, a diacylated lipopeptide (TLR2/6). Optimal concentrations of Pam3 (100ng/ml), FSL-1 (1ng/ml), Poly(I:C) (20µg/ml) and LPS (10ng/ml) were used for stimulation of synovial fibroblasts in subsequent experiments.
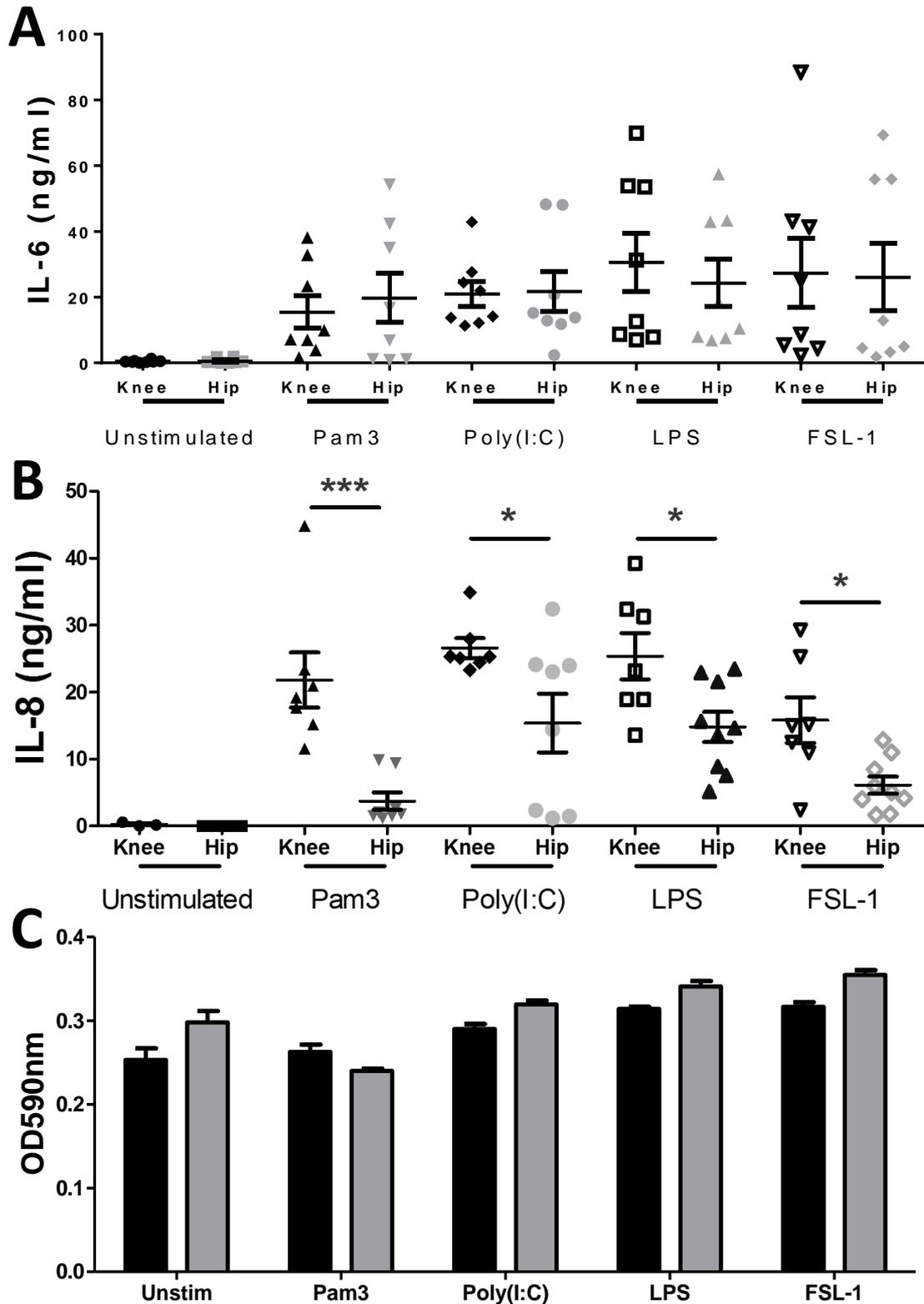
Figure 3.12: Osteoarthritic synovial fibroblasts from TKR and THR differ in response to TLR ligands. OASFs stimulated production of **A)** IL-6 and **B)** IL-8 through incubation with 100ng/ml Pam3sK, 20µg/ml Poly(I:C), 10ng/ml LPS or 1ng/ml FSL-1for 24 hours. Data is shown as mean ±SEM from 16 separate experiments of 8 knee OASFs (Black) and 8 hip OASF (Grey) cultures. **C)** Representative MTT. Un-paired student t-test, 2 –tailed *P <0.05, *** P<0.001.

TLR-induced IL-6 levels were similar between hip and knee OASF groups whilst IL-8 production was significantly higher in knee than in hip stimulated with any TLR agonist used (Figure 3.12, A-B).

The difference in Pam3 induced production of IL-6 was not statistically significant in hip (22.48±8.1ng/ml) compared to knee (14.3±5.6ng/ml) (P=0.8048). However, Pam3-induced IL-8 production was significantly higher (P=0.0007) in knee OASFs (21.8±4.1ng/ml) than hip (3.7±1.3ng/ml).

Poly(I:C)-induced IL-6 secretion was similar between groups with knee (20.6±4.4ng/ml) and hip (24.4±6.2ng/ml) (P=0.7104), however, induced IL-8 production was significantly increased (P=0.0386) in knee OASFs (26.6±1.5ng/ml) than in hip (15.4±4.4ng/ml).

LPS-induced IL-6 production was not significantly altered between groups (P=0.9015), IL-8 production was again significantly increased (P=0.0191) in knee OASF cultures (25.3±3.5ng/ml) compared with hip (14.8±2.3ng/ml).

FSL-1-induced IL-6 secretion from knee OASFs (27.7±12.1ng/ml) (29.5±11.1ng/ml) was not significantly different from hip (P=0.8048). However, once again, IL-8 secretion, was significant increased (P=0.0111) in knee (15.8±3.4ng/ml) compared with hip (6.1±1.3ng/ml).

None of the TLR agonists tested was toxic in our experimental condition as measured by the MTT cell viability assay (Figure 3.12, C). Suggesting the result was not due to toxicity issues but rather through an increased, TLR induced, IL-8 secretion from knee synovial fibroblast cultures.

3.7   TLRs expressed by synovial fibroblasts may attribute to OA pathogenesis

The purpose of these experiments was to compare functional TLR IL-6 and IL-8 secretion from synovial fibroblasts of the OA joint. Spontaneous cytokine production from membrane cultures demonstrated that IL-6 and IL-8 secretion was linked with the higher inflammatory environment of the knee. An increase in chemotaxis, brought about through increased TLR-mediated IL-8 induction in knee OASFs could indicate a contributing factor to disease progression through the migration of peripheral cells into the joint; these infiltrating cells could then be responsible for secretion of TNF. DAMPs/PAMPs present within the joint can therefore contribute to disease pathology in different manners, by stimulating a variety of cell types including synovial fibroblasts and infiltrating monocytes, macrophages or neutrophils. TLR activity of monocytes and macrophages has been demonstrated as a major contributor to inflammatory joint disease progression, however, less is known about the involvement of the synovial fibroblasts (Durand et al., 2013).

As TLR2 forms heterodimers with TLR1 or TLR6, it was important to investigate differences in their roles in OASF cytokine production. Therefore, the use of TLR1/2 and TLR2/6 specific agonists PAM3 and FSL-1 allowed for specific activation of each receptor pairing allowing for comparison. There was no significant difference in the expression of TLR1 or TLR6 at the mRNA (qPCR) or protein level (flow cytometry). However, significant differences in IL-8 induction by either Pam3 or FSL-1 between hip and knee suggest other mechanisms that may be regulating production of IL-8. These findings require

further validation and investigation as to what is very different between these cell types but provide a good basis for analysis.

The only significant difference in the expression levels of tested TLRs in knee compared with hip was that of TLR2 and TLR3. TLR2 was significantly increased in expression at the message level, whilst protein expression was unchanged knee OASFs confirmed by flow. However, the TLR$^+$ population of OASFs was significantly increased in hip OASFs compared with knee. TLR3 receptor was significantly increased at the protein level in knee OASFs, but analysis demonstrated a significantly increased TLR3$^+$ population in hip OASFs. This difference in expression resulted in a reduced response to TLR2 and TLR3 ligands and subsequent IL-8 production from hip OASFs. However, the difference did not affect IL-6 induction, suggesting the presence of other mechanisms regulating its production as mentioned previously in the case of IL-8.

In OA, inflammation associated with the disease is milder than that of classical inflammatory arthrides such as RA (Berenbaum, 2013). However, potent pro-inflammatory cytokines such as TNF and IL-6 as well as chemotactic agents such as IL-8, are present and inducible. They potentially play a larger part in disease activity than originally thought confounded by results demonstrating the correlation of these specific cytokines with disease activity and their ability to promote an active disease state in models of OA. (Maksymowych et al., 2012, Stannus et al., 2010, Kaneko et al., 2000, Takahashi et al., 2015).

Spontaneous cytokine production from RA and OA cultures has previously demonstrated the potential for OA to be a TNF-α-driven disease with

comparable levels of secretion (Brennan et al., 1989). Whilst later attributed to unknown endotoxin presence, my data supports the findings and potential importance for TNF in OA pathogenesis with sufficient exclusion of endotoxin contamination. Due to the lack of efficacy of anti-TNF studies in therapeutic treatment of OA anti-TNF therapy is not used in treatment (Grunke and Schulze-Koops, 2006, Guler-Yuksel et al., 2010). However, there are still clinical trials ongoing exploring the benefits of subcutaneous and intra-articular injection of etanercept (soluble TNF receptor) after promising results of the effect of intra-articular injection for alleviating OA associated pathology, limited only by its reduced bioavailability and quick clearance from the joint. (Clinical trial numbers: NCT02722772, NCT02722811).

This study shows that there is a significant difference in cytokine induction dependent on the joint locations tested. One should bear in mind, however, that a limitation of our study was the relatively low patient number (n=16; 8 knee-affected and 8 hip-affected patients) as well as the fact that we only looked at two type of joints. If validated in a larger cohort, this opens an avenue for designing clinical trials in selected patient subgroups discriminating based on joint location.

3.8 <u>Summary</u>

- Knee synovial membrane isolates produce significantly more IL-6, IL-8 and TNF compared to hip membrane cultures, which subsequently, produce significantly more MMP13.

- TLR2 mRNA is significantly increased in knee compared with hip OASFs.

- TLR3 and TLR4 protein expression is significantly increased in knee OASFs compared to hip, although hip has a higher percentage of TLR3$^+$ TLR4$^+$ cells.

- TLR mRNA expression does not correlate with protein expression in matched patient OASF samples.

- TLR1 and TLR6 protein expression positively correlates with percentage expressing cells in OASFs.

- TLR3 and TLR4 protein expression negatively correlates with percentage expressing cells in OASFs.

- OASFs from knee produce significantly more TLR induced IL-8 than those derived from hip.

Several differences between knee and hip OASFs in their TLR expression and activation were discovered. Foremost amongst these differences was significantly elevated production of inflammatory cytokines; IL-6, IL-8 and TNF by knee OASF synovial membrane cultures (Figure 3.9). Levels of MMP13 were increased in synovial membrane cultures isolated from hip suggesting a more degenerative environment. I also discovered that TLR-induced IL-8

secretion from knee OASFs was markedly increased over their hip counterparts. IL-6 induction was similar in the populations.

These data demonstrated that TLR expression in OASFs is not uniform amongst joint locations and variations in joint architecture and cellular composition may contribute to disease pathology differently in individual, depending on the afflicted joint.

Chapter 4 - Expression and activation of TLRs in chondrocytes from degenerated cartilage

Hypothesis: TLR expression is altered in chondrocytes from regions of OA progression. This contributes to an increased production of pro-inflammatory cytokines and degenerative enzymes in chondrocytes, thereby driving disease development.

4.1 Introduction

Isolated from the extra-cellular environment by a barrier of highly dense extracellular matrix, the chondrocytes maintain the cartilage through regulation of matrix turnover (Muir, 1995). Chondrocytes originate from mesenchymal stem cells and contribute less than 10% of the total volume of articular cartilage (Cohen et al., 1998). The majority of the volume is made up from synovial tissue fluid with the dry weight being accounted for by various forms of collagens and proteoglycans. During the progression of OA this dense matrix is degraded exposing the chondrocytes to a variety of foreign stimuli, including growth factors, hydrostatic pressures, altered oxygen content, PAMPs and DAMPs (Bush and Hall, 2003, Hooiveld et al., 2003, Notoya et al., 2000). De-differentiation of chondrocytes to cells of a more fibroblastic nature is common when attempting culture in a 2D *in vitro* environment. It was theorised that this effect may also occur *in vivo* with detrimental effects caused by cellular expansion and invasion as demonstrated through increased vascularisation of cartilage (Schnabel et al., 2002). The effect of this change in cell morphology *in vitro* would also be analysed in tandem with the expression and activation

of TLRs from regions of cartilage affected to different levels by the progression of OA. These regions were characterised into non-degenerate (grade 0), early stage (grade 1) and late-stage OA (grade 2).

4.2 TLR mRNA expression by chondrocytes is significantly altered through disease progression

Macroscopically graded articular cartilage was surgically isolated from the femoral head of the hip or the tibial and fibula plateaus of the knee. After being snap frozen in liquid nitrogen total RNA was isolated from the resident cells and analysed for TLR expression by qPCR (Figure 4.1).

It should be noted that, unlike what we did previously with synovial fibroblasts, we did not distinguish between those of knee or hip origin. There were two reasons for this. The first was that in a majority of hip cases, the disease was severely advanced and it was difficult to obtain sufficient cartilage for analysis. The second, was that the purpose of this investigation was not to compare different anatomical locations but rather to characterise, within the same patient, regions of cartilage at different stages of degradation: no sign of OA (grade 0) early OA (grade 1) and end-stage OA (grade 2). Therefore, for a vast majority of experiments documented in this chapter, only chondrocytes isolated from knee joints were used.
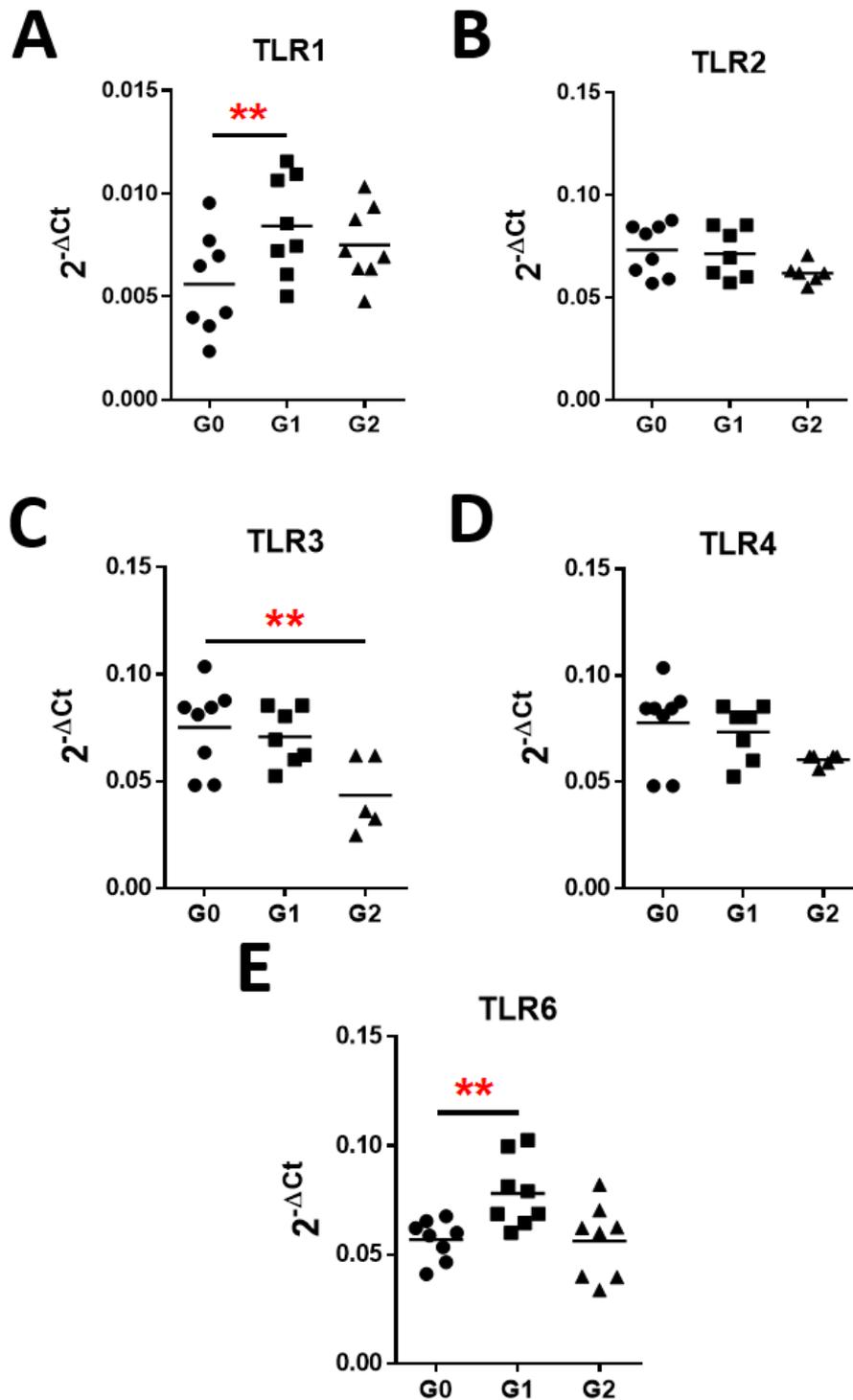
Figure 4.1: Chondrocyte TLR mRNA expression alters significantly through the progression of OA. Macroscopically graded cartilage (G0=no sign of OA, G1=signs of early OA, G2=end-stage OA) were isolated from the joint, snap frozen in liquid nitrogen and cryo-pulverised for RNA extraction. These samples were then analysed for: **A)** TLR1, **B)** TLR2, **C)** TLR3, **D)** TLR4 and **E)** TLR6 mRNA. Expression represented as the 2-ΔCt with the two housekeeping genes (HPRT1 and GAPDH). **A-E)** Data is of 8 patient samples (7 Knee 1 Hip) **B-D)** Data is from 8 samples (7 Knee, 1 Hip). ** P<0.01 paired student t-test, 2-tailed.

4.2.1 TLR mRNA is altered through progression of OA

In an attempt to observe TLR expression as close to an *in vivo* environment as possible RNA extraction was performed on cryo-preserved patient samples. Analysis of TLRs present within the cartilage demonstrated that TLR1 was significantly increased in G1 cartilage (P=0.0012) along with TLR6 relative to G0 (P=0.0224). TLR3 however, was significantly reduced in expression in G2 cartilage relative to G0 (P=0.0025). No significant differences were observed between G0, G1 or G2 cartilage for TLR2 or TLR4.

4.3 TLR2 and TLR4 expression of *in situ* cartilage chondrocytes (immunofluorescence)

The organisation of chondrocytes within normal articular cartilage is divided into superficial, transitional and deep zones (Buckwalter and Mankin, 1998). Cells are visually compartmentalised into these regions dependent on cell orientation (Figure 1.5). Superficial chondrocytes appear to lie on their side whilst transitional cells are semi-ellipsoidal. Deep zone chondrocytes however, appear in columnar like structures and border the calcified cartilage region before the bone itself.

Ironically, during the progression of OA and associated matrix degradation, features of cartilage development are noted. In regions of OA-related degradation it was commonly noted that cells had undergone clonal expansion forming multinucleated or multicellular pockets of chondrocytes. Unfortunately, these expansion events were sometimes within regions of interest and, as the ECM degrades, the recognisable stratified zones become blurred or in fact lost

entirely in agreement with findings as described by others (Pritzker et al., 2006). This can be seen in Figure 4.2 A-C, where the zones become less easy to distinguish thereby making it appear that the transitional and deep zones combine. To determine whether the different zones of the cartilage possessed different TLR expression, analysis of TLR2, TLR3 and TLR4 location within the chondrocytes of the cartilage was undertaken (Figure 4.2 and 4.3).
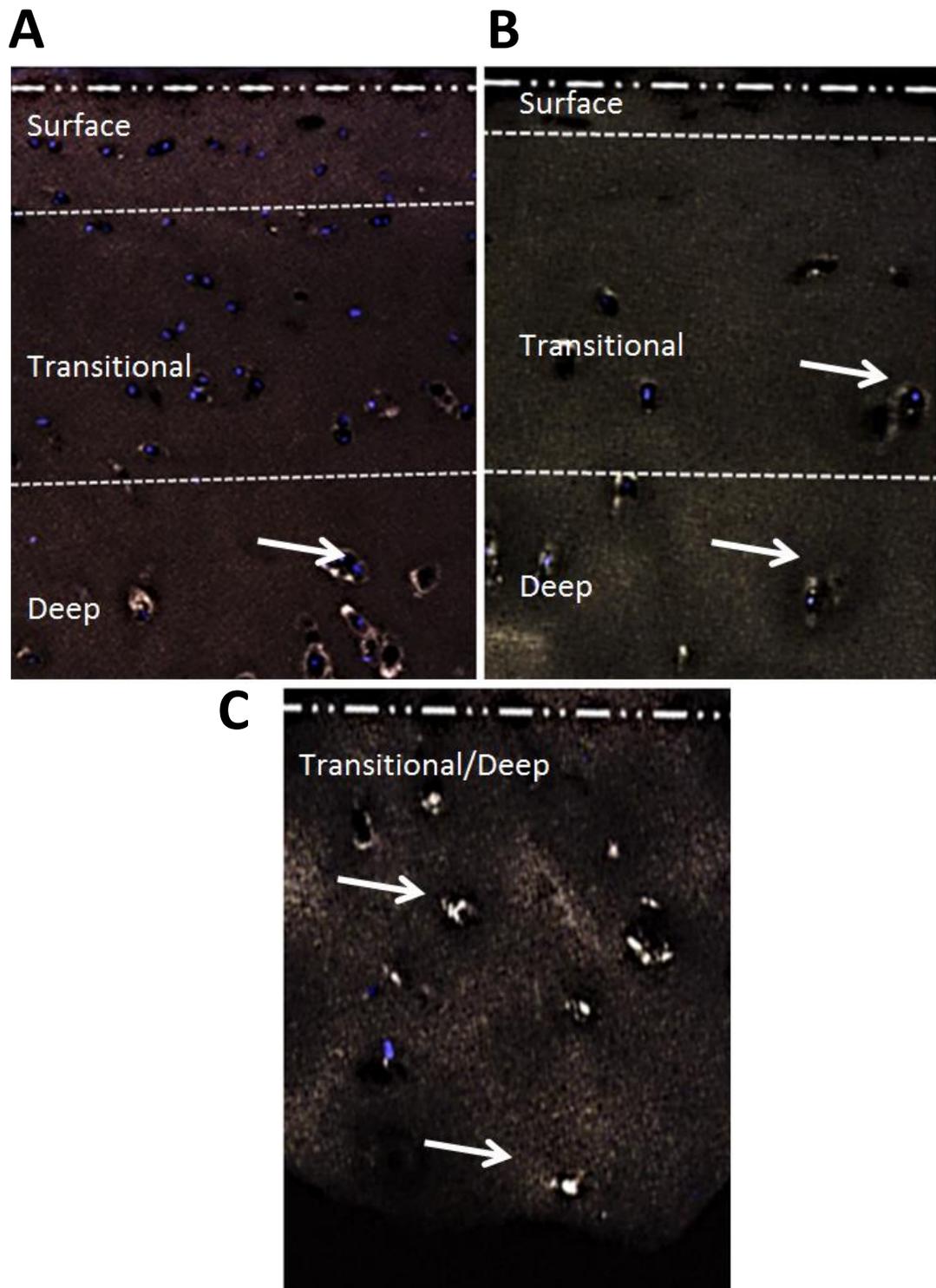
Figure 4.2: TLR2 and TLR4 in articular cartilage are predominantly co-expressed in chondrocytes of the deep zone. TLR2 and TLR4 immuno-staining of **A)** Grade 0, **B)** Grade 1and **C)** Grade 2 regions of articular cartilage from a representative patient. Superficial, transitional and deep zones of chondrocyte locations are marked with progressive loss of superficial and transitional zone through OA progression. Magnification **A-C)** x200. Image is a representative example of 3 separate patients. Arrows indicate examples of TLR2 and TLR4 co-expressing cells (yellow). Scale bar = 100μm.
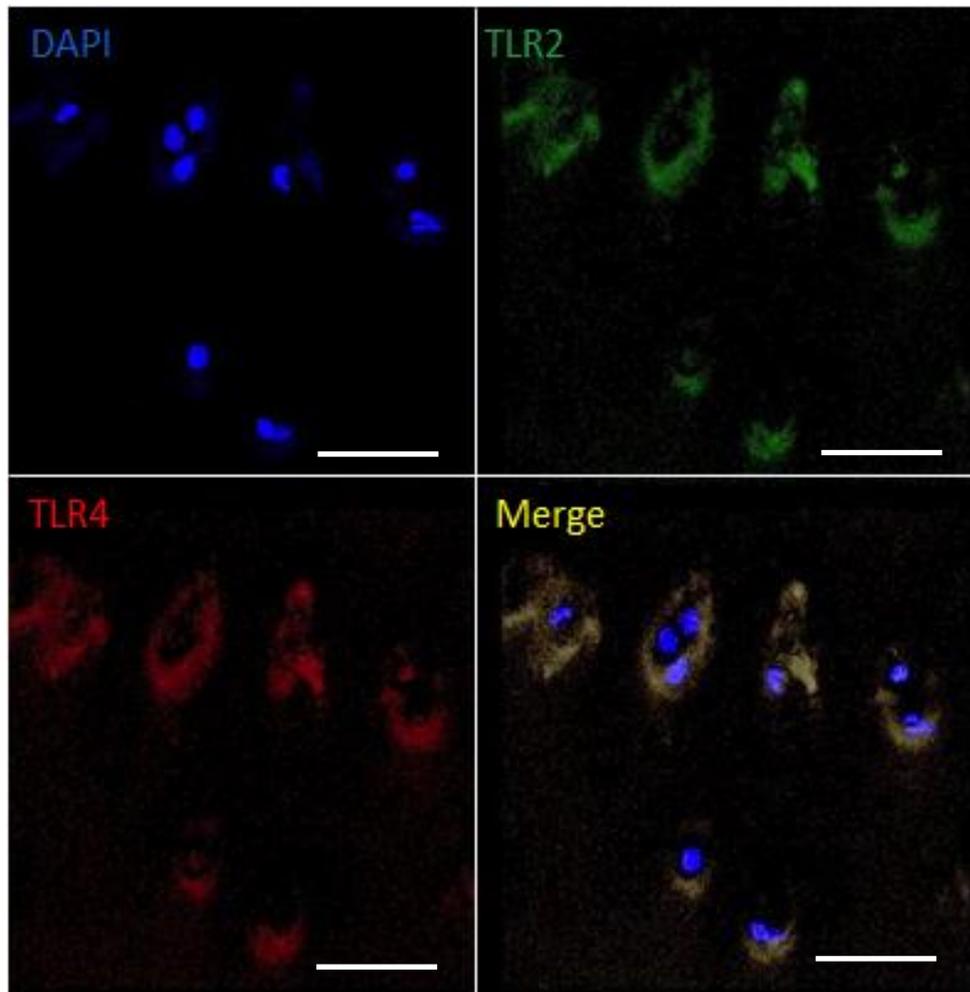
Figure 4.3: TLR2 and TLR4 co-expression in chondrocytes. TLR2 and TLR4 immuno-staining of Grade 0 cartilage from a representative patient. Magnification **=** x630. Image is a representative example of 3 separate patients. TLR2 – Alexa488 (Green), TLR4 – PE (red), co-expressing cells (yellow). Scale bar = 100µm. Blue (DAPI staining).

4.3.1 Expression of TLR2 and TLR4 is localised to the deep zone of cartilage.

TLR2 and TLR4 were observed to be primarily expressed at a much greater occurrence in the deep zone cartilage chondrocytes close to the calcified regions and bone plate (Figure 4.2). However, sub-stratification of the cartilage into these regions became difficult due to the degradation of the cartilage matrix. Because of this process, the superficial region was indistinguishable and in collaboration with clonal expansion of chondrocytes meant distinguishing between zones was difficult but not impossible.

Due to the known activation of TLR2 and TLR4 through DAMPs generated through ECM particulates, it would make sense to shield those cells, which if activated, are known to generate sterile inflammation through components of the ECM such as hyaluronan and fibronectin (Gondokaryono et al., 2007, Okamura et al., 2001). Activation of these receptors is known to trigger MMP induction from chondrocytes which would trigger the remodelling of the joint (Buckwalter and Mankin, 1998). The production and removal of ECM components is balanced to prevent joint destruction or overgrowth. However, the chronic inflammation associated with the disease, could act as a trigger for early OA. Exposure of these TLR2/4$^{+/+}$ cells, to synovial fluid DAMPs produced through mechanical trauma, could theoretically trigger joint remodelling which, if imbalanced, might lead to joint destruction.

4.4 TLR3 expression in chondrocyte changes during OA progression

TLR3 is documented to be expressed with the endosome of cells, as activation of the receptor requires the acidic environment (Chaturvedi and Pierce, 2009). My previous data demonstrated that this was indeed the case with expression in OASFs (Figure 3.4). However, to our current knowledge this has been unexplored in chondrocytes. Therefore, macroscopically graded cartilage from intact regions of cartilage (grade 0), early evidence of OA (grade 1) and severely degraded (grade 2) were analysed (Figure 4.4).
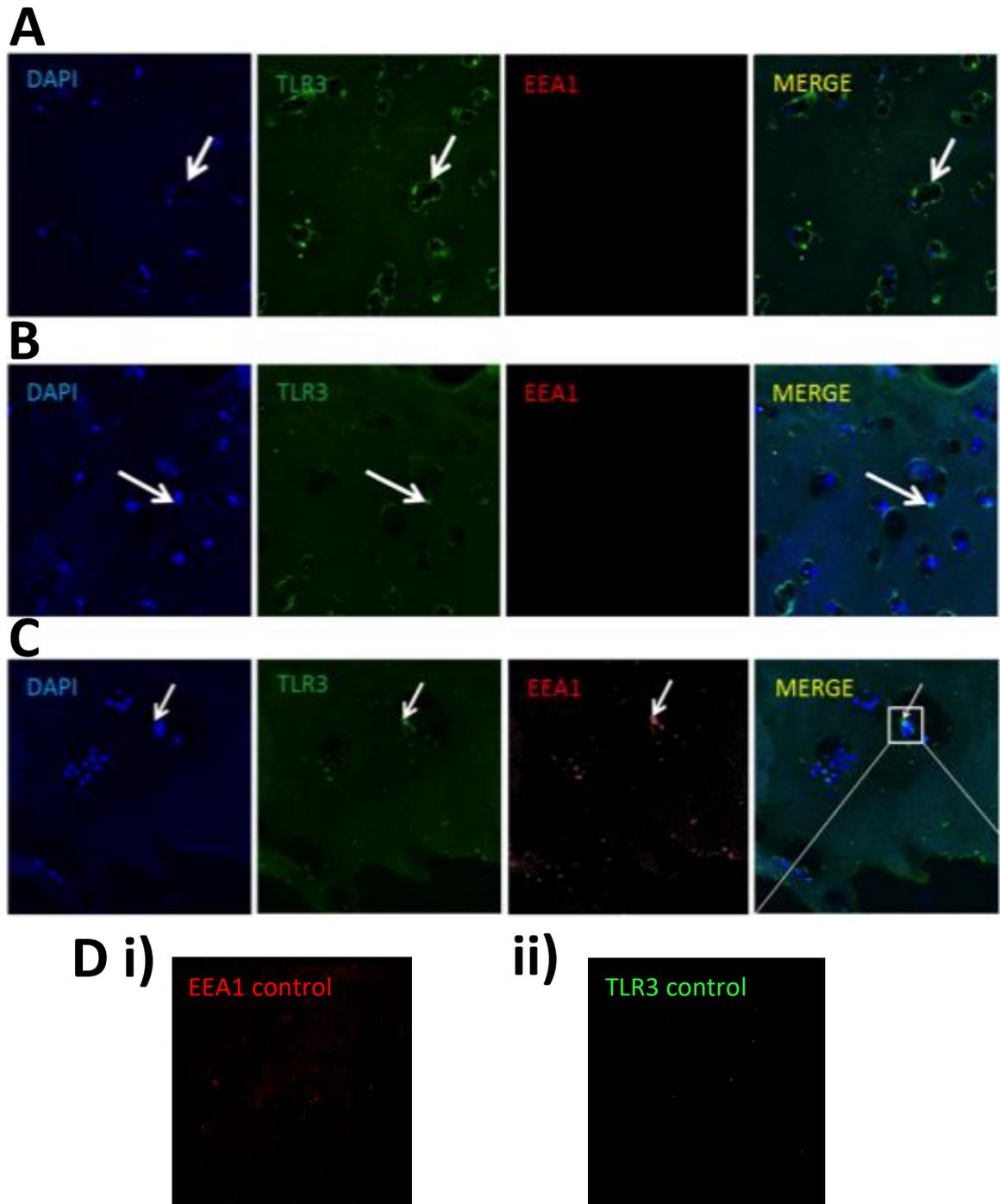
Figure 4.4: Human articular chondrocytes show altered cellular localisation of TLR3 in degenerate cartilage. Sectioned 10µm cartilage slices were stained for fluorescent confocal microscopic analysis of **A)** Grade 0, **B)** Grade 1 and **C)** Grade 2 OA cartilage. **D)** Negative controls examples from sample **A) i)** TLR3 and **ii)** EEA1. (Magnification x200). Images are representative of 3 biological donors. Arrows indicate example regions for TLR3 (green), EEA1 (red) and DAPI (Blue) staining. MERGE is overlay of TLR3 and DAPI staining. Scale bar = 100µm.

4.4.1 TLR3 translocation in G2 chondrocytes

TLR3 is predominantly bound within the endosome, requiring the acidification of the endo-lysosome to trigger cathepsin cleavage of the receptor for activity (Garcia-Cattaneo et al., 2012). This data demonstrates that TLR3 localises to two separate locations in chondrocytes. In Grade 0 "healthy" cartilage, unaffected by the progression of OA, TLR3 stained heavily to the cell surface whilst in G2 "degenerate" chondrocytes TLR3 localised to the endosome, as depicted by co-localisation with EEA1 (Figure 4.4, C). Whilst this is uncommon, translocation of TLR3 has been documented to occur with overexpression of trafficking proteins, such as UNC93B1 (Pohar et al., 2013, Tatematsu et al., 2015). Cell surface expression of active TLR3 has also been reported in human skin fibroblasts (Matsumoto et al., 2002) and this, along with our observation, could suggest that TLR3 is bioactive on both the cell surface and inside the cell, a hypothesis tested later in this thesis. TLR3 was expressed by all chondrocytes and was independent of joint strata unlike TLR2 and TLR4 expression mentioned earlier (Figure 4.3).

4.5   TLR activation in primary isolated chondrocytes from regions of OA degeneration

The progression of OA is seen to be a multivariate attack with the disease progressing from the outside-in with stimulation from the joint but also from the inside-out (Aigner et al., 2004). The latter theory attributes the cartilage degradation associated with the disease to the resident cells of the cartilage, the chondrocytes (Goldring, 2012).

Chondrocyte hypertrophy is a marker of osteoarthritis progression and pathology. Alterations in chondrocyte behaviour due to hypertrophy have been demonstrated to include a reduction in type II collagen production and increased MMP13 secretion (van der Kraan and van den Berg, 2012). TLR-induced IL-6, IL-8, MMP1, MMP3 and MMP13 secretion from G0, G1 and G2 isolated chondrocytes was therefore measured to ascertain how degrading chondrocytes could be contributing to disease pathology (Figure 4.5).
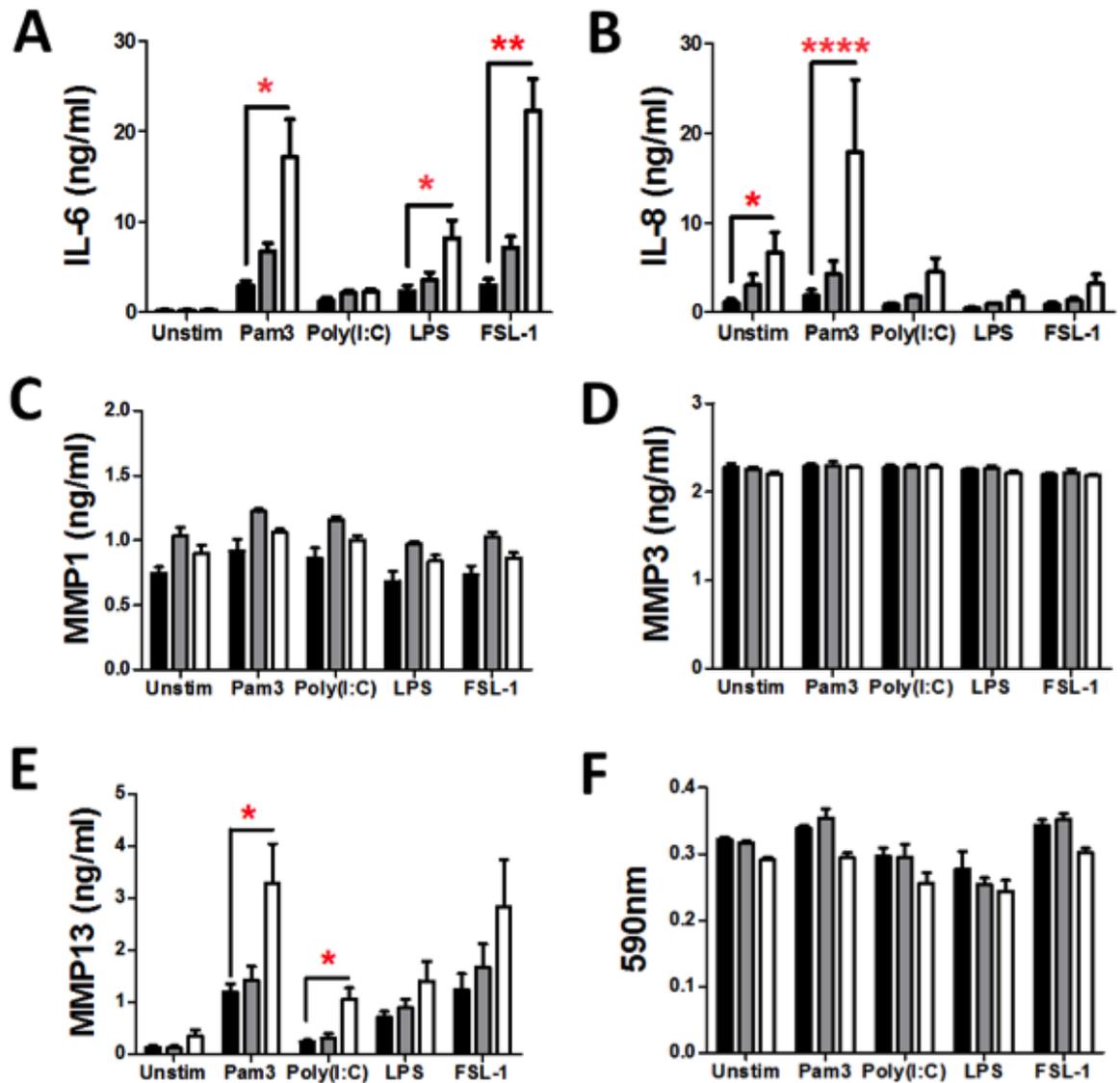
Figure 4.5: Chondrocytes isolated from severely degenerated regions of cartilage produce higher levels IL-6, IL-8 and MMP3 or MMP13 under TLR stimulation. Primary isolated chondrocytes from macroscopically graded cartilage were stimulated for 24 hours with either 100ng/ml Pam3, 20µg/ml Poly(I:C), 10ng/ml LPS, 1ng/ml FSL-1 or media alone (unstim). Supernatants were assayed by ELISA for **A)** IL-6, **B)** IL-8, **C)** MMP1, **D)** MMP3 or **E)** MMP13. Data are the mean ± SEM of samples from 5 individual patients assayed in triplicate. * P<0.05, ** P <0.01, **** P <0.0001 2-way repeated measures Anova. **F)** MTT cell viability assay in one representative patient. **Solid black** (grade 0) **Grey** (grade 1) **White** (grade 2)

4.5.1 Increased TLR activity in chondrocytes isolated from degenerate cartilage.

Chondrocyte hypertrophy is one of the defining characteristics of OA. During hypertrophy, reduced production of matrix proteins and increased collagenase activity are concurrent events (van der Kraan and van den Berg, 2012).

Many studies have demonstrated active TLRs in chondrocytes, however many use cultured cells that do not appropriately represent cells *in vivo* (Kim et al., 2006, Sillat et al., 2013, Bobacz et al., 2007). Therefore, cells were immediately stimulated after isolation from cartilage rather than grow the sample to a larger volume. It is for this reason that patient replicates were limited as yield from a patient varied greatly and allowed for only a select few experiments.

The data shown in Figure 4.5, demonstrated spontaneous cytokine production from unstimulated cells and furthermore, an increased level of IL-8 from cells isolated from regions of degeneration (G0 = 1.057ng/ml, G2 = 6.645ng/ml) (P=0.0365). Likewise, MMP13 secretion was higher in areas of degradation (G0 = 0.2146ng/ml, G2 = 0.6136ng/ml) (P=0.0096).

Both Pam3- and FSL-1-induced IL-6 production were significantly increased in G2 chondrocytes compared to G0 stimulated cells from the same joint (P=0.0151 and 0.0242 respectively). LPS-induced IL-6 secretion was also increased significantly (P=0.0205). IL-8 production was increased in G2 chondrocyte isolates (unstimulated P=0.0365) but also significantly so under Pam3 stimulation (P<0.0001). No variation in TLR induced MMP1 or MMP3 production was noted (Figure 4.5, C-D). However, MMP13 secretion from G2

chondrocytes was significantly increased by Pam3 (P=0.0141) and Poly(I:C) (P<0.01) compared to G0 isolates. There was no significant difference in LPS- and FSL-1-induced MMP13 secretion by different samples although there was a trend toward an increase.

4.7 Collagen ratio analysis of cartilage explants.

Autologous chondrocyte implantation is a widely-used treatment for large defects of the joint without pre-existing OA and/or any other complications (Minas et al., 2010, Ruano-Ravina and Jato Diaz, 2006). Efficacy of the treatment, however, is highly variable with surgical intervention commonly resulting in short to mid-term relief for patients ultimately resulting in the need for further treatment. The technique was hoped to be used in the treatment of OA, through replenishing degraded cartilage, although current trials have been widely ineffective. This is due to a feature of the technique by which isolated cells from donors are expanded *in vitro*. Chondrocytic differentiation *in vitro* has been widely documented as a feature of monolayer chondrocyte expansion that has the unfortunate characteristic of producing mechanically inferior ECM, with high collagen type I content, which is unsuitable for implantation (Caron et al., 2012). During the de-differentiation process and cellular expansion, the chondrocytes become fibroblast-like cells, characterised by an increase in type I collagen and a decrease in production of cartilage specific markers such as type II, XI, IX collagens and aggrecan (Schnabel et al., 2002). The major purpose of the chondrocyte is to maintain the ECM by keeping a delicate balance of cartilage turnover. An imbalance in the regulation of these anabolic and catabolic factors could well play a critical

role in chondrocyte phenotype maintenance, while failure may result in chondrocyte de-differentiation. These catabolic factors, such as inflammatory cytokines and proteases, participate in cartilage degradation and are major factors in the progression of OA (Fernandes et al., 2002).
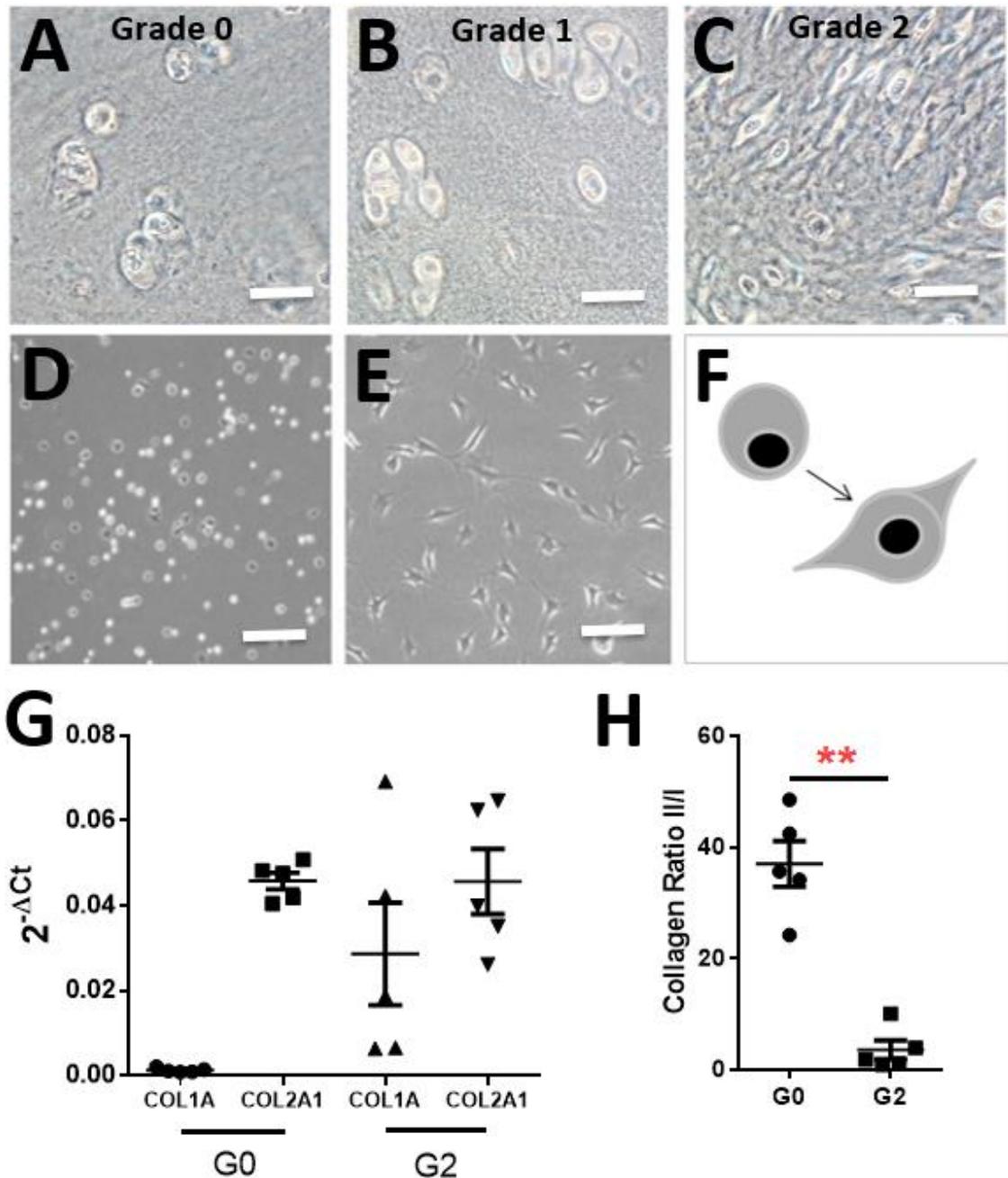
Figure 4.6: Chondrocytes from degenerate cartilage exhibit a more fibroblastic like phenotype as observed by Collagen type I and II ratio. Wide field light microscope images of **A)** grade 0 **B)** grade 1 **C)** grade 2 cartilage chondrocytes. **D)** grade 0 chondrocytes freshly isolated and **E)** cultured for 2 weeks. **F)** diagrammatical representation of morphological change in chondrocytes. **G)** qPCR analysis of Type I and Type II collagen expression in G0 and G2 cartilage explants. **H)** Collagen ratio represented as collagen type II production over collagen type I. Data in **G and H**) are the mean ± SEM from 5 independent donors. Photomicrographs are representative images from 3 donors. Magnification **A-C)** x400 **D and E)** x200. ** $P<0.05$ paired student t-test, 2-tailed. Scale bar **A-C)** ~50µm, **D-E)** ~200µm.

4.7.1 Chondrocytes are more fibroblastic in late stage OA cartilage

Chondrocytes from unaffected regions of cartilage express type II collagen mRNA at a much higher ratio than type I collagen (average ratio = 36:1) in agreement to what the literature describes (Marlovits et al., 2004). However, in regions of OA-associated cartilage degeneration the balance shifts with type I collagen production increasing significantly shifting the ratio to be type I dominant (average ratio = 4:1) (P=0.0042).

This data demonstrates a visual phenotypic change in chondrocytes accompanied by a shift in collagen production. Morphologically, chondrocytes were seen to be rounded and of sparse density in healthy cartilage regions (Figure 4.6, A), in agreement with published data (Costa Martinez et al., 2008). During disease progression evidence for chondrocyte clonal expansion can be seen in samples tested (Figure 4.6, B) as described elsewhere (Martin and Buckwalter, 2001). However, this phenomenon was much more prominent in the severely degenerate regions. Along with this expansion the cells appear morphologically altered looking more fibroblastic (Figure 4.6, C). This change was more prominent when isolated cells underwent de-differentiation when cultured *in vitro* (Figure 4.6, D and E) and correlates with published data (Caron et al., 2012).

This shift in production to type I collagen predisposes the existing cartilage to poor structural integrity and increased degradation through disease progression associated with several joint arthrides (Poole et al., 2002, Poole et al., 1995).

4.8   Chondrocyte involvement in OA pathogenesis.

- Chondrocyte expression of TLR mRNA alters across states of OA degeneration
- TLR3 receptor expression changes from the cell membrane to the endosome in late stage degeneration
- Grade 2 chondrocytes produce significantly more IL-6, IL-8 and MMP13 under TLR stimulation
- Chondrocytes appear to undergo a differentiation process characterised by the significant reduction in collagen type II expression

These data demonstrate that other resident cells of the joint, including chondrocytes, could contribute to the pathogenesis of OA through the secretion of inflammatory cytokines and metalloproteases. G2 chondrocytes were noted to resemble isolated chondrocytes which, when cultured in a 2D, undergo a de-differentiation process and take on a more fibroblastic nature (Figure 4.6, C) (Hong and Reddi, 2013, Caron et al., 2012). It appears that these cells are much more reactive to TLR stimulation, as evidenced by an increased cytokine and MMP13 production, key contributors to disease progression.

Chapter 5 – Effect of synovial fluid on TLR activity

Hypothesis: OA synovial fluid contains substances that will activate TLRs on synovial fibroblasts.

5.1   Introduction

Synovial fluid from healthy joints is a clear straw-coloured viscid that has the consistency of egg whites. It is a plasma dialysate which is suited for purpose by containing large quantities of proteins secreted by the joint tissues (Curtiss, 1964). The most notable difference between synovial fluid and any other fluid of the body is the high content of hyaluronic acid. The exact source of the hyaluronic acid has been subject for serious debate; however, thought to be produced mainly by fibroblasts from the synovial intima and structural cells from the membrane lining (Ogston and Stanier, 1953). As well as reducing the friction felt upon the joints for smooth articular movement, the synovial fluid acts as a nourishing media for the cells of the joint. This is important because chondrocytes, buried in the dense cartilaginous matrix, have very limited access to oxygen or nutrients. Therefore, synovial fluid acts as a nutrient delivery system and supplies such through the compression and relaxation of the cartilage which acts in similarity to that of a sponge.

When cartilage is damaged through trauma, or the progression of diseases such as OA, this usually clear fluid can become saturated with debris including matrix and necrotic cellular components. These fragments can then in turn act as endogenous DAMPs for PRRs, such as TLRs, thereby triggering sterile inflammatory responses (Brentano et al., 2005). This part of the project aimed

to investigate the relationship between synovial fluid and the response of the synovial fibroblasts, specifically in respect to TLR activation.

5.2 MMP1 is present at a significantly increased level in hip synovial fluid compared with knee

MMPs are a group of zinc dependent extracellular enzymes that play a key role in tissue remodelling and combined, have the ability to degrade all components of the ECM (Visse and Nagase, 2003). Specific focus was dedicated to the collagenases MMP1 and MMP3 and the stromelysin MMP3 that, in addition to its ability to degrade ECM components such as aggrecan, or collagen types III, IV, IX and X, can also cleave pro-forms of MMPs 1, 7, 8, 9 and 13 into their active states (Vincenti and Brinckerhoff, 2002, Okada et al., 1992). To measure MMPs within the synovial fluid of OA patients undergoing total joint replacement, samples were diluted, clarified by centrifugation and analysed by specific ELISA assays for total MMP1 and MMP3 (Figure 5.1, A). To further analyse differences in the precursor and mature forms of the proteins western blot analysis of patient matched samples were analysed (Figure 5.1, B).

Measurements of IL-6, IL-8, TNF and MMP13 was attempted in synovial fluid samples. However, due to the dilution factor could not all cytokines could be accurately determined in multiple patient samples due to assay sensitivity, those that could are shown in Figure 5.5.
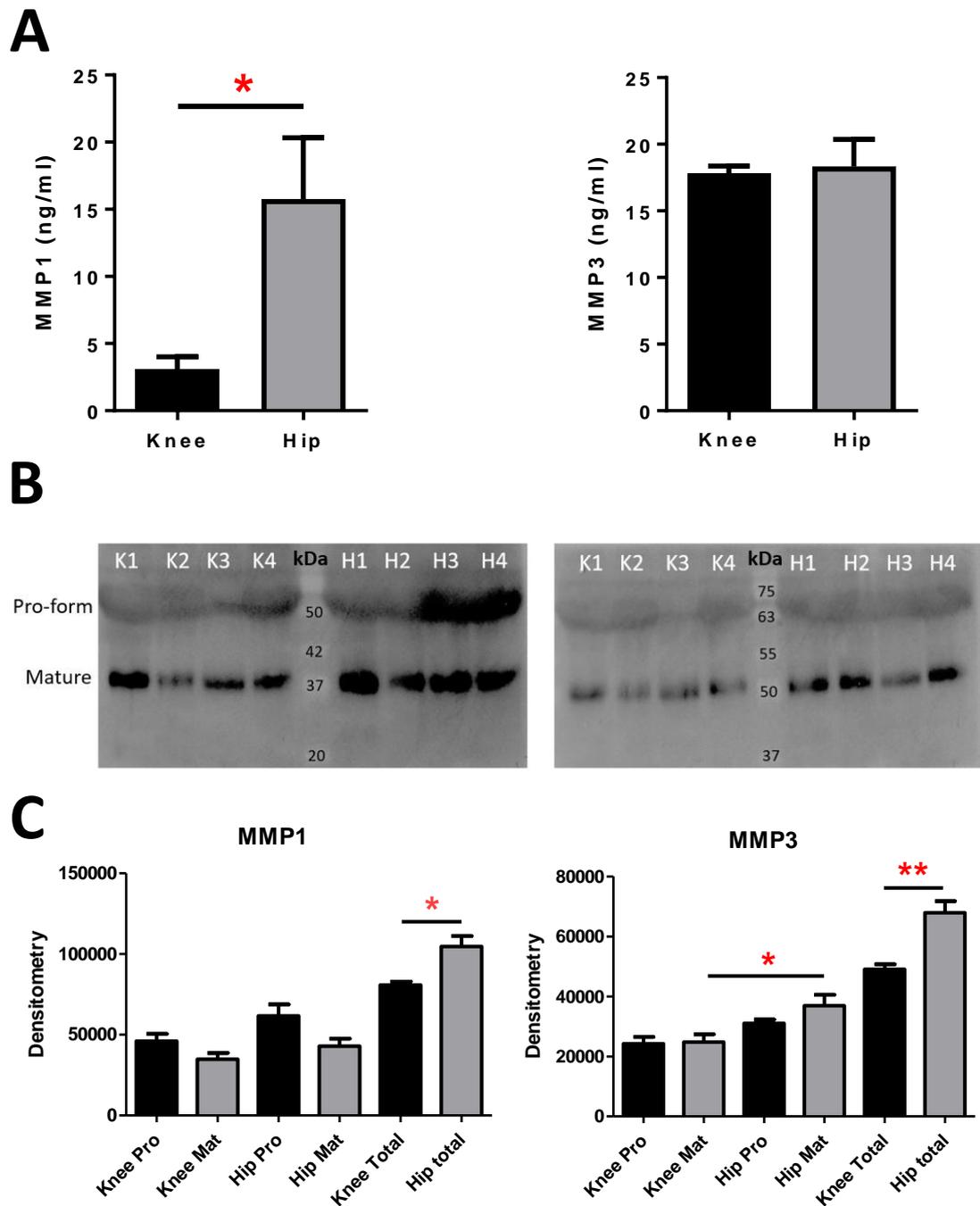
Figure 5.1: MMP1 is present at much higher levels in synovial fluid derived from hip OA joint replacements than knee. Synovial fluid from TKR and THR were diluted 1:8 in complete DMEM and centrifuged at 300g to remove debris. **A)** Samples were analysed for total MMP1 and MMP3 by ELISA. **B)** Western blot analysis of pro and mature forms of MMP1 and MMP3 and **C)** Densitometry analysis. Data is pooled (±SEM) from 4 knee (K1-4) and 4 hip donors (H1-4). *P <0.05 **P<0.01 unpaired student t-test, 2-tailed.

5.2.1 MMP1 and MMP3 presence in synovial fluid is significantly altered between the knee and hip

There were significantly higher levels of MMP1 in synovial fluid of the hip relative to the knee (P=0.0268) (Figure 5.1, A) while MMP3 levels were not different between joints (P=0.8107) (Figure 5.1, A). However, this technique did not allow for discrimination between the pro and mature form of the protein. We therefore performed a Western blot protein for both MMPs. Western blot, followed by densitometry analysis confirmed the significantly higher levels of MMP1 (P=0.0137) (Figure 5.1, B) in synovial fluid of the hip compared to the knee. In this case, Western blot analysis of MMP3 levels demonstrated to be significantly higher in hip synovial fluid (P=0.037) (Figure 5.1, C) in contrast to the results found in the ELISA.

Western blot analysis of MMP1 levels showed no discernible difference between the precursor and mature form of the protein in either the knee or hip synovial fluid (P=0.8995 and 0.0922 respectively). However, mature MMP3 presence was significantly increased in the synovial fluid of hip compared with knee (P=0.0412) (Figure 5.1, C).

5.3   OA synovial fluid interferes with endosomal TLR signalling in HEK293 cell lines

In order to study the effect of synovial fluid with a focus on TLR activation, HEK293 cells transfected to overexpress individual TLR were used. Synovial fluid contains matrix component DAMPs along as necrotic cell debris that can activate several TLRs including TLR2, TLR3 and TLR4 through matrix components such as fibronectin, hyaluronan and necrotic cell debris and host nucleic acids demonstrated elsewhere (Brentano et al., 2005). Investigation of the interaction of OA synovial fluid rich in theoretical DAMPs, and individual TLRs was therefore determined (Figure 5.2). The suppressive effect of synovial fluid addition on Poly(I:C) HEK293-TLR3 cells was further investigated to determine the effects time-course (Figure 5.3).
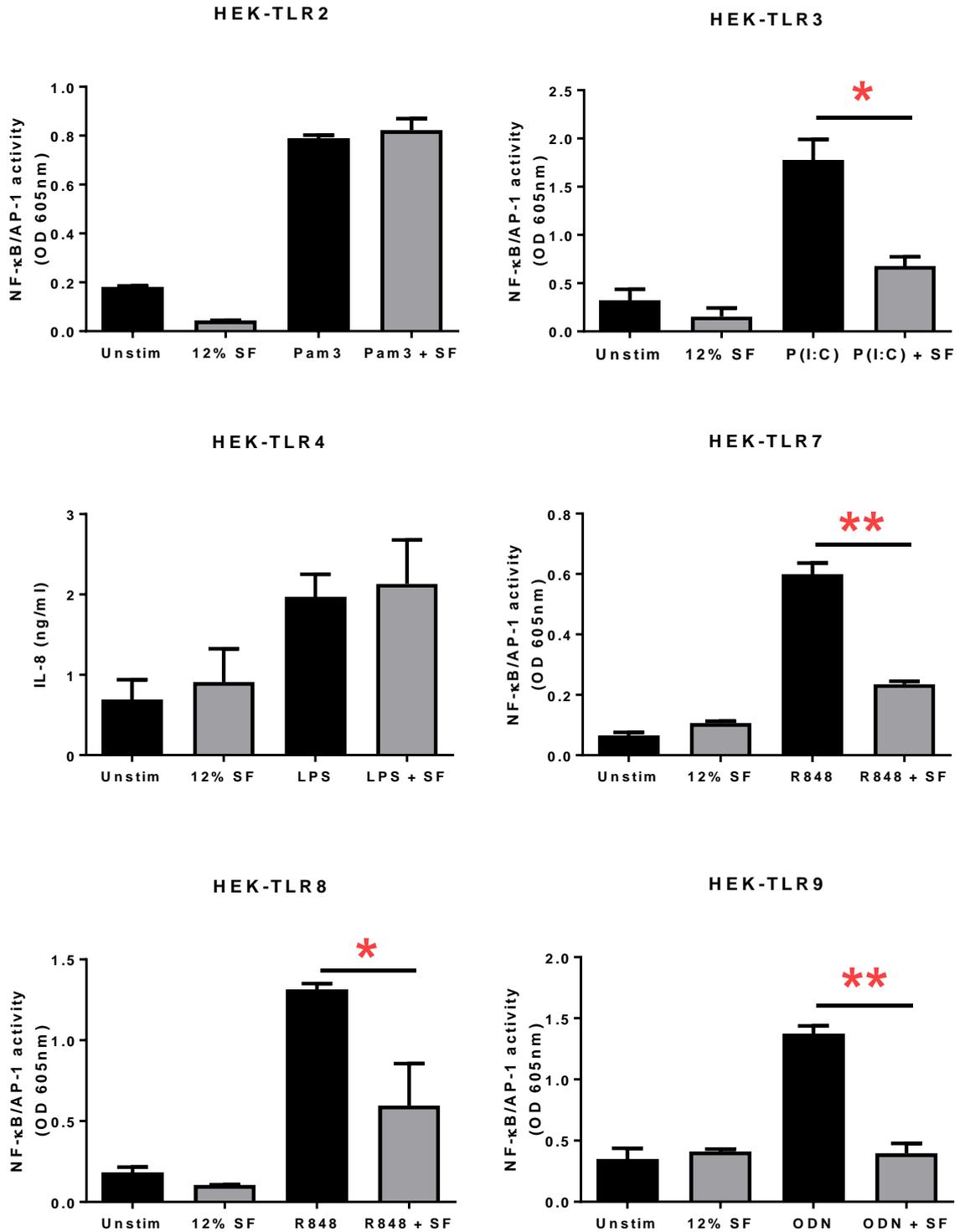
Figure 5.2: Osteoarthritic synovial fluid represses the activity of endosomal TLRs. Stably transformed HEK293 cell lines were stimulated with either: 100ng/ml Pam3, 20µg/ml Poly(I:C), 10ng/ml LPS, 2µg/ml R848 or 5µM ODN2216 with or without 12% osteoarthritic synovial fluid. Data is mean ±SEM of 3 unrelated knee synovial fluid (SF) donors performed as 3 separate experiments. Activation recorded via IL-8 ELISA or NF-kB/AP-1 SEAP reporter QuantiBlue assay (Invivogen) *P <0.05, **P<0.01 Paired student t-test 2-tailed.
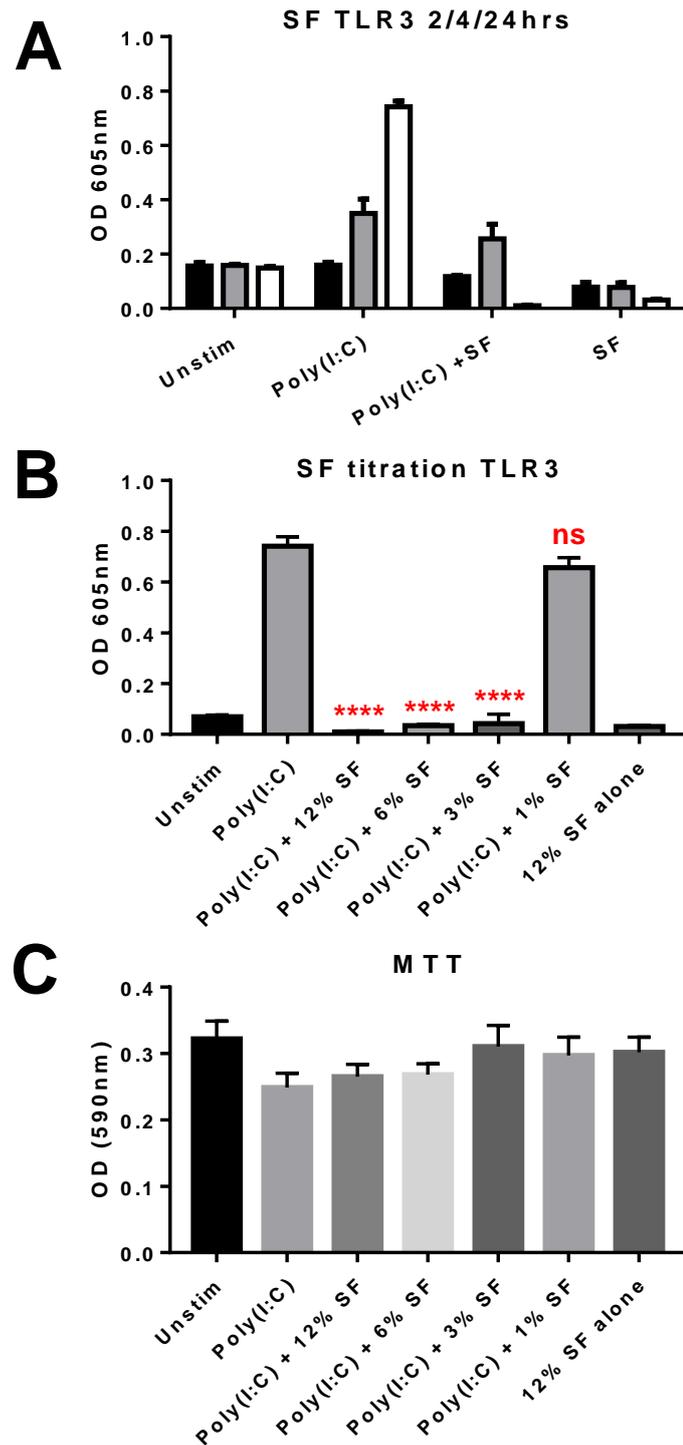
Figure 5.3: Synovial fluid inhibits NF-kB/AP-1 activation through TLR3.
HEK293-TLR3 cells were stimulated at for 24hrs with Poly(I:C) in addition to
or absent of diluted knee synovial fluid (12%, 6% or 3%) in complete DMEM.:
A) Poly(I:C) SEAP production could be measured within 4 hours and peaked
at 24hrs but was absent under the co-stimulation with 12% synovial fluid (SF).
B) Poly(I:C) induced SEAP production from the cells measured by QuantiBlue
at 12%, 6%, 3% and 1% synovial fluid dilutions. **C)** Representative MTT assay
for cell toxicity of experiment **B**. Data is mean ±SD of 3 synovial fluid (SF)
donors performed as 3 individual experiments in triplicate. Significance
determined against Poly(I:C) alone.

5.3.1 Synovial fluid inhibition of endosomal TLRs

To study the potential regulatory effect of synovial fluid from OA patients on TLR activity 12% v/v, knee derived, synovial fluid was added to HEK293 cells transfected to overexpress individual TLRs. Respective TLRs were then stimulated with their corresponding agonists and TLR activity was measured using the NF-kB/AP-1 SEAP reporter gene system. TLR4 activation was measured with IL-8 as the readout as the cells were additionally transfected with co-receptors CD14 and MD2. The average volume of synovial fluid is highly variable between individuals and joint type, and during this project varied between 1 and 12ml from knee, whilst hip isolates were usually less than 1ml (100-350µl). Therefore, for practical reasons, only knee synovial fluid was used in these experiments from 3 individual donors.

OA synovial fluid inhibited the activation of all tested endosomal TLRs in transformed HEK293 cell lines. TLR3 was markedly supressed (P=0.0248) as was TLR7 (P=0.0049) TLR8 (P=0.0485) and TLR9 (P=0.0048). There was neither observable activation of any TLR tested nor inhibition of TLR2 (P=0.4227) and TLR4 (P=0.4139) activation through Pam3 and LPS respectively. The time course experiment shown in Figure 5.3 shows the inhibitory effect of synovial fluid requires between 4-12 hours in order to supress activity, suggesting that the effect is long lived. Dilution of the synovial fluid demonstrated that the inhibitory effect of synovial fluid was still detectable at a 3% dilution suggesting the interfering agent was present at a high concentration although all effect was lost at 1% dilution (Figure 5.3, B). MTT

assays also demonstrated that the SF was not toxic to any of the cells tested (Figure 5.3, C).

5.4 OA synovial fluid supresses TLR3-induced cytokine production in OASFs

In order to determine whether the suppressive effect seen on endosomal TLR activity was just an artefact of the artificial system represented by transfected HEK293 cells primary cells were tested. Synovial fibroblasts from knee synovial membranes were stimulated with TLR ligands with or without OA synovial fluid from knee. Production of IL-6 and IL-8, measured by ELISA, was used as a marker for TLR activation (Figure 5.4). As previously determined, OASFs do not respond to TLR7/8 or TLR9 ligands. Therefore, TLR3 ligand Poly(I:C) and TLR4 agonist LPS were used.
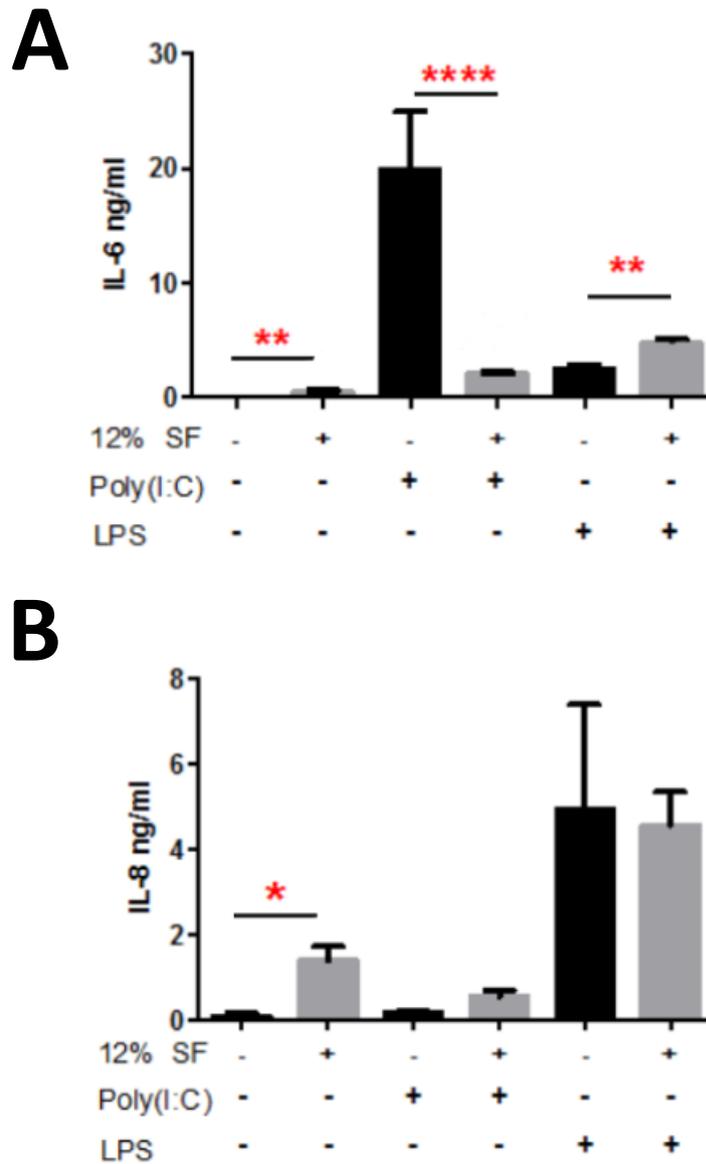
Figure 5.4: OA synovial fluid represses TLR3 induced cytokine production in human primary synovial fibroblasts. P3 harvested OASFs were co-stimulated with 12% synovial fluid and either 20µg/ml Poly(I:C) or 10ng/ml LPS for 24 hours. Supernatants were analysed using ELISA for **A)** IL-6 and **B)** IL-8 production. Data expressed mean ±SEM of 3 separate experiments of 3 individual donors, (3 knee OASFs). *P <0.05, **P<0.01, ****P<0.0001 Un-paired student t-test 2-tailed.

5.4.1   Synovial fluid inhibits TLR3 in primary OASFs

As shown in Figure 5.4, TLR3- but not TLR4-induced IL-6 production was significantly reduced through incubation with OA synovial fluid. The inhibition of TLR3 ($P<0.0001$) was significant however, TLR4 induced IL-6 production in primary synovial fibroblasts was predominantly unaffected (Figure 5.4, A) ($P=0.1175$). There was a significance increase in IL-6 secretion between LPS and LPS+SF treated cells however, this was not significantly different between the additive levels of IL-6 present within the SF (Figure 5.4, A and Figure 5.5).
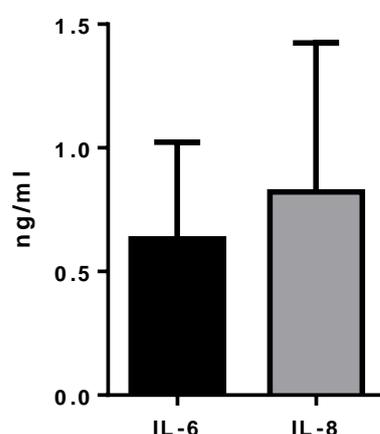
Figure 5.5: Concentrations of IL-6 and IL-8 in knee 12% synovial fluid isolates. IL-6 and IL-8 content of 12% synovial fluid was determined using ELISA. Data expressed mean ±SEM of 3 separate experiments of 3 individual donors, (3 knee OA synovial fluid).

It was concluded that the significance was due to an additive effect of the residual cytokine presence with LPS induced levels. Interestingly, IL-8 induction was not significantly altered through TLR3 stimulation ($P=0.1157$) as was TLR4 driven IL-8 release ($P=0.7897$) (Figure 5.4, B). There was a slight induction of IL-8 secretion from OASFs incubated with 12% synovial fluid alone ($P=0.0486$) however, the effect, whilst significant could be attributed to cytokine levels contained within the synovial fluid itself not a product of stimulation of OASFs.

5.5   Synovial fluid has potent endonuclease activity upon Poly(I:C)

In an attempt to determine why synovial fluid has a repressive effect upon signalling the fluid was further analysed for nuclease properties which would explain the specificity toward TLR3, TLR7/8 and TLR9 nucleic acid receptors. Activity of Poly(I:C) is dependent on its size; with TLR3 not being activated by shortened fragments or certain forms of low molecular weight Poly(I:C). *In vivo* TLR3 has a cut off, of naturally occurring dsRNA, at 20-23bp. If any shorter, will bind the receptor but not cause the dimerization of the TIR domains resulting in the lack of downstream activation (Liu et al., 2008, Jelinek et al., 2011, Kleinman et al., 2008).

Due to Poly(I:C)'s similarity to naturally arising nucleic acids it is not without cause to assume that it could be attacked by nucleases and broken down into its constituent components as has been documented previously in human plasma samples (de Clercq, 1979). With this idea in mind Poly(I:C) was incubated with OA synovial fluid for increasing periods of time before being extracted and analysed by gel electrophoresis (Figure 5.6). TLR7/8 and TLR9 ligands, R848 and ODN2216 respectively, are nucleic acid mimics. The nuclease activity documented against Poly(I:C) here, is more than likely the causative agent in the interference with TLR7/8 and TLR9 signalling breaking down the complementary strand of ssDNA required for TLR7/8 signalling and the ssDNA ODN2216 agonist preventing binding to TLR9 and subsequent signalling.

Poly(I:C) was incubated with OA synovial fluid (12%) for 10-30 minutes before being extracted from the solution using phenol chloroform extraction. The isolates were then run on agarose gels in order to determine whether incubation in synovial fluid was causing degradation (Figure 5.6). This was in turn compared with Poly(I:C) incubated in medium not containing OA synovial fluid.
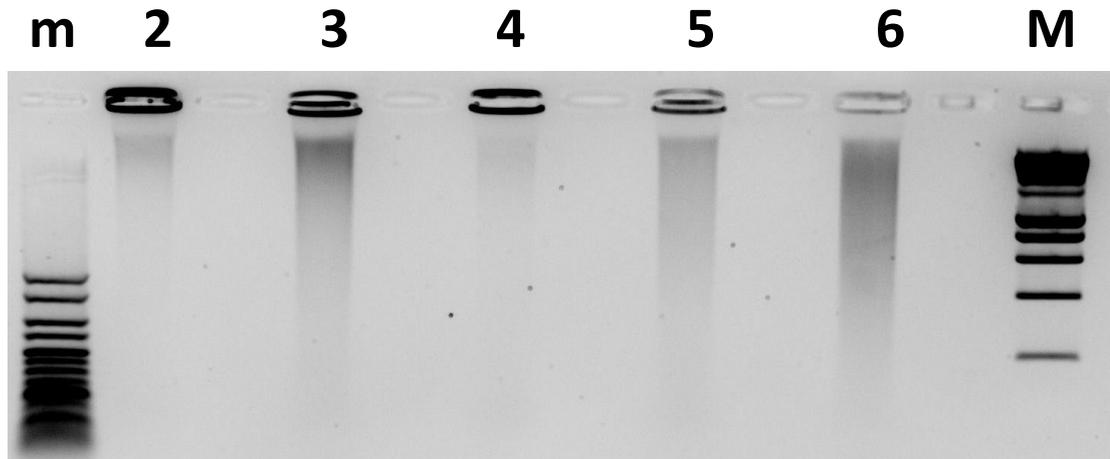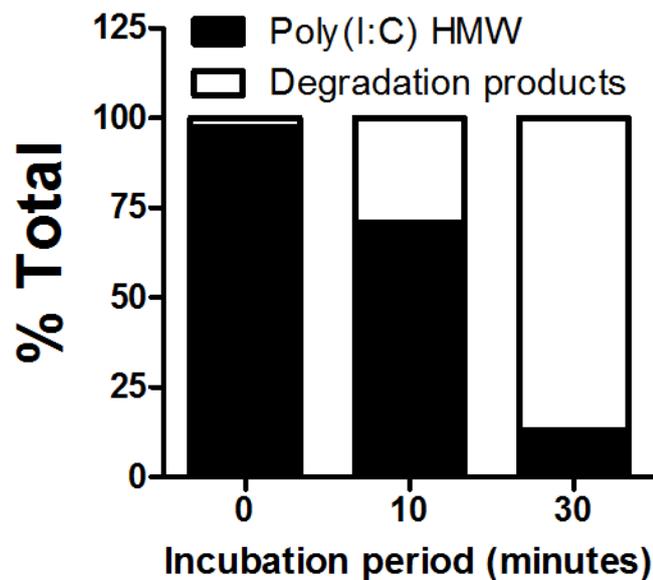
Figure 5.6: OA synovial fluid contains endonucleases which degrades Poly(I:C). Synovial fluid (12%) from knee patients was incubated with 20µg/ml of unlabelled Poly(I:C) for 10 or 30 minutes at 37°C before being extracted from solution (methods 2.8.1). **A)** Samples were resolved on an Ethidium Bromide stained 1.5% Agarose gel to determine degradation products (Lane 1 – LMW Marker 100bp-1Kbp, lane 2 - Poly(I:C) (no treatment), lane 3 – Poly(I:C) incubated with 12% synovial fluid for 30 minutes, lane 4 – Poly(I:C) heat treated for 30 minutes in DMEM (no synovial fluid), lane 5 – Poly(I:C) treated for 10 minutes in 12% synovial fluid, lane 6 – Poly(I:C) heat treated for 30 minutes in 12% synovial fluid and lane 7 – HMW marker 1Kbp-100Kbp. Data is representative of 3 separate experiments utilising synovial fluid from 3 separate donors. **B)** Ratio of percentage Poly(I:C)/degradation products to well, determined through densitometry analysis. Markers – **M)** Hyper-ladder I and **m)** Hyper-ladder IV for arbitrary sizing.

When incubated with synovial fluid, Poly(I:C) was degraded (Figure 5.6). The degradation of the ligand provides an explanation for reduced activation of TLR3 in synovial fibroblasts incubated in the presence of patient derived synovial fluid. This observation, in addition to the documented ability of synovial fluid to inhibit TLR3-induced cytokine induction suggests the presence of a nuclease within synovial fluid. Due to the chemical composition of Poly(I:C) and the degradation profile imaged through agarose gel analysis we can hypothesise that an endonuclease would be acting upon the molecule as complete degradation was not observed (Figure 5.6).

This is one of several working hypotheses for the observed effect as it makes the most theoretical sense. However, initial attempts to inhibit nucleases with EDTA were unsuccessful, hypothesised to be caused by endotoxin contaminated EDTA. Therefore, further work needs to be performed to clarify whether it is indeed nucleases contained within the synovial fluid that is causing the observed inhibition of endosomal TLR activation. Examples of future planned work are detailed in the further work section of this thesis.

5.6 OA synovial fluid contains both disease inducing and disease reducing substances

While previous studies focused on synovial fluid as a source of DAMPs, this data suggests that synovial fluid from OA patients can also have a protective effect on disease activity, possibly by limiting endosomal TLR activity through nuclease interference. Synovial fluid isolated during knee and hip replacement procedures proved to be significantly different in their content and potential contribution to disease activity.

The first result demonstrating this dichotomy was the measured levels of MMP1 and MMP3. In fact, MMP1 was significantly higher in synovial fluid isolated from hip than knee (P=0.0137) whilst MMP3 was not different. Western blot analysis of the samples differentiated between the precursor and the mature form of the protein, which further indicated a much more actively destructive environment found in the hip. MMP1 is a potent collagenase whose involvement in the progression of OA is theorised to be very important through the destruction of the articular cartilage. The presence of high levels of MMP1 and MMP3 in the hip of OA patients is enlightening as, whilst MMP1 and MMP3 were detectable in both locations, there was a significant variation in presence dependent on the joint tested. Western blot analysis of synovial fluid confirmed that the abundance of MMP1 in hip synovial fluid was due to a combination of both pro and mature MMP1. However, MMP3 analysis indicated that levels of mature MMP3 in the hip were significantly higher than in the knee. One possible explanation to this discrepancy between results obtained with MMP3 Western blot and ELISA could be that the ELISA detected some non-specific

products or peptide fragments associated with the use of polyclonal antibodies that target multiple epitopes. It is also likely that the increased sensitivity of western blot methodology allowed the observation of much smaller concentration differences that would otherwise go unnoticed utilising ELISA.

Observed here is evidence of MMP1 and MMP3 presence within the knee and hip synovial fluid. Synovial fluid contains several MMPs and mechanisms are in place, particularly TIMPs, to stop an uncontrolled destruction of the joint (Nagase et al., 2006, Zhang et al., 2004). Chondrocytes are supposed to maintain healthy cartilage through the balanced removal and addition of cartilage components. However, as demonstrated above (Figure 4.6, A-C), chondrocytes isolated from regions of cartilage degradation are no longer morphologically or biologically the same as healthy cells. Altered collagen production from these cells could thus create an imbalance within the matrix furthering OA progression in the affected joint.

The data also suggests that the collagenase MMP1 may be a contributing factor to disease progression in the case of hip OA. Its presence combined with high levels of MMP3, also theorise the possibility of increased cleavage of pro-MMP1 to Mat-MMP1.

Discrimination between early and late stage OA cannot be defined in this study as all patients involved being at the terminal stage of the disease (joint replacement). However, early intervention with joint effusion therapies may alleviate disease activity in the early stages of the disease by regular removal of MMPs and debris from the joint space.

Analysis of the ability of synovial fluid to interfere with TLR activation was demonstrated by a marked inhibition of endosomal TLR activity in transformed HEK293 cells. This phenomenon was not observed with TLR2- or TLR4- transformed cells and indicated the potential for interference with nucleic acid binding by endosomal TLRs. Confirmation of the findings in the primary OASFs cells showed marked reduction of Poly(I:C) induced IL-6 production. Interestingly, synovial fluid did not affect Poly(I:C)-induced IL-8 production. Gel electrophoresis resolution of Poly(I:C), demonstrated that synovial fluid seems to have potent nuclease activity, breaking down HMW Poly(I:C) into smaller degradation products. These data provide an explanation for the inhibition of TLR3 activation by synovial fluid, being that the ligand is degraded and therefore inactivated. However, there is also the possibility that there are other effects of the synovial fluid on endosomal TLR internalisation and/or signalling including possible inhibition of clathrin dependent endocytosis, a process by which endosomal TLR signalling is reliant upon for activation (Mbengue et al., 2016). Future work could focus on investigating the exact process of interference and why had only affected IL-6 but not IL-8 secretion.

5.7   <u>Summary</u>

- Synovial fluid from hip contains significantly more MMP1 than knee although mature MMP1 levels are the same.
- MMP3 levels are increased overall in hip synovial fluid through western blot analysis although ELISA disagrees with the result.
- Synovial fluid inhibits TLR3, TLR7, TLR8 and TLR9 signalling in HEK293 cell lines but not TLR2 or TLR4.

- Synovial fluid activates IL-6 and IL-8 in OASFs.

- Synovial fluid supresses TLR3 induced IL-6 production in OASFs but not IL-8

- HMW Poly(I:C) is degraded by incubation with synovial fluid

MMP expression within synovial fluid of OA joints has been previously documented to be significantly increased from healthy controls and highly dependent or arthritic disease type (Tchetverikov et al., 2005, Balakrishnan et al., 2014b). However, this study is the first demonstration to our knowledge that knee and hip synovial fluid contain MMP1 in significantly different levels. This proposes the existence of a more actively degenerative environment found within the hip joint. Higher MMP1 levels in hip may partially explain the increased cartilage destruction so commonly noted with hip OA opposed to that found in the knee.

We also report for the first time that synovial fluid can interfere with endosomal TLR signalling in both HEK293 and primary OASFs. This data has also demonstrated potent nuclease activity within the fluid degrades Poly(I:C), significantly IL-6 secretion from primary synovial fibroblasts independent of joint location. However, the data also has shown that IL-8 secretion is unaffected by the synovial fluid suggesting the presence of another pathway by which Poly(I:C) can initiate IL-8 production.

Overall, these data reveal the importance of the synovial fluid and a portion of its constituents in their ability to modulate disease pathology and furthermore, how this can vary dependent on joint location, a concept that arises throughout this project.

Chapter 6 - Complement and its crosstalk with the TLR associated pathogenesis of OA.

Crosstalk of the complement system with TLR signalling has been documented by the interaction of several shared signalling pathways, in particular the shared use of the MAP kinases and documented inhibition of P38 phosphorylation in monocytes by co-stimulation of TLR4 and C3aR (Song, 2012, Holst et al., 2012). Whilst this interaction has been demonstrated in immune cells such as peripheral monocytes and differentiated macrophages, investigation into non-immune cells has to our knowledge been largely neglected.

6.1   Complement anaphylatoxin expression in HEK293 cells

With the concern that anaphylatoxins produced in a bacterial *E.coli* system may possess contaminating LPS, C3a and C5a peptide fragments production was attempted in a mammalian system. In addition to the potential for LPS contamination it was feared that there was reduced activity of the peptide due to the lack of glycosylation of the peptide. Bacterially-produced anaphylatoxins are widely used by the research community for complement research and whilst correct glycosylation may not be important for complement activity, TLR signalling may be altered.

Glycosylation of human proteins is extremely important for their correct structure and function and is the primary cause of desired micro-heterogeneity associated proteins (glycoforms) (Ucakturk, 2012). Changes in these structures have been noted to reflect and result in physiological changes characterised in some cancers, mental health conditions and even RA (Stanta et al., 2010). The correct glycosylation is even more poignant in acute phase proteins which undergo changes at the time of inflammation. Several groups have demonstrated that alterations in glycosylation may be involved in chronic inflammatory conditions and correlate with disease activity (Malhotra et al., 1995, Stanta et al., 2010, Gindzienska-Sieskiewicz et al., 2016). It was with this knowledge in mind, along with potential LPS contamination from production in *E.coli,* that we attempted to produce anaphylatoxins C3a and C5a in a mammalian system (Figure 6.1).
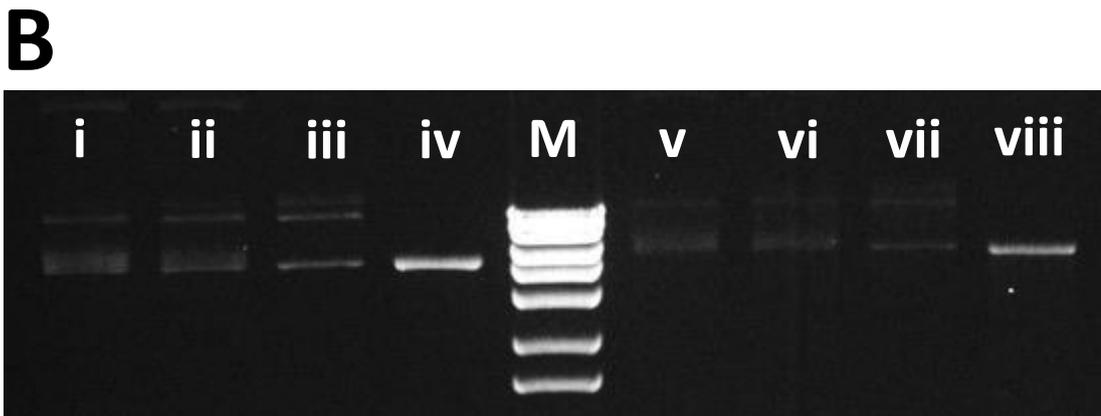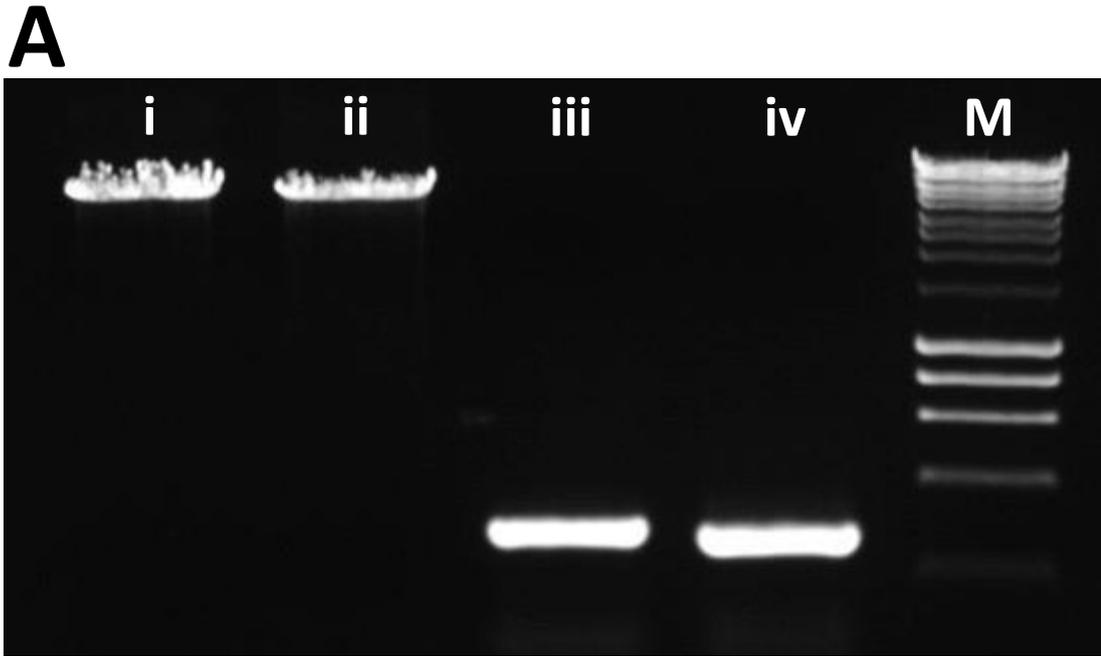
Figure 6.1: Complement C3a and C5a cloning. A) Restriction enzyme digested pcDNA6 plasmid with BAMHI/XhoI and HindIII/XhoI restriction sites and respective C3a and C5a gene fragments. **B)** Subsequent ligation into pre-digested plasmids. Samples were run on 1.5% agarose gels and stained with ethidium bromide. **Ai)** pcDNA6 BAMHI/XhoI digest **Aii)** pcDNA6 HindIII/XhoI digest **Aiii)** C3a BAMHI/XhoI digest **Aiv)** C5a HindIII/XhoI digest **B1)** 1:1 ratio ligation pcDNA6 BAMHI/XhoI digest + C3a BAMHI/XhoI digest **B2)** 2:1 ratio ligation pcDNA6 BAMHI/XhoI digest + C3a BAMHI/XhoI digest **B3)** 3:1 pcDNA6 BAMHI/XhoI digest + C3a BAMHI/XhoI digest **B4)** pcDNA6 BAMHI/XhoI digest **B5)** 1:1 ratio ligation pcDNA6 HindIII/XhoI digest + C5a HindIII/XhoI digest **B6)** 2:1 ratio ligation pcDNA6 HindIII/XhoI digest + C5a HindIII/XhoI digest **B7)** 3:1 ratio ligation pcDNA6 HindIII/XhoI digest + C5a HindIII/XhoI digest **B8)** pcDNA6 HindIII/XhoI digest.

Whilst the cloning and ligation successfully produced viable clones for transfection, carboxypeptidase B (CBP) impeded protein expression. CPB is a basic carboxypeptidase that cleaves C-terminal basic residues of arginine or lysine from protein and peptides (Bajic et al., 2013). This proved to be detrimental as the activity of the anaphylatoxins C3a and C5a are diminished by the removal of the C-terminal arginine. An additional issue is that once in the des-Arg form the proteins are rapidly degraded and removed from the system meaning that any measurable amount of the protein was removed from the cell lysate meaning it could not be purified.

The reason HEK293 cells were chosen as the most viable cell line to use was the fact that the system itself has been used by others in our lab in the successful expression of biologically active proteins and peptide fragments. An alternate expression system such as CHO cells would have been another option although as we did not own the cell line the un-transformed HEK293 line was the most viable. Thus, due to the unforeseen issues with the expression of the C3a and C5a peptide fragments, commercially available C3a and C5a were used in the experiments in this chapter. Both the C3a and C5a peptide fragments were produced in *E.coli* and are purchased endotoxin low <0.01EU/µg.

6.3 Membrane-bound complement regulators CD46, CD55 and CD59 are differentially regulated by TLR activation in isolated articular chondrocytes

The terminal stage of complement activation is the formation of the membrane attack complex (MAC). A cytolytic pore, MAC deposits on the cell surface membrane of invasive pathogens and disrupts the lipid raft culminating in osmolysis of the targeted cell (Muller-Eberhard, 1984). Previous studies have described that sub-lytic levels of MAC deposition on host cells can actually trigger signal induction pathways activating events such as proliferation (Niculescu et al., 1999).

Increased MAC deposition on lesioned cartilage and synovial membrane tissue has been previously reported in patients affected by OA, RA and acute joint trauma (Cooke, 1987, Cooke et al., 1980, Corvetta et al., 1992). These observations were the early suggestions of a local immune reaction present within OA joints. As the disease presents no evidence of systemic illness, it was theorised that these immune reactions reflected past events in the host's response to mechanical damage. The infiltration of mononuclear cells did not suggest that OA was initiated by an autoimmune mechanism, but was rather perpetuated by repeated immune insults which results in prolonged clinical inflammation accelerating cartilage damage (Cooke, 1987).

The regulation of MAC is inevitably important as uncontrolled deposition of host cells would lead to self-lysis of both chondrocytes and synovial cells damaging the joint. Therefore, complement regulators exist in the fluid phase or at the cell membrane to prevent sterile response (Noris and Remuzzi, 2013).

Fluid phase regulators control the MAC complex prior to its formation and are isolated to different compartments of the tissue or distributed within the plasma and bodily fluids. Such proteins, like factor H, are involved in the regulation of the alternative pathway complement activation including caboxypeptidase-N, which cleaves the carboxy-terminal arginine from anaphylatoxin peptides resulting in reduced efficacy in triggering the complement cascade (Hugli et al., 1981, Mueller-Ortiz et al., 2009)

Membrane-bound complement regulators act in a variety of manners in order to prevent the formation of MAC. CD46 (membrane cofactor protein) in collaboration with serum factor I, has the ability to aid in co-inactivation of complement components C3b and C4b inhibiting the upstream formation of MAC (Oglesby et al., 1992). CD55 (decay accelerating factor) accelerates the decay of the complex once deposited on the cell membrane whilst CD59 (protectin) prevents final assembly of the MAC through physical blocking of C9 polymerisation (Zipfel and Skerka, 2009).

The purpose of these experiments was to determine the effect of complement anaphylatoxins and TLR ligands upon the expression of membrane-bound complement inhibitors CD46, CD55 and CD59 in knee chondrocytes (Figure 6.2, 6.3and 6.4). The results would allow us to determine whether increased MAC deposition on the cartilage of OA patients demonstrated in the literature could be attributed to TLR activation.
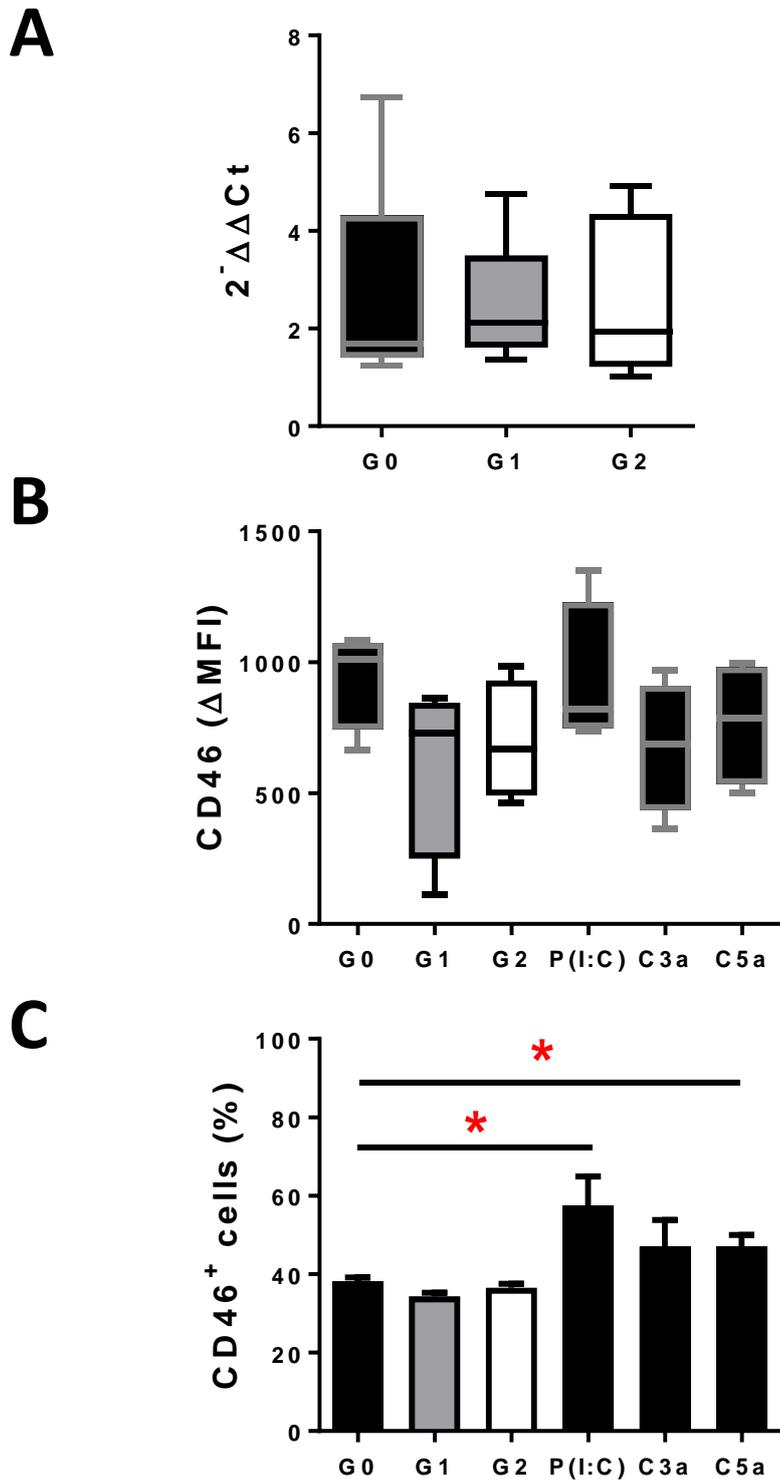
Figure 6.2: Complement membrane regulator CD46 is significantly increased through Poly(I:C) and C5a stimulation. A) mRNA expression of CD46 **B)** protein expression of CD46, including representative histogram **C)** CD46 positive cell populations and the effect of stimulation, including representative scatter plot. Data **(B-C)** was obtained using flow cytometric analysis of G0 (grade 0) G1 (grade 1) and G2 (grade 2) articular chondrocytes. Data is mean ±SEM of 4-5 unrelated donors performed under a minimum of 2 separate experiments. P(I:C) = 20μg/ml Poly(I:C), C3a and C5a = 100ng/ml. *P<0.05 un-paired student t-test 2-tailed.
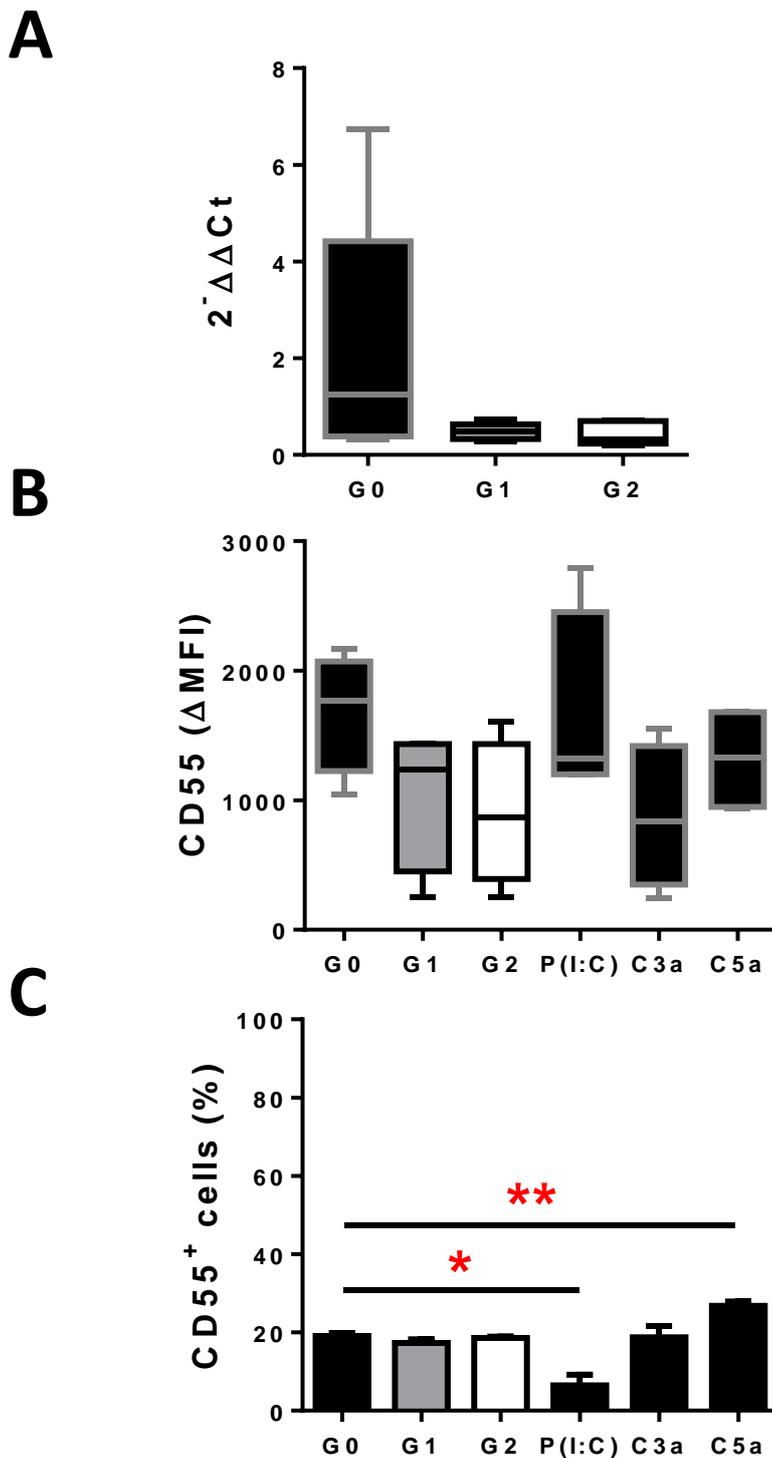
Figure 6.3: Complement membrane regulator CD55 is significantly altered through TLR3 and C5aR activation. A) mRNA expression of CD55 **B)** protein expression of CD55, including representative histogram **C)** CD55 positive cell populations and the effect of stimulation, including representative scatter plot. Data **(B-C)** was obtained using flow cytometric analysis of G0 (grade 0) G1 (grade 1) and G2 (grade 2) articular chondrocytes. Data is mean ±SEM of 4-5 unrelated donors performed under a minimum of 2 separate experiments. P(I:C) = 20µg/ml Poly(I:C), 100ng/ml C3a and 100ng/ml C5a. *P<0.05 un-paired student t-test 2-tailed.
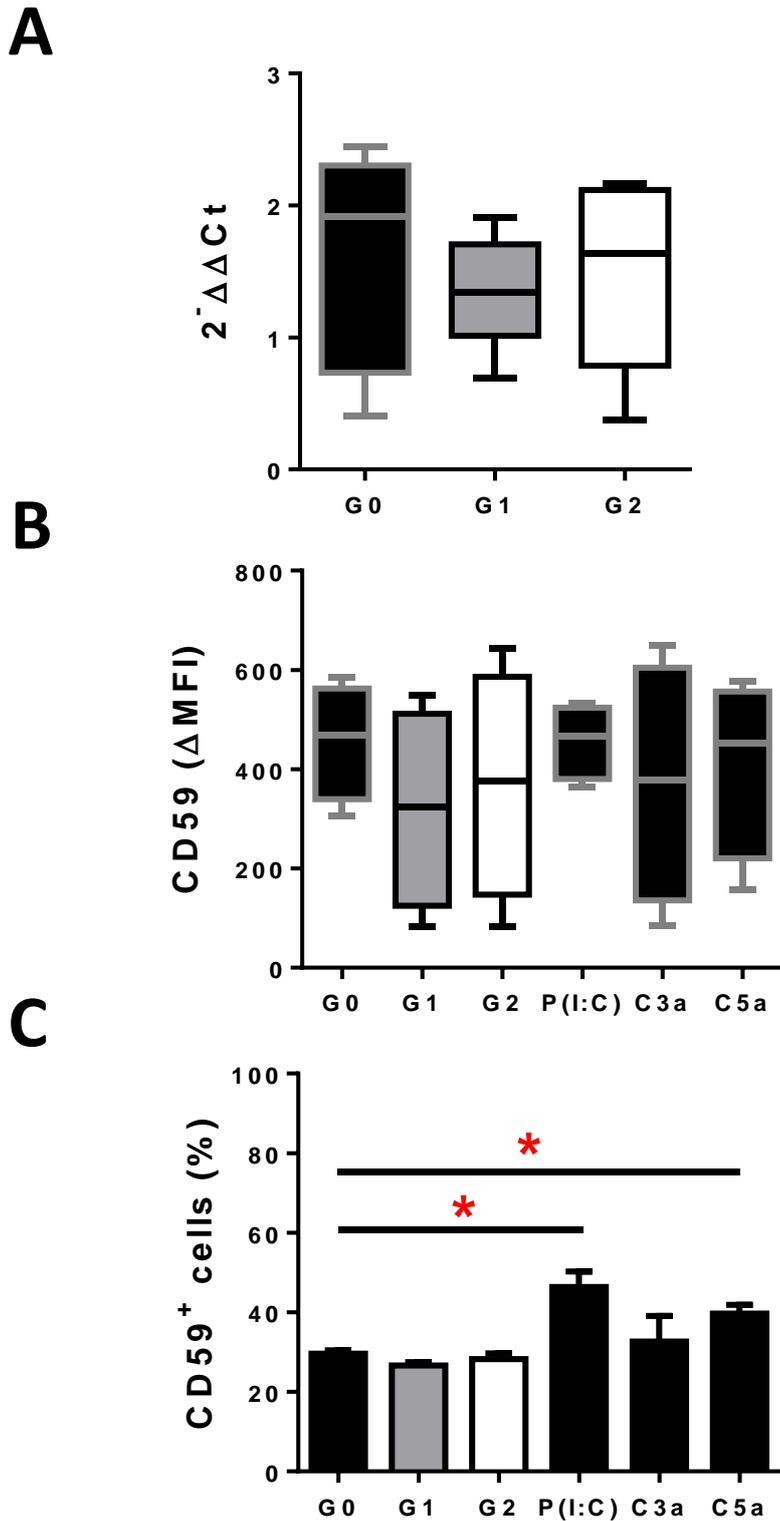
Figure 6.4: **Cells positive for CD59 is significantly increased through TLR3 and C5aR activation. A)** mRNA expression of CD59 **B)** protein expression of CD59, including representative histogram **C)** CD59 positive cell populations and the effect of stimulation, including representative scatter plot. Data **(B-C)** was obtained using flow cytometric analysis of G0 (grade 0) G1 (grade 1) and G2 (grade 2) articular chondrocytes. Data is mean ±SEM of 4-5 unrelated donors performed under a minimum of 2 separate experiments. P(I:C) = 20µg/ml Poly(I:C), 100ng/ml C3a and 100ng/ml C5a. *P<0.05 un-paired student t-test 2-tailed.

Poly(I:C) and C5a significantly alter CD46, CD55 and CD59 positive populations in chondrocytes

Findings of increased membrane attack complex deposition upon chondrocytes of lesioned OA cartilage suggested a potential deficit in regulator mechanisms of the host cell (Wang et al., 2011). We therefore analysed the expression of CD46, CD55 and CD59 in chondrocytes.

CD46 is a co-factor of serum factor I which mediates the degradation of C3b upstream of C3b4a production thereby limiting the cascades progression (Bjorge et al., 1996). There was no difference in the mRNA expression across the cartilage grades (G0-G1, P=0.7722) (G0-G2, P=0.99) (Figure 6.2, A), and this was confirmed to be unchanged at the protein level using flow cytometric analysis (G0-G1, P=0.205) (G0-G2, P=0.1041) (Figure 6.2, B). Stimulation with Poly(I:C), C3a or C5a did not alter protein expression of CD46 significantly. However, the percentage of CD46-positive cells was significantly increased by Poly(I:C) (P=0.0432) and C5a stimulation (P=0.0253) (Figure 6.2, C).

Decay accelerating factor (CD55) increases the degradation of C3 and C5 convertases from the classic and alternative pathway of complement activation to reduce the production of damaging anaphylatoxins and aberrant production of MAC (van Beek et al., 2005). There was a trend for a reduced CD55 expression in degraded (G1 and G2) cartilage, although this difference was not statistically significant (G0-G1, P=0.2305) (G0-G2, P=0.2033). This was observed at both the mRNA (Figure 6.3, A) and protein level (G0-G1, P=0.1245) (G0-G2, P=0.1576) (Figure 6.3, B).

Stimulation of G0 chondrocytes with TLR3 agonist Poly(I:C), resulted in a significantly reduced positive cell population (P=0.0288) (Figure 6.3, C). Conversely stimulation with C5a led to a significant increase in the positive cell population (P=0.0037) (Figure 6.3, C).

CD59 acts by blocking the interaction between the C5b-8 complex and subsequent polymerisation of C9, thereby preventing the construction of the MAC (Huang et al., 2006). Both mRNA and protein expression (Figure 6.4, A-B) of CD59 was unaffected across erosion gradings or through TLR3, C3aR or C5aR stimulation. However, stimulation of isolated G0 chondrocytes with Poly(I:C) (P=0.0125) or C5a (P=0.0429) significantly increased the CD59 positive cell population (Figure 6.4, C).

These data demonstrate that TLR3 activation has a statistically significant effect on complement regulation in chondrocytes. However, the results do not demonstrate a clear reduction in complement regulators CD46, CD55 or CD59 that could be attributed to the documented increased deposition of MAC in lesioned OA cartilage chondrocytes (Wang et al., 2011). They do show that activation of TLR3 can alter these regulatory components by significantly altering positive cell populations. In the case of CD55 Poly(I:C) stimulation of TLR3, significantly reduced the positive populations of G0 cells opposed to untreated controls. If the data set was enlarged through increased recruitment of OA patients, the reduced CD55 mRNA expression found within G2 chondrocytes may prove to be a significant feature of end stage cartilage degradation in OA.

Chapter 7 – Discussion

7.1 Summary of results

OA is a disease dominant in the aged. With an aging population, the incidence of OA is only increasing, but with little understanding of disease initiation and progression, treatments have remained unchanged for many years. However, with the acceptance of OA as a disease of inflammation, many avenues of research have arisen with therapeutic targets. This study aimed to investigate the expression and subsequent activation of TLRs in synovial fibroblasts and chondrocytes from late-stage OA. Furthermore, determining the possible involvement of synovial fluid on TLR signalling and disease progression and the way in which complement membrane regulators are altered by TLR activation.

Synovial membranes TLR expression and inflammatory cytokine production is dependent on joint location

RA synovial membrane cultures, without any addition of external stimuli, have been documented to spontaneously produce pro-inflammatory cytokines and MMPs of interest (Brennan et al., 1989, Sacre et al., 2008). Many of these cytokines and matrix degenerative enzymes are linked to the progression of bone and cartilage erosion in addition to their inflammatory effects. Whilst the OA joint appears to be much less inflammatory compared with an RA joint, the production of many of these factors have been noted to be present within the synovial fluid of OA patients, with the potential to contribute to disease progression. In order to clarify whether OA synovial membrane cultures

produced some of these factors spontaneously, production of several pro-inflammatory cytokines and MMPs were measured. The purpose of this experiment was to determine whether the OA synovial membrane itself was perpetuating disease activity through production and maintenance of the chronic inflammatory state within the synovium.

Data demonstrated that OA synovial membrane cultures had distinct inflammatory patterns dependent on joint location with knee isolates being more pro-inflammatory than hip with increased TNF, IL-6 and IL-8 spontaneous production. Additionally, MMP13 production from hip OA synovial membrane cultures was significantly higher than from knee suggesting an environment able to readily degrade cartilage components at a much greater incidence than found in the knee. The spontaneous production of TNF from synovial membrane cultures has been previously documented to be within the levels shown demonstrated here, although some of the original findings were attributed to endotoxin contamination (Brennan et al., 1989). Therefore, during this project endotoxin low collagenase was used for isolation protocols and samples of collagenase type I and II used in the data set, were LAL tested for endotoxin contamination. Whilst the examination of collagenases proved endotoxin levels were below the threshold of the test it is possible that the digestion process itself creates endogenous DAMPs from ECM components and necrotic debris which in turn can activate TLRs. This phenomenon has been observed in the literature with the production of hyaluronan, S100 proteins and fibronectin fragments which can trigger TLR2 and TLR4 activation in chondrocyte and synovial membrane isolates (Schelbergen et al., 2015, Hwang et al., 2015). Additionally, cytoplasmic proteins such as HMGB1-

complexes and host nucleic acids (created through destruction of cells) have been demonstrated to regulate TLR2, TLR3, TLR4 and TLR9 activity in both RA and OA models and should cross-over regardless of disease or cell type originally mentioned (Brentano et al., 2005, Wahamaa et al., 2011, Pearson and Jones, 2016).

The production of TNF from synovial membrane cultures was an unexpected observation especially at the levels found within knee synovial cultures which were comparable to RA membrane cultures, a disease demonstrated to be driven, in part, by TNF (Maini et al., 1997). Synovial fibroblasts do not produce TNF but monocytes can. Therefore, the TNF production seen in the OA synovial membrane cultures is possibly due to the presence of infiltrating monocytes from the periphery. This hypothesis is supported by publications demonstrating infiltration of activated B cells and T lymphocytes into the synovium and their contribution to OA pathogenesis through production of TNF and other inflammatory cytokines including IL-6 and IL-8 (Wenham and Conaghan, 2010).

It is also important to note that the potential artefact due to the presence of endotoxin, or possibly other contaminants, in the collagenase preparation, used for cell isolation cannot be ruled out. Likewise, we cannot rule out the possibility that danger signals produced by tissues during the digestion with collagenase may contribute to the induction of TNF observed here. Therefore, although this is a potentially interesting finding, it would require future studies to confirm, or not, these findings as they cannot be extrapolated as such into the clinical settings.

It may be possible that the ineffectiveness of anti-TNF therapy for OA may be, in part, due to research not discriminating between joint location. Variable effectiveness of TNF blockade in clinical trials has meant that it is not currently used for treatment. Several studies have demonstrated significant efficacy of the drug etanercept in alleviating OA associated pain and joint erosion in knee and hand OA trials (Guler-Yuksel et al., 2010). However, with larger cohort studies, using a multitude of joint locations, a beneficial effect has not been seen (Li et al., 2009, Chevalier et al., 2015). Ongoing trials add to the hope that targeting TNF may be beneficial for the treatment of OA and may be dependent on drug delivery mechanisms and joint type. Sub-categorisation of effected joints may demonstrate significant differences in efficacy of treatment, but is not currently observed due to large changes in cellular infiltration and population dynamics, depending on joint location and architecture.

The examination of TLR expression in OA synovial fibroblasts demonstrated several interesting observations. Expression of TLRs at the mRNA and protein level was significantly altered between pure populations of isolated synovial fibroblasts derived from the knee or the hip. Several other groups have documented TLR expression within synovial fibroblasts, although none to current knowledge have compared expression within groups isolated from separate joint locations (Ospelt et al., 2008, Hu et al., 2014).

The increased expression of TLR3 and TLR4, in knee compared with hip OASFs, correlated to a significantly increased IL-8 production from knee OASFs under stimulation. Whilst TLR1, TLR2 or TLR6 levels were not significantly altered at the protein level, induction of IL-8 was again increased

through stimulation. Interestingly, IL-6 production was unaffected between groups. Heterogeneity between samples demonstrated how variable individual patient samples were and, whilst the results for comparison were significant, increasing the cohort would aid in validating the results. These data support the hypothesis that hip and knee OASFs are inherently different in their TLR expression characteristics and suggest therapies for the treatment of OA may be more effective targeting specific joints rather than focusing on ubiquitous treatments.

Degenerate chondrocytes have altered TLR expression and activity due to de-differentiation processes

In order to determine the involvement of articular chondrocytes in disease progression, cells were isolated from varying regions of OA-associated cartilage degradation and analysed for TLR expression and activity. A modified scaling system was used to visually assess cartilage erosion according to OA progression and was denoted grade 0, grade 1 and grade 2, scaled from no-visible signs of OA to late stage (Mankin et al., 1971). Altered TLR mRNA and protein expression has been previously documented in severe regions of degradation associated with the progression of OA and the results documented here support several of the publications (Kim et al., 2006, Zhang et al., 2008, Sillat et al., 2013). However, many of these publications use chondrocytes cultured *in vitro* for long periods of time; a poor comparison to the cells in their natural environment. For the experiments documented within this thesis, when isolated from the cartilage ECM, chondrocytes were typically a rounded structure indicative of a healthy live cell. However, several anomalous multinucleated cells were amongst the isolates demonstrating the

active clonal expansion within diseased cartilage. Within 48 hours the cells would adhere to the culture surface and over the space of 1-2 weeks differentiate from rounded spheres into fibroblastic like cells. This phenomenon of dedifferentiation has been widely studied and so was not unexpected (Schnabel et al., 2002). However, white-light microscopic investigation of cartilage explants revealed that several G2 cartilage samples contained differentiated looking cells within the remaining cartilage ECM. It was hypothesised that the cells seen within the ECM of degenerate cartilage could be those of infiltrating synovial fibroblasts dissociated from the synovial membrane. However, it seemed more likely that the chondrocytes themselves had de-differentiated into a fibroblast-like cell like those developed during *in vitro* culture. Therefore, in order to analyse cells in an environment as close to in the body as possible samples were analysed as quickly as possible after isolation from the patient either through snap freezing samples for RNA isolation or a maximum culture time of 24 hours post-isolation for stimulatory experiments. Furthermore, in order to reduce patient variability as much as possible, analysis of TLR expression was determined between individual patients using non-degenerate, grade 0, regions as intra-patient control groups.

Analysis of TLR expression demonstrated numerous differences, primarily focused on the expression and activation of the TLR1/2/6, 3 and 4 in OA cartilage samples. Location of TLR expression was first determined with TLR2 and TLR4 predominantly expressed within deep zone chondrocytes whilst TLR3 was expressed primarily in the superficial zone. Secondly, it was noted that TLR3 was expressed on the cell surface of those chondrocytes rather than

in the endosome. Whilst uncommon, some types of cells express TLR3 on the cell surface as well as within in the cytoplasm (Erdinest et al., 2014). Interestingly, the location of TLR3 changed in grade 2 degenerate cartilage samples, whereby the receptor became localised with the early endosome marker EEA1. Whether this was a marker of activation or an alteration in cell phenotype is unknown but can be hypothesised. This phenomenon had been observed in published findings on rabies virus infected cells, whereby TLR3 localisation moved from the cell surface to the endosome, supporting the hypothesis that TLR3 in chondrocytes of degenerate cartilage were activated (Menager et al., 2009).

Analysis of TLR mRNA expression demonstrated that TLR1, TLR3 and TLR6 were altered in expression significantly across the modified scaling system denoting OA severity. The significant decrease in TLR3 expression between G0 and G2 cartilage corresponded with the change in cellular location of TLR3 from the cell surface, to the endosome. This change may also indicate a phenotypic change in the cell, which has been documented to occur in the literature when chondrocytes are cultured *in vitro* (Caron et al., 2012). This change in TLR3 expression is a novel observation to our knowledge and suggested that cells may be differentially active compared to intra-patient controls.

To discern whether the observed changes in TLR expression led to altered induction of cytokines, chondrocytes from macroscopically graded cartilage were enzymatically isolated and stimulated with TLR ligands. Stimulation of these cells revealed that chondrocytes isolated from regions of degradation

(grade 2) produced significantly higher levels of IL-6, IL-8 and MMP13 than those from unaffected areas (grade 0) upon stimulation with TLR2 or TLR4 ligands.

The findings of *in vitro* differentiation of chondrocytes, along with the data reported within this thesis, suggest that during the progression of OA, *in vivo* chondrocytes undergo a similar process within the cartilage and become more fibroblastic. This subsequently creates a more aggressive cell type which, under TLR2, TLR3 or TLR4 stimulation, can produce significantly increased levels of disease relevant inflammatory cytokines IL-6, IL-8 and cartilage destructive enzyme MMP13.

The initial impact trauma which has been widely accepted as the initiating step in OA could act as a gateway to the usually encased chondrocytes, which, when exposed to signals usually devoid in the cartilage, transform the cells into the fibroblastic nature documented in these results. Alteration in TLR expression, through phenotypic and morphological changes, appears to create a more TLR ligand sensitive cell type. These findings indicate that there may be a contribution of chondrocytes in TLR mediated OA pathogenesis.

From this data it is feasible to hypothesise that TLR activation of chondrocytes may contribute to OA pathology due to increased receptor expression and ligand availability. The de-differentiation of the cells and subsequent pro-inflammatory cytokine production, increased peripheral blood cell migration and matrix degradation through MMP13 induction are all markers of disease perpetuation. Studies into fibroblast like chondrocytes demonstrate their aggressive nature and suggest an increased role in OA pathogenesis

compared with undamaged cartilage chondrocytes (Tesche and Miosge, 2005). During OA progression, and subsequent cartilage degradation, cellular components and ECM DAMPs are secreted into the synovial fluid. With several TLR DAMPs documented to be present within the synovial fluid of OA patients, including HMGB1, fibronectin fragments and host nucleic acids, the effect of synovial fluid on TLR signalling was investigated (Okamura et al., 2001, Hwang et al., 2015, Wahamaa et al., 2011, Qin et al., 2014).

Endosomal TLR inhibition by synovial fluid

Synovial fluid, contrary to published studies, did not activate TLR expressing HEK293 cell lines or primary synovial fibroblasts. However, the addition of synovial fluid to TLR transformed HEK293 cell lines showed the complete ablation of endosomal TLR signalling. When incubated with primary human synovial fibroblasts the same phenomenon occurred with the inhibition of TLR3 but not TLR4 IL-6 induction. Stimulation with Poly(I:C) in the presence of knee synovial fluid, significantly reduced IL-6 induction from synovial fibroblasts suggesting that activation of TLR3 was inhibited.

The inhibition of TLR3 signalling led to two testable hypotheses, the first being that something in the synovial fluid was binding the ligand, the other that the ligand was being degraded. This latter hypothesis was most feasible as nucleases are known to be present within blood and other bodily fluids so would be likely to be found in the synovial fluid. However, the small molecule R-848 would not likely be effected by a nuclease. Recent discoveries into the requirement of a secondary binding site in TLR8 activation gave some hypothetical explanation. TLR7/8 usually requires the partial degradation of

ssRNA to release a single Uridine which binds to an epitope in the hydrophobic LRR domain of the TLR8 homodimer. However, it also requires a remaining length of ssRNA to bind and stabilise the complex. If these ssRNAs are degraded by nuclease activity, signalling may not occur as the stabilisation of the dimer is required for TIR domain activated receptor signalling (Tanji et al., 2015). This is one hypothesis however, and there may be other mechanisms regulating endosomal TLR activation and internalisation that were not explored during this project.

Further investigation revealed that it was indeed nuclease activity within the synovial fluid that digested Poly(I:C) into smaller fragments. This observation suggested that the degradation may be one of the methods as to why Poly(I:C) in addition with synovial fluid did not stimulate TLR3. High molecular weight Poly(I:C) is described to form complex lengths of between 1.5-8kb. However, TLR3 itself can signal following binding dsRNA of 20-50bp or greater. The experiments demonstrated that the ligand being degraded through incubation in synovial fluid and was theorised to be caused by nuclease activity. There is potentially another mechanism, other than the predicted endonuclease, that is inhibiting activation of endosomal TLRs but identification of this mechanism is beyond the scope of this project.

As complete signalling pathways for the induction and processing of both IL-6 and IL-8 are unavailable at this current time, only hypotheses for future studies can be made. These works may look into identifying not only the receptor associated with the activation of cytokine induction but also the ligand for

synovial fluid activation of an inflammatory response might aid in the ablation of a portion of the inflammatory component of OA.

Differential MMP content of OA synovial fluid

Synovial fibroblasts isolated from both knee and hip joints did not produce detectable levels of MMP1, MMP3 or MMP13 when stimulated with TLR1/2, TLR3, TLR4 or TLR2/6 ligands. However, chondrocytes induced readily detectable levels both constitutively and under TLR stimulation. It was therefore predicted that OA synovial fluid would contain these MMPs secreted from exposed chondrocytes (grade 1/2) providing an environment of destruction, further promoting OA-related cartilage degradation from the cartilage articular surface.

Analysis of synovial fluid via ELISA showed that hip synovial fluid contained much higher levels of MMP1 than the knee and, whilst MMP3 was readily detectable, there was no difference between joint location. The ELISA antibody pairings however, did not distinguish between precursor and mature forms of the protein. This meant that the same samples, when analysed through western blot, demonstrated additional findings in that mature MMP3 was present at a significantly higher level in hip than knee. However, mature MMP1 was similar in levels in both hip and knee. Furthermore, MMP13 could not be detected using ELISA antibody pairs, possibly due to the necessary dilution of the viscous synovial fluid for stimulation assays.

MMP1 and MMP13 are thought to be key components in the progression of OA. They are both highly ubiquitous collagenases and as this data demonstrates MMP1 to be present at a significantly increased concentration in hip synovial fluid suggested a much more destructive environment opposed to the knee. Furthermore, as an interstitial collagenase, MMP3, has the ability to cleave not only several forms of collagen, but also to cleave the pro-domain, or signal domain, from other inactive MMPs releasing active forms (Chen et al., 2014). The presence of resident MMPs within the joint is known and accepted in order to regulate and maintain the joint matrices. However, these metalloproteases are generally in their inactive, precursor form, to prevent degeneration of the joint architecture. The increased presence of MMP3 within the hip gives rise to the possibility that many, usually inactive MMPs, are being cleaved into their active form, promoting cartilage degradation and in turn disease progression.

Regulation of the complement system by TLR3

Published data also has demonstrated increased MAC deposition on chondrocytes within areas of severe OA progression as well as in the inflamed synovium (Corvetta et al., 1992, Wang et al., 2011). These observations suggested that there may be an issue with regulation of the complement cascade. Reduced complement regulators such as CD55 have been documented to be altered by activation of cytoplasmic nucleic acid receptors including TLR3, RIG-I and ZBP1 (Karpus et al., 2012). Therefore, the effect of TLR3 activation on several of these complement plasma-membrane bound regulators was investigated. Analysis of complement membrane bound

regulators CD46, CD55 and CD59 demonstrated that TLR3 and C5a stimulation could significantly alter positive cell populations within grade 0 (relatively healthy) articular chondrocytes without significantly altering protein expression.

CD46 (Membrane cofactor protein/MCP) is a ubiquitously expressed C3b-binding protein that acts primarily as a co-factor in the Factor-I mediated proteolytic cleavage of C3b and C4b. The vital role it plays in preventing host deposition of MAC complex has been noted by deficiency studies indicating predisposition of several disease conditions arising from its absence (Bjorge et al., 1996, Cardone et al., 2011, Brocklebank et al., 2014). In relation to disease, the role of CD46 in regulation of complement activity has been evidenced in several pathologies including the findings of a heterozygotic mutation causing atypical haemolytic uremic syndrome. Contrastingly, several tumours overexpress CD46 to increase resistance to complement driven attack (Geis et al., 2010). The role of CD46 in the mediation of signalling in macrophages and dendritic cells, hinted that non-immune cells such as chondrocytes, which express the protein could function in a similar or interesting manner.

CD55 (Decay accelerating factor/DAF) is one of, if not the most important member of, the membrane bound complement regulator proteins. CD55 inhibits complement activity through interference with C3 and C5 convertase activity (Miwa and Song, 2001). The reduction in positive cell population, through TLR3 stimulation with Poly(I:C), suggests a regulated mechanism for anti-viral purposes. It is possible that when TLR3 is stimulated, the expression

of CD55 decreases to allow MAC induced cell-lysis of host cells to destroy an intracellular virus. However, in chondrocytes where the induced lysis can release intracellular pathogens, nucleic acids and other metabolites the danger that the virus presents may be less than the total destruction created through MAC induced lysis. Additionally, the loss of chondrocytes from the cartilage matrix could lead to the formation of micro-fractures in the cartilage, furthering contributing to erosion.

CD59 regulates the polymerisation of the C5b-8 complex to the C9 component in the formation of the cytolytic MAC (Davies et al., 1989). TLR3 stimulation in isolated chondrocytes was demonstrated to significantly upregulate CD59$^+$ cell populations. To see a similar effect in C5a treated cells indicated the potential for TLR3 to regulate membrane bound complement regulators in a manner similar to the complement anaphylatoxin involved in chemotaxis. This data also demonstrated that the chondrocytes CD59 expression is stable between G0 and G2 regions of degeneration giving little support to this thesis' hypothesis, that a reduced level contributes to host MAC deposition as seen in OA.

This data demonstrates the potential for TLR3 activation to alter complement activation and host cell MAC deposition through this pathway and opens avenues for further analysis to confirm whether TLR activation of chondrocytes promotes MAC deposition.

Summary

The project has clearly identified differences in TLR expression and function in OA in relation to joint location and cell type. The synovium and the cartilage of patients with OA appear to be contributing to the progression of the disease through both the production of pro-inflammatory cytokines and matrix metalloproteases, but in manners individual to the affected joint. The observation of nuclease activity within the synovial fluid of patients whilst not new, demonstrates the natural defence of the body in limiting sterile inflammation driven through nucleic acid receptors such as TLR3 by possible degradation of agonists. Additionally, through the progression of the disease and associated destruction of the cartilage matrix, the usually inactive chondrocyte appears to undergo a de-differentiation process becoming increasingly aggressive characterised by altered TLR expression and increased induction of pro-inflammatory cytokine and MMP production upon stimulation. Furthermore, Poly(I:C) and anaphylatoxin's C3a and C5a activation of chondrocytes, significantly alter complement membrane regulators CD46, CD55 and CD59 positive cell populations, potentially attributing to disease progression through MAC deposition, and associated joint erosion through cellular necrosis.

Chapter 8 - Further work

In order to further elucidate the results in chapter 3, whereby TLR expression and subsequent response to TLR stimulation was significantly altered between joint locations, an increased patient cohort should be recruited. Inclusion of other joint procedures common in OA could include the shoulder and interphalangeal joints where surgery is required. I would theorise that if joint architecture is important in TLR expression profiles; the hip and shoulder, being ball and socket joints, should express in a similar manner. However, as they differ immensely in joint loading this could prove incorrect.

In addition to an increased cohort of patient one of the next stages of this should be to perform a full cytokine screen. As well as validating the findings within this data set, the ability to screen a wide array of cytokines would allow determination of whether synovial fibroblasts from different joints are inherently different in their ability to respond to TLR activation. These experiments could be performed using a variety of methods to monitor transcriptional and/or translational production. Whilst transcriptional analysis of TLR induced cytokine production would be perhaps easier; the overall result would be inconclusive and as such should rather be performed using multiplexing ELISA or protein arrays for end point cytokine secretion to include both long and short lived inflammatory responses.

Spontaneous secretion of several pro-inflammatory cytokines and MMPs were noted in both synovial membrane and cartilage digests. With documented research demonstrating TLR activation through ECM fragments it is distinctly possible that, during the enzymatic digestion process, DAMPs are created as

a by-product leading to TLR activation. In order to determine whether this is indeed the case, TLR inhibitory substances such as Chloroquine or monoclonal antibodies could be used to specifically inhibit TLRs and determined their true involvement in the process.

Autologous cartilage transplantation therapy is a promising technique in the treatment of OA, aiding in cartilage repair through *ex vivo* expansion of patient derived chondrocytes and subsequent implantation of new tissue (Minas and Chiu, 2000). Unfortunately, due to currently unknown mechanisms the artificially produced cartilage implants are prone to fail (Minas et al., 2010). The observed de-differentiation of chondrocytes in grade 2 regions of cartilage coincided with an increased response to TLR stimuli and subsequent inflammatory and degenerative enzyme production. Therefore, a new chapter in this project would be determining whether it is possible that this process can be reversed through the use of a 3D culture system or chondrogenic differentiation media. These cells would then be compared to grade 0 chondrocytes and would demonstrate whether a healthy phenotype can be restored in respect to TLR driven inflammatory responses.

The presence of a potent nuclease within the synovial fluid was not a new observation, however its activity upon Poly(I:C) was. Degradation of Poly(I:C) by other bodily dialysates such as plasma and cerebral spinal fluid is known but has yet to our knowledge been clearly documented in synovial fluid (de Clercq, 1979, Kovacs, 1953). Future work into this area of research could include conformation of the presence and subsequent classification of the hypothesised nuclease. In addition to determining which nuclease is responsible, it may also be prudent to attain as to whether it is significantly

altered in the synovial fluid of OA patients opposed to healthy controls. Due to ethical considerations, obtaining healthy synovial fluid is difficult, although joint effusions of early stage OA requires aspiration of synovial fluid which could, in theory, be used for comparison. Due to the presence of only one endosomal TLR in fibroblasts the investigation into the digestion of TLR: 7, 8 and 9's ligands were not performed. However, it would be interesting to test whether the inhibition demonstrated in the HEK293(TLR) cell lines was due to ligand degradation or perhaps another more ubiquitous mechanism.

Complement anaphylatoxins and other components of the complement cascade are present at a significantly increased concentration in the synovial fluid of patients with OA opposed to healthy control (Wang et al., 2011). Of these components, the presence of C3a and the des-Arg form were of particular interest. The anaphylatoxin C3a is a potent pro-inflammatory agent which is regulated by the cleavage of the carboxy-terminal arginine, which results in a des-Arg form which has been documented to bind the C3aR receptor with only a 5-10% efficiency compared to its un-cleaved form (Bajic et al., 2013). C3a is involved in many immune response mechanisms including chemotaxis and macrophage activation (Schraufstatter et al., 2009, Khan et al., 2015).

Many non-immune cells express functional forms of the C3aR and with shared TLR signalling components possible TLR-C3aR crosstalk was preliminarily investigated in synovial fibroblasts. To that end, OASFs were stimulated with specific TLR agonists with or without the addition of the anaplatoxin C3a and monitored for altered IL-6 and IL-8 production (Appendix 1.1).

A

TLR + C3a OASFs

B

TLR + C3a OASFs
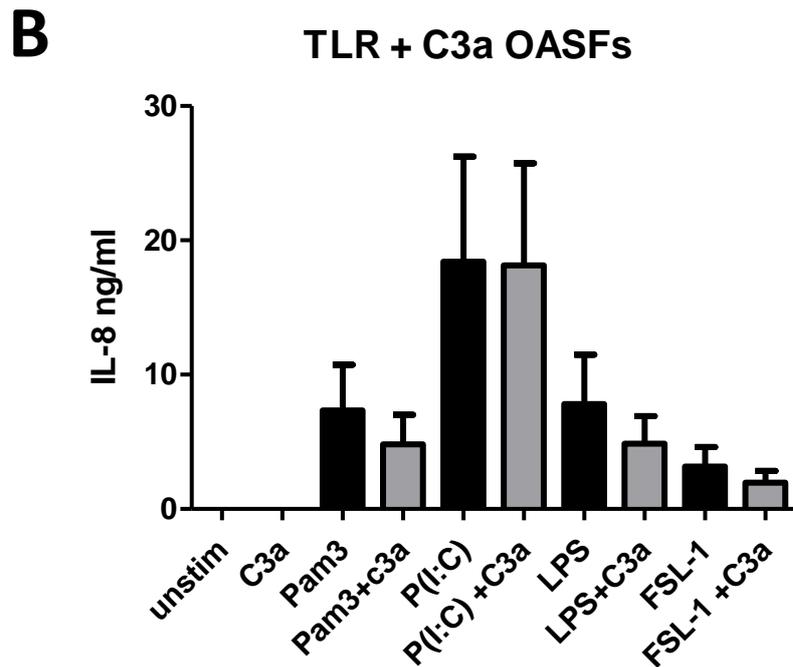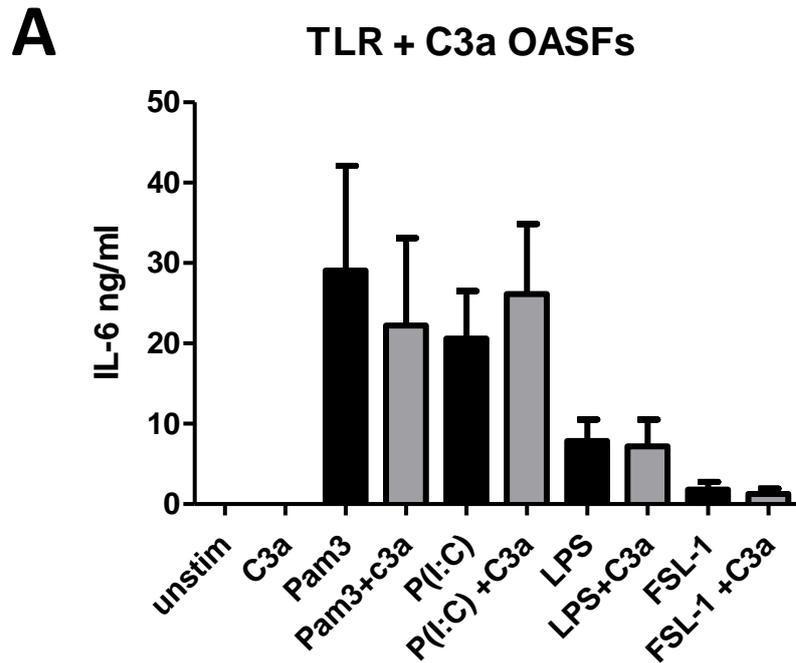
Appendix 1.1: Complement anaphylatoxin C3a does not interfere with TLR mediated cytokine production. A) IL-6 and **B)** IL-8 production. Synovial fibroblasts stimulated at 10×10^4 cells/well in a 96 well plate with 100ng/ml Pam3, 20μg/ml Poly(I:C), 100ng/ml LPS or 1ng/ml FSL-1 with or without the addition of 100ng/ml of recombinant C3a. Data represent mean ±SEM from 3 separate donors performed in triplicate. *P<0.05.-paired student t-test, 2 tailed.

Stimulation of OASFs with TLR1/2, TLR2/6, TLR3 and TLR4 ligands, in tandem with the anaphylatoxin C3a, demonstrated no significant change in IL-6 or IL-8 production (Appendix 1.1). There was a slight trend to a reduced Pam3-(TLR1/2) induced IL-6 and IL-8 production, although was not significant ($P=0.116$ and $P=0.211$ respectively)

Levels of C3 and C3a-des Arg are documented to be significantly upregulated in proteomic analysis of synovial fluid from patients with OA compared to healthy controls (Balakrishnan et al., 2014b, Ritter et al., 2013). However, the concentration of purified recombinant C3a was considerably less than that found in physiological conditions, which can range into thousands of nanograms per millilitre, and may not represent accurately the physiological environment.

Proteomic analysis of synovial fluid performed by several independent groups describes altered complement components within the joint fluid of patients with OA compared to healthy controls (Ritter et al., 2013, Gobezie et al., 2007, Wang et al., 2011). The anaphlatoxins C3a and C5a are two such proteins shown to be markedly increased in concentration in the synovial fluid of OA patients in both their active and des-Arg forms. Previously documented crosstalk between complement receptors and TLR signalling, along with this significantly increased presence, warranted further investigation (Holst et al., 2012).

The production of complement anaphylatoxins within a mammalian cell has, to our knowledge, never been successfully carried out. Bacterial derived proteins were originally thought to be a poor choice for the experiments as potential

endotoxin contamination may alter cytokine production through stimulation of TLR4. Therefore, an attempt to produce anaphylatoxins C3a and C5a for use in these experiments was made. Cloning of customised gene fragments into the pcDNA6 plasmid vector was successful. However, production of the peptide fragments from transiently transfected HEK293T cells was not. Due to the functional relevance of the c-terminal arginine in the activity of C3a and C5a, a secretory tag was omitted. Unfortunately, enzyme degradation of the peptide fragments within the cell cytoplasm, resulted in inefficient production of either anaphylatoxin confirmed by western analysis. Instead, the project utilised *E.coli* derived complement peptide fragments C3a and C5a for use in all of the experiments. Contrary to published data, there was no observable effect on IL-6 or IL-8 production from TLR and C3a co-stimulated synovial fibroblasts. This does not disprove the crosstalk between TLR and C3aR but rather suggest that potentially certain non-immune cells, such as synovial fibroblasts, may not regulate signalling in the same manner as monocytes, those used in the published work demonstrating the effect (Raby et al., 2011, Holst et al., 2012).

Future expansion of this work aim to optimise the cloning of C3a and C5a in order to produce usable quantities of anaphylatoxins mimicking levels found within the body. It may also be poignant to use suboptimal concentrations of TLR ligand in order to cross titrate anaphylatoxins and TLR ligands to distinguish whether there is indeed an effect on TLR activity in these cell types. Additionally, isolation of C3a and/or C5a from OA patient synovial fluid may be feasible if large enough volumes were obtained. Comparisons of the effect of naturally occurring, and artificially cultured peptides could aid in determining

whether there is an additional effect of anaphylatoxin-TLR interaction that is currently unexplored.

Concluding remarks

These data support the hypothesis that the molecular mechanisms active in OA may differ significantly dependent on joint location and this is potentially a factor that should be taken into account when designing clinical trials. The project's aims and investigation targets were broad. Far from being obstructive to progression it was found to be required as the basic disease pathology assumed by many other groups would have greatly hindered analysis. At the beginning of the project, in order to increase significance, samples were grouped together independent of joint type. Further analysis of TLR expression, and activation, led to the discovery that knee and hip should be analysed separately, a factor that not originally considered. This seemingly obvious factor, could perhaps open avenues for research into OA developed treatments tailored specifically to the affected joint.

With an increasingly aging population, a disease as common as OA requires better treatment. Research into effective treatments of OA needs to be as heterogeneous as the disease itself. This project demonstrates the potential involvement of TLRs in OA progression through the production of several disease relative cytokines. However, it cannot be surmised that the selected cytokines studied *in vitro* can accurately represent a complete joint nor should be. It does however; give a basis for further expansion of knowledge and questioning.

Appendix 1:

Common buffers

- **Phosphate buffered saline (PBS)**
  - 137mM NaCl (Fisher Scientific, Loughborough, UK)
  - 1.4mM KCl (Fisher)
  - 4.3mM Na_2PO_4 (Fisher)
  - 1.4mM KH_2PO_4 (Fisher)
  - Made up to one litre of distilled water; pH7.4.
    - *(Commonly made at a ten times concentration and diluted as required)*
- **PBS-Tween (PBS-T)**
  - PBS supplemented with 0.1% (v/v) Tween-20 (Fisher)
- **Tris buffered saline (TBS)**
  - 20mM Tris-HCl (Sigma-Aldrich, Poole, UK)
  - 137mM NaCl
  - 10mM EDTA
  - pH7.4
- **TBS-Tween (TBS-T)**
  - TBS supplemented with 0.1% Tween-20.
- **Tris Acetate-EDTA buffer (TBE)**
  - 89mM Tris-base (Sigma)
  - 89mM boric acid (Sigma)
  - 0.2mM EDTA (Fisher)
    - *(Commonly made at a five times concentration and diluted as required)*

- **FACS wash buffer (FwB)**
  - PBS supplemented with 5% (v/v) human serum pooled from AB positive male donors (Sigma)
  - 0.1% (v/v) sodium azide (Sigma)
- **Permeabilising FACS wash buffer (pFwB)**

- o FwB supplemented with 0.01% (w/v) Saponin (Sigma).

- **SDS-PAGE resolving gel**
  - o 250mM Tris-HCl pH 8.8
  - o 10% (v/v) Acrylamide/Bis solution
  - o 0.1% (v/v) SDS
  - o 0.1% (v/v) APS
  - o 0.1% (v/v) tetramethylethylenediamine (TEMED)

- **SDS-PAGE stacking gel buffer**
  - o 250mM Tris-HCl pH6.8
  - o 5% (v/v) Acrylamide/Bis solution
  - o 0.1% (v/v) SDS
  - o 0.15% (v/v) APS
  - o 0.1% (v/v) TEMED

- **SDS-PAGE running buffer**
  - o 25mM Tris-base
  - o 192mM glycine
  - o 0.1% (w/v) SDS (Sigma).

- **Western blot transfer buffer**
  - o 25mM Tris-base
  - o 192mM glycine (Sigma)
  - o 20% (v/v) methanol (Fisher).

- **ELISA wash buffer**
  - o 1x PBS with 0.01% (v/v) Tween-20

- **ELISA blocking buffer**
  - o 1x PBS with 2% BSA (Sigma)

Tissue culture reagents

- **Dulbecco's Modified Eagles Medium (DMEM)**
  - o Containing 25mM HEPES and 4.5g/l glucose.

- **Roswell Park Memorial Institute (RPMI)**
  - o Medium containing 2mM L-glutamine.

- **Hanks Buffered Salt Solution (HBSS)**

- Containing 0.35g/L sodium bicarbonate (without calcium or magnesium).

- **Foetal Bovine Serum (FBS)**
  - Supplemented at 5-10% (v/v) to the above media following 30 minutes of heat inactivation at 55°C.
  - 10EU/ml (manufacturers MSDS) Sigma F9665

- **Penicillin (100U/ml) and streptomycin (100µg/ml)** (Pen/Strep)
  - Were supplemented to all growth media.

- **Gentamicin (100U/ml)**
  - Was supplemented in enzymatically digested cultures of synovial membrane explants.

Chapter 9 - Bibliography

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